Differential Effect of Isotype on Efficacy of Anti-Tumor Necrosis Factor α Chimeric Antibodies in Experimental Septic Shock

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Summary

Immune complexes containing human gamma (g)1 or murine g2a antibodies generate secondary effector mechanisms via Fc receptor binding or complement activation, whereas those containing human g4 or murine g1 antibodies generally do not. Therefore, isotype selection of therapeutic antibodies may have important clinical consequences. In a rabbit model of recombinant human tumor necrosis factor (rhuTNF)-induced pyrexia, a murine/human chimeric g4 anti-human TNF-α monoclonal antibody (mAb) (cCB0011) showed a dose-dependent inhibition of pyrexia, whereas a g1 isotype variant of the same mAb gave a marked pyrexia that was seen at all doses indicative of an immune complex-mediated response. To investigate whether isotype difference could influence mAb efficacy in pathological disease states, hamster/murine chimeric g1 and g2a anti-murine TNF-α mAbs (TN3g1, TN3g2a) were studied in experimental shock in mice and rats. In lipopolysaccharide-induced shock in mice, treatment with TN3g1 mAb at 30 and 3 mg/kg resulted in 90% survival by 72 h (p < 0.004), and prolonged survival to 45 h (p < 0.05), respectively, compared with 100% mortality by 27 h in controls. In contrast, a g2a isotype variant of the same mAb (30 mg/kg) resulted in only 10% survival by 72 h (p < 0.05). In a neutropenic sepsis model in rats there was greater survival in animals receiving the g1 isotype of TN3 compared with g2a isotype variant (70 vs. 27%; p < 0.005) with 100% mortality in the controls. These differences were not due to the pharmacokinetic profiles of the mAbs. In models of experimental shock antibody isotype can affect outcome with inactive isotypes (human g4 and murine g1) being more efficacious than active isotypes (human g1 and murine g2a).

Clinical septic shock continues to be associated with a high mortality rate, despite the availability of potent broad spectrum antimicrobial agents (1, 2). As a result, much interest has centered on additional therapeutic strategies that address the systemic inflammatory consequences of infection in the shocked patient.

It is known that many of the features of septic shock are caused by endotoxin or LPS, a component of bacterial cell walls (3–5). Endotoxin exerts many of its effects indirectly by triggering the release of a complex cascade of endogenous mediators. It is the effect of these mediators on the tissues that elicits the clinical symptoms of septic shock.

TNF has been identified as a major proinflammatory cytokine synthesized primarily by macrophages/monocytes but also by other activated cells such as lymphocytes, natural killer cells, and endothelium (5–7). It can be stimulated by a wide variety of infectious or inflammatory stimuli (8, 9), and will itself trigger the release of other inflammatory cytokines, such as IL-1 and IL-6.

TNF is one of the principal cytokines involved in the pathology of septic shock. When administered to animals and humans TNF causes hemodynamic and pathophysiological symptoms that resemble the responses to endotoxemia (4, 10–12). In addition, controlled administration of LPS into human volunteers leads to a rapid rise in serum levels of TNF (13), and in some sepsis and septic shock diseases TNF levels are elevated (9, 14, 15). Indeed, anti-TNF immunotherapy using either polyclonal antibodies or mAbs has been shown...
to reduce mortality from lethal doses of LPS or massive intravenous doses of *Escherichia coli* in animal models (16–18). These results form the basis for an immunotherapy strategy for treating septic shock in humans with antibodies against TNF for which the selection of the most appropriate and effective antibodies is of paramount importance.

Although studies using animal models have focused on the feasibility of using anti-TNF mAb immunotherapy either alone or in combination with known treatments, none has addressed the question of the effect of isotype on the efficacy of an antibody. The antibody isotype (Fc region) is known to determine the ability of antibody/antigen complexes to direct immune responses, for example, by initiating the complement cascade or interacting with Fc receptors on cells of the reticuloendothelial system (19–22). Therefore the efficacy of an antibody may be directly related to its Fc function. Immune complexes containing human gamma1 (g1) or murine gamma2a (g2a) antibody isotypes are efficient at binding C1q and hence initiating the complement cascade. In addition, they interact with and activate Fc receptors on cells such as macrophages, neutrophils, and lymphocytes (21–24). In contrast, immune complexes containing human gamma4 (g4) or murine gamma1 (g1) antibody isotypes do not bind to Fc receptors and C1q effectively. Therefore these antibody isotypes do not mediate clearance of antigen by activating components of the reticuloendothelial system, but neutralize the antigen by forming antigen–antibody complexes.

The effect of human g1 and g4 isotypes on the efficacy of an anti-human TNF (cCB0011) mAb was initially studied in a rabbit model of recombinant human TNF (rhuTNF)-induced pyrexia using murine/human chimeric anti-TNF mAbs. This study indicates that different isotypes can modify the pyrexia seen. Furthermore, to determine whether isotype difference can influence therapeutic outcome, hamster/murine chimeric anti–murine TNF mAbs were made by replacing the entire constant regions of the heavy and light chains of hamster mAb, TN3-19.12 (25), with either murine g1 or g2a constant regions. The relative protective efficacy of these antibodies was compared in a LPS-induced shock model in mice, and in a model of *Pseudomonas aeruginosa* bacteremia in immunocompromised rats.

**Materials and Methods**

*rhuTNF-induced Pyrexia Model in Rabbits*

_Dose–Response Curve._ Male NZW rabbits (18–22 kg) (Rosemead Rabbits, Waltham Abbey, UK) were placed in restraining stocks and a temperature thermo probe was inserted ∼4 cm into the rectum. Rectal temperature was recorded every 5 min using an Isothermex monitoring device (Columbus Instruments, Columbus, OH). Drugs were administered via the femoral vein and blood samples withdrawn via the femoral artery, both vessels having been cannulated 24 h previously. After a 60-min stabilization, rabbits were given one of the following pretreatments: (a) saline only (n = 4); (b) cCB0011g1, 1–100 μg/kg (n = 4–5); and (c) cCB0011g4, 1–100 μg/kg (n = 4–7). This was followed 15 min later by 1 μg/kg rhuTNF (Saxon Biochemical Gmbh, Hanover, Germany). Saline–saline, cCB0011g1 (100 μg/kg)–saline, and cCB0011g4 (100 μg/kg)–saline groups served as controls (n = 3–4). Blood samples were taken every 60 min up to 5 h for plasma rhuTNF measurement by ELISA (26) and L929 assay (27), after which the animals were killed.

_Effect of Preformed Immune Complexes._ In two further groups of rabbits (n = 6 per group), preformed TNF/anti-TNF immune complexes were administered via the femoral vein and measurements continued for 300 min. One group received cCB0011g1 (100 μg/kg)/rhuTNF (1 μg/kg) and the other received cCB0011g4 (100 μg/kg)/rhuTNF (1 μg/kg) immune complexes. The antibodies and rhuTNF were mixed and allowed to stand for 60 min before administration.

**LPS-induced Shock Model in Mice.** Male BALB/c mice (18–20 g) (Harlan Olac Ltd., Bicester, UK) were housed in a thermoneutral environment with a 12-h light/dark cycle, and allowed to eat and drink ad libitum.

LPS (Serotype 0111:B4; Sigma Chemicals Ltd., Poole, UK) was freshly prepared to the required concentration before the start of the experiment by dilution in sterile saline. In a pilot study, a LPS dose of 5 mg/kg caused 100% mortality within 48 h and was chosen for further experiments.

For investigation into the effect of mAb treatment, groups of 10 mice were anaesthetized using halothane in 5% O2 1 h before receiving LPS, and injected with either 0.15 ml (i.v.) saline or 0.15 ml (i.v.) of TN3g1 or TN3g2a, then allowed to recover. The antibodies were used at doses of 30, 3, and 0.3 mg/kg/1 h later (time 0) the animals were anaesthetized and received either 0.1 ml of saline or 0.1 ml of LPS (50 mg/kg i.v.). Animals were assessed at least four times daily and surviving animals were killed after 72 h.

In a separate experiment, groups of 10 mice received either MOPC21 (30 mg/kg) or TAL14.1 (30 mg/kg) 1 h before LPS challenge. These antibodies served as nonspecific antibody controls for TN3g1 and TN3g2a, respectively.

**Neutropenic Rat Sepsis Model.** Female, albino, pathogen-free Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 125–150 g were maintained in filtered biosafety cages and allowed to eat and drink ad libitum. Animals were pretreated with intramuscular cefamandole (Eli Lilly, Indianapolis, IN) at 100 mg/kg on an every other day schedule, and then rendered neutropenic by intraperitoneal administration of cyclophosphamide (Bristol-Myers, Evansville, IN) at a dose of 150 mg/kg at time 0 followed by a second dose of 50 mg/kg 72 h later. The details of this animal model have been described previously (28).

Animals were inoculated orally with ∼10^8* P. aeruginosa* strain 12.4.4 at 0, 48, and 96 h after the initial dose of cyclophosphamide.

At the onset of fever, antibody therapy with either TN3g1 (n = 20), TN3g2a (n = 22), or control mAb (L23D9; Celltech Ltd., Slough, UK) (n = 18) was given at 20 mg (i.v.). Blood cultures and serum specimens for endotoxin and TNF levels were obtained at onset of fever and 24 h after onset of fever in each animal. Endotoxin levels were measured by a quantitative turbidimetric Limulus lysate method (Associates of Cape Cod, Inc., Woods Hole, MA), and bioactive circulating TNF levels were measured by the L929 bioassay (27). Animals were examined daily for a 12-d period and all animals that did not survive the study period underwent necropsy examination with quantitative bacterial cultures of liver, lung, heart, and spleen tissue.

**Monoclonal Antibodies**

_Chimeric Anti–murine TNFα mAb (TN3)._ TN3 is a murine/hamster chimeric anti–murine TNFα with hamster variable

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1. Abbreviations used in this paper: g1, gamma1; g2a, gamma2a; g4, gamma4; rhu, recombinant human.
regions and murine heavy and light chain constant regions of either g1 (TN3g1) or g2a (TN3g2a) isotype. cDNA clones containing genes for the heavy and light chains of the hamster antibody, TN3-19.12 (25), were isolated (29, 30). Chimeric (hamster-mouse) genes were constructed joining the hamster V1 gene to mouse Ck and the hamster Vh gene either to mouse C-g-gamma1 (g1) or mouse C-gamma2a (g2a) (31, 32). These chimeric genes were subcloned for expression and cell lines established by transfecting linearized plasmid DNA into NSO cells and selecting cells that grew in the absence of glutamine (33). The hamster/murine chimeric mAbs (TN3g2a and TN3g1) were purified from 100 liters of tissue culture supernatant by protein A affinity chromatography and shown to be >98% monomeric IgG by HPLC on a Zorbax GF250 size exclusion column (DuPont, Wilmington, DE) and reducing and nonreducing SDS-PAGE (34). Endotoxin levels were <2 IU/mg (35).

In vitro bioassay, using the L929 mouse fibroblast assay (27), showed that both mAbs were equipotent to hamster TN3-19.12 (25).

Nonspecific Control mAbs. TAL14.1 is a murine g2a mAb directed against HLA-DRα (Imperial Cancer Research Fund, London, UK) (36). MOPC21 is a murine g1 mAb derived from X63P3 with no known specificity. L23D9 is a hamster-derived mAb directed against murine recombinant IL-2 that does not react with natural murine or rat IL-2 (25). Endotoxin levels were <0.1 IU/mL.

Chimeric Anti-human TNF-α mAb (cCB0011). cCB0011 is a murine/human chimeric anti-human TNF-α with murine variable regions and human constant regions of either g1 (cCB0011g1) or g4 (cCB0011g4) isotype. The antibodies were prepared as described above from murine anti-human TNF-α mAb, 101/4 (National Institute of Biological Standards and Control, London, UK). Endotoxin levels were <0.7 IU/mg.

Pharmacokinetics of Chimeric Anti-murine TNF mAb and Determination of Immune Response to Antibodies. Groups of male BALB/c mice (Harlan Olac Ltd.) (n = 4-5) received 10 mg/kg (i.p.) of antibody with serum samples obtained at 0, 8, and 24 h, 3, 6, 7, 10, 13, 14, 17, 20, and 21 d. Groups of male Lewis rats (Harlan Olac Ltd.) (n = 3) received 10 mg/kg (i.p.) of antibody with plasma samples taken at 0, 8, and 24 h, and 3, 4, 6, 7, 8, 10, 13, 16, 19, and 21 d.

Serum or plasma concentrations of TN3g1 and TN3g2a were measured using an ELISA. Plates were coated with rabbit anti-TN3-19.12 antibody at 2.5 μg/ml. Samples were serially diluted and assayed in duplicate with the relevant isotype standard in each assay. A biotin-conjugated antibody to the hypervariable regions of TN3-19.12 (rabbit anti-εTN3-19.12 g2a affinity purified on TN3-19.12-Sepharose) was then added, and the assay completed with horseradish peroxidase-streptavidin (HRP-SA) followed by tetramethylbenzidine (TMB) substrate.

A specific double-antigen sandwich ELISA was used to detect immune responses. Plates were coated with the TN3-19.12 isotype under investigation at 1 μg/ml. Isotype-specific, affinity-purified rabbit polyclonal standards were used. Samples were diluted 1:10 and assayed in duplicate. Biotin-conjugated TN3-19.12 (same isotype) was then added, followed by HRP-SA and TMB substrate. Results were expressed as U/mL, where 1 U is equivalent to 1 μg/ml rabbit anti-TN3-19.12.

Data Analysis. For the rabbit pyrexia model differences between groups were assessed by one-way analysis of variance (ANOVA). Differences were considered significant when p < 0.05. Statistical analysis of the Kaplan-Meier survival curves from the LPS-induced shock model in mice was performed using the log-rank test and the Wilcoxon test. Differences were considered significant when p < 0.004, and borderline significant when p < 0.05. Kruskel-Wallis one-way ANOVA was used to analyze the survival of animals in the neutropenic rat model. Differences were considered significant when p < 0.05.

Results

Effect of Chimeric Anti-human TNF (g1 and g4) mAb on rhuTNF-induced Pyrexia in Rabbits

Dose-Response Curve to rhuTNF. None of the control groups showed any rise in temperature during the experimental period. In the saline plus rhuTNF-treated group there was a transient rise in rectal temperature with a peak of +0.69 ± 0.1°C at 60 min. In the presence of cCB0011g4 mAb there was a dose-dependent inhibition of the rhuTNF-induced pyrexia, the pyrexia being completely ablated by 100 μg/kg of antibody (Fig. 1 A).

With the g1 isotype, however, a marked pyrexia was seen at all doses. Indeed, with the lowest dose of antibody, the pyrexia was greater than that seen in the saline plus rhuTNF group (Fig. 1 B). Furthermore, there was a persistent pyrexia in the highest dose group (100 μg/kg) plus rhuTNF with the temperature remaining raised until the end of the experiment (5 h).

In contrast to the pyrexia data, the effect of both isotypes on the removal of rhuTNF from the plasma was the same. Both gave a dose-dependent reduction in immunoreactive and nonimmunoreactive rhuTNF.
bioactive rhuTNF in the plasma with none being detected at either 10 or 100 μg/kg of each antibody (Fig. 2).

**Effect of Preformed Immune Complexes.** With preformed immune complexes of cCB0011g4/rhuTNF there was no change in temperature during the course of the experiment. In the case of cCB0011g1/rhuTNF immune complexes, there was an initial rise in temperature followed by a further progressive rise, reaching +0.5 ± 0.2°C by 5 h (Fig. 3). At the end of the experiment, the change in temperature was significantly higher (p <0.05) in the g1 compared with the g4 immune complex–treated group. No free bioactive TNF was detectable in either preparation of immune complexes.

**Effect of Chimeric Anti–murine TNF mAb on LPS-induced Shock in Mice.** All animals receiving saline only survived the duration of the experiment. In contrast, all saline-treated control animals receiving LPS had died by 27 h, with overt symptoms of shock such as piloerection, hunched posture, moribundity, and listlessness appearing by 6–8 h after LPS challenge. Pretreatment with nonspecific control mAb (MOPC21 or TAL14.1) had no effect on survival.

TN3g2a antibody at 30 mg/kg gave minimal protection with 10% survival at 72 h (p <0.05) (Fig. 4 A). No protection was seen with 3 mg/kg (100% mortality by 25.5 h) or 0.3 mg/kg (100% mortality by 21 h).

Figure 2. Dose-dependent reduction in immunoreactive rhuTNF in plasma. (A) cCB0011g4; (B) cCB0011g1. Data are geometric mean and 95% confidence interval (C.I.).

Figure 3. Effect of preformed immune complexes of cCB0011g1 and g4 plus rhuTNF on pyrexia in rabbits (n = 6). Data are mean ± SEM.

Figure 4. Effect of different isotypes of TN3 mAb on survival after LPS-induced shock in mice. (A) TN3g2a (n = 10); (B) TN3g1 (n = 10).
Treatment with both TN3 mAbs did not reduce the frequency of bacteremia when compared with animals receiving the control mAb. Necropsy examination of animals that did not survive the 12-d study period uniformly revealed multiorgan infection with \textit{P. aeruginosa} 12.4.4. This finding was not affected by the administration of anti-TNF mAb. Serum bioactive TNF levels were not detectable by the L929 assay after administration of either TN3 mAbs. Control animals had a mean of 2.25 ± 2.2 IU/ml of TNF detected in serum after administration of the control mAb (p <0.0001). Circulating endotoxin levels were comparable in septic animals regardless of the mAb treatment received (1.18 ± 1.17 ng/ml TN3g1; 1.35 ± 1.1 ng/ml TN3g2a; 1.36 ± 1.4 ng/ml control; not significant).

Despite similar levels of bacteraemia, circulating TNF levels, and endotoxin levels, animals that received TN3g1 mAb had a significantly greater survival rate than animals that received TN3g2a mAb (70 vs. 27% survival; p <0.005). TN3g2a mAb offered marginal survival benefit over the control group (27 vs. 0% survival; p <0.05). The g2a isotype was clearly not as efficacious as the g1 isotype (Fig. 5).

**Pharmacokinetics of Anti-TNF Antibodies.** In mice, the \(C_{\text{max}}\)’s for 10 mg/kg chimeric TN3 mAbs were similar; 75 \(\mu\)g/ml for g1 and 87 \(\mu\)g/ml for the g2a (Fig. 6). The elimination phase half-life of both mAbs in the circulation was also similar at 9.3 d calculated from the peak of concentration to the final value at 21 d. No measurable immune response was made against the antibodies by the mice from days 6 to 21.

In rats, the \(C_{\text{max}}\)’s for the mAbs were similar; 40.6 \(\mu\)g/ml for g1 and 71.3 \(\mu\)g/ml for g2a with a steady elimination phase to day 10, after which clearance was more rapid (Fig. 7). The elimination phase half-life calculated to day 8 for TN3g1 was 5.4 d, and for TN3g2a was 6.1 d. The difference in half-life between the g1 and g2a isotypes was not significant. The rapid clearance after day 10 coincided with the development of a measurable immune response (20 ng/ml) against the g2a isotype detectable from day 6, though none was detected against the g1 isotype.

**Discussion**

In this study, we have investigated the effect of isotypes by making two matched pairs of antibodies. One pair uses human g1 and g4 constant domains with an anti-human TNF binding site, and the second pair uses murine g1 and g2a constant domains with an anti-murine TNF binding site. In terms of Fc effector function, the human g1 and murine g2a are similar, both having the capacity to actively elicit immune responses via complement and Fc receptors. The human g4 is matched to the murine g1 in being functionally relatively inactive (22-24). In both cases, each member of the pair has the same TNF neutralizing activity in vitro. For the murine pair, the plasma clearance rates have been shown to be similar in both mice and rats.

Against this background, the effect of isotype differences on therapeutic efficacy was studied in a series of animal shock models in which anti-TNF therapy had previously been shown to be effective (16, 25, 37, 38). The human antibodies (cCB0011g1 and cCB0011g4) were tested in a model of rabbit pyrexia induced by exogenous rhuTNF. The murine antibodies (TN3g1 and TN3g2a) were tested in a murine lethal LPS challenge model and a model of \textit{Pseudomonas} bacteremia in neutropenic rats.

In the rabbit pyrexia model exogenous rhuTNF induced
concentration of the rhuTNF. Indeed, under gl treatment the presence of the gl isotype pyrexia was seen at all doses, despite dose-dependent manner by cCB0011g4. However, in the presence of complexes of g1 but not g4 isotype. These data indicate that, in addition to neutralizing the TNF bioactivity, the human g1 isotype containing immune complexes elicits other physiological effects, presumably via Fc receptor binding and/or complement fixation. It was important to determine whether these effects of isotype difference could affect treatment efficacy in a pathological situation. To this end, hamster/murine chimeric mAbs were compared in lethal models of septic shock in mice and rats.

The murine g1 mAb isotype effectively protected mice against the lethal effects of LPS challenge with 90% survival at 72 h compared with 100% mortality in the untreated controls. In contrast the murine g2a isotype gave only minimal protection against lethality with 10% survival by 72 h in the high dose (30 mg/kg) antibody-treated group only. The superior effect of TN3g1 observed in the endotoxin treated mice was confirmed in an infection model of Pseudomonas bacteremia in immunocompromised rats, where the differential effects of the two mAbs were not related to their ability to remove TNF from the circulation of infected animals. Indeed both mAbs successfully neutralized bioactive TNF in the circulation of neutropenic rats.

These differences could be due to differing pharmacokinetic profiles in vivo. However, TN3g1 and TN3g2a have similar half-lives in the circulation of between 5–8 d in rats and 9 d in mice, indicating that accelerated clearance does not account for the lack of effect of the murine g2a antibody. In mice no immune response against the antibodies was detected that could have led to decreased efficacy. Although an immune response was detected in rats from day 6, it is unlikely to have had an effect on efficacy since most of the deaths in this model occurred within 6 days of antibody treatment. These results suggest that the Fc region of each antibody directly influences its ability to prevent TNF-mediated pathology. Kipps et al. (39) have demonstrated that the isotype of murine antibodies will affect their ability to implement antibody-dependent, cell-mediated cytotoxicity (ADCC) by human peripheral blood lymphocytes. They compared IgG1, IgG2a, and IgG2b antibody isotypes and showed that the murine IgG2a was most effective at mediating ADCC, with IgG2b being intermediate and IgG1 inactive. In addition, Oi et al. (40) demonstrated that the capacity to fix complements was greatest for IgG2b, intermediate for IgG2a, and least for IgG1. Taken together, this suggests that immune complexes containing the g2a antibody isotype may be exacerbating the symptoms of septic shock by activating complement and effector cells through their C3b or Fc receptors. This activation causes the release of additional inflammatory cytokines such as IL-6, IL-1, and TNF, which in turn potentiates the effects of TNF. In a model system where most of the pathological and hemodynamic changes are occurring systematically, further activation of the immune and inflammatory networks may offset any beneficial effects of anti-TNF treatment and lead to reduced survival. Certainly at low dose (0.3 mg/kg), the g2a antibody isotype showed 100% mortality by 21 h compared with 27 h in the untreated controls in the LPS-induced shock model. However, when used at sufficiently high doses the benefits of anti-TNF therapy may outweigh the negative effects mediated by antibody Fc function.

This study clearly demonstrates the importance of antibody isotype on the functional antagonism of circulating TNF. In septic shock models, where systemic inflammatory reactions are occurring, an antibody that does not lead to further potentiation of the inflammatory network (i.e., murine g1 and human g4) is more effective than an antibody capable of fixing complements and interacting with Fc receptors (i.e., murine g2a and human g1). Clearly an understanding of the interactions between different isotypes and effector mechanisms of the immune system will aid in choosing the best strategy for immunotherapy in various human diseases.

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