An anti-human recombinant tumor necrosis factor alpha (TNFα) monoclonal antibody recognizes an epitope in feline TNFα

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Abstract – It is likely that the murine response to human recombinant TNFα (hrTNFα) may generate antibodies (Ab) to epitopes present in TNFα from other species. Here, we demonstrate that F5 anti-hrTNFα monoclonal antibody (mAb) recognizes feline TNFα while E8 anti-hrTNFα mAb failed to do so. In order to demonstrate that E8 and F5 mAb recognize different epitopes in the hrTNFα molecule, a constant concentration of E8 and variable concentrations of F5 were incubated with solid phase bound hrTNFα. Binding of E8 and F5 to hrTNFα was determined with anti-µ and γ chain specific Ab. F5 bound equally to hrTNFα in the presence or absence of E8 and the same amount of E8 bound to hrTNFα, in spite of the presence of F5. When using the E8 and F5 mAb for capturing the TNFα from the equine, canine, feline and bovine species, in supernatants of an ex vivo lipopolysaccharide (LPS)-stimulated whole blood cell culture, we only detected the feline TNFα by F5 mAb (p = 0.001). By a cytotoxic assay on L929 fibroblasts, we indeed demonstrated the feline TNFα production after the LPS stimulus. In an inhibition assay, the human and feline cytokines competed for F5, although the inhibition of native human TNFα binding to F5 was significant but only about 20% (p = 0.001). In conclusion, most likely the F5 anti-hrTNFα mAb recognizes an epitope in feline TNFα. Its immunomodulatory potential in the feline model remains to be studied.

monoclonal antibodies / tumor necrosis factor alpha / cross-reactivity

1. INTRODUCTION

TNFα is a 17 kDa, highly pleiotropic, non species-specific cytokine secreted mainly by activated monocytes and macrophages. Its production is triggered by a wide range of infectious and inflammatory stimuli, the most potent being the endotoxin lipopolysaccharide (LPS), from the cell wall of Gram (−) bacteria. TNFα has affinity for cell surface receptors of 55 and 75 kDa. Both receptors are released to the plasma where they combine with the cytokine [22].

At physiological concentrations, TNFα induces the expression of molecules that promote the adherence of neutrophils, macrophages and lymphocytes to the
endothelial cells, at the site of inflammation [11, 24]. High concentrations of the cytokine raise the body temperature, inhibit the bone marrow and promote disseminated intravascular coagulation and irreversible shock. Chronic high levels can induce a caquectic stage [5, 14, 23].

The quantification of TNFα is difficult due to its short (6 to 20 min) half-life, the existence of two active forms (seric and membrane associated), and the presence of soluble receptors [4]. A useful tool for the study of TNFα is the measurement of its in vitro and/or ex vivo over-expression, by peripheral mononuclear cells, isolated [18] or present in whole blood [25], respectively. The lack of anti-species-specific animal TNFα monoclonal antibodies (mAb) has encouraged the development of assays based on cross-reactive antibodies (Ab) [10, 15].

The effects of monoclonal and polyclonal Ab to TNFα and the use of soluble receptors, alone or fused to Fcγ fragments, have been the main strategies to modulate TNFα activity [6, 7, 12, 16]. Although the results of TNFα immunomodulation in the human model are rather controversial, the results obtained in the animal model are more encouraging [3].

With the long-term goal of providing an immunomodulatory model with a prophylactic or therapeutic value in the animal model, we evaluated the capacity of two anti-human recombinant TNFα (hrTNFα) mAb to recognize the cytokine of several animal species, with an economic and/or affective value.

2. MATERIALS AND METHODS

2.1. Generation of anti-hr TNFα mAb

Briefly, according to Köhler and Milstein [9], BALB/c female mice were immunized with hrTNFα (Genetech, San Francisco, Ca, USA). Two clones, IgM (E8) and IgG (F5) producers, were selected for in vitro and in vivo expansion. Purification was done by precipitation with ammonium sulfate, followed by sizing and Sepharose-Protein G affinity chromatography, respectively. Purity was assessed by SDS-PAGE.

2.2. The immunoradiometric assay (IRMA) for epitopic recognition by the anti-hrTNFα mAb

Polyvinyl chloride (PVC) microtitration plates were sensitized with hrTNFα (150 ng/mL). After blocking the solid phase, serial dilutions of F5 (2570, 257, 25.7, and 2.57 pg/µL) and constant concentrations of E8 (4 ng/µL) were added. The differential detection of μ (E8) and γ (F5) heavy chains was done with goat IgG, affinity purified and radiolabeled, anti-μ and anti-γ chain specific Ab, respectively (SIGMA, St. Louis, Mo, USA).

2.3. Over-expression of TNFα in whole blood from the human, canine, feline, bovine and equine species. Reactivity with the mAb by IRMA

An ex vivo, whole blood culture system (WBCS) was used [25]. Briefly, 2–5 mL of blood were diluted five times with RPMI-1640 (supplemented with 200 mM L-glutamine, 200 IU/mL penicillin, 200 mg/mL streptomycin and 2 IU/mL heparin) and 360 µL were placed in flat-bottomed microtitration plates. After 4 h of pre-incubation, 40 µL of LPS (Escherichia coli, serotype 026:B6, SIGMA MR, USA) to a final concentration of 10 µg/mL were added. After incubation for 12 h, the TNFα content was determined as follows. PVC microtitration plates were sensitized with E8 or F5 (3 µg/mL), followed by the addition of supernatants from human, equine, canine, feline and bovine origin. Then polyclonal rabbit anti-hrTNFα antiserum was added and, for detection, a goat IgG, affinity purified and radiolabeled, anti-rabbit IgG was used.
2.4. Cytotoxic assay for human and feline TNFα

As described [8], 100 µL of a suspension of 4 x 10^5 L929 fibroblasts (ATCC CCL 1; American Type Culture Collection, Rockville, MD, USA) were seeded in RPMI 10% FBS in 96-well flat bottomed microtitration plates. The culture medium was eliminated and 100 µL of each supernatant in triplicate, obtained from ex-vivo LPS-stimulated WBCS, were added (three samples for each species). hrTNFα (1 ng/mL) and RPMI-1640 were used as positive and negative controls, respectively. Next, 100 µL of medium containing actinomycin D was added to each well yielding a final concentration of 5 µg/mL. After incubating, the culture medium was removed, and 0.05% crystal violet in 20% ethanol was added. To wash the stained cells, 100% methanol was added and the absorbance at 600 nm was determined.

2.5. Competition solid phase immunoassay for the detection of native feline TNFα

PVC plates were sensitized with F5 and mixtures of supernatants (SN) from the human and the feline over-expressed WBCS were added (%v/v human SN / %v/v feline SN: 100/0, 78/22, 66/34, 50/50, 0/100). In the absence of feline SN, RPMI was incorporated into human SN (100%v/v human SN was prepared by a mixture of the same volumes of human SN and RPMI) while, in the absence of human SN, RPMI was incorporated into feline SN (100%v/v feline SN was prepared by a mixture of the same volumes of feline SN and RPMI). After incubating, radiolabeled E8 was added for the detection of bound human TNFα. If human and feline TNFα compete for the F5, as feline TNFα increases a diminished E8 radioactive signal should be detected since the E8 mAb does not recognize feline TNFα.

2.6. Statistical methods

In the experiments to determine the sensitivity of the calibration curves and in the competition assays, a two criteria variance analysis was used. In the antigenic capture assays to determine if E8 or F5 recognize TNFα from other species, a 2 × 6 factorial analysis was employed. All the experiments were performed three times.

2.7. Bioethical considerations

All animal species were handled with permanent veterinary supervision. Trained personnel performed venous punctures and the minimum necessary blood volumes were obtained.

3. RESULTS

3.1. Epitopic recognition of the anti-hrTNFα mAb

By a specially designed competition assay, E8 and F5 mAb most likely recognized different epitopes on the TNFα molecule. A constant concentration of E8 and variable concentrations of F5 were simultaneously incubated with solid phase bound TNF. Binding of E8 and F5 to TNFα was determined with anti-µ and γ chain specific Ab. Figure 1 shows that F5 (IgG) bound equally well to TNFα, in the presence or absence of E8 (IgM). Moreover, the same amount of the E8 mAb was bound to TNFα, in spite of the presence of increasing concentrations of F5.

3.2. Reactivity of E8 and F5 mAb with over-expressed-TNFα from the human, canine, feline, bovine and equine species.

Cytotoxic and competition assays for detecting feline TNFα

As shown in Figures 2a and 2b, when we used the E8 and F5 mAb to detect the TNFα from the equine, canine, feline and
bovine species, in supernatants of an ex vivo LPS-stimulated whole blood cell culture, only the feline TNFα reacted significantly ($p = 0.001$) above the baseline with the F5 mAb (Fig. 2b). However, the differences between the LPS-treated and non-treated cultures were marginal.

In order to demonstrate the feline TNFα production, after the LPS stimulus, we analyzed the TNFα present in both human and feline supernatants by a cytotoxic assay on L929 murine fibroblasts. As shown in Figure 3, at the same supernatant volumes, supernatants from human LPS-stimulated cells induced a higher mortality on L929 cells than supernatants from feline blood ($p = 0.001$). We observed that LPS indeed stimulates feline TNFα production.

As depicted in Figure 4, in an inhibition assay where microtitration plates were sensitized with F5 mAb and mixtures of supernatants from human and feline LPS-stimulated whole blood cell cultures, the human and feline TNFα competed for the mAb. The feline TNFα inhibited approximately 20% of the binding of the native human TNFα to F5 mAb ($p = 0.001$). In human supernatants, when feline supernatants were replaced by 50% v/v of RPMI, the binding of human TNFα to F5 mAb was unaltered (Fig. 4, first bar to the left).

4. DISCUSSION

Susceptibility to septic shock is highly variable among vertebrate species. Thus, in the feline, equine, ovine and swine models, this susceptibility derives from large numbers of resident macrophages within the pulmonary capillaries. At low doses of Gram (−) endotoxins, large alveoli damage can be produced. In contrast, in the canine model and in laboratory animals, a smaller number of these macrophages have been described and thus a lower susceptibility to this kind of damage is found. TNFα is a key molecule in these pathologies [19].
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The use of anti-LPS mAb may prevent the septic shock or in vivo animal correlates, such as the Shwartzman reaction [1]. Similarly, anti-TNFα mAb may block the biological activity of the cytokine, preventing the manifestation of the shock syndrome [2, 17].

TNFα is the cytokine with the lowest species-specific restriction [21]. Thus, mouse and human TNFα share about 86% of the aminoacidic sequences [20]. It is likely, therefore, that the murine response to hrTNFα may generate antibodies to epitopes present in TNFα from other species. Such antibodies may represent valuable tools for immunomodulation.

In spite of the fact that E8 and F5 belong to different classes (M and G, respectively), they most likely recognize different epitopes on the TNFα molecule, as

Figure 2. TNFα over-expression in an ex vivo whole blood culture system from the animal species. Using the E8 (a) and F5 (b) mAb, TNFα from human, equine, canine, feline, and bovine origin was captured. Diluted blood was incubated in the presence (dashed bars) or absence (white bars) of LPS. As negative and positive controls, RPMI and hrTNFα were used. TNFα was measured by immunoradiometric assay. Line bars represent standard deviations.
shown in Figure 1. The two mAb did not interfere with each other in their binding to TNFα, over a wide range of F5 dilutions.

From the 4 animal species studied, only the feline showed levels of TNFα significantly above the baseline (Fig. 2b). Three alternative or concomitant explanations may be proposed to understand why TNFα levels detected in the cell culture supernatant of this species were about half of those detected in the over-expressed human cell culture. First, the affinity of F5 for the feline epitope may be lower or the actual number of the relevant epitopes may be less; second, the polyclonal rabbit anti-hrTNFα may recognize a lower number of epitopes in the feline cytokine and, third, the type of LPS used in the experiments described here may be less effective in inducing over-expression of TNFα in the feline than in the human model.

As we hypothesized above, the effect of *E. coli* LPS on TNFα induction was higher in human cells than feline cells (Fig. 3). This differential capacity of LPS induction has also been reported depending on the bacterial origin of the LPS [8, 13]. This phenomenon may explain the diminished TNFα levels observed in Figure 2b, after LPS-induction.

When human and feline cytokines competed for F5, the inhibition of native human TNFα binding was only about 20% (Fig. 4). Considering the possibility that F5 may have a lower affinity for the feline epitope, this inhibition is acceptable. Moreover, if F5 had the same affinity for both the human and feline epitopes, a maximum inhibition of 50% should be expected in the protocol used. The marginal signal for 100%v/v feline TNFα shown in Figure 4, compared to those shown in Figure 2b, are not contradictory,
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Given the different nature of the assays. Thus, Figure 4 shows results obtained with radiolabeled E8, a reagent that does not recognize feline TNFα, while in Figure 2b detection of TNFα, captured by F5, is done with rabbit immunoglobulins, followed by the goat immunoprobe.

In competition experiments using hrTNFα (1 ng/mL as a limiting concentration) and supernatants derived from feline blood stimulated by LPS, we demonstrated a similar pattern as observed for native TNFα present in the supernatants derived from human blood (results not shown).

Additionally, in experiments not shown, we also demonstrated that binding of feline TNFα to F5 can be blocked by pre-incubating the feline’ supernatants with the rabbit polyclonal anti-hrTNFα serum. In these conditions, the native human TNFα binding to F5 remained unaltered.

In conclusion, we describe an anti-human TNFα mAb that recognizes TNFα from vertebrate species different from humans. This assay does not require the laborious purification of the native cytokine, or the generation of the recombinant product, to be used for detecting the feline cytokine. Studies to determine the immunomodulatory capacity of the F5 mAb will soon begin.

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REFERENCES


