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Inflammation Research

Anti-tumor necrosis factor α F(ab')$_2$ antibody fragments protect in murine polymicrobial sepsis: Concentration and early intervention are fundamental to the outcome

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Abstract. *Background:* Negative results are frequent using anti-TNFα antibodies in sepsis models and clinical trials.
Methods and Results: Different prophylactic doses of anti-TNFα F(ab')$_2$ antibody fragments were compared for the prevention of death by sepsis induced by cecal ligation and puncture (CLP) in mice. High (10 mg/kg) and very low (0.01 and 0.1 mg/kg) concentrations of anti-TNFα antibody fragments were not the most adequate for treating polymicrobial sepsis, since they did not significantly improve survival. To the contrary, intermediate doses (1 mg/kg) significantly protected the challenged animals. Protective activity was also observed when administration of the antibody fragments was initiated early (up to 30 min) after CLP.
Conclusions: These results suggest that in processes where excessive production of cytokines is involved, the aim should be to return them to their physiologically acting range but not to inhibit their production. The timing of initiating therapy should also be considered in order to maximize the possible benefits.

Key words: Polymicrobial sepsis – Immune response regulation – F(ab')$_2$ fragments – Anti-TNFα

Introduction

TNFα is produced by a large variety of cell populations, including monocytes, macrophages and dendritic cells [1]. There is evidence involving this cytokine in the development of lymphoid organs [2–4], in the formation of germinal centers in the spleen and in the maturation of the antibody response [5–7]. In physiological conditions, it is considered as a main mediator of events involved in inflammation and immunity [8], playing a preponderant role in defense against infections by fungi [9, 10], bacteria [11–14] and even parasites [15]. Paradoxically, its exacerbated production during the septic process, together with the presence of other cytokines like IL-1β, IL-12, IL-8 and IFNγ [16–25], contributes to the development of tissue damage and multiple organ dysfunction [26]. This occurs through such mechanisms as overproduction of nitric oxide, with the consequent decrease in peripheral vascular resistance [27] which facilitates extracellular extravasation of liquids, activation of the coagulation cascade associated with the development of disseminated intravascular coagulation [28] and the migration of PMN to zones of inflammation with subsequent degranulation and direct tissue damage [29–31]. The above described pro-inflammatory process has been termed the systemic inflammatory response syndrome (SIRS) that, in turn, induces the development of a compensatory anti-inflammatory response syndrome (CARS), characterized by the production of anti-inflammatory factors such as IL-10 and TGFβ, among others [32]. CARS has been associated with suppression of the pro-inflammatory response, loss of bactericidal capacity and immunoparalysis. Due to the key role of TNFα in the development of SIRS, experimental immunotherapies in sepsis have sought to eliminate this and other pro-inflammatory cytokines. In this context, it has been reported that while treatment with anti-TNFα has provided protection in endotoxic

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shock induced by the administration of purified lipopolysaccharides, in other murine models such as polymicrobial sepsis [33, 34] and even in controlled clinical trials, negative results have frequently been obtained [35, 36]. This is probably because the approach aimed at eliminating TNFα and other cytokines underestimates their homeostatic and microbicide role. We consider that elimination of TNFα could accelerate the development of a state of CARS. In this study, we analysed the hypothesis that low concentrations of anti-TNFα represent a better option than high concentrations in the treatment of polymicrobial sepsis. We also explored the usefulness of administering the antibody after performing a sepsis inducing procedure (cecal ligation and puncture).

Materials and methods

Immunization and isolation of anti-TNFα F(ab')$_2$ antibody fragments.

Healthy male horses (3 years of age) kept in Instituto Bioclon's farming facilities (Huehuetoca, México) under standard conditions, were subcutaneously immunized with human recombinant TNFα (PeproTech Inc; Rocky Hill, NJ). After the immunization scheme, the horses were bled and plasma was obtained to evaluate by ELISA the presence of specific anti-TNFα antibodies. In order to obtain F(ab')$_2$ fragments, the IgG fraction was purified and digested with pepsin, according to protocols described [37–39] under the Instituto Bioclon's GMP facilities (Mexico, DF Mexico) and the titers were again evaluated by ELISA.

Bioactivity of recombinant murine TNFα (rmTNFα)

Cytotoxic activity of rmTNFα was determined on L929 mice tumor cells (ATCC CCL-1). 24,000 cells/well were seeded in a volume of 100 µl per well in a 96 well plate (Corning) in modified Dulbecco Medium (DME) with 10 % horse serum (HS). Eighteen hours later, 100 µl of the same medium plus 2 µg/ml actinomycin D were added, containing serial dilutions of rmTNFα to a final concentration of: 10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.013, 0.004, 0.0015, 0.0005, 0.0002 ng/ml. Cells were incubated for other 18 h at 37 °C with 5 % CO_2 in a humid atmosphere. Viability of the cells was determined measuring their metabolic activity with a tetrazolium salt assay, adding 20 µl of CellTiter96® [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] [40]. The plate was incubated for 4 h and read by spectrophotometry (Molecular Devices, Sunnyvale CA) at 490 nm.

Neutralizing activity of F(ab')$_2$ anti-TNFα antibody fragments.

In order to define the neutralizing activity of F(ab')$_2$ antibody fragments anti-TNFα 24,000 L929cells/well seeded in a volume of 100 µl per well in a 96 well plate (Corning) in modified Dulbecco Medium (DME) with 10 % HS. Cells were incubated at 37 °C with 5 % CO_2 in a humid atmosphere. Eighteen hours later, 3 lethal doses of rmTNFα were added along with serial dilutions of F(ab')$_2$ antibody fragments anti-TNFα in a volume of 100 µl of the same medium plus actinomycin D. The final volume of each well was 200 µl, containing F(ab')$_2$ antibody fragments anti-TNFα at several concentrations (1,111; 370; 123; 41; 13; 4.5; 1.5; 0.5 µg/ml), rmTNFα at 180 pg/mL and actinomycin D at 1 µg/mL. The plate was incubated for 18 h at 37 °C with 5 % CO_2 in a humid atmosphere. Viability of the cells was determined measuring their metabolic activity with a tetrazolium salt assay, adding 20 µl of CellTiter96®. The plate was incubated for 4 h as described before, and read at 490 nm.

Induction of sepsis by Cecal Ligation and Puncture (CLP)

Male Balb/c mice, 8–12 weeks of age, were acquired from Harlan México (Mexico City, Mexico) and kept in standard animal house conditions. The animals were anesthetized with sodium pentobarbital (25 mg/kg) administered intraperitoneally (IP). A 1 to 2 cm midline incision was done in the mice, the cecum was exposed, its base was ligated without occluding it with a 4-0 black silk suture and it was punctured twice with a 21 gauge needle. The cecum was squeezed and placed back in the peritoneal cavity. The wound was sutured in two layers (muscular and dermal) with 4-0 black silk. A laparotomy was performed on a sham group (S) in which the cecum was manipulated but not ligated nor punctured. All groups were processed simultaneously in order to avoid day to day variations. The Ethics Committee of the institution approved this study.

Treatments

Prophylactic. The animals received F(ab')$_2$ antibody fragments anti-TNFα ip in a sterile physiological saline solution 2 h before the induction of sepsis and every 24 h up to a total of 5 doses in one of the following concentrations: *a)* 0.01 mg/kg, *b)* 0.1 mg/kg, *c)* 1 mg/kg, or *d)* 10 mg/kg. Sham (S) and control (C) groups received sterile physiological saline solution with the same scheme. The complete surgical procedure was performed on C group.

Therapeutic. Since the most protective dose was 1 mg/kg, in order to define its capacity to protect after the septic process has initiated, other groups of mice were treated with an initial dose of 1 mg/kg of F(ab')$_2$ anti-TNFα administered 30 min, 2 h or 24 h after CLP. This was followed by subsequent equal doses every 24 h until a total of 5 was completed. With comparative purposes, groups were included whose initial dose was applied 2 h or 30 min before the surgical procedure. Alacramyn® is a preparation of polyvalent F(ab')$_2$ antibodies fragments against the venom of *Centruroides spp* (Instituto Bioclón, Mexico DF, Mexico), and it was administered as a control non-related F(ab')$_2$ antibody fragment at a concentration of 1 mg/kg, 2 h before the induction of sepsis.

Blood cultures

Bacteremia was documented 24 and 48 h after the induction of sepsis, obtaining blood by cardiac puncture. During this process, 200 µl of blood were obtained and were cultivated in Bac Tec Peds Plus/F Mod. 9240 (Becton Dickinson; Sparks, MD) culture media. The media were incubated for 7 days and positivity was evaluated by the Bactec automated system (Becton Dickinson Microbiology System; Sparks, MD). Pathogens were identified through specific microbiological batteries [41].

Measurement of serum cytokines

Blood was extracted from mice at 3, 24 and 48 h after CLP by cardiac puncture. Levels of TNFα, IL1β, IL-10, TNFRI and TNFRII were determined through sandwich enzyme-linked immunosorbent assay (ELISA), using the commercial duoSet system (R&D Systems; Minneapolis, MN) and following the protocol suggested by the manufacturer.

Statistical analysis

Survival curve comparisons were performed using a Mantel-Haenzel log-rank test. The cytokine levels were analyzed with one-way ANOVA and unpaired t test using Prism 3.0 software (Graphpad Software Inc; San Diego, CA). Significant differences among the groups were considered when $p < 0.05$.

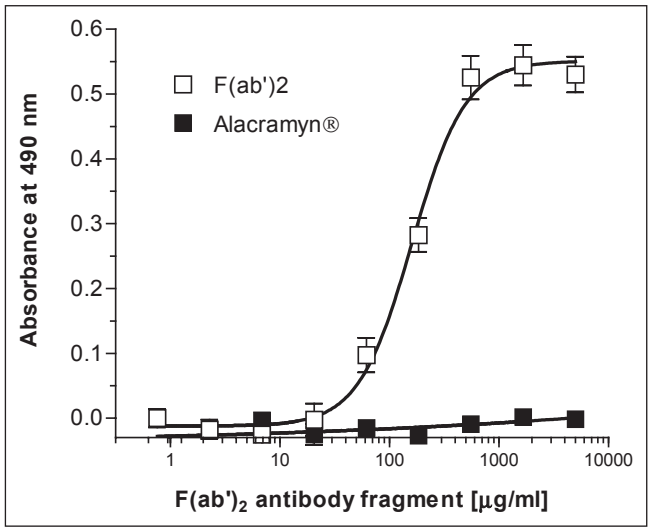

Fig. 1. Neutralization of TNF-mediated cytotoxicity by F(ab')$_2$ antibody fragments. The cytotoxic effect of rmTNFα on L929 cells was measured in the presence of various amounts of anti-TNFα F(ab')$_2$ antibody fragments as described in material and methods. The results with unrelated F(ab')$_2$ antibody fragments (Alacramyn®) are shown.

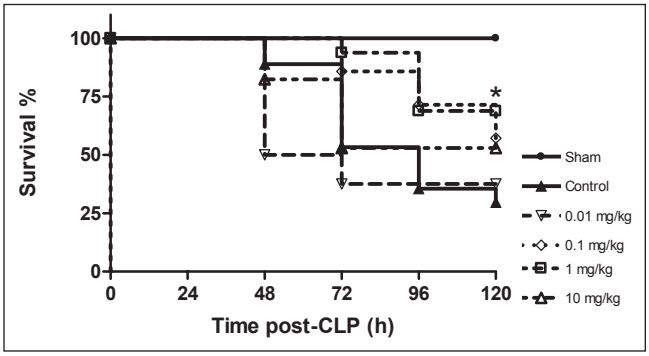

Fig. 2. Survival of animals with prophylactic administration of F(ab')$_2$ antibody fragments anti-TNFα. Saline solution (sham and control), 0.01, 0.1, 1 or 10 mg/kg of F(ab')$_2$ anti-TNFα were administered 2 h before and every 24 h after inducing sepsis until a total of five doses. The figure is a compilation of two independent experiments. (number of animals: Sham = 15, control = 18, 0.01 mg/kg = 8, 0.1 mg/kg = 7, 1 mg/kg = 16, 10 mg/kg = 17). Survival comparisons were analyzed with the Mantel-Haenzel Log Rank test,* $p < 0.05$ vs control.

Results

Anti-TNF F(ab')$_2$ antibody fragment characterization

L929 cell line is sensitive to death by TNFα [42], and was used to demonstrate the neutralizing capacity of anti-TNFα F(ab')$_2$ antibody fragments. A dose-dependent cytolytic effect was observed with rmTNFα. The lethal dose 50 (LD_{50}) was found to be 60 pg/mL (data not shown). Figure 1 shows a dose-dependent neutralizing effect of F(ab')$_2$ anti-TNFα in the cytotoxicity bioassay on L929 cells using 3 LD_{50} (180 pg/mL). The neutralizing dose 50 (ND_{50}) typically was 160 µg/mL. An unrelated F(ab')$_2$ (Alacramyn®) did not have any effect on the bioassay. Even when this anti-TNFα was raised against rhTNF this assay shows neutralizing activity against rmTNFα. This result supports the use of our anti-TNFα F(ab')$_2$ in murine models.

Survival

After 120 h the sham group showed 100 % survival (Fig. 2), while in the control group only 29 % of the animals survived. Treatments with anti-TNFα in doses of 0.01 and 0.1 mg/kg increased survival to 37 and 57 % respectively (not significant (n.s.)). Doses of 10 mg/kg increased survival to 53 % (n.s.). Only the group treated with 1 mg/kg of the anti-TNFα was significantly protected ($p < 0.05$), presenting 69 % survival (Fig. 2).

Blood cultures

In all the groups subjected to CLP, blood cultures turned positive between 7 and 10 h (data not shown). Polymicrobial infection was corroborated with the presence of Gram-negative (*Escherichia coli*) and Gram-positive bacteria (*coagulase-negative Staphyloccocus*). Sham animals presented negative blood cultures.

Serum cytokines

Three hours after surgery the Sham group (Fig. 3a), produced mainly IL-1β and IL-10. This was followed at 24 h by an increase in serum levels of IFNγ, the maintenance of IL-1β and the disappearance of IL-10. A further decrease of these cytokines was observed at 48 h. In the C group (Fig. 3b), there was also an early (3 h) but more intense production of pro-inflammatory cytokines with a clear predominance of IL-1β ($p < 0.05$ vs all other groups) and IL-6 ($p < 0.05$ vs S and 10 mg/kg). This was followed at 24 h and 48 h by a notable decrease in the production of IL-1β, IL-6 and IFNγ to the extent that the last two could no longer be detected. A simultaneous persistent increase of IL-10 was observed ($p < 0.05$ vs S). Compared with the C group treatment with both doses of F(ab')2 anti-TNFα significantly reduced the initial (3 h) production of IL-1β ($p < 0.05$ C vs 1 and 10 mg/kg) and IFNγ ($p < 0.05$ C vs 10 mg/kg) (Figs. 3c and 3d). Notably, IL-6 was high after 1 mg/kg ($p < 0.05$ vs S and 10 mg/kg). Furthermore, at all times the production of IL-6 and IFNγ was greater in the group treated with 1 mg/kg than in the 10 mg/kg group. Indeed, in the group with the highest dose, at no time could production of IFNγ be observed. At 24 h, IL-6 and IFNγ were significantly higher ($p < 0.05$) in the 1 mg/kg group compared to the control group, but production of IL-10 was very similar. To the contrary, at 24 and 48 h the production of cytokines was notably similar between controls and those treated with 10 mg/kg. As seen in control mice, in both treated groups the production of IL-10 remained high throughout the study.

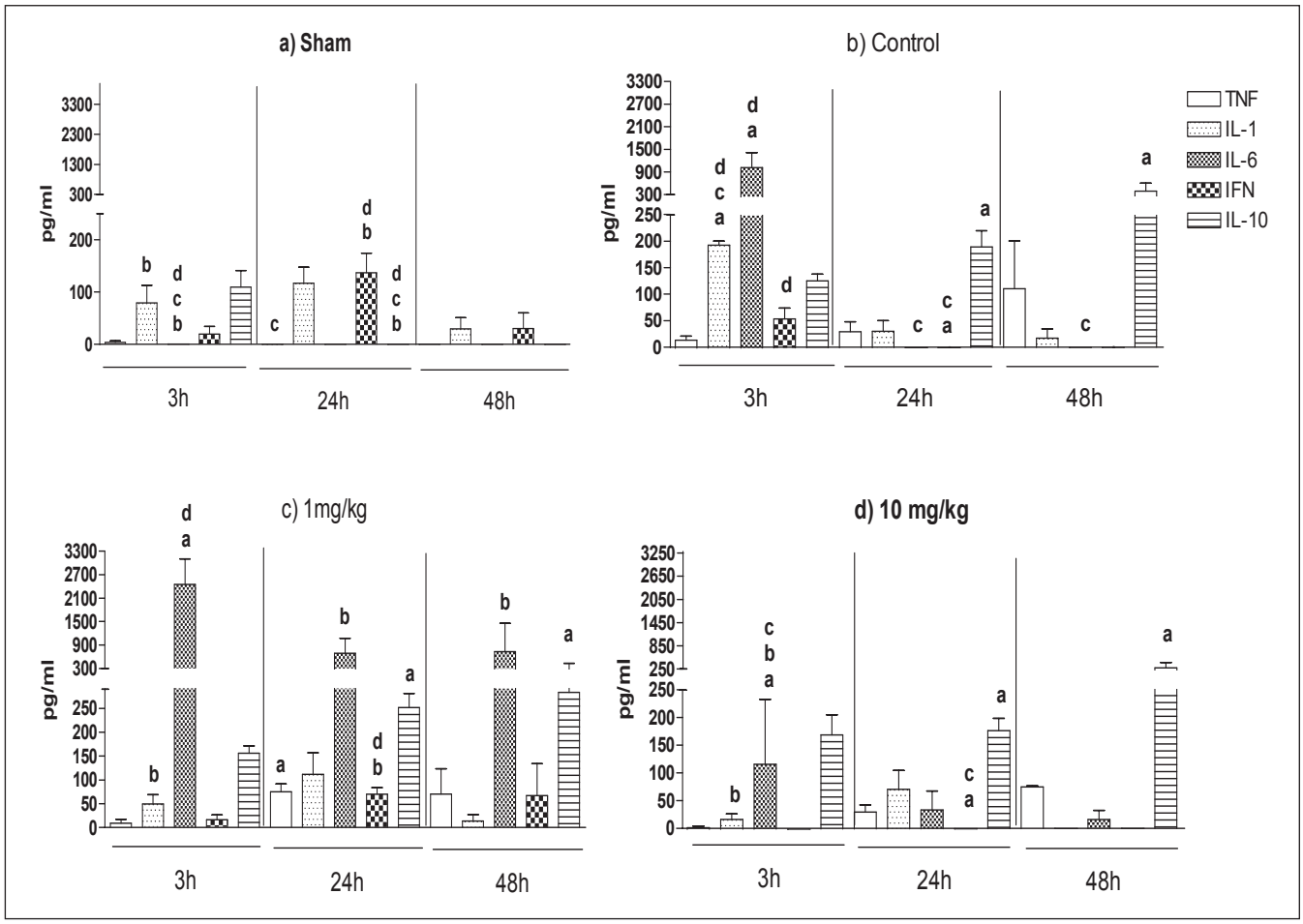

Fig. 3. Profile of serum cytokines during the septic process. Serum samples were obtained from septic animals and the presence of cytokines at different times was measured. a) Sham; b) Control; c) 1 mg/kg and d) 10 mg/kg. Saline solution or F(ab')$_2$ anti-TNFα were administrator as stated in legend to Figure 2. The differences among groups were determined using a one-way ANOVA test and a Mann-Whitney U test; they were considered significant when $p < 0.05$. a = $p < 0.05$ vs Sham for the same time point; b = $p < 0.05$ vs Control for the same time point; c = $p < 0.05$ vs 1 mg/kg for the same time point; d = $p < 0.05$ vs 10 mg/kg for the same time point. N = 5 or 6 animals for each group and time point.

Soluble TNFRI and TNFRII

Given the low concentrations of TNFα observed even in control animals 3 h after CLP, we decided to measure the levels of soluble receptors for TNFα (TNFRI and II) as a possible explanation for these findings. Indeed, the presence of TNFRI and II were detected in high concentrations during the 48 h of screening in all animals (data not show).

Therapeutic role of anti-TNFα F(ab')$_2$ antibody fragments

Once the 1 mg/kg concentration of F(ab')$_2$ anti-TNFα was established as the most protective dose, its therapeutic usefulness was evaluated by administering it at different times subsequent to the surgical procedure. This was compared to the protection achieved by administering it 2 h or 30 min before the surgical procedure. Corroborating the above results, the administration of the initial dose 2 h before CLP gave a significant protection ($p < 0.001$ vs. C), increasing survival to 78 % compared to the 42 % found in the control groups (without treatment and treated with unrelated F(ab')$_2$ antibodies fragments) (Fig. 4). The greatest protection, however, was observed in the groups treated with the antibody fragments 30 min before or 30 min after CLP, obtaining a survival of 93 and 92 % respectively ($p < 0.001$ vs. C). The protection observed on administering the F(ab')2 antibody fragments 2 and 24 h after the surgical procedure did not prove to be significant in the final survival rate (61 % and 57 % survival, respectively) (Fig. 4).

Discussion

In the present study, we compared different doses of anti-TNFα F(ab')$_2$ antibody fragments in preventing death after CLP. Our data demonstrate that when administered prophylactically, from before CLP and continued after it, high doses (10 mg/kg) of F(ab')$_2$ antibody fragments of anti-TNFα do not represent the most adequate treatment strategy, since10 times smaller doses (1 mg/kg) achieved a significant protection of the mice. Very low doses (0.01 and 0.1 mg/kg) did

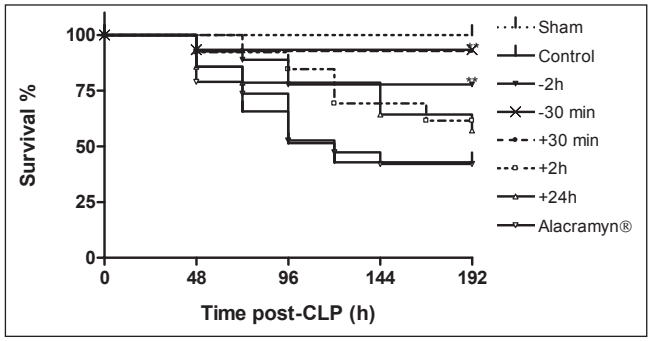

Fig. 4. Survival of animals on therapeutic administration of F(ab')$_2$ antibody fragments of anti–TNFα. All experimental animals were treated with 1 mg/kg initiated at the stated times relative to CLP, and then every 24 h for a total of 5 doses. As a non related F(ab') antibody fragment, Alacramyn® was administrator at a concentration of 1 mg/kg, initiated 2 h before CLP and every 24 h for a total of 5 doses. Sham and control mice received saline solution under the same scheme. The figure is a compilation of two independent experiments. N: Sham = 20, Control = 35, –2 h = 26, –30 min = 15, +30 min = 14, +2 h = 13, +24 h = 14, Alacramyn® = 19. Survival comparisons were performed with the Mantel-Haenzel Log Rank test,** $p < 0.01$ vs control.

not prove to be adequate either. Elimination of TNFα is an experimental strategy that has given protection in the endotoxemia model [43], in which lethal doses of lipopolysaccharide are administered. This induces production of high levels of TNFα and activation of pathophysiological mechanisms characteristic of sepsis [44]. With the inhibition of this cytokine the induction of these events is avoided. In contrast, the elimination of TNFα in a polymicrobial sepsis model [33, 34] and in clinical trails [35, 36] has frequently yielded negative results regarding protection. We believe that in this model, the excessive decrease in TNFα can modify the immune response, affect the antimicrobial protection mechanisms and, as a consequence, make the animals more susceptible. This hypothesis agrees with the results obtained in this study, since the mice treated with doses of 10 mg/kg of F(ab')$_2$ antibody fragments anti-TNFα showed a lower rate of survival than that obtained with lower doses of the antibodies fragments (1 mg/kg). As expected serum cytokines of control animals showed an initial production of pro-inflammatory cytokines followed by a decline of IL-1β simultaneous with increasing amounts of IL-10. In contrast, protected animals (treated with 1 mg/kg) showed a more stable production of pro- and anti-inflammatory cytokines throughout the study, and animals treated with the dose of 10 mg/kg at 24 and 48 h showed a similar pattern of pro- and anti-inflammatory response to that of the control mice. The lower production of pro-inflammatory cytokines could be the result of the partial neutralization of circulating TNFα because of its important influence on the production of IFN-γ, IL-6 and IL-1β [45, 46]. It is noteworthy that not even the high dose used in this study was sufficient to completely inhibit the production of TNFα from 24 h on. It is even possible that in all cases, real serum levels were underestimated due to the presence of raised levels of soluble TNFRI and II. Nonetheless, in contrast to the animals treated with the F(ab')$_2$ antibody fragments anti-TNFα, an early pro-inflammatory response (3 h) mainly represented by IL-6 and IL-1β predominated in the control group. It has been suggested that the pro-inflammatory response is necessary during the septic process, since it triggers the activation of microbicidal mechanisms, while an excessive anti-inflammatory response could lead to a state of immunoparalysis [47]. It is also clear that the anti-inflammatory response is necessary to regulate the exacerbated pro-inflammatory response that is triggered during sepsis, since if the former does not appear there is a risk of activating mechanisms leading to multiple organ dysfunction [26]. Our results in animals treated with 1 mg/kg of the anti-TNFα suggest that, by allowing a sustained pro-inflammatory response in co-existence with an anti-inflammatory response, and thus maintaining a balanced ratio between antagonistic cytokines, survival of the animals that underwent CLP could be significantly improved. Furthermore, the further this balance is disrupted the worst seems to be the outcome. The IL-6/IL-10 ratio could represent a good example of this: it was incalculably low in the control group by 24 and 48 h; it was below united and descended as the experiment advanced in the 10 mg/kg group; remaining above unity in the protected 1 mg/kg group. The previous demonstration that increased IFN-γ levels increase lethality after CLP in rats [48] is opposed to the finding of a restoring effect of IFN-γ treatment on monocyte function in septic patients [49], and along with our finding of small amounts of IFN-γ only in the protected group, supports the balance hypothesis. Another finding of this study that must be mentioned is the demonstration that, at a dose of 1 mg/kg, the F(ab')$_2$ anti-TNFα protected not only when its administration was started 2 h before the surgical procedure but, to an even greater extent, when it was started 30 min before or 30 min after CLP. In contrast, no significant protection was observed if treatment began 2 or 24 h after surgery. This clearly suggests that the regulation of circulating levels of TNFα must begin in the early stages of the septic process for it to be of use. Thus as discussed before, the negative results obtained in controlled clinical trials could be at least in part related to the administration of too high concentrations of anti-TNFα antibodies. Furthermore, by not considering the immunological state of the patient when the antibody is administered, treatment may be initated too late. In other words, if the patient is in a late phase of SIRS or even has established CARS it is likely that benefits will not be obtained from anti-TNFα therapy. We consider that subsequent studies must be addressed at reassessing doses and timing at which a biological therapy is initiated. To do this it is necessary to standardize markers that make it possible to assess the immunological status of the patients in order to evaluate if any therapy with anti-cytokine antibodies could be beneficial or not to a particular patient. Recent results, which show that chimeric anti-TNFα monoclonal antibodies administered chronically to patients with rheumatoid arthritis increase susceptibility to infection in some patients [50–54], support the need to reassess doses, timing and possible duration of the anti-cytokine therapies. In conclusion, this study demonstrates that neither high nor very low concentrations of anti-TNFα are the most adequate for treating polymicrobial sepsis, since they did not significantly improve survival of mice challenged with CLP. To the contrary, treatment with intermediate doses of F(ab')$_2$ anti-TNFα did significantly protect. The finding that the administration of 1 mg/kg of

anti-TNFα was able to protect even after the septic process had been initiated but only if started up to 30 min after induction of sepsis by CLP is noteworthy. These results suggest that in dealing with processes where excessive production of cytokines is involved, the aim should be to return them to their physiologically acting range but not to inhibit their production. Likewise, if regulation of an intense pro-inflammatory response is needed, this approach should start very early, or else other strategies should be studied, for example regulating the secondary anti-inflammatory response.

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