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ENHANCEMENT OF IN VIVO IMMUNE RESPONSE BY TUMOR NECROSIS FACTOR

PAOLO GHIARA,[1]* DIANA BORASCHI,* LUCIANO NENCIONI,* PIETRO GHEZZI,† AND ALDO TAGLIABUE*

*From the *Laboratorio di Immunofarmacologia, Centro Ricerche Sclavo, Via Fiorentina 1, I-53100 Siena, Italy, and †Istituto di Ricerche Farmacologiche "Mario Negri," Via Eritrea 62, I-20157 Milano, Italy*

Interleukin 1 (IL-1) has been shown to regulate several immunologic functions. Since tumor necrosis factor (TNF) shares many biologic properties with IL-1, we have investigated here the role of TNF in the modulation of the immune response. We have thus tested low doses of human recombinant TNF-α (hu rTNF-α) for its capacity to enhance the in vivo antibody responses evaluated at the cellular level in the hemolytic plaque assay. It was found that hu rTNF-α, like human IL-1β, is able to enhance the immune response to a T cell-dependent antigen (sheep red blood cells). Interestingly, at variance with human recombinant IL-1β, hu rTNF-α was not able to enhance the in vivo antibody response to a T cell-independent antigen (type III pneumococcal polysaccharide). These results suggest that low levels of TNF may have a role in the modulation of the immune response in vivo and shed new light on the biologic significance of this mediator.

Tumor necrosis factor (TNF),[2] initially described as a product of endotoxin-activated macrophages causing hemorrhagic necrosis of tumors in vivo (1–3), has recently been shown to be identical to cachectin (4, 5), a protein that, when injected in large amounts in vivo, mimics the metabolic derangement leading to shock and cachexia often observed during severe invasive diseases (6). TNF has been included in the group of soluble factors, such as interleukin 1 (IL-1) and interferon (IFN), that are released mainly by the activated cells of the reticuloendothelial system during host reaction to infection or injury, but the precise role of this molecule in the host's defense mechanisms still remains to be defined. In particular, the in vivo role of TNF is unlikely to be confined to the necrosis of tumors and to the ill-effects on metabolism, because these activities are observed only with very high doses of the protein. Because TNF shares with IL-1 many common biologic properties (7), and IL-1 plays a major role in the onset of the immune response (8), we wanted to verify whether TNF also has a role in the modulation of immune responses in vivo. The results presented here show that human recombinant TNF-α (hu rTNF-α) is able to potently enhance the antibody response in vivo in a much lower range of doses than those reported to cause cachexia and severe tissue toxicity.

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[1] Address all correspondence and requests for reprints to Dr. P. Ghiara, Laboratorio di Immunofarmacologia, Centro Ricerche Sclavo, Via Fiorentina 1, I-53100 Siena, Italy.

[2] Abbreviations used in this paper: TNF, tumor necrosis factor; hu rTNF-α, human recombinant TNF-α; IL-1 and -2, interleukin 1 and 2; hu rIL-1β, human recombinant IL-1 β; IFN, interferon; SIII, type III pneumococcal polysaccharide.

MATERIALS AND METHODS

Mice. C3H/HeN Crl BR mice, obtained from Charles River Breeding Laboratories (Calco, Italy) were bred in our animal facilities and housed in air-conditioned rooms on a 12-hr light-dark schedule (illumination: 7.00 a.m. to 7.00 p.m.). Food and water were available ad libitum. Male mice between 10 and 12 wk of age, weighing approximately 25 g, were used in all the experiments. In some experiments, C3H/HeJ male mice (The Jackson Laboratory, Bar Harbor, ME) of the same age and weight as above were used.

Cytokines. The hu rTNF-α used in this study (kindly donated by Dr. A. Galazka, Biogen Research Corp., Geneva, Switzerland, batch No. 30 Ly 1839/182; and by Dr. L. S. Lin, Cetus Corp., Emeryville, CA, batch No. LYM12-101485B) was more than 95% pure and had a specific activity of 1.5×10^7 U/mg of protein (measured on a 24-hr L929 bioassay with actinomycin D). The hu rTNF-α contained less than 0.06 ng endotoxin/μg. A specific rabbit anti-TNF-α serum with a titer of 400,000 TNF neutralizing U/ml (Cetus Corp., lot No. 57-030685) was obtained from Dr. L. S. Lin. The human recombinant IL-1β (hu rIL-1β) had a specific activity of 1×10^8 U/mg of protein and was purchased from Genzyme Corp., Boston, MA.

Antigens and immunization. Sheep red blood cells (SRBC; Sclavo, Siena, Italy) were washed three times, resuspended in pyrogen-free saline, and inoculated i.v. at 1 to 2×10^8/0.2 ml/mouse. Purified type III pneumococcal polysaccharide (SIII; kindly provided by Phillip J. Baker, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD), was diluted in pyrogen-free saline and inoculated i.p. at the previously determined optimal dose of 0.5 μg/0.5 ml/mouse. At the time of antigen inoculation, groups of three mice received an i.p. injection of saline alone or containing hu rIL-1β (20 ng/kg), or different doses of hu rTNF-α.

Plaque-forming cell (PFC) assay. Four of five days after immunization, direct PFC were detected by Cunningham and Szenberg's slide technique (9), by using splenocyte suspensions as the source of antibody-secreting cells. Guinea pig serum (Sclavo) at the final dilution of 1/64 was used as a source of complement. For the detection of anti-SIII PFC, SIII was coupled to SRBC as described (10). Briefly, 0.5 ml of pelleted SRBC were resuspended in 1 ml of saline containing 1 mg of SIII. Then 1 ml of 0.1% chromium chloride (Sigma Chemical Co., St. Louis, MO) in saline was added to the mixture and incubated for 5 min at room temperature under gentle stirring. Cells were then washed four times with 20 vol of saline and used as indicator cells in the PFC assay.

Statistical analysis. Results are expressed as means after logarithmic transformation ± SE or as geometric means of PFC/spleen from three mice assayed individually. Statistical significance was calculated by Student's *t* test.

RESULTS

It has been shown that IL-1 is able to increase the immune response in vivo (11), and we have recently demonstrated that hu rIL-1β can enhance the antibody responses to different antigens as measured by the he-

molytic plaque assay (12). Here we analyzed the effect of hu rTNF-α on in vivo antibody responses by using hu rIL-1β as positive control.

Administration of hu rTNF-α at the same time as the antigen enhances the primary immune response measured in the spleen cells of C3H/HeN mice 4 days after immunization with SRBC (Fig. 1). The adjuvant activity of hu rTNF-α on the primary response to SRBC was dose-dependent, and the effective doses (1.3×10^1 to 8×10^3 ng/kg) caused more than a twofold increase in the number of anti-SRBC PFC per spleen. As shown in Figure 1, the maximal effect obtained with hu rTNF-α was comparable to the potentiating activity of an optimal dose of hu rIL-1β. Differences in splenic cellularity were never observed in mice treated with hu rIL-1β or hu rTNF-α when compared with control animals. However, changes in the composition of spleen cell populations cannot be excluded, inasmuch as accurate studies of the nature of cells present in the spleens were not performed.

The possibility that endotoxin contamination could be responsible for the observed biologic activity is unlikely,

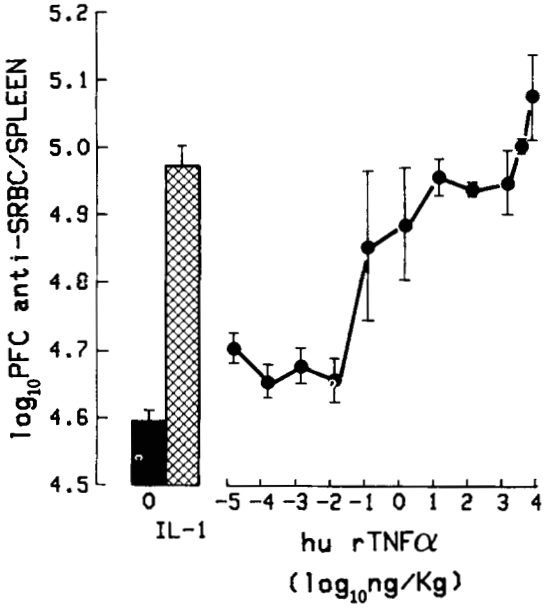

Figure 1. Splenic PFC anti-SRBC of control saline-treated C3H/HeN mice (*filled column*) and of mice treated with 20 ng/kg of hu IL-1β (*hatched column*) or with different doses of hu rTNF-α. Results represent the mean of four experiments performed. *Vertical bars* represent standard errors (SE) of the means. PFC anti-SRBC response of hu IL-1β-treated mice was significantly different ($p \leq 0.01$) from that of control mice, whereas only mice receiving doses of hu rTNF-α ranging from 1.3×10^1 to 8×10^3 ng/kg developed a PFC anti-SRBC response significantly different ($p \leq 0.01$) from that of controls.

TABLE I

Adjuvant activity of hu rIL-1β and hu rTNF-α on immune response to SRBC in endotoxin-insensitive C3H/HeJ mice

Treatment[a]	Direct PFC Anti-SRBC/Spleen[b]		p[c]
	Mean $\log_{10}$ values ± SE	Geometric mean	
Saline	4.193 ± 0.057	15,596	
hu rIL-1β	4.523 ± 0.003	33,343	≤0.05
hu rTNF-α	4.653 ± 0.045	44,978	≤0.01

[a] Groups of three male C3H/HeJ mice (10 wk of age) received an i.p. injection of saline alone or containing hu rIL-1β (20 ng/kg) or hu rTNF-α (13 ng/kg) at the same time as antigen administration.

[b] Splenic PFC anti-SRBC were detected 4 days after immunization as described in Figure 1.

[c] Significance vs saline-treated animals.

TABLE II

Effect of boiling and of treatment with a specific antiserum on the adjuvant activity of hu rTNF-α

Treatment	Direct PFC Anti-SRBC/Spleen[a]		p[b]
	Mean $\log_{10}$ values ± SE	Geometric mean	
Expt. 1[c]			
Saline	4.677 ± 0.012	47,533	
hu rTNF-α	4.973 ± 0.037	93,972	≤0.01
hu rTNF-α, 100°C, 20 min	4.663 ± 0.041	46,026	NS[d]
Expt. 2[e]			
Saline	4.493 ± 0.032	31,117	
hu rTNF-α	4.850 ± 0.025	70,795	≤0.01
hu rTNF-α + anti-hu rTNF-α	4.467 ± 0.024	29,309	NS
hu rTNF-α + NRS	4.870 ± 0.040	74,131	≤0.01

[a] Splenic PFC anti-SRBC were measured in groups of three male C3H/HeN mice (10 to 12 wk of age) 4 days after i.v. immunization. PFC were detected as described in the legend to Figure 1.

[b] Significance vs saline-treated animals.

[c] Two hours after immunization with SRBC, mice received an i.p. injection of 0.2 ml of saline alone or containing hu rTNF-α (133 ng/kg) or the same amount of hu rTNF-α boiled for 20 min in a water bath.

[d] NS, not significant.

[e] Mice received an i.p. injection of 0.2 ml of saline alone or containing hu rTNF-α (13 ng/kg) or the same dose of hu rTNF-α preincubated for 5 hr at 37°C with a 1/250 dilution of a rabbit anti-TNF-α serum, or with the same amount of normal rabbit serum (NRS).

because the maximal endotoxin content of the hu rTNF-α inocula (see *Materials and Methods*) is far below the minimal bioactive dose reported for this experimental system (13). In addition, as reported in Table I, hu rTNF-α also maintained its adjuvant activity in endotoxin-insensitive C3H/HeJ mice. Furthermore, results in Table II show that the adjuvant capacity of hu rTNF-α is completely abolished by boiling, as well as by treatment with a specific rabbit anti-TNF-α serum (14).

To further characterize the mechanism of the adjuvant activity of TNF, we tested the capacity of hu rTNF-α to enhance the PFC response to SIII; this is considered a helper T cell-independent antigen, in that it triggers the production of antibodies by B cells apparently without the cooperative interaction with helper T cells (15). Interestingly, at variance with hu rIL-1β, hu rTNF-α proved completely unable to enhance the antibody response to SIII (Fig. 2).

DISCUSSION

The results presented here demonstrate that hu rTNF-α is able to potently enhance the immune response in vivo in a range of doses that is much lower than that required to observe in vivo antitumoral activity (16, 17), and far below those reported to be able to cause cachexia and extensive tissue damage (6). The immunostimulation induced in vivo by hu rTNF-α is likely to be the result of an increase in the frequency of specific antibody-secreting cells in the spleen, because we never observed any increase of splenic cellularity or splenomegaly.

The possibility that a contaminant might account for the observed biologic activity of hu rTNF-α can be reasonably excluded on the basis of several experimental data hereafter summarized. Highly purified preparations of hu rTNF-α from two different sources (see *Materials and Methods*) were used without observing any difference in the immunopotentiating activity. Furthermore, a possible role of endotoxin can be ruled out on the basis of the purity of hu rTNF-α preparations, the effect of boiling and the effect of a specific antiserum. Moreover, the

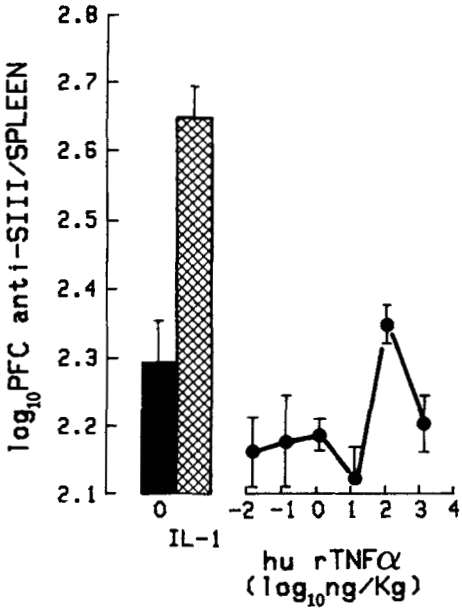

Figure 2. Splenic PFC anti-SIII response of control saline-treated C3H/HeN mice (*filled column*) and of mice treated with 20 ng/kg of hu IL-1β (*hatched column*) or with different doses of hu rTNF-α. PFC anti-SIII were measured 5 days after immunization. *Vertical bars* represent SE of the mean PFC/spleen of replicate mice in a single experiment, representative of two performed. Only hu IL-1β-treated mice developed a PFC anti-SIII response significantly different ($p \leq 0.05$) from that of control mice.

capacity to enhance the in vivo immune response is also maintained in endotoxin-insensitive C3H/HeJ mice.

A precise assessment of the possible mechanism of action of hu rTNF-α in enhancing the immune response in vivo is quite complex mainly because, so far, the effects of TNF on immunity have been only marginally investigated. Indeed, specific binding sites for TNF have been shown on the membrane of some T cell tumor lines but not on a B cell tumor line, suggesting that only T cells among the lymphocytes might be responsive to TNF (18). Furthermore, it has recently been reported that interaction of hu rTNF-α with its receptors on activated normal T lymphocytes leads to an increase in the number of interleukin 2 (IL-2) receptors as well as to an augmented IL-2-dependent production of IFN (19). Thus an in vivo regulatory activity of TNF on T lymphocytes is conceivable and it can be hypothesized that the observed adjuvant activity on the T cell-dependent antibody response may be due to an amplification of the antigen-specific helper T cell population, as a consequence of its increased sensitivity to endogenously produced IL-2, due to the increase in IL-2 receptor number.

Another possible consequence of the interaction of TNF with helper T cells could be the release of soluble factors that can affect B cell proliferation and/or differentiation. Interestingly, TNF has been shown to induce the release in vitro of IFN-β_2 (20, 21). This molecule recently turned out to be identical to the B cell stimulating factor-2 (22), a helper T cell-derived lymphokine that has been shown to induce the final maturation of B cells into antibody-secreting cells (23). The possible induction in vivo of B cell stimulating factor may thus represent an additional mechanism of the adjuvant activity of hu rTNF-α on the immune response to SRBC.

We have recently reported (12) that IL-1 is able to exert a potent adjuvant activity on antibody response against SRBC and it is known that IL-1 is able to synergize with B cell-specific factors during the activation and proliferation of antibody-producing cells (reviewed in Reference 24). TNF has been reported to induce IL-1 synthesis and release both in vitro and in vivo (25–28); thus the presence of circulating IL-1 after the injection of hu rTNF-α cannot be excluded and may play an important role in mediating the TNF-induced immunostimulation described here. However, at variance with its ability to exert an adjuvant activity on a T cell-dependent immune response, hu rTNF-α lacks any potentiating activity on an antibody response that apparently does not involve helper T cells. In contrast, as shown in Figure 2 and as described more extensively elsewhere (12), hu rIL-1β is able to potently enhance the immune response to SIII. This difference between the adjuvant capacities of IL-1 and TNF indicates that these two factors might have distinct mechanisms of in vivo immunopotentiation.

Recently, many reports have appeared in the literature suggesting that TNF might be a pleiotropic mediator of the reticuloendothelial system. In fact, it enhances the expression of HLA antigens on endothelial cells and fibroblasts (29) as well as on T cells (19), it activates some neutrophil functions (30, 31), it induces the release of colony-stimulating factors (32, 33), it induces prostaglandin E_2 and collagenase release in fibroblasts and synovial cells (34), it has been proposed as the mediator of some kinds of cell-mediated cytotoxicity against tumors (14, 35, 36), and it has also been described as a potent antiviral agent (37, 38). When injected in vivo, TNF has been shown to be a powerful pyrogen (28) and to exert a potent antitumoral activity (16, 17), a property that supports its possible clinical use. TNF has also been proposed as a major mediator of cachexia and metabolic derangements observed in endotoxin-induced acute toxicity in experimental animals (5, 6). However, the in vivo physiologic role of TNF still remains to be well understood (39).

The results reported here represent the first evidence that low doses of TNF may stimulate the immune system in vivo. We would like to propose that the primary role of TNF in vivo is the modulation of the immune response, whereas the inflammatory and toxic effects can be seen only when it is released in large amounts, as a consequence of a persistent and/or massive invasive challenge.

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