Brief Definitive Report

Interleukin 10 Reduces the Release of Tumor Necrosis Factor and Prevents Lethality in Experimental Endotoxemia

By Catherine Gérard,*[1] Catherine Bruyns,* Arnaud Marchant,§ Daniel Abramowicz,‖ Peter Vandenabeele,¶ Anne Delvaux,* Walter Fiers,¶ Michel Goldman,§ and Thierry Velu*‡

*From the *Institut de Recherche Interdisciplinaire, Services de ‡Génétique Médicale, §d'Immunologie, de ‖Néphrologie, Campus Erasme, Université Libre de Bruxelles, B-1070 Brussels; and the ¶Laboratorium voor Moleculaire Biologie, Rijksuniversiteit van Gent, B-900 Gent, Belgium*

Summary

Because of its ability to efficiently inhibit in vitro cytokine production by activated macrophages, we hypothesized that interleukin (IL) 10 might be of particular interest in preventing endotoxin-induced toxicity. We therefore examined the effects of IL-10 administration before lipopolysaccharide (LPS) challenge in mice. A marked reduction in the amounts of LPS-induced tumor necrosis factor (TNF) release in the circulation was observed after IL-10 pretreatment at doses as low as 10 U. IL-10 also efficiently prevented the hypothermia generated by the injection of 100 μg LPS. Finally, pretreatment with a single injection of 1,000 U IL-10 completely prevented the mortality consecutive to the challenge with 500 μg LPS, a dose that was lethal in 50% of the control mice. We conclude that IL-10 inhibits in vivo TNF secretion and protects against the lethality of endotoxin in a murine model of septic shock.

Endotoxin (LPS) from gram-negative bacteria is a major causative agent in the pathogenesis of septic shock (1). A shock-like state can indeed be induced experimentally by a single injection of LPS into animals. These toxic effects of LPS are mostly related to macrophage activation leading to the release of multiple inflammatory mediators. Among these mediators, TNF appears to play a crucial role, as indicated by the prevention of LPS toxicity by the administration of neutralizing anti-TNF antibodies (2–5).

IL-10 is a recently described cytokine that efficiently blocks the in vitro production of TNF as well as of other cytokines by LPS-activated monocytes/macrophages (6–8). IL-10 might therefore be an efficient agent to downregulate LPS toxicity in vivo. To test this hypothesis, we analyzed the effects of IL-10 administration before LPS challenge in mice, focusing on the systemic release of TNF, hypothermia, and lethality.

Materials and Methods

Animals. 10–15-wk-old BALB/c mice were purchased from the KUL Proef Dieren Centrum (Leuven, Belgium).

Reagents. LPS from *Escherichia coli* (serotype O55:B5) was purchased from Sigma Chemical Co. (St. Louis, MO). The JES5-2A5 mAb, a neutralizing rat anti-mouse IL-10 IgG1 mAb, was kindly given by Tim Mosmann (Department of Immunology, University of Alberta, Edmonton, Canada) (9). The LO-DNP mAb, a rat IgG1 antibody used as control, was a kind gift from H. Bazin (Experimental Immunology Unit, Université Catholique de Louvain, Brussels, Belgium).

Mouse Recombinant IL-10: Cloning and Expression. Specific oligonucleotides for the murine IL-10 cDNA were synthesized according to its sequence (10). Restriction sites were added to their 5′ end for subcloning: HincII/SacII for the sense primer 5′-CTCCATCATGCCTGGCTCA-3′ (nucleotide 69-87) and SmaI/XhoI for the antisense primer 5′-TACACACTGCAGGTGTTTTAGC-3′ (nucleotide 608-629). Total RNA was prepared from the spleen of mice injected with the hamster anti-CD3 mAb 145-2C11 as a nonspecific stimulator of cytokine transcription (11). 1 μg RNA was reverse transcribed using the antisense oligonucleotide (1 μg) as primer and 200 U Moloney-murine leukemia virus (Mo-MuLV) reverse transcriptase (RT) (Promega Corp., Madison, WI) in RT buffer (50 mM KCl, 20 mM Tris HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml acetylated BSA, dNTPs 2.5 mM each, RNasin 20 U [Promega Corp.]) in a final volume of 20 μl. To this were added 2.5 U Taq DNA polymerase, and 1 μg of each sense/antisense primer, in the same buffer, and in a total volume of 100 μl. A PCR was performed using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 35 cycles: 1 min denaturation at 94°C, 2 min annealing at 55°C, and 3 min extension at 72°C. A band with the 560-bp predicted size was obtained. The specificity of the amplified cDNA was validated by its restriction pattern. Sequencing was also performed following the dideoxynucleotide chain termination method (12). Nucleotides 322, 465–468, 522, and 523 were different from those published (10), but the amino acids remained unchanged. The SacII-XhoI restriction fragment, containing the

[1] C. Gérard and C. Bruyns contributed equally to this study.

murine IL-10 cDNA, was inserted in the Harvey murine sarcoma virus–derived retroviral vector pCO6-HX in place of the coding sequence of p21 *ras* (see Fig. 1) (13). In this construction, called pTG1H-mIL10, the IL-10 cDNA is the only functional gene. A similar vector, with no inserted IL-10 gene, was used as negative (mock) control. To derive individual colonies of cells expressing high levels of IL-10, pTG1H-mIL10 was cotransfected with the plasmid pSV2-neo, which contains the neomycin resistance gene used as a selectable marker. Transfection was performed in the CHO-K1 (Chinese hamster ovarian K1) cell line using a modified calcium phosphate method as described, except that no carrier DNA was present (14). Selection for transfectants was initiated by adding 400 μg/ml G418 (Geneticin; Gibco, Paisley, Scotland). 10 d later, individual resistant colonies were isolated and tested for IL-10 expression using a specific enzyme-linked immunosorbent assay (Pharmingen, San Diego). IL-10 concentrations were determined by reference to a standard curve of recombinant IL-10 expressed in COS cell supernatant (470 U/ml) (Pharmingen). The supernatant collected after 24 h culture of a colony with high IL-10 level (200 U/ml) was concentrated to 5,000 U IL-10/ml on an ultrafiltration membrane (Amicon Corp., Danvers, MA). Control (mock) supernatant from CHO-K1 cells transfected with the control plasmid and with pSV2-neo has been selected, collected, and concentrated similarly. The endotoxin content of the injected preparations was below 1 ng/ml.

Determination of Serum TNF Levels. Blood samples were obtained from individual mice by retroorbital puncture. Serum levels of TNF were estimated by a cytotoxicity assay on actinomycin-D–treated WEHI-164 clone 13 cells as previously described (15). Results were expressed in pg/ml in reference to the cytotoxic activity of a standardized (National Biological Standards Board, Hertfordshire, UK) preparation of recombinant murine TNF expressed in *Escherichia coli* (16). This preparation had a specific biological activity of 2.25×10^8 IU/mg, with 1 IU representing about 4 pg murine TNF. We found that IL-10 was not interfering with this bioassay (data not shown).

Experimental Protocol. Mice were injected intravenously with 100 μg of LPS 30 min after an intraperitoneal administration of either CHO-IL10 or mock supernatants. Serum TNF levels were determined 1.5, 3, and 6 h after LPS challenge, whereas rectal temperature was measured at regular intervals with a digital thermometer. In another series of experiments, we evaluated the effects of IL-10 on the lethality induced by a single dose of 500 μg LPS.

Statistics. TNF levels and temperatures were compared using the Mann-Whitney *U* test. Lethality data were analyzed with the Fisher's exact test.

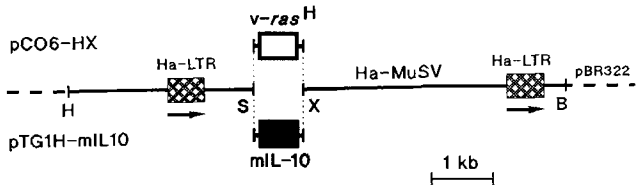

Figure 1. Construction of the murine IL-10 retroviral vector. Plasmid pTG1H-mIL10 was derived from the Harvey murine sarcoma virus (Ha-MuSV) cloned in pBR322 (pCO6-HX) (13). The entire coding region of v-*ras*^{H} (SacII-XhoI) was removed and replaced by the 560-bp fragment coding for the murine IL-10 cDNA. (*Solid lines*) Ha-MuSV sequences; (*broken lines*) pBR322. Restriction endonucleases sites: (*H*) HindIII; (*S*) SacII; (*X*) XhoI; (*B*) BamHI. (*Black box*) Coding sequences of IL-10; (*open box*) coding sequences of v-*ras*^{H}; and (*crosshatched boxes*) Ha-MuSV LTRs.

Table 1. *Effects of Various Doses of IL-10 on the systemic release of TNF Induced by LPS*

Pretreatment	Challenge	TNF serum levels 1.5 h	TNF serum levels 3 h
		pg/ml	
Medium	–	<10	<10
Medium	LPS	5268 ± 1118	<10
Mock	LPS	5270 ± 1283	<10
IL-10 (10 U)	LPS	1216 ± 469*	ND
IL-10 (100 U)	LPS	1846 ± 1295‡	ND
IL-10 (1,000 U)	LPS	416 ± 70§	<10
IL-10 (1,000 U)	–	<10	ND

BALB/c mice (n = 5 in each group) were injected intraperitoneally with the indicated doses of IL-10, 30 min before an intravenous injection of 100 μg LPS. As control, mice were pretreated with either the medium used for culturing CHO-K1 cells, or the mock supernatant. Serum levels of biologically active TNF (mean ± SEM) were determined 1.5 and 3 h after LPS challenge.

* $p < 0.01$, ‡ $p < 0.05$, and § $p < 0.005$, as compared with mice pretreated either with control medium or with mock supernatant before LPS challenge.

Results and Discussion

To assess the in vivo effects of IL-10 administration on LPS-induced TNF release, three different doses of IL-10 (10, 100, or 1,000 U) were given intraperitoneally 30 min before the intravenous injection of 100 μg LPS. Since previous studies showed that TNF levels peaked 1.5 h after LPS challenge, blood samples were taken at that time (17). IL-10 pretreatment resulted in a significant reduction in the amounts of

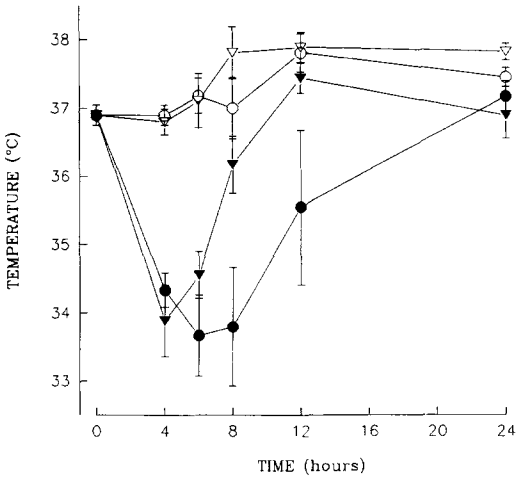

Figure 2. Time course of rectal temperature (mean ± SEM) in BALB/c mice (6–21 per group) injected with either medium alone (○); LPS (100 μg i.v.) without pretreatment (●); LPS (100 μg i.v.) after pretreatment with 1,000 U IL-10 (▽); or LPS (100 μg i.v.) after pretreatment with supernatant from mock-transfected CHO-K1 cells (▼).

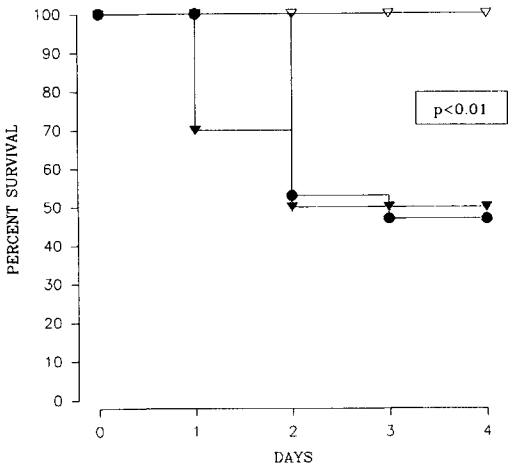

Figure 3. Lethality after injection of 500 μg LPS in three groups of BALB/c mice: (●) mice with no pretreatment (n = 15); (▽) mice pretreated intraperitoneally with 1,000 U IL-10 (n = 15); (▼) mice pretreated intraperitoneally with supernatant from mock-transfected CHO-K1 cells (n = 10). Two-tailed p value has been determined with the Fisher's exact test.

TNF released in the circulation after LPS challenge, as shown in Table 1 representing one out of three experiments which gave similar results. This in vivo effect of IL-10 was already observed with doses as low as 10 U (p <0.01 as compared with LPS after pretreatment with control medium). As 1,000 U had a more pronounced and reproducible activity, this dose was chosen for further studies. The specificity of the effect of the IL-10 preparation was assessed by pretreating mice with a supernatant from mock-transfected CHO-K1 cells. As shown in Table 1, this control preparation had no effect on TNF release. TNF serum levels returned to baseline values 3 h after LPS challenge in all groups of mice, indicating that IL-10 did not merely delay the release of TNF (Table 1).

As a first approach to evaluate in vivo the effect of IL-10 on LPS-induced toxicity, we monitored rectal temperature for 24 h after challenge with 100 μg LPS. Indeed, as previously reported by others, LPS caused a profound hypothermia which is probably related to TNF release (18). The data presented in Fig. 2 indicate that IL-10 pretreatment efficiently prevented LPS-induced hypothermia (p <0.0001 at 4 h, and p <0.005 at 6 h, as compared either with LPS alone or LPS after pretreatment with mock supernatant), whereas control supernatant from mock-transfected CHO-K1 cells had no significant effect. A substantial variation in the recovery from hypothermia was seen among the animals injected with 100 μg LPS, either given alone or after pretreatment with mock supernatant. As an additional control of specificity for the effect observed after pretreatment with CHO-IL10 supernatant, we added 1 mg of the neutralizing anti-mouse IL-10 JES5-2A5 mAb or of a control rat IgG1 LO-DNP mAb to the IL-10 preparation. The anti-IL-10 completely abrogated the protective effect of IL-10 on hypothermia whereas the control rat mAb had no effect (data not shown).

To evaluate the effect of IL-10 pretreatment on LPS-induced lethality, mice were challenged with 500 μg LPS, a dose which is lethal within 72 h in 50% of the animals. All mice pretreated with 1,000 U IL-10 survived the LPS injection, whereas pretreatment with supernatant from mock-transfected CHO-K1 cells did not modify the LPS-induced lethality (Fig. 3).

Taken together, these data indicate that pretreatment with IL-10 prevents the toxicity of LPS in a murine model of endotoxin shock. The beneficial effect of IL-10 could be due at least in part to the reduction of TNF release. Indeed, a similar level of protection against LPS-induced lethality has been obtained by the use of neutralizing anti-TNF antibodies (2–5). In vitro data suggest that IL-10 might also prevent the release of other monocyte/macrophage-derived cytokines involved in the pathogenesis of septic shock, especially IL-1 (1, 6, 19, 20).

IL-10 can therefore be added to the potential immunointervention strategies for the prevention and/or treatment of septic shock, which already include anti-TNF mAb (2–5), IL-1 receptor antagonist (21–23), differentiation factor/leukemia inhibitory factor (24), and G-CSF (25). Because of its deactivation effect on macrophages, IL-10 therapy might be of particular interest, possibly in combination with some of these other biological agents.

We thank Freya Van Houtte for technical assistance.

This work was supported by grants from the Fonds National de la Recherche Scientifique/Télévie, and the Association Contre le Cancer, Brussels, Belgium. Contribution of the Laboratory of Molecular Biology (LMB) was supported by the Fonds voor Geneeskundig Wetenschappelijk Onderzoek (FGWO), and by the Interuniversitaire Attractiepolen (IUAP).

Address correspondence to Dr. Thierry Velu, Institut de Recherche Interdiciplinaire, Campus Erasme, Bat. C, Route de Lennik, 808, B-1070 Brussels, Belgium.

Received for publication 5 November 1992.

References

1. Glausner, M.P., G. Zanetti, J.D. Baumgartner, and J. Cohen. 1991. Septic shock: pathogenesis. *Lancet.* 338:732.
2. Beutler, B., I.W. Milsark, and A.C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science (Wash. DC).* 229:869.
3. Tracey, K.J., Y. Fong, D.G. Hesse, K.R. Manogue, A.T. Lee, G.C. Kuo, S.F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (Lond.).* 330:662.
4. Mathison, J.C., E. Wolfson, and R.J. Ulevitch. 1988. Participation of tumor necrosis factor in the mediation of gram-negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* 81:1925.
5. Silva, A.T., K.F. Bayston, and J. Cohen. 1990. Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-alpha in experimental gram-negative shock. *J. Infect. Dis.* 162:421.
6. Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147:3815.
7. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage deactivation by interleukin 10. *J. Exp. Med.* 174:1549.
8. de Waal Malefyt, R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. de Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174:1209.
9. Mosmann, T.R., J.H. Schumacher, D.F. Fiorentino, J. Leverah, K.W. Moore, and M.W. Bond. 1990. Isolation of monoclonal antibodies specific for IL-4, IL-5, IL-6, and a new Th2-specific cytokine (IL-10), cytokine synthesis inhibitory factor, by using a solid phase radioimmunoadsorbent assay. *J. Immunol.* 145: 2938.
10. Moore, K.W., P. Vieira, D.F. Fiorentino, M.L. Trounstine, T.A. Khan, and T.R. Mosmann. 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Science (Wash. DC).* 248:1230.
11. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84: 1374.
12. Sanger, F., and A.R. Coulson. 1978. The use of thin acrylamide gels for DNA sequencing. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 87:107.
13. Velu, T.J., L. Beguinot, W.C. Vass, M.C. Willingham, G.T. Merlino, I. Pastan, and D.R. Lowy. 1987. Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene. *Science (Wash. DC).* 238:1408.
14. Velu, T.J., L. Beguinot, W.C. Vass, K. Zhang, I. Pastan, and D.R. Lowy. 1989. Retroviruses expressing different levels of the normal epidermal growth factor receptor: biological properties and new bioassay. *J. Cell Biochem.* 39:153.
15. Espevik, T., and J. Nissen Meyer. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods.* 95:99.
16. Fiers, W., P. Brouckaert, A.L. Goldberg, I. Kettelhut, P. Suffys, J. Tavernier, B. Vanhaesebroeck, and F. Van Roy. 1987. Structure-function relationship of tumour necrosis factor and its mechanism of action. *Ciba Found. Symp.* 131:109.
17. Brouckaert, P.G., C. Libert, B. Everaerdt, N. Takahashi, and W. Fiers. 1989. A role for interleukin 1 in the in vivo actions of tumor necrosis factor. *Lymphokine Res.* 8:269.
18. Bauss, F., W. Droge, and D.N. Mannel. 1987. Tumor necrosis factor mediates endotoxic effects in mice. *Infect. Immun.* 55:1622.
19. Okusawa, S., J.A. Gelfand, T. Ikejima, R.J. Connolly, and C.A. Dinarello. 1988. Interleukin 1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *J. Clin. Invest.* 81:1162.
20. McIntyre, K.W., G.J. Stepan, K.D. Kolinsky, W.R. Benjamin, J.M. Plocinski, K.L. Kaffka, C.A. Campen, R.A. Chizzonite, and P.L. Kilian. 1991. Inhibition of interleukin 1 (IL-1) binding and bioactivity in vitro and modulation of acute inflammation in vivo by IL-1 receptor antagonist and anti-IL-1 receptor monoclonal antibody. *J. Exp. Med.* 173:931.
21. Ohlsson, K., P. Bjork, M. Bergenfeldt, R. Hageman, and R.C. Thompson. 1990. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature (Lond.).* 348:550.
22. Alexander, H.R., G.M. Doherty, C.M. Buresh, D.J. Venzon, and J.A. Norton. 1991. A recombinant human receptor antagonist to interleukin 1 improves survival after lethal endotoxemia in mice. *J. Exp. Med.* 173:1029.
23. Wakabayashi, G., J.A. Gelfand, J.F. Burke, R.C. Thompson, and C.A. Dinarello. 1991. A specific receptor antagonist for interleukin 1 prevents *Escherichia coli*-induced shock in rabbits. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:338.
24. Alexander, H.R., G.G. Wong, G.M. Doherty, D.J. Venzon, D.L. Fraker, and J.A. Norton. 1992. Differentiation factor/leukemia inhibitory factor protection against lethal endotoxemia in mice: synergistic effect with interleukin 1 and tumor necrosis factor. *J. Exp. Med.* 175:1139.
25. Gorgen, I., T. Hartung, M. Leist, M. Niehorster, G. Tiegs, S. Uhlig, F. Weitzel, and A. Wendel. 1992. Granulocyte colony-stimulating factor treatment protects rodents against lipopolysaccharide-induced toxicity via suppression of systemic tumor necrosis factor-alpha. *J. Immunol.* 149:918.