SURGICAL INFECTIONS
Volume 14, Number 1, 2013

DOI: 10.1089/sur.2011.093

Effects of Infliximab and Hydrocortisone on in Vitro Cytokine Responses After Stimulation with Lipopolysaccharide

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Abstract

Background: Both glucocorticosteroids and biologic drugs such as the tumor necrosis factor (TNF)-α antagonist infliximab are used often in the treatment of rheumatoid arthritis or inflammatory bowel disease. In severe disease, or if allergic reactions occur during treatment with infliximab, combined therapy with these drugs often is instituted. Combining infliximab and glucocorticosteroids may increase substantially the risk of severe opportunistic infections or dissemination of malignancies because of their additive effects as immunosuppressors.
Methods: In a whole-blood in vitro model, we studied the influence of different doses of infliximab and hydrocortisone, either separately or in combination, on the synthesis of selected cytokines after stimulation with lipopolysaccharide (LPS).
Results: Hydrocortisone in therapeutic serum concentrations significantly inhibited the expression of a majority of the cytokines tested. Infliximab, in serum concentrations relevant to clinical situations, significantly inhibited TNF-α activity. This effect was potentiated when infliximab was combined with hydrocortisone. Similar effects were found using a low dose of infliximab combined with hydrocortisone. Infliximab alone inhibited the expression of the cytokines interleukin (IL)-1 receptor antagonist, monocyte chemoattractant protein-1, IL-8, and IL-12. Hydrocortisone in combination with low-dose infliximab potentiated the suppressive effects on TNF-α, IL-1β, IL-8, and macrophage inflammatory protein-1α synthesis.
Conclusions: Immune-modulating effects of infliximab were found both in clinically relevant doses and, most notably, in low doses reflecting serum concentrations commonly found in patients several months after the last injection. Infliximab potentiates the suppressive effects of hydrocortisone on cytokine synthesis.

Patients with inflammatory bowel diseases (IBDs), rheumatoid arthritis (RA), or other autoimmune diseases commonly are treated with immune modulators such as corticosteroids or with biologics such as the anti-tumor necrosis factor (TNF)-α agent infliximab. Patients receiving these drugs should be considered to have potentially altered immune competence. The degree of immune deficiency generally is dose dependent and differs among the drugs.

Infliximab has a long half-life, remaining in the circulation for as long as 6 mos. As treatment with combinations of immune modulators is common, this long half-life could reinforce the immune deficiency and result in a higher incidence of opportunistic infections [1–3]. In a study from the Mayo Clinic, the odds ratio for opportunistic infections associated with the use of corticosteroids, azathioprine/6-mercaptopurine, or infliximab was increased significantly when two or three of these drugs were combined [4]. Combined treatment with immune modulators also may result in the development of malignancies or more pronounced dissemination of existing malignancies [5,6].

In vitro studies on the possible additive effects on the immune system of combinations of such agents are largely lacking. The aim of the present study was to investigate the effects on cytokine responses in vitro after challenging whole blood with lipopolysaccharide (LPS). Prior to LPS stimulation,

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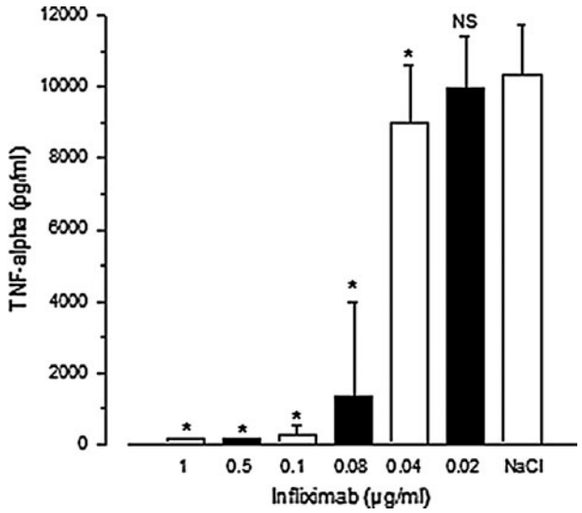

FIG. 1. Concentrations of tumor necrosis factor-α in plasma after stimulation with LPS and treatment with different doses of infliximab. Data from five healthy persons are presented as mean values and standard deviation. *$p < 0.05$ vs. NaCl; NS = not significant vs. NaCl.

infliximab or hydrocortisone or both, in clinical relevant concentrations, were added to the whole blood.

Materials and Methods

Freshly drawn blood from five healthy female donors, mean age 30 years (range 27–40 years), was tested in a whole-blood in vitro model. The blood was anticoagulated using heparin sodium (30 IU/mL, CP Pharmaceuticals, Wrexham, UK), and 0.6-mL volumes of blood were placed in plastic tubes (Monovette, Sarstedt, Germany). The tubes were incubated with gentle rotation at 37°C. To each tube was added

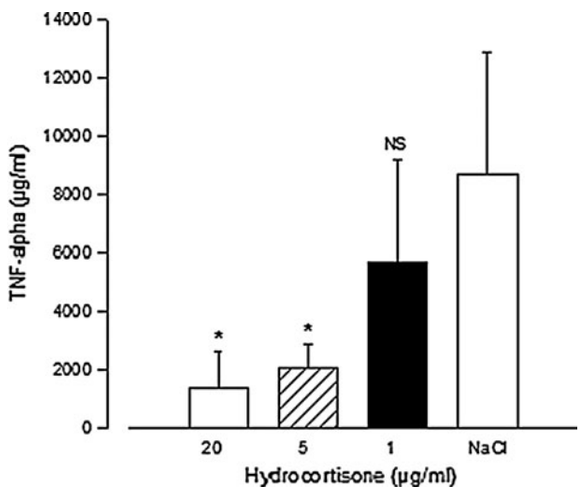

FIG. 2. Concentrations of tumor necrosis factor-α in plasma after stimulation with LPS and treatment with different doses of hydrocortisone. Data from five healthy persons are presented as mean values and standard deviation. *$p < 0.05$ vs. NaCl; NS = not significant vs. NaCl.

6 mcL of infliximab solution containing 0.012 mcg to 120 mcg (0.02–200 mcg/mL in whole blood) or 6 mcL of hydrocortisone solution containing 0.6–12 mcg (1–20 mcg/mL in whole blood). In a parallel experiment, a combination of infliximab 0.6–0.12 mcg (1–0.2 mcg/mL) and hydrocortisone 0.6–3 mcg (1–5 mcg/mL) was added to each tube. Thirty minutes after incubation with the drugs, 6 mcg of 1 mcg/mL solution (final concentration in whole blood 10 ng/mL) of *Escherichia coli* LPS (Sigma, Poole, Dorset, UK) was added to each tube. Each experiment included a control sample in which 0.9% saline was added in place of the drugs. After stimulation with LPS, the tubes were incubated for 6 h before centrifugation, with the resulting plasma being stored at −20°C for subsequent measurement of cytokine concentrations. A multiplex antibody bead kit (Cytokine 25-plex, Luminex, Biosource, Camarillo, CA, USA) was used for simultaneous measurement of the cytokines selected.

Data are presented as mean values and standard deviations and were tested for normal distribution. Thereafter, differences between values were estimated with one-way repeated-measures analysis of variance or one-way repeated-measures analysis of variance on ranks as appropriate. The results were elaborated using the Dunnett or Student-Newman-Keul post hoc test. Any p values < 0.05 were considered statistically significant.

Results

After challenging whole blood with LPS, infliximab attenuated TNF-α release significantly ($p < 0.05$) (Fig. 1). Infliximab in higher concentrations (200–50 mcg/mL) also reduced the plasma concentrations of interleukin (IL)-1 receptor antagonist (ra), the chemokine ligand (CCL)2/monocyte chemoattractant protein (MCP)-1, and the chemokine ligand CXCL8/IL-8 and IL-12 significantly (data not shown). Hydrocortisone lowered the plasma concentrations of several cytokines after stimulation with LPS (TNF-α [Fig. 2], IL-1β, IL-1ra, CCL2/MCP-1, IL-6, IL-8, and IL-12). With the combination of hydrocortisone 5 mcg/mL and a low dose of infliximab (0.08–0.02 mcg/mL), additive effects were found on the plasma concentrations of TNF-α (Fig. 3), IL-1β, macrophage inflammatory protein (MIP) CCL3/MIP-1α, and CXCL8/IL-8 ($p < 0.05$) (Table 1).

Discussion

Using an in vitro whole-blood model, we demonstrated immunosuppressive effects of even low concentrations of the anti-TNF-α agent infliximab. Combining infliximab with clinical relevant concentrations of hydrocortisone resulted in additive effects on several cytokine concentrations in plasma after a challenge with LPS.

Using a similar whole-blood in vitro model, our group earlier described cytokine modulation during experimental endotoxemia. Challenging whole blood with LPS induces the release of several cytokines from leukocytes: TNF-α; IL-1β, IL-6, IL-8, IL-10, and IL-12; CCL3/MIP-1α, CCL4/MIP-1β, and CCL2/MCP-1 [7–9]. Experience gained from previous studies defined the doses of LPS used for the present study.

Infliximab is a chimeric monoclonal immunoglobulin IgG_1 antibody to TNF. The monoclonal antibody has significant effects against the symptoms of several chronic inflammatory diseases, including IBD and RA. It also is a potent inhibitor of

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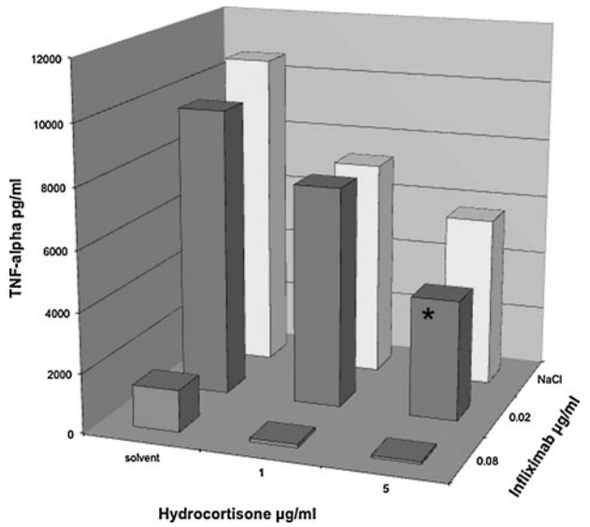

FIG. 3. Concentrations of tumor necrosis factor (TNF)-α in plasma after LPS stimulation and treatment with infliximab, hydrocortisone, or both. Data from five healthy persons are presented as mean values. Combining infliximab 0.02 mcg/mL and hydrocortisone 5 mcg/mL significantly decreased TNF-α concentration compared with either drug given alone (*$p < 0.05$).

both soluble and membrane-bound human TNF-α [10]. The pharmacokinetics of infliximab after intravenous (IV) infusion includes a long half-life (9.5 days). However, complete removal of infliximab from the circulation may take as long as 6 months, and after 12 weeks, serum concentrations > 0.1 mcg/mL commonly are detected [11]. A dose-dependent maximum serum concentration in patients amounts to 118 mcg/mL after an IV dose of 5 mg/kg, which is common in clinical use.

Neutralizing TNF-α is not the sole mechanism of action of infliximab. In therapeutic concentrations, the monoclonal antibody induces apoptosis of monocytes in peripheral blood [12] and of lamina propria T cells [13]. It also reduces vascular cell adhesion molecule-1 (VCAM-1) and CD-40 expression on mucosal endothelium [14], as well as influencing wound healing by increasing the production of tissue inhibitor of metalloproteinase-1 (TIMP-1), thus reducing matrix metalloproteinase (MMP) activity. Furthermore, infliximab inhibits myofibroblast migration in vitro [15].

Tumor necrosis factor has both pro-inflammatory and immunoregulatory functions. The diverse activities are mediated by ligand interaction with two receptors (p55/TNFRI and p75/TNFRII) expressed on human cells. Tumor necrosis factor is expressed as a transmembrane precursor (tm' TNF), which, after proteolysis, forms a soluble homo-trimer (s' TNF). The binding of both forms to their receptors induces other inflammatory mediators and cell adhesion molecules [16].

Infusion of infliximab neutralizes TNF and has been validated for the treatment of a number of immune-mediated disorders. Using antibody kits, we have shown that infliximab significantly attenuates the release of TNF-α in plasma, probably secondary to binding to infliximab. However, this effect probably is not the only mechanism of the clinical action. The clinical relevance of attenuated TNF release has yet to be elucidated. It is unclear whether the TNF immunoassay (Luminex) detects TNF bound to infliximab in addition to free TNF in the blood. However, TNF bound to infliximab is physiologically inactive. It is reasonable to believe that the active TNF molecule is the most important to measure because of its role in the pathophysiological mechanisms seen during the inflammatory processes.

Lipopolysaccharide exerts its effects mainly by activation of white blood cells, endothelial cells, and tissue macrophages [17]. In whole blood, LPS induces cytokine production mostly in monocytes, although other cells do contribute. For instance, terminally differentiated cells such as polymorphonuclear granulocytes (PMNs) were thought to lack transcriptional activity and perform little, if any, protein synthesis [18]. At present, a large body of evidence points to PMNs as important contributors to cytokine serum values, principally because of their great number.

Infliximab binds to the cell-surface receptors Toll-like receptor (TLR) 4 and CD14 and recently was shown to reduce these surface markers significantly in vitro [19]. This effect could explain some of the reduced responsiveness of

Table 1. Effects of Low Doses of Infliximab, Hydrocortisone, and the combination on Selected Cytokine Concentrations in Plasma After Stimulation with Lipopolysaccharide[a]

		Infliximab		*Hydrocortisone*		
Cytokine pg/mL	*NaCl*	*0.02 mcg/mL*	*0.08 mcg/mL*	*5 mcg/mL*	*5 mcg/mL and infliximab 0.02 mcg/mL*	*5 mcg/mL and infliximab 0.08 mcg/mL*
TNF-α	10,635 (1,735)	9,620 (2,200)	1,398 (2,781)	5,682 (1,747)	4,013[b] (1,534)	80 (28)
MIP-1α	2,529 (445)	2,469 (393)	2,479 (594)	2,151 (1,747)	1,965[b] (270)	1,595[b] (420)
IL-1β	2,240 (253)	2,122 (388)	2,007 (221)	906 (357)	673[b] (102)	723[b] (311)
IL-8	7,208 (3,257)	7,614 (3,257)	7,135 (2,967)	5,651 (2,040)	5,183 (1,878)	3,859[b] (1,515)

[a]Data from five healthy persons given as mean values (standard deviation).
[b]Combination of infliximab and hydrocortisone is significantly different ($p < 0.05$) from the same dose of either infliximab or hydrocortisone alone.
MIP = macrophage inflammatory protein; IL = interkeukin; TNF = tumor necrosis factor.

leukocytes to LPS, although it probably is not the only mechanism [20].

This in vitro study reveals significant immunosuppressive effects of infliximab in concentrations as low as 0.04 mcg/mL. Additive effects on the TNF-α concentration are detected when low doses of infliximab and hydrocortisone are combined, which could indicate more pronounced immunosuppression. Even if glucocorticosteroids are administrated more than 12 weeks after an injection of infliximab, such combined treatment may contribute to opportunistic infections. Furthermore, if a patient develops a malignant disease during such treatment, the tumor cells could be predisposed to dissemination.

Using an in vitro model, Nesbitt et al. saw a significant decrease in the IL-1β concentration in purified human monocytes if infliximab was added after stimulation with LPS [21]. In our study, IL-1β concentrations were altered insignificantly at all infliximab doses tested. This difference may be explained by the different time intervals examined.

Glucocorticosteroids commonly are used in several acute and chronic inflammatory disorders because of their immunosuppressive effects. According to the producer's manual (OE Jacobsen, Pfizer Ltd., Oslo, Norway; personal communication), an intravenous (IV) dose of hydrocortisone (200 mg) in normal subjects results in a maximum concentration in plasma of 2.3 mcg/mL, and the concentration provided by hydrocortisone 400 mg IV peaks at 6.3 mcg/mL in the plasma of adults. In our study, hydrocortisone 5 mcg/mL significantly decreased the concentrations of the cytokines TNF-α, IL-1β, IL-1ra, CCL2/MCP-1, CXCL8/IL-8, and IL-6 and IL-12 after stimulation with LPS. The anti-inflammatory and immunosuppressive effects of glucocorticoids rely on several molecular mechanisms. Similar to infliximab, glucocorticoids exert their effects by inhibiting the expression of cytokines and adhesion molecules. Glucocorticoids bind to cytosolic receptors, an action which modulates transcription activation. Glucocorticoid receptors antagonize the activity of transcription factors such as nuclear factor (NF) κB [22]. Inflammatory signals commonly activate mitogen-activated protein kinase (MAPK) cascades. Glucocorticoids induce the production of the anti-inflammatory protein MAPK phosphatase 1. Glucocorticoids thus act through several mechanisms to control inflammation [23].

Glucocorticosteroids also affect apoptosis [24]. Thus, several anti-inflammatory effects of infliximab and glucocorticosteroids are similar in the modulation of cytokine responses and apoptosis. Therefore, the main message of this study is the demonstration that even minute amounts of infliximab have important additive effects on glucocorticoid innate immune system repression. These plasma concentrations are found several months after infliximab injections have been discontinued.

A limitation of our whole-blood model is the fact that only the effects of LPS on blood cells incubated for 6 h were investigated. The model is well suited to study the effects of immune modulation on a mixed leukocyte population [7]. It retains some of the interplay between blood cells and plasma components. As an ex vivo model, immunologic changes seen in endothelial cells or tissue macrophages or at different time spans as the result of stimulation with LPS are not revealed.

When using a whole-blood in vitro model, infliximab, even in subclinical doses, modulates cytokine signaling after challenge of whole blood with LPS. Combined with hydrocortisone, additive effects on the concentrations of selected cytokines are revealed. Such effects may contribute to a higher incidence of opportunistic infections or malignancies in patients receiving combined therapy with immunomodulatory agents.

Acknowledgments and Disclosure

The authors thank Grethe Dyrhaug and Signe Flood Kjeldsen for excellent technical assistance.

The authors have no competing financial interests.

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DOI til publisert versjon / DOI to published version: 10.1089/sur.2011.093