Infliximab Induced T Lymphocyte Apoptosis in Crohn's Disease

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ABSTRACT. Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract of unknown origin. Therapies include immune modulating agents, biological therapies, and surgery. The activity and efficacy of the anti-tumor necrosis factor (TNF) therapies infliximab and etanercept have proved to be different: infliximab is effective to induce and maintain remission in refractory CD, while etanercept is not. This brief review considers the question of whether this disparity can be explained by the different structure of the proteins, their different binding affinities, or the subsequent effects on T lymphocytes. (J Rheumatol 2005;32 Suppl 74:26-30)

Key Indexing Terms:
TUMOR NECROSIS FACTOR
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EFFICACY OF TNF ANTAGONISTS IN CROHN'S DISEASE

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal (GI) tract of unknown origin. Available therapies include immune modulating drugs, therapy with biological compounds, or surgery. Anti-tumor necrosis factor (TNF) therapy has become a drug of major importance in remission and endoscopic remission induction, which was not shown previously with available therapies. Two anti-TNF molecules are approved for clinical therapy of inflammatory disorders. Infliximab (Remicade), a chimeric anti-TNF-α antibody, is registered for both rheumatoid arthritis and Crohn's disease. Etanercept, a recombinant TNF receptor/IgG fusion protein, is registered for rheumatoid arthritis only.

Interestingly, the activity and efficacy profiles of infliximab and etanercept in Crohn's disease have proved to be different. Infliximab is effective to induce and maintain remission in steroid-refractory CD; it is also used to treat fistulizing disease. Targan, *et al* showed in a multicenter, randomized, double-blind, placebo-controlled trial that infliximab induced a clinical response by 61% of patients at Week 2, 65% at Week 4, and 41% at Week 12 in patients with moderate to severe, treatment-resistant CD compared to response rates in the placebo group of 17%, 17%, and 12%, respectively[1].

Rutgeerts, *et al* showed that response can be maintained with repeated administration. In a randomized, double-blind, placebo-controlled study, 62% of patients treated with infliximab maintained clinical response compared to 37% of patients who received placebo every 8 weeks for 36 weeks[2].

In contrast, etanercept is not effective in the treatment of CD. Response rates following the administration of etanercept 25 mg or placebo subcutaneously twice weekly for 8 weeks in a randomized, double-blind, placebo-controlled trial were similar at all timepoints (2, 4, and 8 weeks). Clinical response at Week 4 (the primary study endpoint) was observed in 39% of patients in the etanercept group and 45% of the placebo group[3].

The question arises whether these disparities can be explained by the different structure of the proteins, the different binding affinities, and subsequent effects on T lymphocytes.

T CELL APOPTOSIS IN CD

Defective T cell apoptosis appears to play a role in the development of inflammation in patients with CD. Lamina propria T cells isolated from patients with CD show a resistance to apoptosis that is based on several disturbances compared to controls. Indeed, these lamina propria T cells show increased interleukin 2 (IL-2) dependent proliferation and are relatively resistant to apoptosis induced by CD2 activation, IL-2 depletion, nitric oxide induced apoptosis, and engagement of Fas[4,5]. This phenomenon is in part explained by an increased ratio of intracellular expression of the proteins Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic)[6].

BINDING OF TNF ANTAGONISTS TO MEMBRANE-BOUND TNF-α

Infliximab and etanercept bind differently to transmembrane TNF. Using a transfection model, Scallon, *et al* showed that infliximab has a higher binding affinity to transfected cells with the uncleavable form of transmembrane TNF than etanercept. Moreover, in their experiments infliximab was shown to bind both soluble and membrane-bound TNF, whereas etanercept bound primarily to soluble TNF[7].

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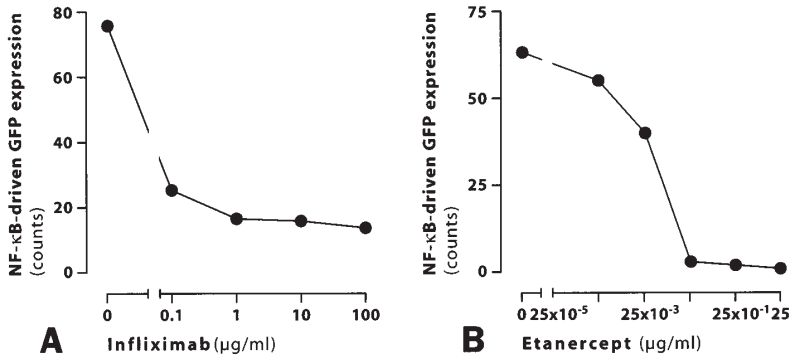

Figure 1. Infliximab (A) and etanercept (B) neutralized recombinant human TNF-α. From Van den Brande, *et al. Gastroenterology* 2003;124:1774-85; with permission.

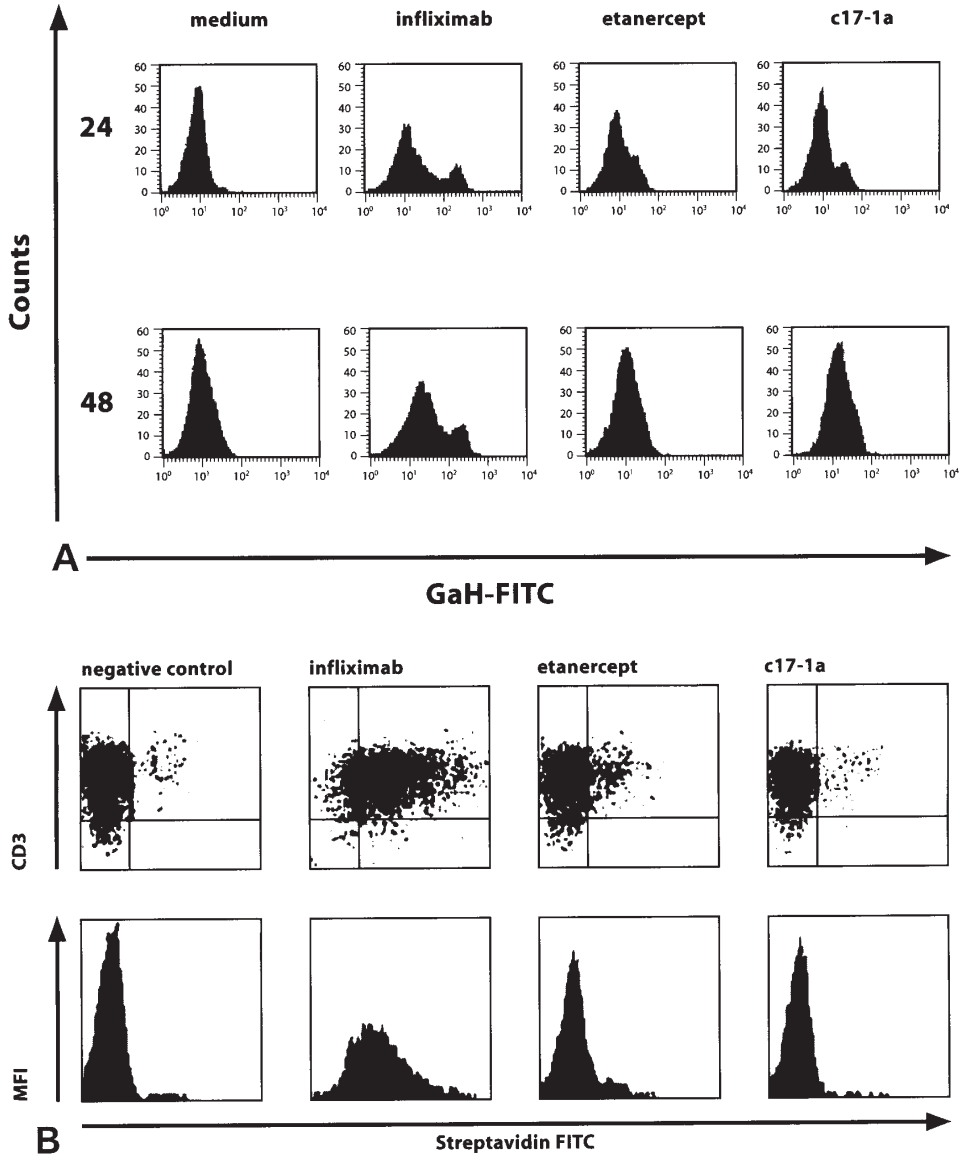

Figure 2A and B. Only infliximab bound transmembrane TNF-α on activated lamina propria lymphocytes from patients with Crohn's disease. From Van den Brande, et al. *Gastroenterology* 2003;124:1774-85; with permission.

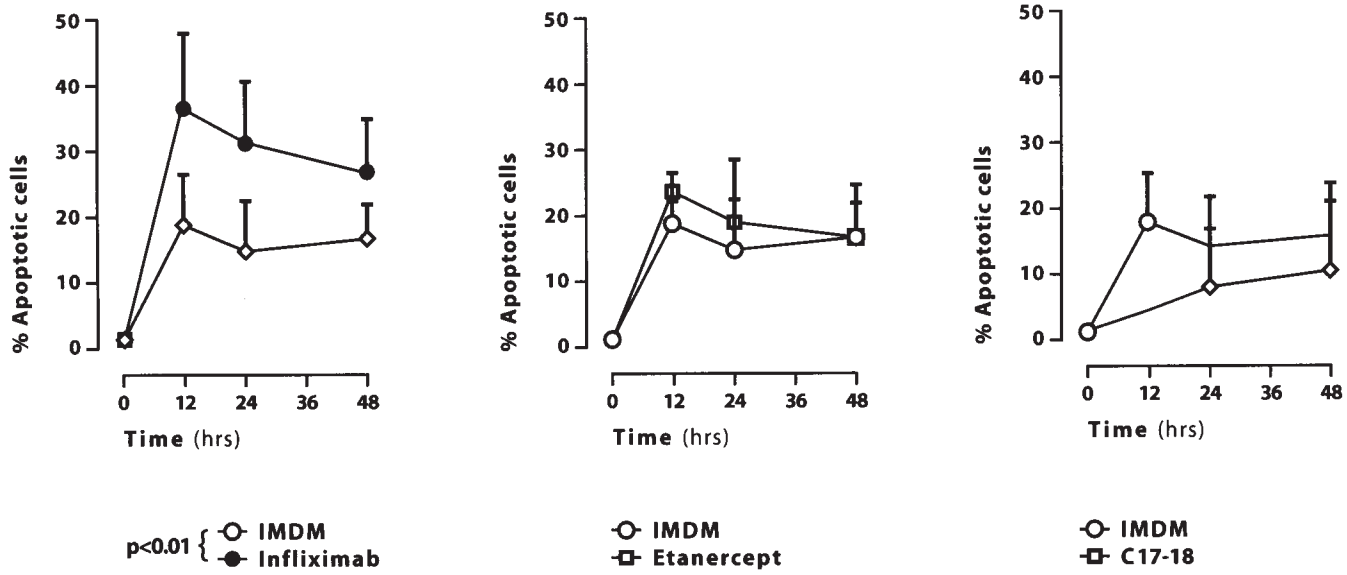

Figure 3A. Only infliximab induced apoptosis in peripheral lymphocytes activated by mixed lymphocyte reaction. *Gastroenterology* 2003;124:1774-85; with permission.

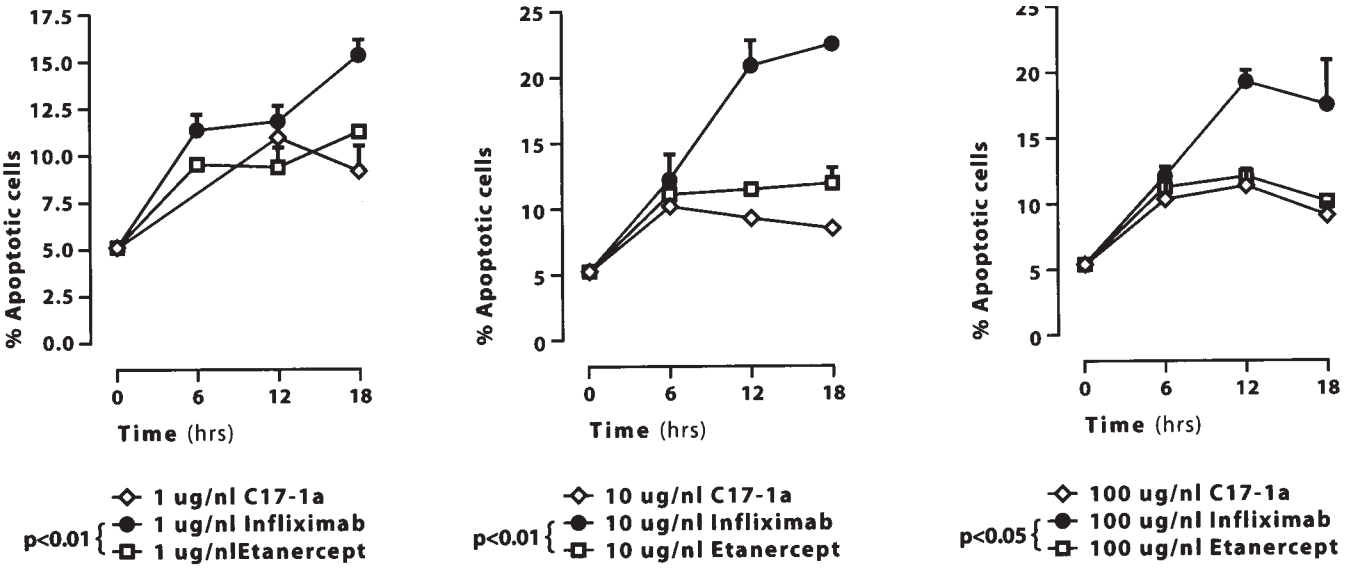

Figure 3B. Infliximab but not etanercept induced apoptosis in activated Crohn's lamina propria mononuclear cells in a mixed lymphocyte reaction. *Gastroenterology* 2003;124:1774- 85; with permission.

INFLIXIMAB INDUCED APOPTOSIS

Apoptosis induction by infliximab has been suggested in 2 earlier reports. Lugering and colleagues demonstrated significant infliximab binding to membrane-bound TNF, dose-dependent increases in apoptosis, and caspase-3 proteolytic activity in monocytes from patients with chronic active CD[8]. Caspase-3 activation and apoptosis following infliximab administration occurred rapidly and were significantly increased as early as 4 hours after treatment ($p = 0.002$ and $p = 0.003$ vs before treatment, respectively). Interestingly $F(ab)_2$ fragments of infliximab (which lack the Fc domain) also induced dose-dependent apoptosis. Second, ten Hove, *et al* showed a significant increase in TUNEL-positive lamina propria (CD3+) T cells in tissue taken from patients 24 hours after infliximab infusion[9].

WHY IS INFLIXIMAB, BUT NOT ETANERCEPT, EFFECTIVE IN CROHN'S DISEASE?

This background information raises a number of questions. Do infliximab and etanercept inhibit activation of target cells by neutralization of soluble TNF-α and subsequently prevent translocation of nuclear factor-κB (NF-κB) and DNA transcription of cytokines? Or do infliximab and etanercept bind to membrane TNF-α on target cells and alter function by inducing apoptosis? We performed a number of experiments in an attempt to answer some of these questions.

Do infliximab and etanercept differ in the capacity to neutralize TNF-α?

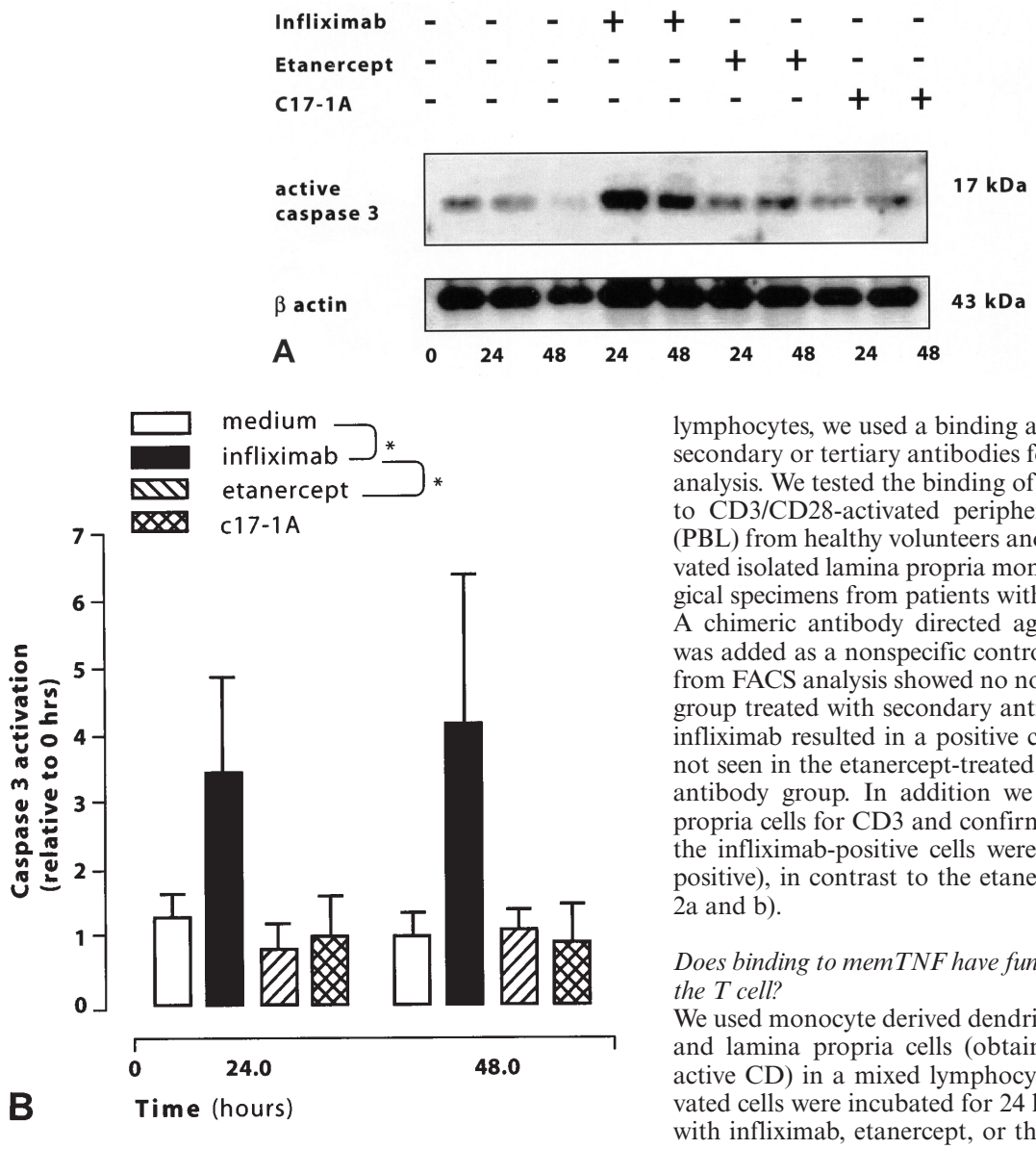

Figure 4A and B. Induction of cleaved caspase-3 upon treatment with infliximab. *Gastroenterology* 2003;124:1774-85; with permission.

We assayed TNF-α neutralizing potential of these TNF antagonists with respect to TNF-α dependent transactivation of a NF-κB-driven reporter construct. After stimulation with TNF-α, green fluorescent protein (GFP) is transcribed along with NF-κB and can be detected by FACS analysis. Infliximab-neutralized TNF-α induced GFP expression in NF-κB reporter construct transfected HeLa cells at a concentration of 1 g/ml, and etanercept neutralized TNF-α at a concentration of 0.25 g/ml (Figure 1)[10]. Thus, both infliximab and etanercept neutralized TNF-α effectively in this assay.

Do infliximab and etanercept differ in the capacity to bind to memTNF-α?

To investigate the difference between infliximab and etanercept with respect to their capacity to bind to transmembrane TNF-α (memTNF-α) expressed by activated lymphocytes, we used a binding assay with FITC-labeled secondary or tertiary antibodies for detection with FACS analysis. We tested the binding of these TNF antagonists to CD3/CD28-activated peripheral blood lymphocytes (PBL) from healthy volunteers and PMA-ionomycin-activated isolated lamina propria mononuclear cells from surgical specimens from patients with active Crohn's disease. A chimeric antibody directed against EpCAM, c17-1a, was added as a nonspecific control antibody. Histograms from FACS analysis showed no nonspecific binding in the group treated with secondary antibody only. Addition of infliximab resulted in a positive cell population that was not seen in the etanercept-treated group or in the control antibody group. In addition we double-stained lamina propria cells for CD3 and confirmed that the majority of the infliximab-positive cells were T lymphocytes (CD3-positive), in contrast to the etanercept condition (Figure 2a and b).

Does binding to memTNF have functional consequences for the T cell?

We used monocyte derived dendritic cells to activate PBL and lamina propria cells (obtained from patients with active CD) in a mixed lymphocyte reaction. These activated cells were incubated for 24 h in separate conditions with infliximab, etanercept, or the above mentioned isotype control antibody. Next, lymphocyte apoptosis was determined using a well established apoptosis assay with annexin V-7AAD double-staining. In brief, annexin V binds to externalized phosphatidyl serine, an early marker of impermeable apoptotic cells, and 7AAD stains the nucleus of permeable necrotic cells. In this assay, the annexin V-positive/7AAD-negative cells represent the apoptotic cells and the double-positive cells the necrotic cells. Addition of infliximab induced a significant increase of apoptosis of the activated lymphocytes (compared to the medium control), whereas etanercept and the isotype control were inactive in this respect (Figure 3a and b). Apparently, the binding of infliximab to memTNF-α on activated T cells correlates with the ability to induce apoptosis of these clinically relevant immune cells.

To further investigate markers of apoptosis we investigated the activation state of caspase-3. Caspase-3 is a downstream cleaving protein that can be activated by cleavage itself either through the intrinsic (mitochondrial)

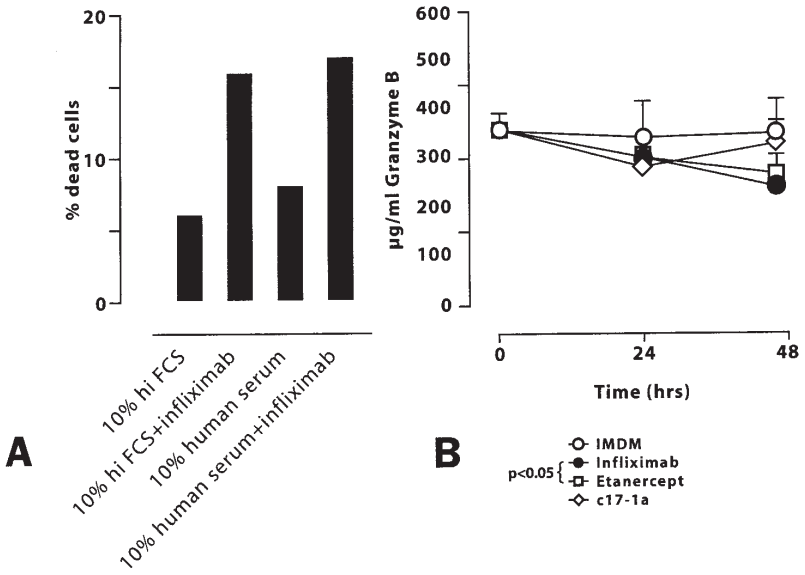

Figure 5. Infliximab-induced apoptosis is not mediated via the antibody-dependent cytotoxicity reaction (ADCR). *Gastroenterology* 2003;124:1774-85; with permission.

or by the extrinsic (caspase-8) cell death pathway. Figure 4 shows a representative experiment of a Western blot of cells in a mixed lymphocyte reaction treated with infliximab, etanercept, and a chimeric control antibody at 24 and 48 h after treatment. Strong induction of cleaved caspase-3 can be seen upon treatment with infliximab. These results show that the induction of apoptosis by infliximab correlates with activation of caspase-3.

THE COMPLEMENT ISSUE

Our experiments involved immune cells in heat-inactivated fetal calf serum-supplemented medium; that is, we considered the complement system to be deactivated. Indeed, we showed that replacing fetal calf serum with complement-competent human serum did not increase infliximab effects on apoptosis, as measured with iodine blue staining (Figure 5A) and by granzyme-B ELISA (Figure 5B).

CONCLUSION

In our assays both infliximab and etanercept neutralize soluble TNF effectively. However, in activated lamina propria lymphocytes, only infliximab binds to membrane TNF, induces apoptosis, and activates caspase-3.

FUTURE PLANS

We intend to perform SPECT scans of patients treated with infliximab to visualize apoptosis. Response to infliximab, about two-thirds in steroid-refractory CD, might be correlated to the degree of apoptosis induction. Characterizing the nonresponder population could increase cost-effectiveness.

The role of apoptosis in remission induction and maintenance should be investigated in powerful therapy regimens such as combinations of immune modulators methotrexate and infliximab.

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