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ten Hove, T.; van Montfrans, C.; Peppelenbosch, M.P.; van Deventer, S.J.H.

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Background and aims: Treatment with infliximab induces remission in about 70% of patients with steroid refractory Crohn’s disease. Because Crohn’s disease is considered to be mediated by uncontrolled activation of mucosal T lymphocytes, we hypothesised that infliximab could induce apoptosis of T lymphocytes.

Methods: Induction of apoptosis in vivo was studied in 10 patients with therapy refractory Crohn’s disease. In vitro, resting or stimulated Jurkat T cells were incubated with infliximab.

Results: Infusion of infliximab (5 mg/kg) in steroid refractory patients with Crohn’s disease induced a clinical response in 9/10 patients but did not influence expression of activation markers, homing receptors, memory cells, Fas expression, or Bax/Bcl-2 expression on peripheral blood T lymphocytes. In contrast, a significant increase in CD3 and TUNEL positive cells within colonic biopsies was detected 24 hours after infusion of infliximab, suggesting that infliximab stimulates apoptosis of activated T lymphocytes but not of resting T cells. To test this hypothesis, the effects of infliximab on Jurkat T cells were investigated. We observed that infliximab induced apoptosis and an increase in the Bax/Bcl-2 ratio of CD3/CD28 stimulated Jurkat T cells but not of unstimulated Jurkat cells.

Conclusions: Our data indicate that infliximab treatment causes a rapid and specific increase in apoptosis of T lymphocytes in the gut mucosa. These findings may explain the rapid and sustained therapeutic effects of infliximab in Crohn’s disease.
MATERIALS AND METHODS

Patients
Ten consecutive patients with active Crohn’s disease were included (mean age 28.6 years (range 21–38)). Mean disease duration was 7.5 (2.7) years (range 4–12) and mean Crohn’s disease activity index (CDAI) was 404 (range 182–808). Localisation of disease activity was assessed by endoscopy (sigmoid and rectum (n=5), left colon (n=2), neoterminal ileum (n=3)). Treatment with corticosteroids for at least four weeks (10 mg/day, orally) had failed in all patients. Concomitant treatment with other immunosuppressive drugs was allowed but the dosage had to be stable for at least eight weeks before enrolment. Other exclusion criteria were a positive human immunodeficiency virus test result or treatment with a drug that could potentially modulate TNF synthesis (thalidomide, pentaxivaline, and embrel). The local ethics committee granted approval for the study and all patients gave informed consent.

Treatment and assessments
A single intravenous dose (5 mg/kg) of the anti-TNF chimeric antibody (infliximab; Centocor, Malvern, Pennsylvania, USA) was administered as a two hour infusion. Patients were admitted to hospital for 24 hours and follow up was one week and six weeks after the initial infusion. Blood pressure, temperature, and pulse were measured before infusion. Thirty minute serial determinations were made after starting the infusion. Routine haematology, blood chemistry, and FACScan analysis were performed at 0, 2, 4, 8, 12, and 24 hours and at week 1 and week 6. Endoscopy was performed immediately before infusion and 24 hours later, and biopsies were taken from involved mucosa and not from ulcers. Location was verified by videotaping the entire procedure so that biopsies obtained 24 hours after the start of the treatment were taken at the same location as immediately before treatment. Patients used a daily diary system before and during the six week study period for assessment of CDAI. CDAI scores were determined within one week before infusion and after infusion at weeks 1 and 6. Clinical response was defined as a decrease in CDAI by 70 points or more, or induction of complete remission defined as a CDAI <150 points.

Cell culture and stimulation
The human Jurkat T cell line was cultured in RPMI 1640 (Bio Whittaker, Belgium) containing 10% fetal calf serum and the antibiotic ciprofloxacin (10 µg/ml), and subcultured twice a week. Cells were stimulated (concentration 1x10⁶ cells/ml) in RPMI 1640 supplemented with 5% pooled human serum (Bio Whittaker). T cells were unstimulated or stimulated with soluble anti-CD3 and anti-CD28 antibodies (2 ng/ml; CLB, Amsterdam, the Netherlands) and incubated with anti-TNF antibodies (10 µg/ml, infliximab; Centocor, Malvern, USA) or its isotype matched control. In parallel experiments, cells were incubated with mouse antihuman anti-CD95 antibody (IgG 1 µg/ml; CLB).

TUNEL assay
The cell pellets of Jurkat cells were washed and cytocentrifuged on superfrost slides in phosphate buffered saline (pH 7.4) supplemented with 1% bovine serum albumin (Sigma Chemical Co, St Louis, Missouri, USA). Slides were air dried and stored at −20°C until use. Human intestinal mucosal biopsies were embedded in Tissue-tek (Sakure Finetech Europe BV, Zoeterwoude, the Netherlands), subsequently snap frozen in liquid nitrogen, and kept at −70°C until use. Frozen sections (6 µm) were cut on a cryostat and kept at −20°C until use. TUNEL assay was performed according to the manufacturer’s instructions (Boehringer Mannheim, Germany). In short, terminal deoxynucleotidyl transferase was used to label the DNA strand breaks with modified fluorescein labelled nucleotides. For light microscopy detection, the incorporated fluorescein was detected by antifluorescein antibody conjugated with AP. After substrate was added, slides were analysed by microscopy and image analysis to quantitate the staining.

Immunohistochemistry
Slides with 6 µm thick human intestinal biopsy sections were fixed with 1% paraformaldehyde, and mouse-antihuman CD3 (CLB) was used. Goat-antimouse-AP labelled antibody was used as a secondary antibody. Detection was done using Fast Red (Dako, Glostrup, Denmark).

Flow cytometry
Jurkat cells were washed twice after stimulation. Cells were permeabilised using lysing and permeabilising solution (Becton Dickinson, Rutherford, New Jersey, USA). Antibodies used were antihuman-Bax (Immunotech, Marseille, France) and antihuman-Bcl-2 (Dako). After addition of the primary antibody, cells were incubated for 30 minutes on ice and washed twice in cold FACS buffer (phosphate buffered saline containing 1% bovine serum albumin, 0.3 mmol/l EDTA, 0.01% sodium azide). Subsequently, cells were incubated for 30 minutes with goat-antimouse-FITC labelled antibody for another 30 minutes on ice. After two washes, cells were analysed by FACScan (Becton Dickinson) and 10 000 cells were counted. After correcting for control IgG fluorescence, specific antibody binding was expressed as mean fluorescence intensity (MFI). Bax/Bcl2 ratios are expressed as MFI. During the six week period, blood samples of patients and six healthy volunteers (mean age was 31 years (range 26–43), four females, two males) were analysed for several markers.

Erythrocytes were lysed with ice cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 10 minutes. Cells were centrifuged at 600 g for five minutes at 4°C. The remaining cells were washed twice with cold FACS buffer. For staining, 1x10⁶ cells/well (96 well microplate; Greiner BV Labor Techniek, Alphen aan de Rijn, the Netherlands) were incubated with the following fluorescent labelled mouse monoclonal antibodies against humans: CD4, CD3, CD95, CD134, CD154, Bax, CD45RO, CD45RA (Immunotech), CD25, CD8, (CLB), Bcl2 (Dako), A487, CD120a, and CD120b (Sanbio). After addition of the primary monoclonal antibody to the cell suspension, the cells were incubated for 30 minutes at 4°C and washed twice in FACS buffer. TNF binding potential was measured using a FITC labelled human TNF-α. The appropriate isotype controls were included in all experiments. Lymphocytes were gated by forward and side scatter using a FACScan flow cytometer in conjunction with the FACScan software (Becton Dickinson, Mountain View, USA). A total of 5000 cells were counted. Results are expressed as the percentage of gated cells positive for the monoclonal antibodies used or as MFI after subtraction of control IgG fluorescence.

Interferon γ production by activated Jurkat cells
Interferon γ (IFN-γ) levels in Jurkat supernatants were measured by ELISA according to the manufacturer’s instructions (CLB).

Detection of anti-DNA antibodies
Sera were tested for DNA binding using an immunofluorescence technique on Crithidia luciliae. As FITC conjugates, antibodies to human Ig (SH17-01-F5; diluted 1/100), to human IgG (KH16-107-F3; 1/100), and to human IgM (KH15-F1; 1/50), all from CLB, were used.

Statistics
For the in vitro Jurkat experiments, statistical analysis was performed using the Wilcoxon test in SPSS for Windows version 7.5. For other experiments and the clinical study results, ANOVA and the Student’s t test were performed where appropriate.
RESULTS
Clinical outcome of infliximab treatment of Crohn’s disease patients
Ten patients with severe steroid refractory Crohn’s disease were treated with a single intravenous dose (5 mg/kg) of infliximab. Infusion of infliximab was well tolerated and no febrile or allergic reactions were observed. Nine patients had a clinical response, as defined as a reduction in CDAI of 70 points or more.

Figure 1 Clinical responses. (A) Boxplot of the Crohn’s disease activity index (CDAI) score of the 10 treated patients before (Pre) and one and six weeks after intravenous infusion of infliximab. *Non-responding patient. (B) Boxplot of C reactive protein (CRP) concentrations measured during the study period. *Relapsing patient.

Infliximab treatment induces anti-dsDNA antibodies
Sera of all patients were tested for DNA binding using an immunofluorescence technique on Crithidia luciliae.

Table 1 Differences in CD95 and CD134 expression (%), and tumour necrosis factor (TNF) binding potential

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=10)</th>
<th>Controls (n=6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD95/CD4⁺</td>
<td>69.2 [8.3]</td>
<td>40.1 [4.0]</td>
<td>0.026</td>
</tr>
<tr>
<td>CD134/CD4⁺</td>
<td>37.7 [6.8]</td>
<td>12.6 [8.6]</td>
<td>0.012</td>
</tr>
<tr>
<td>TNF binding granulocytes</td>
<td>39.7 [10.4]</td>
<td>63.1 [8.2]</td>
<td>0.036</td>
</tr>
<tr>
<td>TNF binding lymphocytes</td>
<td>22.1 [3.9]</td>
<td>86.3 [21.6]</td>
<td>0.012</td>
</tr>
</tbody>
</table>

Values are mean (SEM).

Six weeks after infusion of infliximab, four of 10 patients became positive for IgM anti-DNA antibodies.

No change in peripheral T lymphocyte markers after infliximab treatment
Peripheral T lymphocytes were frequently analysed throughout the study period for changes in expression of various membrane activation markers and intracellular Bax/Bcl-2 levels. No significant changes over time were noted for expression of activation markers (CD23, CD134, CD154), homing receptor (CD62L), memory cells (CD45RO, CD45RA), Fas expression (CD95), or Bax/Bcl-2 ratio. Expression of most of these markers was not different from normal healthy controls. However, compared with controls, baseline percentage of CD134 (patients 37.4%, controls 12.6%; p<0.05) and percentage of CD95 (patients 69.2%, controls 40.1%; p<0.05) positive T lymphocytes was increased in patients with Crohn’s disease. Infliximab treatment did not alter expression of these markers (table 1). Expression of both TNF receptors (p55 and p75) was similar in patients and controls but the TNF binding capacity of lymphocytes and granulocytes, as measured by FACScan analysis, was significantly decreased in patients with active Crohn’s disease (table 1). Infliximab treatment caused no significant change in the TNF binding capacity of lymphocytes or granulocytes during the six week study period. Hence the beneficial effects of infliximab treatment of active Crohn’s disease were not reflected in major changes in properties or apoptosis of circulating T lymphocytes.

Infliximab induces apoptosis of CD3 positive lamina propria T lymphocytes
Subsequently, we investigated whether cells present at the site of active disease were a target of infliximab induced apoptosis. To this end, the intestinal biopsies taken during endoscopies performed immediately before and 24 hours after infliximab infusion were analysed for apoptotic cells using the TUNEL assay. A quantitative image analysis method was used to
Infliximab treatment in Crohn’s disease

**DISCUSSION**

In the present study, a single infusion of infliximab resulted in a rapid clinical response in 9/10 patients with severely active Crohn’s disease. Activation markers and intracellular Bax/Bcl-2 concentrations were frequently assessed during the six-week study period using FACS analysis of peripheral blood mononuclear cells but no changes were observed. In contrast, in intestinal biopsies a significant increase in the number of apoptotic cells was found 24 hours after treatment, and by analysis of serial sections the vast majority of TUNEL positive cells were found to be CD3 positive. Furthermore, in vitro experiments with Jurkat cells suggest that this effect is mediated by a change in the Bax/Bcl-2 ratio whereas the functional consequences of infliximab treatment were reflected in a significant reduction in IFN-γ production by stimulated Jurkat cells. Hence our data indicate that infliximab treatment can induce apoptosis of activated but not resting T lymphocytes, and that infliximab treatment of Crohn’s patients causes a rapid and specific increase in the number of apoptotic T lymphocytes in the gut mucosal compartment, but not of peripheral blood mononuclear cells.

It has been reported previously that infliximab can cause complement dependent lysis of mononuclear cells but it is unlikely that this mechanism occurs in vivo. Firstly, infusion of infliximab is well tolerated and patients do not develop symptoms of a cell lysis syndrome. Secondly, in contrast with the effects of CD4 depleting antibodies, no significant decrease in peripheral blood mononuclear counts occurred after infliximab treatment. Indeed, as has been reported in rheumatoid arthritis patients, infliximab caused a transient lymphocytosis, which may be a result of acute inhibition of T lymphocyte trafficking to areas of inflammation. We interpret our results to indicate that the effects of infliximab in patients with Crohn’s disease are secondary to specific induction of apoptosis of lamina propria T lymphocytes. Apoptosis resistance of T lymphocytes has been implicated as a pathogenic mechanism in several immune mediated diseases, and it has been speculated that such a mechanism may constitute a genetic basis for multiple sclerosis. In Crohn’s disease, mucosal T lymphocytes are less susceptible to Fas mediated apoptosis, which coincides with an increased Bcl-2 concentration. It is uncertain whether Crohn’s disease patients have a genetic defect in activation induced T lymphocyte apoptosis, or whether the increased Bcl-2 concentration within mucosal T lymphocytes is a result of mucosal inflammation. Indeed, cytokines that activate the interleukin 2 common γ chain (interleukin 2, interleukin 7) are known to increase Bcl-2 concentrations within T lymphocytes. In the search for a mechanism of action, we have extended our clinical study with several ex vivo experiments. We found that incubation of CD3/CD28 stimulated Jurkat T lymphocytes with infliximab caused a significant reduction in IFN-γ production. Because infliximab does not recognise

[Figure 3: Infliximab induces apoptosis of CD3 positive T lymphocytes in the lamina propria of treated patients. (A) Boxplot of total number of apoptotic cells measured in biopsies taken before and 24 hours after treatment. (B) Immunohistochemical staining of two consecutive slides of a biopsy taken one day after treatment of one patient. The TUNEL positive cells (left) are located on the same site as the CD3 positive cells (right).]
IFN-γ, an effect on T lymphocyte survival was suspected. Indeed, very similar effects on CD3/CD28 stimulated IFN-γ production were observed when methotrexate, which is known to induce T lymphocyte apoptosis, was added. Indeed, using TUNEL assays, it became clear that infliximab caused significant induction of activation induced apoptosis, which was associated with an increase in the Bax/Bcl2 ratio.

Although we have found that binding of infliximab to activated Jurkat cells results in a change in the Bax/Bcl-2 ratio, the precise mechanism of induction of apoptosis remains unclear, and it may seem paradoxical that neutralisation of TNF-α, a cytokine that is involved in apoptosis induction in certain types of cells, leads to apoptosis of T lymphocytes. On the other hand, TNF-α also induces the transcription factor nuclear factor κB that has strong antiapoptotic effects in T lymphocytes. Hence it is conceivable that neutralisation of soluble TNF-α would reduce nuclear factor κB activation which in activated T lymphocytes is known to protect against apoptosis induction, leaving the cells exposed to induction of apoptosis through Fas/Fas ligand interactions. Infliximab binds to membrane expressed TNF-α that has an unusually long intracellular tail, but is not thought to contain known signal transducing domains. None the less, it is possible that engagement of transmembrane TNF-α may cause “reverse signal transduction” through outside-in signal transduction. It is conceivable that such a mechanism results in a change in the Bax/Bcl-2 ratio, either directly by interfering with upstream elements of the apoptosis pathways in T lymphocytes, such as FLIP, or by upregulation of expressed molecules such as CTLA-4 that are known to alter T lymphocyte sensitivity of membranes to activation induced apoptosis.31 32

Several other antibodies have been reported to increase activation induced T lymphocyte apoptosis. Activation of the human HLA-1 α domain caused ceramide-caspase mediated Fas independent apoptosis, whereas horse antilymphocyte serum at a low concentration stimulates apoptosis of phytohaemagglutinin stimulated T lymphocytes through induction of Fas.33 34 Methotrexate also induced Fas independent apoptosis of phytohaemagglutinin stimulated but not resting peripheral blood lymphocytes, and ex vivo activation of peripheral blood lymphocytes of methotrexate treated rheumatoid arthritis patients resulted in apoptosis.35 It has been reported recently that both etanercept, a TNF-α neutralising molecule, and infliximab are strongly synergistic with methotrexate in the treatment of patients with rheumatoid arthritis.36 Although it is not known whether etanercept, like infliximab, alters the susceptibility of T lymphocytes to activation induced apoptosis, it is tempting to speculate that combinations of drugs that alter T lymphocyte apoptosis through different mechanisms may become effective strategies for the treatment of diseases that are associated with local T lymphocyte activation, such as rheumatoid arthritis, multiple sclerosis, and Crohn's disease.37 Such combinations may effectively eliminate the steroid refractory T lymphocyte clones that sustain chronic inflammation in these conditions, and thereby have the potential to modify the natural course of these diseases.

Infliximab treatment has been reported previously to (transiently) induce low titre anti-dsDNA antibodies in 3% of treated patients. The much higher incidence of anti-dsDNA antibodies in our study (4/10 patients) is presumably a result of earlier and more frequent sampling in the present study. The mechanism of anti-dsDNA antibody induction by infliximab remains uncertain, but in view of our finding that treatment causes rapid apoptosis of a significant number of lamina propria T lymphocytes, it is tempting to speculate that acute exposure to DNA is one of the causes.

In conclusion, infliximab induced activation dependent apoptosis of Jurkat T lymphocytes, possibly altering the Bax/Bcl-2 ratio. In patients with Crohn's disease, infliximab rapidly increased the number of apoptotic T cells in the inflamed mucosa, without affecting peripheral blood mononuclear cells. These data indicate that infliximab functions, in part, as an “immunotoxin” that specifically targets the mucosal T lymphocytes that are involved in the pathogenesis of Crohn's disease.

Authors’ affiliations
T ten Hove, C van Montfrans, Peppelenbosch, et al

Figure 4  Effects of infliximab on Jurkat T cells. [A] Bax/Bcl2 ratio in Jurkat cells determined by FACSscan analysis. Data are expressed as mean (SEM) of six independent experiments. Jurkat cells were incubated for 24 hours with IgG isotype control, infliximab (Inflix), RPMI, and anti-Fas antibody. Cells were either not stimulated (left) or stimulated with anti-CD3/anti-CD28 antibodies (right). (B) Quantitative results of a TUNEL assay on cytospins of Jurkat cells. TUNEL positive cells were counted in non-stimulated (left) and stimulated (right) cells.
REFERENCES