Differences in the Intrinsic Capacity of Peripheral Blood Mononuclear Cells to Produce Tumor Necrosis Factor Alpha and Beta in Patients with Inflammatory Bowel Disease and Healthy Controls

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Although the etiology of the inflammatory bowel diseases (IBD), Crohn's disease (CD), and ulcerative colitis (UC) is still unknown, clinical and experimental observations have elucidated several factors that play an important role in the pathogenesis of IBD. It is widely held that in these diseases, there is only one pathogenetic mechanism responsible for tumor necrosis factor alpha and beta in patients with inflammatory bowel disease and healthy controls. Scand J Gastroenterol 1995;30:1095–1100.

Background: Tumor necrosis factor alpha (TNFa) and beta (TNFβ) appear to play an important role in the regulation of the inflammatory response. The aim of the present study was to investigate the intrinsic capacity of peripheral blood mononuclear cells (PBMC) to produce these cytokines. Methods: PBMC from 41 patients with Crohn’s disease (CD), 32 with ulcerative colitis (UC), and 23 healthy controls (HC) were cultured for 48 h in the presence or absence of the T-cell activators anti-CD3 and anti-CD28. Biologically active total TNF (TNFa and β), TNFa, and TNFB production were measured using a bioassay for biologically active TNF and specific TNFa and TNFB enzyme-linked immunosorbent assays. Results: Large interindividual differences in TNF production were observed. Production of biologically active TNF after T-cell stimulation was significantly decreased in UC patients as compared with HC and CD patients (median, 337 U/ml, 800 U/ml, and 1050 U/ml, respectively). Stimulated TNFa production in UC patients (median, 432 U/ml) and in CD patients (median, 337 U/ml, 800 U/ml, and 1050 U/ml, respectively). Stimulated TNFB production in UC patients (median, 337 U/ml, 800 U/ml, and 1050 U/ml, respectively). In contrast, stimulated TNFB production was statistically significantly from HC (median, 730 U/ml). In contrast, stimulated TNFB production was statistically significantly from HC (median, 730 U/ml). In contrast, stimulated TNFB production was statistically significantly from HC (median, 730 U/ml). In contrast, stimulated TNFB production was statistically significantly from HC (median, 730 U/ml). In contrast, stimulated TNFB production was statistically significantly from HC (median, 730 U/ml). Conclusions: These findings support the concept that UC and CD are not two homogeneous, clearly distinguishable disease entities but rather a heterogeneous group of diseases. Studies directed to assess the immunogenetic background of these different disease manifestations in IBD are underway.

Key words: Crohn’s disease; cytokines; inflammatory bowel disease; tumor necrosis factor; ulcerative colitis

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Table I. Demographic data and clinical characteristics of patients with inflammatory bowel diseases and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Patients, n</th>
<th>Age, years (range)</th>
<th>Sutherland score</th>
<th>CDAI</th>
<th>Medication</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(female/male)</td>
<td></td>
<td></td>
<td></td>
<td>Prednisone</td>
<td>Azathioprine</td>
</tr>
<tr>
<td>CD</td>
<td>41 (31:10)</td>
<td>32 (20–65)</td>
<td>&lt;150; n = 39</td>
<td>&gt;150; n = 2</td>
<td>n = 15</td>
<td>n = 4</td>
</tr>
<tr>
<td>UC</td>
<td>32 (15:17)</td>
<td>40 (19–75)</td>
<td>&lt;8; n = 31</td>
<td>&gt;8; n = 1</td>
<td>n = 7</td>
<td>n = 1</td>
</tr>
<tr>
<td>HC</td>
<td>23 (13:10)</td>
<td>34 (22–54)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n = Number of patients; CD = Crohn’s disease; UC = ulcerative colitis; HC = healthy controls; 5-ASA = 5-aminosalicylic acid.

ground might predispose to expression of a particular form of inflammatory response. There have been several studies on HLA associations in IBD. Although associations are not strong, an association between the HLA-DR2 allele and UC has been found earlier and was recently confirmed (10–13).

TNFα production in patients with IBD has been evaluated in several studies. These studies focused mainly on TNFα as an inflammatory mediator, and results of these studies are not consistent (14–29). This inconsistency may be due to measurement of different kinds of specimens, different methods of cell activation and measurement of TNFα, and patient selection. No studies on TNFβ in IBD have been performed until now. To evaluate whether IBD patients have primary abnormalities in the ability to produce the cytokines TNFα and TNFβ, we wished to evaluate the intrinsic capacity of peripheral blood mononuclear cells (PBMC) to produce these cytokines, both spontaneously and after stimulation with T-cell activators, in patients with IBD and healthy controls (HC).

PATIENTS AND METHODS

Patients

Blood samples were obtained from 73 IBD patients attending the outpatient department of gastroenterology over a 2-month period and who were in clinical remission. Blood samples from 23 unrelated healthy persons served as controls. Diagnosis of CD or UC was based on the conventional clinical, radiologic, endoscopic, and pathologic criteria, as described by Lennard-Jones et al. (30). UC disease activity was assessed using the Sutherland score (31). The Crohn’s disease activity index (CDAI) in accordance with Best et al. (32) was used for CD. Demographic data and clinical characteristics are summarized in Table I.

Methods

From each patient 10 ml of heparinized blood was collected. Vials were coded, and PBMC isolated within 2 h after blood collection. PBMC were isolated after density gradient centrifugation of heparinized blood on Lymphoprep (Nycomed A/S, Oslo, Norway), and washed three times with phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) (Gibco, Paisley, GB). After being washed, cells were suspended in RPMI 1640 with 25 mM Hepes (Gibco), with the following additives: l-glutamine, 2 mM (Gibco); penicillin, 100 U/ml; streptomycin, 100 μg/ml; and 10% human pooled serum (CLB, Amsterdam).

The isolated cells were cultured for 48 h at 37°C under 5% CO2 in humidified air in sterile culture flasks (Nunc, Roskilde, Denmark) in 5 ml culture medium at a concentration of 1 x 10⁶/ml. Cells were cultured in the presence or absence of both 1 μg/ml anti-CD3 (monoclonal 15E8) and 1 μg/ml anti-CD28 (monoclonal 16A9, both kindly provided by Dr. van Lier, CLB). Under these conditions a maximal T-cell response is achieved. After the culturing, cell culture supernatants were harvested, aliquoted, and stored at −80°C until assay.

TNFα and TNFβ measurements

Biologically active total TNF (TNFα plus TNFβ) was measured using the L929 bioassay, as previously described, with a few modifications (33). The murine L929 cell line is sensitive for both TNFα and TNFβ. In short, L929 cells (ATCC CCL 1; American Type Culture Collection, Rockville, Md., USA; 40,000 cells/well) were cultured 24 h in 96-well flat-bottom culture plates (Greiner, Alphen a/d Rijn, The Netherlands) at 37°C under 5% CO2 humidified air in 100 μl well of iscove’s culture medium enriched with 10% fetal calf serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml l-glutamine. Then, 100 μl of culture supernatant or rTNFα (Cetus, Emeryville, Calif., USA), diluted in Iscove’s medium, were added to each well, as was 25 μl/well of actinomycin D (0.02 mg/ml, diluted in Iscove’s medium) (ICN, Cleveland, Ohio, USA), and incubation proceeded for 24 h at 37°C. Living cells were fixed with 1% paraformaldehyde (Merck, Darmstadt, Germany) in PBS and stained with 0.5% crystal violet (ICN, diluted 1:4 in a methanol/water solution). Extinction was read at 590 nm in a Multiscan spectrophotometer (Titertek). Concentrations of total TNF are expressed as units per milliliter.

TNFα and TNFβ were determined using specific TNFα and TNFβ enzyme-linked immunosorbent assays (ELISAs). The TNFα ELISA has been reported to have a sensitivity of 10 pg/ml and an inter- and intra-assay coefficient of variance of less than 10% (34). According to the manufacturer, the TNFβ ELISA has a sensitivity of less than 50 pg/ml.
Fig. 1. Box-and-whisker plot showing the spontaneous and stimulated production of biologically active total tumor necrosis factor (TNFα and TNFβ) after 48 h of culture. Horizontal lines represent medians; boxes represent interquartile ranges; whiskers represent 10th to 90th centiles; and individual points represent outlying data. HC = healthy controls; UC = ulcerative colitis patients; CD = Crohn's disease patients. Values in units per milliliter; s = stimulated production.

For TNFα measurements, Nunc Maxisorp ImmunoPlates were incubated overnight at 4°C with 100 μl/well of anti-TNFα, 1.25 μg/ml (monoclonal 61E71, kind gift from Dr. Buurman, Maastricht, The Netherlands). After blocking with 1% BSA in PBS, samples were added and incubated for 3–4 h at 20°C. Plates were subsequently incubated with 100 μl/well of polyclonal rabbit anti-TNF serum (1:1000, Buurman) for 1 h and 100 μl/well horseradish-labeled swine anti-rabbit (1:500, Dako). Finally, 100 μl/well of substrate (3,3,5,5-tetramethylbenzidine; Sigma, St. Louis, Mo., USA) in acetate buffer, pH5.5, together with H2O2 was added. Incubation was carried out for 30 min in the dark at room temperature. Reactions were stopped by adding 100 μl/well 1 N H2SO4. Absorption was read out at 450 nm. Concentrations of TNFα are expressed as units per milliliter.

For TNFβ determination the same procedure as described for TNFα determination was followed, with a few exceptions. The monoclonal anti-TNFβ was obtained from Boehringer (monoclonal 9B9, Boehringer Cell Biology, Mannheim, Germany; 2 μg/ml); the polyclonal anti-TNFβ serum was a kind gift from Dr. Scuderi (Miles Inc., Pharm. Div., West Haven, Conn., USA) and was used in a dilution of 1:1000. All incubations were carried out in 50 μl. Concentrations of TNFβ are expressed as units per milliliter.

Statistical analysis

Differences between groups were examined with the Mann–Whitney test. A p value <0.05 was considered statistically significant (35).

RESULTS

Production of biologically active TNF

The production of biologically active total TNF on stimulation with anti-CD3 and anti-CD28 was significantly decreased in UC patients as compared with HC (median, 337 U/ml, versus 800 U/ml in HC). Stimulated total TNF production in CD patients (median, 1050 U/ml) did not differ statistically from HC. It is interesting to note that there is a subgroup of CD patients who produced very high levels of biologically active TNF. The diminished capacity of UC patients to produce TNF was also reflected in the spontaneous production of biologically active TNF (median, 45 versus 125 U/ml in HC) (Fig. 1).

TNFα production

The spontaneous and stimulated TNFα production, as measured with a specific TNFα ELISA, are depicted in Fig. 2. Large interindividual differences in TNFα secretion were observed. In a subgroup of CD patients very high levels of TNFα were found. A tendency towards lower TNFα production in UC patients as compared with HC (median, 432 versus 730 U/ml) was observed. However, the differences in stimulated production did not reach statistical significance when groups were compared.

There were no statistically significant differences in spontaneous TNFα production between the three groups studied.

TNFβ production

The stimulated TNFβ production, as detected by a specific TNFβ ELISA, was significantly higher in CD patients than in HC (median, 1637 versus 800 U/ml) (Fig. 3). Median stimulated TNFβ production in UC patients (837 U/ml) did not differ from that in HC. Spontaneous TNFβ production was not measurable in any of the individuals tested.
TNF production in correlation to disease factors and medication

As shown in Table I, we have studied mainly patients who were in clinical remission. In fact, only two patients with CD had an activity index (CDAI) higher than 150, and of the ulcerative colitis patients only one had moderate–severe active disease, according to the Sutherland score. Therefore, no correlation between disease activity and TNF secretion could be analyzed.

With regard to the influence of therapy on cytokine production, Table II shows that steroids had no significant effect in patients with UC and CD. No significant differences were observed in relation to the total TNF production, unstimulated TNFα production, or treatment with mesalazine (5-aminosalicylic acid) (results not shown).

DISCUSSION

The intrinsic capacity of PBMC to produce the cytokines TNFα and TNFβ, spontaneously and after stimulation with T-cell activators, was studied. Spontaneous and stimulated production of biologically active TNF was significantly decreased in UC patients as compared with HC. Although statistically not significant, there was also a tendency towards
lower TNFα production in UC patients as compared with HC. The stimulated TNFβ production was significantly increased in CD patients as compared with HC (Figs. 1–3). More importantly, large interindividual differences in TNFα and TNFβ secretion were observed. As these interindividual differences in production were found in a group of patients who, with regard to disease activity, was homogeneous, this strongly suggests that the differences observed are not simply due to the inflammatory process but rather reflect interindividual differences in the ability to produce these cytokines.

There have been several studies on TNFα in IBD. TNFα levels have been studied in serum, in stools, in the mucosa of the gut, and in isolated PBMC. The results of these studies are not consistent (14–29). When TNF production by PBMC was investigated, lipopolysaccharide (LPS) was usually used as stimulus. LPS predominantly activates monocytes. As T cells are thought to be the essential cells in the pathogenesis of autoimmune diseases, we activated the PBMC with specific T-cell activators. To achieve maximal T-cell responses, we cultured the cells for 48 h.

Why do some individuals produce large amounts of TNFα and TNFβ, whereas others do not? Several studies have demonstrated that there are associations between HLA-class II alleles and TNFα inducibility. It is now established that HLA-DR2 is associated with low TNFα production, whereas HLA-DR3 and HLA-DR4 are associated with high TNFα production (36,37). For TNFβ, these associations have not yet been established. The exact reason for the association between certain HLA alleles and TNF inducibility is unknown. HLA-linked polymorphic variations within the TNF genes have been claimed to be responsible for differences in secretion (38–42). Preliminary results from our laboratory strongly support this suggestion (43).

What is the importance of the present findings for the predisposition to IBD? The inflammatory bowel diseases are traditionally divided in two clinical forms, CD and UC. Individual CD or UC patients, however, can differ greatly in their disease course, prognosis, and response to medical treatment. This suggests that UC and CD are not two homogeneous, clearly distinguishable disease entities but rather a heterogeneous group of diseases. Therefore, clinical subgroups have been defined (44,45). Immunogenetic studies may be helpful to clarify the biologic basis of these subgroups. In support of this, it was recently shown that associations exist between intractability of UC and certain HLA class-II subtypes. Patients with extensive and intractable UC had more commonly the HLA-DR2 (DRB1*1502) and HLA-DRw11 subtypes than those with limited colitis or without intractability (46). The idea that a different immunogenetic background may underlie these different disease manifestations in IBD is attractive and may give a better understanding of the pathogenesis and be helpful in designing a more rational therapeutic approach for patients with these diseases.

ACKNOWLEDGEMENTS

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REFERENCES


