T cell specificity and cross reactivity towards enterobacteria, *Bacteroides*, *Bifidobacterium*, and antigens from resident intestinal flora in humans

R Duchmann, E May, M Heike, P Knolle, M Neurath, K-H Meyer zum Büschenfelde

**Abstract**

**Background**—T cell responses to normal intestinal bacteria or their products may be important in the immunopathogenesis of chronic enterocolitis.

**Aims**—To investigate the T cell specificity and cross reactivity towards intestinal bacteria.

**Patients/Methods**—T cell clones were isolated with phytohaemagglutinin from peripheral blood and biopsy specimens of inflamed and non-inflamed colon from five patients with inflammatory bowel disease (IBD) and two controls. T cell clones were restimulated with anaerobic *Bacteroides* and *Bifidobacterium* species, enterobacteria, and direct isolates of aerobic intestinal flora. T cell phenotype was analysed by single-cell immunocytoassay.

**Results**—Analysis of 96 T cell clones isolated from peripheral blood and biopsy specimens from two patients with IBD showed that both *Bifidobacterium* and *Bacteroides* species specifically stimulate proliferation of CD4+TCRβ+ T cell clones from both sites and that cross reactivity exists between these anaerobic bacteria and different enterobacteria. Analysis of 210 T cell clones isolated from three patients with IBD and two controls showed that indigenous aerobic flora species specifically stimulate T cell clones from peripheral blood and biopsy specimens from a foreign subject. Some of these flora specific T cell clones were cross reactive with defined enterobacteria. In addition, T cell clones stimulated by their own indigenous aerobic flora were identified in patients with IBD.

**Conclusion**—Immune responses to antigens from the intestinal microflora involve a complex network of T cell specificities.

(Keywords: inflammatory bowel disease; T cells; intestinal flora; mucosal immunity)

Inflammatory bowel disease (IBD) comprises two forms of chronic intestinal inflammation, Crohn’s disease and ulcerative colitis. The cause of IBD is still not known, but there are numerous indications that T cells are important in IBD pathogenesis. The antigens that drive T cell activation and clonal expansion in IBD still need to be defined but both clinical and experimental evidence strongly incriminates normal luminal bacteria as a source of antigen. Normal luminal bacteria comprise an estimated number of 400 different bacterial species and reach their highest concentrations in the terminal ileum and colon. Anaerobes outnumber aerobes by a factor of 103 in the lumen, but are represented in almost equal numbers at the bowel wall. A large variety of observations support the hypothesis that IBD is due to T cell hyperresponsiveness towards otherwise harmless components of the normal intestinal flora. As a clinical observation, IBD manifests itself most frequently in the terminal ileum and colon—that is, those sites with the highest bacterial concentrations in the intestine. It has been found that Crohn’s disease relapses are prevented by diversion of the faecal stream, increases in gut permeability precede acute flares in Crohn’s disease, and antibiotic treatment is effective in both Crohn’s disease and ulcerative colitis. In addition, intestinal lesions of patients with IBD are characterised by increased T cell reactivity towards bacterial antigens, an increase in enterobacteria specific T cells, and a loss of tolerance towards indigenous flora. A role for bacteria in the pathogenesis of chronic intestinal inflammation has also been well established in different inducible animal models of intestinal inflammation. Furthermore, in interleukin–2 knockout mice, interleukin–10 knockout mice, and HLA-B27 transgenic rats, intestinal inflammation improves under germ free or specific pathogen free conditions, whereas germ free HLA-B27 transgenic rats develop chronic intestinal inflammation when colonised with normal luminal bacteria, especially *Bacteroides vulgatus*. As the critical bacteria in most animal models are still not known, it may be of note that *B vulgatus* has also been shown to play an essential role in the pathogenesis of carrageenan induced colitis in guinea pigs. A variety of changes implicating luminal bacteria in disease pathogenesis were observed in rodent models of colitis induced by the hapten reagent trinitrobenzenesulphonic acid. Colitis induced by this reagent in mice is associated with a TH1 cytokine profile and can be transferred to naive recipient animals with purified CD4 T cells from the colon of...
T cell responses to intestinal bacteria

with the Declaration of Helsinki ethical guide,
reviewed by the local ethics committee and all
from closely adjacent areas. The study was
confirmed by examination of biopsy specimens
assessed during endoscopy and histologically
intestinal biopsy samples was macroscopically
day. Inflamed or non-inflamed status of all
were taken during the same endoscopic proce-
dure and blood was withdrawn on the same
day. Inflamed or non-inflamed status of all
were treated with mesalazine or cortico-
steroids. In patients with ulcerative colitis, dis-
eease activity according to criteria established by
Truelove and Witts\(^3\) was moderate (n = 2) or
severe (n = 1). Patients were treated with mesalazine (n = 2) or corticosteroids (n = 1).
None of the patients or controls was treated
with antibiotics. Biopsy specimens from infl-
amed (B+) and non-inflamed (B−) intestine were
taken during the same endoscopic proce-
dure and blood was withdrawn on the same
day. Inflamed or non-inflamed status of all
intestinal biopsy samples was macroscopically
assessed during endoscopy and histologically
confirmed by examination of biopsy specimens
from closely adjacent areas. The study was
reviewed by the local ethics committee and all
patients gave informed consent conforming with
the Declaration of Helsinki ethical guide-
lines.

PREPARATION OF MONONUCLEAR CELLS
Peripheral blood mononuclear cells were iso-
lated from heparinised peripheral blood by
Ficoll-Hypaque (Seromed, Berlin, Germany)
density gradient centrifugation. Lamina pro-
pria mononuclear cells were isolated from
intestinal biopsy specimens by following an
established protocol using collagenase
treatment.\(^3\) For cryopreservation, mono-
nuclear cells and T cell clones were stored in
90% fetal calf serum and 10% dimethyl
sulphoxide in liquid nitrogen. For generation of
monocyte- and B-cell-enriched mononuclear
cells (E−), T cells were removed by rosetting
with neuraminidase treated sheep erythrocytes
as described.\(^5\)

GENERATION OF T CELL CLONES
Lamina propria T cells and matched peripheral
blood T cells were cloned using phytohaemag-
glutinin, as reported previously.\(^11\) In brief, T
cells separated by E-rosetting were incubated in
Terasaki plates at 0.3 cells/well together with
12 000 cells/well of allogeneic irradiated (40 Gy)
feeder cells from buffy coats in RPMI
medium containing phytohaemagglutinin (1
µg/ml; Murex, Hannover, Germany), human
AB-Serum (10%) and interleukin-2 (10 U/ml;
Boehringer, Mannheim, Germany). Clonal T
cell populations were identified by eye at day
10 and expanded together with allogeneic irra-
diated (40 Gy) feeder cells. All T cell clones
reported in this study are independent. Clonal-
ity of T cell clones was confirmed on a
randomly selected T cell clone population (n =
10) by polymerase chain reaction (PCR) using
primers specific for 20 different T cell receptor
(TCR)-V\(\beta\) families as described.\(^6\) Phenotyping
of T cell clones was performed using anti-
CD4 (Dianova, Hamburg, Germany), anti-
CD8 (Dianova), and anti-TCR\(\beta\) (ATCC HB
9283) monoclonal antibodies in a single-cell
immunocyte assay.

ANTIGENS
*Bacteroides thetaitaomicron* (ATCC 12290, iso-
lated from purulent material from an incision
during appendicectomy), *Bifidobacterium bifi-
dum* (ATCC 35914, isolated from human faeces),
and *Helicobacter pylori* (NCTC 11637) were
obtained from ATCC. Enterobacteria
were obtained from the Institute of Medical
Microbiology, University of Mainz. *Escherichia
coli* was isolated from a routine agar culture,*Salmonella typhimurium* and *Yersinia enterocol-
tica* 03 were cultured from the stools of
patients with enterogenic reactive arthritis.\(^6\)
Bacteria from the indigenous intestinal flora
were obtained from biopsy material and grown
on blood agar plates as reported previously.\(^6\)
After harvesting, all micro-organisms were
washed, resuspended in phosphate buffered
saline, and sonicated. Sonicates were stored at
\(-80^\circ\text{C}\) and tested for sterility by incubation on
agar plates. Depending on the mononuclear
cells with which they were incubated, sonicates
from bacteria of the intestinal flora were used
as bacteria from autologous intestine (BsA;
sonicates and mononuclear cells from the same
subject) or as bacteria from heterologous intesti-
tine (BsH; sonicates and mononuclear cells
from different subjects). Protein concentra-
tions were determined by the Lowry method
(Bio-Rad protein assay; Bio-Rad, Munich,
Germany). Tetanus toxoid was a gift from
Behring-Werke, Mannheim, Germany.
PROLIFERATION ASSAYS

Proliferative responses were determined in triplicate in 200 µl culture medium (RPMI/10% AB-Serum) containing 5 × 10^4 peripheral blood mononuclear cells or 1 × 10^4 cloned responder T cells and 2 × 10^4 autologous irradiated (40 Gy) E− antigen presenting cells in 96-well microtitre plates (Nunc, Roskilde, Denmark). Bacterial sonicates (E coli, Y enterocolitica, S typhimurium, H pylori, B thetaiotaomicron, B bifidum, BsA, BsH) were added at 50 µg/ml. Medium, tetanus toxoid (10 µg/ml), and phytohaemagglutinin (1 µg/ml) served as controls. Cultures with peripheral blood

**Figure 1** Clonal T cell responses towards Bacteroides and Bifidobacterium. T cell clones were isolated, using phytohaemagglutinin (PHA), from two patients with ulcerative colitis (patient R: peripheral blood n = 14, non-involved colon n = 12, involved colon n = 18; patient B: peripheral blood n = 12, non-involved colon n = 17, involved colon n = 23) and restimulated with the indicated antigens for 42 hours. Mean proliferation of T cell clones from peripheral blood (RP3), non-involved colon (RB−9 and BB−17), and involved colon (RB+2, RB+16, BB+6, and BB+22) stimulated by Bacteroides thetaiotaomicron (ATCC 12290) or Bifidobacterium bifidum (ATCC 35914) is shown as measured by [3H]thymidine incorporation in triplicate cultures. Clone BP6 is representative of other T cell clones reactive with enterobacteria only. All T cell clones were CD4+TCR^+ by single-cell immunocyte assay.

**Figure 2** Clonal T cell responses to aerobic intestinal flora. T cell clones were isolated with phytohaemagglutinin (PHA) from two controls (patient A, bile acid induced diarrhoea: peripheral blood n = 23, colon n = 15; patient W, irritable bowel syndrome: peripheral blood n = 20, colon n = 19) and restimulated with the indicated antigens for 42 hours. Aerobic intestinal flora was obtained from intestinal biopsy specimens touched to blood agar plates and grown under aerobic conditions. Sonicates from outgrowing bacteria were used as BsA (sonicates and mononuclear cells from the same person) or BsH (sonicates and mononuclear cells from different people). Mean proliferation of T cell clones from peripheral blood (WP2 and WP18) and colon (AB−3, WB−5, and WB−15) stimulated by BsA or BsH is shown as measured by [3H]thymidine incorporation in triplicate cultures. All T cell clones were CD4+TCR^+ by single-cell immunocyte assay. Incubation of antigen presenting cells with the indicated stimuli without T cell clones resulted in <500 cpm.
mononuclear cells were pulsed with [3H]thymidine (0.5 µCi/well) at day 5, and cultures with T cell clones were pulsed with [3H]thymidine (0.5 µCi/well) after 24 h. All cells were harvested 18 hours after being pulsed. [3H]Thymidine incorporation measured in triplicate was expressed as (mean (SD)) cpm.

Results

CLONAL T CELL RESPONSES TOWARDS ANAEROBIC BACTEROIDES AND BIFIDOBACTERIUM

We have previously shown that CD4 T cells specifically recognise different enterobacteria and that enterobacteria specific T cell clones are increased in intestinal IBD lesions. Using new T cell clones we now determined the T cell response to major constituents of the anaerobic intestinal flora in humans—that is, *Bacteroides* and *Bifidobacterium*—and assessed whether T cell cross reactivity between anaerobes and enterobacteria occurs. T cell clones were isolated from peripheral blood (n = 26) and lamina propria T cell populations (n = 70) from two patients with IBD (both ulcerative colitis) as described above, and subsequently restimulated with sonicated preparations from enterobacteria, *H pylori*, *B thetaiotaomicron*, and *B bifidum*. Confirming previous data, T cell clones responding to enterobacteria were obtained (fig 1). In addition, [3H]thymidine incorporation identified a total of seven out of 96 T cell clones from peripheral blood and intestine with reactivity towards *B thetaiotaomicron* and *B bifidum*. Of these seven T cell clones, two were reactive towards *B thetaimo-
mean proliferation of triplicate cultures measured by 
then incubated with T cell clone FB−10 for 48 hours at optimal concentration. Data show colonies were isolated, expanded in Luria-Bertani medium, and sonicated. Sonicates were of aerobic intestinal bacteria used for the generation of the original BsH sonicate, 100

Figure 3 Reactivity of BsH specific T cell clone FB−10. From the heterogeneous mixture of aerobic intestinal bacteria used for the generation of the original BsH sonicate (fig 3). Using these intestinal bacteria used for the generation of enterobacterial colonies. In all, this indicated that antigen(s) recognised by BsH reactive T cell clone FB−10 were shared by different enterobacteria but not expressed by all bacteria of a given genus.

Discussion

The intestinal flora is increasingly incriminated as a source of antigens inducing or perpetuating intestinal inflammation in IBD. Therefore bacteria specific or antigen specific immunomodulation of antibacterial immune responses may represent an option for IBD treatment. The feasibility of this approach, however, will depend on the complexity of the cellular immune response to intestinal bacteria, the nature of the relevant antigens, and whether some bacterial species are more relevant to the inflammatory process than others.24

To address these questions, we determined the complexity of the CD4+ TCRβ+ immune response to antigens from the intestinal flora and show that: (a) both anaerobic intestinal bacteria and enterobacteria express discrete and shared antigens, (b) T cell clones cross react with anaerobic intestinal bacteria and enterobacteria, (c) T cell clones recognising foreign intestinal flora can cross react with enterobacteria, (d) T cell clones recognising indigenous aerobic intestinal flora of the same subject can be isolated in patients with IBD. These data from peripheral blood and lamina propria T cells therefore support the concept that the CD4+ TCRβ+ T cell response to antigens from the resident intestinal flora in controls and patients with IBD is specific but also comprises the potential for broad cross reactivity between different bacterial species.

In IBD, and here especially in Crohn’s disease, abnormalities in the composition of the intestinal flora have been shown for variety of aerobic and anaerobic bacteria.37–42 Providing a link to the pathological immune response associated with Crohn’s disease inflammation and tissue injury, these descriptive studies have been complemented by investigations of the humoral or cellular antibacterial immune response. Thus increased T cell reactivity towards E.coli and other microbial recall antigens and an increase in enterobacteria specific T cells in intestinal lesions of patients with IBD have implicated T cell responses to enterobacterial antigens in IBD. With regard to anaerobic bacteria, luminal concentrations of Peptostreptococci, Eubacteria, and Coprococcus40 and serum antibodies to these bacteria were found to be selectively increased in patients with Crohn’s disease. Furthermore, purified peptidoglycan–polysaccharide polymers from Peptostreptococcus, Eubacteria, Salmonella, E coli, and E.coli were shown to induce chronic inflammation in susceptible rat strains.43

As we had previously determined the response of human CD4+ TCRβ+ T cell clones

<table>
<thead>
<tr>
<th>Non-stimulating bacterial colonies</th>
<th>Stimulating bacterial colonies</th>
</tr>
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<tbody>
<tr>
<td>3 Salmonella, E.coli</td>
<td>9 E.coli, Proteus</td>
</tr>
<tr>
<td>17 Salmonella, Y enterocolitica,</td>
<td>13 E.coli, Citrobacter freundii</td>
</tr>
<tr>
<td>03 or 09</td>
<td></td>
</tr>
<tr>
<td>33 E.coli, Y enterocolitica,</td>
<td>26 E.coli, Proteus</td>
</tr>
<tr>
<td>03 or 09</td>
<td>37 E.coli, Proteus</td>
</tr>
<tr>
<td>44 E.coli, Proteus</td>
<td>56 Proteus, Y enterocolitica,</td>
</tr>
<tr>
<td>03 or 09</td>
<td>57 Proteus, Y enterocolitica,</td>
</tr>
<tr>
<td>50 E.coli, Proteus</td>
<td>70 Y enterocolitica,</td>
</tr>
<tr>
<td>52 Salmonella</td>
<td>92 E.coli, Proteus, Y enterocolitica,</td>
</tr>
<tr>
<td>03 or 09</td>
<td>95 E.coli, Proteus</td>
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<tr>
<td>76 Proteus, Y enterocolitica,</td>
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</tr>
<tr>
<td>03 or 09</td>
<td>80 E.coli, Y enterocolitica,</td>
</tr>
<tr>
<td>87 Proteus, Y enterocolitica,</td>
<td>95 E.coli, Proteus</td>
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From patients with IBD with reactivity towards BsH. In controls, three of five BsH reactive T cell clones were stimulated by BsH only and two of five T cell clones (WB−5 and WB−15) cross reacted with either E.coli or S. typhimurium. In patients with IBD, 15 out of 21 T cell clones were stimulated by BsH only, two out of 21 T cell clones (DB−8 and SP24) showed weak cross reactivity between BsH and H pylori or BsH, H pylori, and E.coli, and four out of 21 T cell clones (DP15, DP18, DB−5 and FB+2) were cross reactive with BsA and BsH. The fraction of T cell clones recognising autologous bacteria was 0/77 in controls and 8/133 in patients with IBD. Four of eight T cell clones (DP15, DP18, DB−5, and FB+2) were cross reactive with BsA and BsH, and four of eight T cell clones (DB+5, DB+8, FP15, and FB+11) reacted to BsA only.

REACTIVITY OF BS H SPECIFIC T CELL CLONE FB−10

Antigens from the indigenous intestinal flora stimulating T cell clones may be expressed by one or different bacterial species or bacterial strains. To investigate this question, we determined the proliferative response of the BsH reactive T cell clone FB−10 to sonicates from 100 different bacterial colonies, all isolated from the heterogeneous mixture of aerobic intestinal bacteria used for the generation of the original BsH sonicate (fig 3). Using these sonicates, 10 out of 100 bacterial colonies were found to stimulate T cell clone FB−10. By microbiological analysis, nine stimulatory colonies and 11 colonies without stimulatory capacity for T cell clone FB−10 were each found to contain a mixture of between one and three different enterobacteria. Thus enterobacteria constituted most of the direct aerobic intestinal isolates used in this study. In addition, the same bacterial species were found in both stimulating and non-stimulating groups of enterobacterial colonies. In all, this indicated that antigen(s) recognised by BsH reactive T cell clone FB−10 were shared by different enterobacteria but not expressed by all bacteria of a given genus.
to different enterobacteria and *H pylori*, we now used *Bacteroides* species, *Bifidobacterium* species, and direct isolates from indigenous intestinal flora to investigate the potential complexity of the immune response to different components of the intestinal flora. Importantly, the bacterial antigens used in this study differ from each other in various ways. Firstly, *Bacteroides* and *Bifidobacterium* are predominant anaerobic isolates from normal faecal— that is, luminal colonic bacteria—whereas direct isolates from indigenous intestinal flora were generated using a technique favouring the growth of aerobic intestinal bacteria from the bowel wall. The bacterial flora at the bowel wall differs considerably from the faecal flora, and in the distal ileum and colon contains a much higher percentage of aerobic bacteria. As it is not known whether luminal bacteria or bacteria at the bowel wall are more relevant to the mucosal immune response in IBD, it may be important to study the two groups of bacteria separately. Secondly, *B thetaiotomicron* had been isolated from purulent material from an incision during appendicectomy, whereas *B bifidum* had been isolated from human faeces. Thirdly, *Bacteroides* and *Bifidobacterium* were established isolates from foreign donors, whereas direct isolates from indigenous intestinal flora allowed us to test the immune response of a subject towards antigens from his/her own flora.

Using these antigens for stimulation of T cell clones, our results provide evidence that not only enterobacteria and *H pylori* but also members of the anaerobic intestinal flora express dominant discrete protein antigens recognised by specific T cells. Cross reactive T cell clones in our study show that anaerobic bacterial species share antigens with one another and *Y enterocolitica*. Thus, complementing T cell specificity, cross-reactivity, as shown between different enterobacteria, different intestinal anaerobic species as well as enterobacteria and intestinal anaerobic species, is a distinct feature of CD4+ TCRβ+ T cell responses to intestinal bacteria. These findings suggest that CD4+ TCRβ+ T cell mediated immune responses to different bacteria within the intestinal flora, relating to either protection or pathogenicity, are regulated by a complex network of T cell specificities. The mechanisms regulating these immune responses are not understood but certainly contrast with the non-specific antibacterial immune response of TCRγδ+ T cells. In this context, it may be interesting that antigenic cross reactivity between autochthonous *Bacteroides* species and intestinal tissue of neonatal mice has been suggested as a mechanism for causing selective unresponsiveness to *Bacteroides* species in adult mice. *B vulgatus* in contrast has recently been identified as a critical bacterium mediating chronic colitis, gastritis, and arthritis in HLA-B27/human β2-microglobulin transgenic rats. In addition, the same bacterium was shown to play an essential role in the pathogenesis of carrageenan induced colitis in guinea pigs. The reason for the increased capacity of *B vulgatus* to induce intestinal inflammation in these animal models is not known and may include immunological and non-immunological causes. In contrast, *Bifidobacterium*, for which there was cross reactivity with *B thetaiotomicron* in this study, has been associated with qualities that are beneficial to the host. Thus it has been suggested that cell association with enterocytes and epithelial invasion by pathogenic enterobacteria are inhibited by *Bifidobacteria*. Furthermore, *Bifidobacteria* have been shown to excrete antimicrobial substances and to reduce the incidence of acute diarrhoea and rotavirus shedding in infants.

As mentioned above, the intestinal flora is a complex ecosystem. It reaches stable expression shortly after birth and its individual composition is influenced by the genetic characteristics of the host. Therefore instead of using established or non-indigenous bacterial isolates, it may be more reasonable to investigate the immune response to bacteria isolated from an individual’s own intestinal flora. Using this approach, we have recently shown using mononuclear cell bulk cultures that controls are specifically tolerant to their own flora and that this tolerance is lost in intestinal lesions of patients with IBD. Investigating the response of clonal T cells, here we show that CD4+ TCRβ+ T cells from peripheral blood and intestinal mucosa specifically recognise antigens from foreign flora and, at least in patients with IBD, also their own intestinal flora. The results showing that clonal T cells cross react with their own and foreign intestinal flora as well as enterobacteria were expected. They reflect the fact that different people share a pool of indigenous bacteria and that our technique for direct isolation of indigenous intestinal bacteria favoured growth of aerobic bacteria from the bowel wall. This was also shown by the fact that enterobacteria were exclusively found in an isolate stimulating BsH specific T cell clone FB-10. Adding a further level of complexity, a more detailed analysis of the bacteria recognised by BsH specific T cell clone FB-10 indicated that the relevant antigens were expressed by different enterobacteria and that antigen expression by these enterobacteria was not constitutive.

T cell clones with specificity for their own intestinal flora in IBD lesions may be the cellular equivalent of the loss of tolerance to BsA observed in mucosal mononuclear bulk cultures. However, BsA reactive T cell clones were also present in peripheral blood mononuclear cells and uninvolved mucosa of these patients, which were tolerant in bulk culture. This indicates that, in these compartments, suppression or anergy rather than deletion may normally be responsible for tolerance to BsA.

T cell cross reactivity between different intestinal bacteria suggests that targeting of single bacterial species may not significantly reduce intestinal inflammation in IBD. This does not, however, exclude the possibility that T cell reactivity in IBD is directed against a limited number of dominant bacterial antigens, especially if there is no strict correlation between expression of stimulating antigens and
bacterial genus or species. Further studies using clonal T cells to define in detail the relevant antigens and their expression among different intestinal bacteria will be required to gain a better understanding of antibacterial immunity. IBD pathogenesis, and whether antigen specific immunomodulation of antibacterial immune responses is a viable option for IBD treatment.

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