Bacterial translocation and immunohistochemical measurement of gut immune function

N P Woodcock, J Robertson, D R Morgan, K L Gregg, C J Mitchell, J MacFie

Abstract

***Aims*—The local immune response in the small bowel mucosa might play a role in bacterial translocation (BT). The aim of this study was to quantify immune cells and secretory antibodies in the small bowel mucosa, and relate this to BT as assessed by culture of a mesenteric lymph node.**

***Methods*—Immunohistochemical techniques were used to measure the frequency of plasma cells and IgA and IgM positive cells in the lamina propria and semiquantitatively to assess mucosal surface IgA and IgM values in small bowel specimens obtained from 11 patients in whom positive evidence of BT had been identified in a mesenteric lymph node harvested at the time of laparotomy. These were compared with similar specimens obtained from 11 patients in whom a similar lymph node had yielded no growth.**

***Results*—BT was associated with a significantly increased median frequency of plasma cells ($p < 0.01$) and IgA positive cells ($p < 0.05$) in the lamina propria. The frequency of IgM positive cells was also higher in these patients, although this difference was not significant. In addition, semiquantitatively scored IgA and IgM concentrations at the mucosal surface were both significantly higher in the patients in whom BT had been identified ($p = 0.006$ and 0.016, respectively).**

***Conclusion*—Higher numbers of plasma cells and higher IgA and IgM values are present in the small bowel mucosa of patients in whom BT has been shown to occur, suggesting an increased local immune response.**

(*J Clin Pathol* 2001;**54**:619–623)

Keywords: bacterial translocation; immunohistochemistry; immune function

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Accepted for publication
16 February 2001

Bacterial translocation (BT) can be defined as the passage of bacteria or their products from the bowel lumen across the lamina propria to local mesenteric lymph nodes, and from there to distant sites.[1] There is now good evidence to suggest that BT is associated with an increased incidence of septic morbidity in patients undergoing surgery.[1 2] In addition, BT has been implicated as a factor in the pathogenesis of multiple organ failure,[3] although its precise role has yet to be determined.[4]

Several factors have been proposed as promoters of BT. These include alterations in gastrointestinal microflora, impairment of gut barrier function, and deficiencies in host immunity.[5] Our own and other studies have established an association between bacterial colonisation of the proximal gastrointestinal tract, BT, and septic morbidity,[6 7] but have failed to confirm a causal relation between alterations in parameters of intestinal barrier function and BT.[8]

A recent study of patients with intra-abdominal sepsis demonstrated a significant reduction in IgA and IgM positive plasma cells in the lamina propria of the small bowel mucosa, and reduced immunoglobulin values at the mucosal surface.[9] This paper proposed that the stress response induced by severe sepsis results in a decrease in immunoglobulin production by mucosal plasma cells, facilitating the adherence of luminal bacteria to the enterocyte surface, which is an important initial step in the process of translocation.[10] However, BT was itself not assessed in this study.

The aim of our study was to investigate whether BT is associated with changes in gut immune function. Standard morphology and immunohistochemical techniques were used to measure the frequency of immune cells in the lamina propria, and immunohistochemistry alone to assess mucosal surface immunoglobulins in specimens of small bowel obtained at laparotomy. The occurrence of BT was determined by culture of a mesenteric lymph node.

Patients and methods

All patients included in our study were under the care of the combined gastroenterology unit at Scarborough Hospital. Approval for the study was obtained from the locally organised research ethics committee.

The patients were identified from a database containing the details of subjects included in a larger prospective study of BT, the results of which have been published previously.[2] Patients were selected if they had undergone a small bowel resection as part of the therapeutic surgical procedure, and stored tissue was available for analysis of gut immune function. These data have not been reported previously. The first group of patients (BT positive group) comprised subjects in whom BT had been confirmed by positive bacterial culture from a mesenteric lymph node (MLN). Previous animal studies have suggested that culture of lymph nodes from the ileocolic mesentery constitutes the gold standard method of assessment of BT.[11] The same number of patients with a negative MLN were selected at random as controls (BT negative group).

Table 1 Demographic data

	BT positive group (n = 11)	*BT negative group (n = 11)*
Male to female ratio	3:8	5:6
Median age (interquartile range)	70 years (54–74)	71 years (60–81)
Diagnosis		
Colorectal carcinoma	3	8
Ulcerative colitis	2	3
Strangulated femoral hernia	2	0
Crohn's disease	1	0
Angiodysplasia	1	0
Benign small bowel stricture	1	0
Enteroenteric fistula	1	0

BT, bacterial translocation.

TISSUE PROCESSING

The segments of normal small intestine removed at laparotomy had been fixed in 10% formal saline for between one and five days from the time of operation, and then processed to a paraffin wax block for storage. Sections were dewaxed in xylene and endogenous peroxidases were blocked using 1% hydrogen peroxide in methanol. Optimal unmasking of antibodies was achieved by incubating sections in protease (10 mg in 100 ml Tris buffered saline (TBS)) at 40°C for five minutes, followed by a five minute wash in running water and a further five minute wash in TBS.

Each section was treated with normal horse serum for 30 minutes to reduce background staining. Rabbit antibodies against IgM and IgA were used at dilutions of 1/1000 and 1/1500, respectively, and sections were incubated for 60 minutes. After TBS washes, biotin labelled secondary antibodies at a dilution of 1/200 were applied for 30 minutes. Slides were then washed in TBS, after which avidin–biotin complex diluted according to the manufacturer's instructions was applied for 30 minutes, followed by further TBS washes.

Demonstration of horseradish peroxidase was achieved using the substrate 3'3-diaminobenzene tetrahydrochloride. Sections were then counterstained with haematoxylin, dehydrated, cleared, and mounted in DPX mountant. Known positive control sections were included in the batch of immunostained specimens, and negative controls were provided by incubating sections from the same block with TBS in place of primary antibody.

To eliminate concerns that the intensity of staining could be influenced by laboratory temperature or duration of fixation of the small bowel specimens,[12] an initial experiment was performed whereby multiple segments from the same length of bowel were fixed at 16°C, 22°C, or 27°C for one, three, or five days before processing to wax and subsequent staining as described above. These criteria were chosen as representative of the range of normal practice in our laboratory. No significant differences in staining intensity for IgA or IgM were observed between the various segments. To avoid the variability of immunohistochemical staining that can occur when multiple staining runs are performed for a particular antigen or epitope, the staining for IgA and IgM were each performed as a single run. This obviously limited the number of patients that could be studied.

QUANTITATION

After staining each specimen was analysed by one consultant histopathologist (DRM), who was blinded as to whether the patient had translocated or not. Five parameters were assessed: (1) plasma cell numbers, (2) frequency of IgA positive cells in the lamina propria, (3) frequency of IgM positive cells in the lamina propria, (4) IgA values at the mucosal surface, and (5) IgM values at the mucosal surface. The plasma cells were identified by cell morphology alone, whereas IgA positive and IgM positive cells were identified according to whether or not they stained positively for the respective immunoglobulins. Cell numbers in the lamina propria were calculated using a 100 mm^2 graticule. To overcome the problem of glands encroaching upon the field of view the median total cell count in a random 10 mm^2 in five separate squares was multiplied by a factor of 10 to give a value in number of cells/cm^2. A semiquantitative method was used for the assessment of IgA and IgM values at the mucosal surface, whereby each specimen was scored 0–3 according to a subjective assessment of intensity of staining for the respective immunoglobulin (0, no staining; 1, low; 2, moderate; 3, high intensity). Until the technology for computer assisted image analysis is validated and becomes widely available this is the acknowledged method of analysis.[13 14]

All non-parametric data are expressed as medians (range). Statistical analysis of the quantitative data was performed using the Mann-Whitney U test. The qualitative data were analysed using Fisher's exact test for small numbers, using the mid p value because it is less conservative and therefore more powerful.[15] A p value of $\leqslant 0.05$ was classed as significant.

Results

In total, 22 patients were studied, 11 of whom were BT positive and 11 BT negative. Table 1 shows the demographic data, including indications for surgery. Fourteen organisms were grown on culture of the MLNs, of which nine were enteric species: *Escherichia coli* (n = 8), *Citrobacter freundii* (n = 1), *Staphylococcus epidermidis* (n = 2), *Streptococcus* spp. (n = 2), and *Pseudomonas aeruginosa* (n = 1).

The median frequency of plasma cells in the BT positive patients was 20/cm^2 (range < 10–70), significantly higher than that in the BT negative patients (median concentration, < 10/cm^2; range, < 10–40; $p < 0.01$, Mann-Whitney U test). The median frequency of IgA positive cells in the BT positive patients (130 cells/cm^2; range, 70–240) was significantly higher than in the BT negative subjects (100 cells/cm^2; range, 70–140; $p < 0.05$, Mann-Whitney U test). The median frequency of IgM positive cells was also higher in the BT positive patients than in the BT negative patients (40 cells/cm^2; range, 20–90 *v* 30/cm^2; range 10–50), although this difference was not significant ($p > 0.05$, Mann-Whitney U test) (fig 1).

Table 2 shows the results obtained from the analysis of IgA and IgM values at the mucosal surface. Some degree of staining was present in

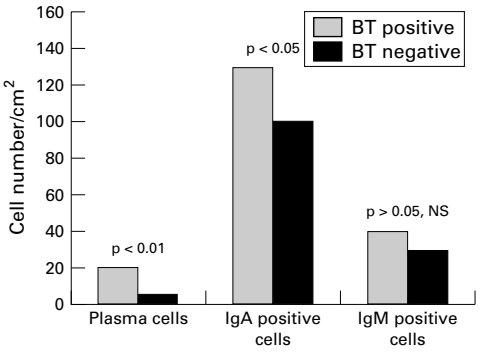

Figure 1 Median number of plasma cells, IgA positive cells, and IgM positive cells in BT positive and negative patients.

Table 2 Semiquantitatively scored IgA and IgM concentrations at the mucosal surface

	BT positive group	*BT negative group*
IgA		
3 High	2	0
2 Moderate	9	5
1 Low	0	6
IgM		
3 High	1	0
2 Moderate	8	3
1 Low	2	8

BT, bacterial translocation.

each of the patients studied. All 11 patients in the BT positive group were deemed to have an IgA score of 2 or 3 (moderate or high degree of staining, respectively), compared with only five of the BT negative patients ($p = 0.006$, Fisher's exact test mid p). Similarly, an IgM score of 2 or 3 was found in nine BT positive patients, compared with only three patients in the BT negative group ($p = 0.016$, Fisher's exact test mid p).

Discussion

Our results suggest that BT, confirmed by positive culture from a mesenteric lymph node, is associated with a significant increase in gut immune function.

In a large prospective study, BT was demonstrated in 15.4% of patients undergoing laparotomy, and was associated with a significant increase in the incidence of postoperative septic morbidity.[2] One mechanism proposed as a promoter of this phenomenon is a deficiency in local gut immune defences. In the normal intestinal mucosa, substantial numbers of plasma cells are present in the lamina propria, producing IgA and smaller amounts of IgM. In its dimeric form, IgA is actively bound to the protein secretory component (SC) while traversing the overlying epithelial layer by endocytosis. SC is produced by the epithelial cells, and facilitates the transport of IgA into secretions and protects it from proteolytic attack. SC is removed by proteolytic cleavage to release free IgA into the mucus layer on the luminal surface of the enterocyte.[16 17] IgM is secreted in its pentameric form and reaches the mucosal surface in a similar manner.

These immunoglobulins at the mucosal surface provide an important part of the normal barrier mechanism of the intestinal mucosa, binding to bacterial antigens and limiting adherence of pathogens to the wall of the enterocyte, the initial step in the process of BT.[10] Alverdy *et al* showed that a significant decrease in IgA was associated with an increase in bacterial translocation in rats given total parenteral nutrition.[18] The beneficial effects of IgA in limiting translocation have also been demonstrated previously in both animal and in vitro studies. A study by Albanese *et al*, using an in vivo model in rats, showed that *E coli* was unable to cross a morphologically intact segment of small bowel tissue while bound to IgA, a phenomenon they termed immune exclusion.[19] Von Specht *et al* found that IgA directed against a *P aeruginosa* outer membrane protein was protective against translocation of this organism in immunosuppressed mice.[20]. An in vitro study by Diebel *et al* demonstrated that the addition of IgA significantly reduced BT across epithelial cell monolayers following an inoculum of bacteria.[21]

Coutinho *et al* took small bowel specimens from 17 patients with intra-abdominal sepsis and stained them immunohistochemically for several markers of immune function, including IgA and IgM.[9] These were compared with control specimens obtained from two transplant donors. They found the numbers of IgA and IgM positive plasma cells to be reduced in the patients with sepsis, although no numerical data were presented in the paper. These findings were explained on the basis of an increase in glucocorticoid secretion as part of the stress response to sepsis causing apoptosis of mucosal plasma cells and a reduction in immunoglobulin production. It was proposed that this predisposes to adhesion of luminal bacteria to the apical surface of the enterocyte and consequent translocation across the epithelium. However, the occurrence of BT in these patients was only presumed; no direct confirmatory evidence was provided.

Our study is the only in vivo study of BT in relation to gut immune function in humans. Our results are at variance with the inferences made from the Coutinho study because we found a significant increase, rather than a decrease, in the gut immune response in those patients in whom BT had been positively identified. We did not stain for SC or J chain because these variables appeared to correspond closely to the immunoglobulin values in the Coutinho study, so we felt that they would not provide any useful additional information. Morphological assessment of apoptosis can be misleading, and in situ hybridisation techniques can be unreliable, particularly on formalin fixed specimens.[22] Furthermore, in situ hybridisation is beyond the technical scope of our laboratory at present. We feel that using numbers of human major histocompatibility complex (HLA) DR positive macrophages in the lamina propria as a measure of apoptosis, as in the Coutinho study, is too simplistic and unreliable. For these reasons we did not attempt to assess plasma cell apoptosis.

A possible explanation for the discrepancy between the results of the two studies could relate to variability of specimen fixation time,

fixative type, temperature of fixation, and differences in processing schedules in the Coutinho study. Such variability is inevitable when specimens are drawn from more than one centre on a retrospective basis. This variability can adversely influence immunohistochemical staining significantly. A further possible explanation is the difference in disease severity between the patients in the two studies—most of our patients were undergoing elective procedures and were thus not septic at the time of surgery. Our results suggest that, at least in these predominantly elective surgical patients, the increased immunoglobulin values seen in those patients with a positive MLN is a secondary phenomenon, occurring in response to the translocation of bacteria across the mucosa. In vitro studies have demonstrated that invasion of colonic epithelial cells by enteric bacteria results in enhanced cytokine expression, which acts as a signal to immunoreactive cells such as B cells in the lamina propria.[23] We cannot confirm the proposed hypothesis that suppression of the immune response, such as that induced by sepsis, predisposes to BT. Furthermore, there is no evidence from our results that BT itself impairs the systemic and intestinal immune response as suggested by previous animal studies.[24 25]

Almost all of the small bowel specimens were from the terminal ileum, removed as part of a right hemicolectomy or total colectomy. This negates the effect of any variation in the local immune response along the length of the small bowel. By analysing only normal segments of intestine we have attempted to remove confounding influences related to the primary disease process, as well as providing a more representative measure of overall gut immune function. It is not known whether the immune response was similar in the diseased intestine, but we consider this to be of little importance because the pathology was very localised in most of our patients. Furthermore, we have previously demonstrated the occurrence of BT in patients with "normal" intestine, such as those undergoing abdominal aortic aneurysm repair.[26] Upregulation of the immune response has been described in patients with inflammatory bowel disease (IBD).[27] We found no significant differences in any of the parameters of immune function in the six patients with IBD compared with the other patients in our study.

It is interesting to note that the frequency of IgA positive cells was greater than that of plasma cells in every case studied, and the frequency of IgM positive cells was greater in all but five cases. We accept the fact that using morphology alone to identify plasma cells may underestimate their true frequency. In addition, some of the cells that stained positively for IgA and IgM might be macrophages rather than plasma cells. However, these considerations do not alter the fact that significant differences were seen between the two patient groups in terms of four of the five parameters measured, including IgA and IgM values at the mucosal surface.

Our findings infer that factors other than intestinal mucosal immunity must be important in the promotion of BT. The most likely of these is bacterial overgrowth within the gut lumen. IgA synthesis by B cells of the gut associated lymphoid tissue increases in response to the overgrowth of bacteria.[28] Colonisation of the proximal gastrointestinal tract is common in critically ill patients, largely as a result of increased gastric pH and reduced peristalsis, and is associated with increased rates of septic complications.[7 29] A recently published paper from our unit describes the association between gastric colonisation, bacterial translocation, and septic morbidity.[6] These findings provide a rationale for the use of selective gut decontamination in those patients at risk of gut derived sepsis, and a recent meta-analysis confirmed a reduction in both nosocomial pneumonia and mortality associated with this technique in patients treated in intensive care units.[30]

In conclusion, we have found a significant increase in immune function in the small bowel mucosa of patients in whom bacterial translocation has been shown to occur. We hypothesise that this represents a response to the occurrence of BT, and challenges previous claims that BT occurs secondary to a reduction in local immune responsiveness. Therapeutic interventions that prevent pathological bacterial overgrowth within the gastrointestinal tract may be the most appropriate means of decreasing BT and thus potentially reducing the incidence of gut derived sepsis.

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