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The Presence of Peptidoglycan–Polysaccharide Complexes in the Bowel Wall and the Cellular Responses to These Complexes in Crohn's Disease

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Interestingly, using a monoclonal antibody, peptidoglycan–polysaccharide complexes (PPC) were detected intracellularly in the mucosa and submucosa of the bowel wall of Crohn's disease (CD) patients. PPC are the main constituents of the gram-positive bacterial cell wall. These PPC were however detected in the normal bowel wall also. Therefore, in this study the hypothesis that an enhanced immune responsiveness to bacterial antigens plays a pivotal role in the induction or the chronicity of CD was tested. As antigens, the peptidoglycan structures of intestinal bacteria (*Eubacterium aerofaciens* or fecal PPC) or of *Streptococcus pyogenes*, the 65-kDa heat shock protein and muramyl dipeptide (MDP), the smallest bioactive subunit of peptidoglycan, were used. The proliferative responses of peripheral blood (PB) mononuclear cells (MNC) of healthy subjects and patients in a remissive stage of CD or an active CD stage were examined. Of this last patient group the MNC responses of the mesenterial lymph nodes that drain the inflamed gut area were measured also. The responses of PB-MNC of the healthy subjects and the patients in a remissive CD stage were not different. Compared to the responses in remissive CD, the PB-MNC responses in active CD to the eubacterial cell wall and streptococcal cell wall antigen were significantly higher. At the inflammation site in active CD, the lymph nodes, the responses to most of the bacterial antigens were significantly higher than in the PB. In summary, the results show the presence of bacterial peptidoglycan in the bowel wall and the immune responsiveness, especially at the inflammation site, to these antigens in active CD and therefore present suggestive evidence for the role of peptidoglycan in the etiology and/or pathogenesis of CD.

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INTRODUCTION

The search for a trigger antigen in the etiology of Crohn's disease (CD)[1] has been the topic of many investigations. A microbial etiology has been suggested. Much interest has been focused on the presence of mycobacteria in the gut and on the immune responsiveness to mycobacterial antigens in CD patients. Chiodini has reported the isolation of a mycobacterium from the gut of CD patients (1, 2). Others have also been able to culture this bacterium from ulcerative colitis (UC) patients, patients with noninflammatory bowel diseases, and healthy subjects (HS) (3). Humoral responses to *Mycobacterium paratuberculosis* have been reported to be greater in patients with CD (4) but this has been contradicted by others (5, 6). Using polyclonal rabbit antisera as a probe, no mycobacterial antigens were found in the tissues of CD or UC patients (6). T cell responsiveness to antigens of various mycobacteria as determined by macrophage inhibition factor production in peripheral blood (PB) was not higher in CD patients compared to UC patients or HS (7) and in a recent study (8) proliferative responses against mycobacterial antigens were not found.

Recently it was shown that the immunodominant proteins involved in the responses to mycobacterial antigens are the so-called heat shock or stress proteins (hsp) (9–11). No enhancement of the humoral response to mycobacterial or human stress proteins was observed in CD patients (12).

The antigens as discussed above are not the most obvious antigens to play a role as the triggering antigen. Here we presume the bacterial cell wall fragments of the normal flora to be a better candidate.

Bacterial cell wall fragments (CWF) are well-known inducers of inflammatory diseases in animal models. In rats the induction of inflammatory joint disease was first described for CWF of *Streptococcus pyogenes* (13). CWF composed of peptidoglycan–polysaccharide complexes (PPC) of the resident anaerobic intestinal flora have also been shown to be arthropatic in rats (14–17).

[1] Abbreviations used: CD, Crohn's disease; CM, complete medium; Con A, concanavalin A; CWF, cell wall fragments; ECW, *Eubacterium aerofaciens* cell walls; HS, healthy subject; hsp, heat shock proteins; LN, lymph nodes; MDP, muramyl dipeptide; MNC, mononuclear cell; PB, peripheral blood; PBS, phosphate-buffered saline; PPC, peptidoglycan–polysaccharide complex; SCW, *Streptococcus pyogenes* cell walls; SEM, standard error of the mean; UC, ulcerative colitis.

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PPC can be isolated in soluble form from human feces (18) and PPC from ileostomy fluid appeared to be arthritogenic in rats (19). This indicates that PPC have the capacity to induce chronic inflammations and are present in the human gut lumen. IgM, G, and A responses to these PPC can be found in all individuals and are elevated in CD patients (20). In the feces of CD patients, the concentration, viscosity, and molecular weight of PPC were less compared to the PPC in feces of HS (18). This change in viscosity and molecular weight could influence the contact and uptake of PPC by the bowel wall.

According to Sartor, "luminal bacterial cell wall polymers with well-described inflammatory and immunoregulatory potential can cross injured colonic epithelia and are capable of initiating and potentiating intestinal inflammation" (21). To verify this statement, we determined the presence of PPC in bowel wall specimens using a monoclonal antibody. Moreover, the cellular responsiveness to several bacterial cell wall antigens in CD patients and HS was studied. This study was focused primarily on intestinal bacterial antigens, as these are the antigens to which the bowel wall is in continuous close contact. The 65-kDa heat shock protein was used as a control bacterial nonpeptidoglycan antigen. The cellular responsiveness obtained from PB or draining lymph nodes (LN) of patients in an active disease stage was compared to that of PB of patients in remission and to HS.

The data show that the presence of and the immunological responses to peptidoglycans might be important in the etiology and/or pathogenesis of Crohn's disease.

MATERIALS AND METHODS

Subjects

The group suffering from active Crohn's disease consisted of 15 patients (12 female, 3 male; median age 38.5 years; range 23–60 years) who were hospitalized for surgery. Peripheral blood samples of the surgical group were taken, with consent of the patient, before surgery. Lymph nodes of patients with active disease were the taken from the inflamed resected bowel directly after surgery. Specimens for immunohistology were also taken from this resected material. The group of 10 CD patients in remission (7 female, 3 male; median age 35 years; range 23–76 years) was obtained from individuals visiting the out-patient department for routine follow-up. The disease activity index according to van Hees (22) was lower than 100 in this group, indicating inactive disease. The healthy subjects were a group of 10 laboratory co-workers (5 female, 5 male; median age 38.5 years; range 23–50 years).

Immunohistochemistry

The monoclonal antibody, 2E9, used in this study was an IgG3 of murine origin directed against bacterial peptidoglycan–polysaccharide complexes from the human fecal flora. The isolation and characterization of 2E9 is described elsewhere (23). Cryosections of 6 μm of bowel wall specimens were made and stored at −20°C until use. After thawing, the sections were fixed for 10 min in acetone (P. A. Merck, Damstadt, Germany). Endogenous peroxidase was blocked by 0.1% (w/v) phenylhydrazinium chloride in phosphate-buffered saline (PBS) for 1 hr at 37°C. After washing in PBS with 0.2% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with 10 μg/ml 2E9 or 10 μg/ml NS7 (ATCC TIB114, specific for sheep red blood cells) as a negative control monoclonal. NS7 is also of the IgG3 isotype. After washing, the sections were incubated for 30 min at room temperature with rabbit anti-mouse immunoglobulin (Dakopatts Z259) diluted 1:20 in PBS with 1% normal human serum. After washing in PBS, a 1:20 dilution of alkaline phosphatase–mouse anti-alkaline phosphatase complex (APAAP, Dakopatts D651) was applied for 30 min. The substrate used to develop the stain consisted of 0.012% (w/v) naphthol AS-MX phosphate, 0.025% (w/v) fast blue BB base, and 0.025% (w/v) levamisole (all from Sigma, St. Louis, MO). After 45 min incubation at room temperature in the dark, the sections were washed in PBS/0.2% BSA and mounted in Kaiser's glycerol gelatin (Merck).

Isolation of Peripheral Blood and Lymph Node Mononuclear Cells (MNC)

Heparinized PB specimens were diluted 1:1 in wash medium (RPMI 1640; Gibco, Paisley, UK) with 1% pooled normal human serum, 25 m*M* Hepes, 1 m*M* sodium pyruvate, 2 m*M* glutamate, 100 U/ml penicillin, and 100 μg/ml streptomycin). The human serum used showed no mitogenic effects on PB-MNC from healthy donors in a [^{3}H]thymidine incorporation assay. MNC were separated by Ficoll (Ficoll–Paque, Pharmacia LKB, Uppsala, Sweden) gradient centrifugation. LN were mashed over a 100-μm nylon gauze filter before ficollation. The separated MNC were washed twice before use in the assays. Live cells were counted by trypan blue dye exclusion.

Proliferation Assays and Antigens/Mitogens

PB and LN-MNC were resuspended in complete medium (CM; wash medium as described above supplemented with 15% human serum) at a concentration of 1×10^6 live cells/ml. One hundred-microliter aliquots were seeded in round-bottom microtiter plates (Costar, Cambridge, UK) and the antigens were added in twice the final concentration in 100 μl CM to triplicate cul-

tures. Streptococcal cell wall (SCW) and eubacterial cell wall (ECW) preparations from respectively *Streptococcus pyogenes* and *Eubacterium aerofaciens* were prepared as described previously (14–16). PPC was isolated from normal feces according to procedures described by Hazenberg (18). Concanavalin A (Con A) and muramyl dipeptide (MDP) were purchased from Sigma. Sixty-five-kilodalton protein was graciously supplied by Dr. J. D. A. van Embden (RIVM, Bilthoven, The Netherlands) (24, 25). ECW, SCW, PPC, and MDP were used at 30 μg/ml while 65-kDa protein and Con A were used at 10 μg/ml. These antigen concentrations were determined to be optimal in a separate study in rheumatoid arthritis (26).

The cells were cultured for 6 days in a humidified Heraeus (Hanau, Germany) incubator at 37°C, 5% CO_2. During the last 17 hr of culture 0.2 μCi [^{3}H]TdR was added (sp act 2 Ci/mmol; Amersham, UK). The cultures were harvested by means of an automatic cell harvester (Costar) and counted in a β counter (Packard, Brussels, Belgium) or a β plate (Pharmacia, LKB, Turku, Finland).

The results are expressed as the net counts per minute (Δ cpm): Mean [^{3}H]TdR incorporation of triplicate cultures with antigen or mitogen minus the mean [^{3}H]TdR incorporation of triplicate cultures in medium. Δ cpm values less than zero were fixed at zero.

Statistics

Significance of the results was determined by the Mann–Whitney *U* test. Differences were considered to be significant if p values were less than or equal to ($\leq$) 0.05.

RESULTS

Staining of CD Bowel Wall Specimens with Monoclonal 2E9, Specific for Peptidoglycan–Polysaccharide Complexes

Figure 1 shows cryosections of colon resection material from a CD patient stained with monoclonal 2E9 which is specific for peptidoglycan–polysaccharide complexes. Intracellular staining was observed in some cells in the mucosa and submucosa. These cells have a dendritic cell-like morphology, and some of the cells double-stain with RFD1, a monoclonal for dendritic cells (data not shown). The negative control monoclonal NS7 was always negative (data not shown). This type of staining was also seen in nine of nine other CD patients studied. Inflammatory infiltrates were present in the mucosa and submucosa of these patients. The cells staining for PPC were more densely distributed in these inflammatory infiltrates compared to the rest of the mucosa and submucosa. In the colon specimen of two of two patients suffering of noninflammatory diseases of the colon, cells staining positively for 2E9 were also demonstrable.

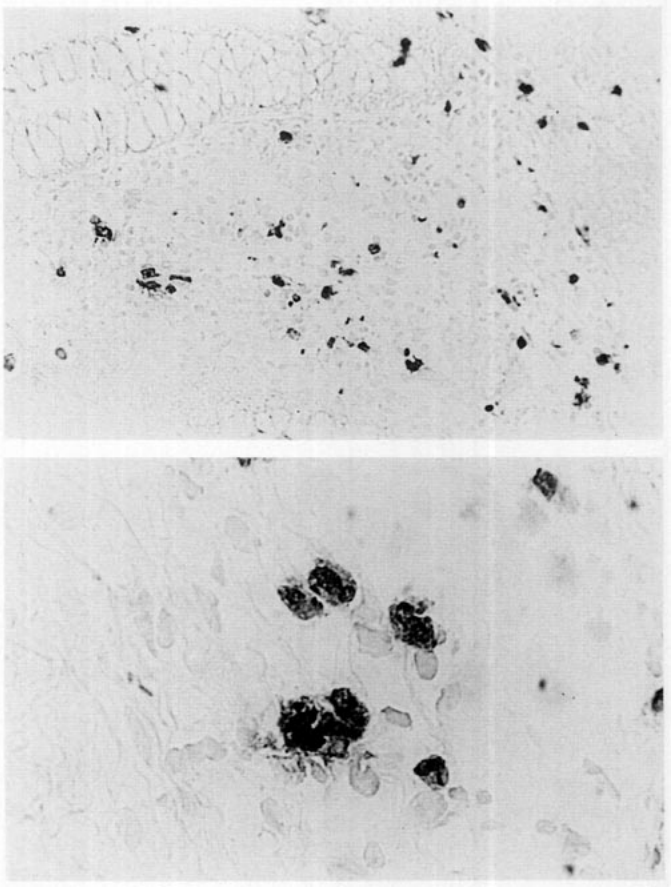

FIG. 1. (Top) Immunohistology of the mucosa of a bowel wall specimen of a patient suffering of active Crohn's disease stained by 2E9. Magnification 194×. (Bottom) Detail of the top. Magnification 582×.

These results raised the question of whether a difference in immune responsiveness to peptidoglycans exists in CD patients in an active compared to a remissive disease stage and to healthy subjects.

Proliferative Responses of Mononuclear Cells to Bacterial Antigens

Table 1 shows the proliferative responses of the mononuclear cells of the peripheral blood to various bacterial antigens; P values comparing the groups tested are shown in Table 2. The PB-MNC were obtained from a group of healthy subjects, a group of CD patients in remission, and a group of patients the day before bowel resection because of active disease. The responses of PB of HS and remissive CD were not significantly different. The only significant difference was an increased responsiveness to ECW in the HS compared to the remissive CD patients. In active disease an increased responsiveness was found in the PB for the antigens ECW and SCW compared to the remissive

TABLE 1
Mononuclear Cell Proliferative Response as Determined by LTT Assay

	Peripheral blood							
	Healthy subjects				Remissive + CD			
	$\bar{x}$ ± SEM	Median	Range	(*n*)	$\bar{x}$ ± SEM	Median	Range	(*n*)
ECW	421 ± 190	216	0–2,117	(10)	28 ± 15	0	0–128	(10)
SCW	220 ± 105	72	0–1,092	(10)	257 ± 120	53	0–996	(10)
PPC	251 ± 103	91	0–910	(10)	680 ± 379	107	0–3,183	(10)
MDP	276 ± 101	88	0–301	(10)	439 ± 222	72	0–597	(10)
65 kDa	94 ± 29	170	0–1,010	(10)	126 ± 57	183	0–2,282	(10)
Con A	6,358 ± 2,025	3,656	1,560–24,299	(10)	6,814 ± 1,245	7,224	484–12,403	(10)
Medium	533 ± 179	278	78–2,031	(10)	501 ± 146	353	56–1,653	(10)

	Peripheral blood				Intestinal lymph nodes			
	Active CD				Active CD			
	$\bar{x}$ ± SEM	Median	Range	(*n*)	$\bar{x}$ ± SEM	Median	Range	(*n*)
ECW	1,044 ± 432	289	0–4,983	(13)	4,797 ± 1,517	3,935	330–16,258	(10)
SCW	5,987 ± 1,716	4,536	3,781–10,222	(5)	10,557 ± 2,699	11,556	881–15,165	(6)
PPC	869 ± 373	365	0–3,838	(13)	6,669 ± 2,675	4,755	0–29,038	(10)
MDP	1,703 ± 26	54	0–2,553	(8)	3,605 ± 701	3,952	487–9,205	(7)
65 kDa	443 ± 309	439	0–5,468	(12)	3,872 ± 1,270	2,927	879–6,832	(8)
Con A	6,041 ± 970	5,544	667–13,224	(13)	3,985 ± 1,650	1,314	0–19,256	(11)
Medium	252 ± 74	157	29–970	(13)	2,375 ± 1,282	661	179–14,703	(11)

Note. Results of the antigens or mitogen are presented as Δ cpm: the medium control of each subject was subtracted from the value of each antigen or the mitogen. The medium values are presented as raw cpm. SEM, standard error of the mean.

CD with the highest responsiveness to the SCW preparation. We did not, however, test this antigen in all patients as at the beginning of our investigations we decided to primarily focus on the intestinal bacterial antigens and there were not always enough MNC available to test all antigens.

As we were interested whether the responses found in the active CD group can also be found at the site of the inflammation, i.e., the gut, the intestinal lymph nodes lymphocytes of active CD were also tested. Significantly higher responses to all bacterial antigens except SCW were found in the LN compared to the PB.

TABLE 2
P Values as Determined by Mann–Whitney *U* Test

R-CD	HS[a]	A-CD[a]	A-CD (LN)	A-CD (PB)[b]
ECW	0.005	0.0008	ECW	0.0004
SCW	1.0	0.002	SCW	0.144
PPC	0.68	0.23	PPC	0.03
MDP	0.85	0.76	MDP	0.005
65 kDa	0.76	0.2	65 kDa	0.03
Con A	0.82	0.5	Con A	0.05
Medium	0.85	0.07	Medium	0.003

[a] *P* values comparing the peripheral blood Δ cpm values of healthy subjects (HS) with those of remissive Crohn's disease (R-CD) and *P* values comparing peripheral blood Δ cpm values of active Crohn's disease (A-CD) with those or remissive Crohn's disease.

[b] *P* values comparing peripheral blood and lymph node Δ cpm values in active Crohn's disease (A-CD).

The responses to SCW were already high in the PB; these were not higher in the LN.

DISCUSSION

The aim of the present study was to investigate the hypothesis that bacterial cell wall polymers are the antigens that play a trigger role in the etiology and/or the pathogenesis of Crohn's disease.

A monoclonal antibody (2E9) directed against PPC from the intestinal flora was used to investigate the presence of these structures in the bowel wall. Cells staining positively for the monoclonal 2E9 were found scattered throughout the gut mucosa and submucosa of the CD patients. Positive cells were also found in the mucosa and submucosa of the bowel wall of the two patients tested suffering of noninflammatory bowel disease (data not shown). The monoclonal 2E9 stained macrophages in the red pulp of spleens of rats (23).

The presence of these PPC in the CD bowel wall and the normal bowel wall raised the question whether disease was caused by an altered immune responsiveness to peptidoglycans in CD. This was studied in proliferation assays to various bacterial antigens.

The results of the proliferation assays demonstrated in Tables 1 and 2 show a significantly higher response to cell wall fragments of the intestinal bacterium *Eubacterium aerofaciens* in active CD patients compared to those in remission. The highest responses were found to streptococcal cell wall antigen. *S. pyogenes* is

not representative of the normal intestinal flora and we do not suggest that *S. pyogenes* is involved in the pathogenesis of CD. The SCW were used in this study because Yamada *et al.* (27) found chronic granulomatous gut inflammation after intramural injection in rats and the preparation was arthropathic in our hands (28).

The antigens used in these experiments do not act as T cell polyclonal stimulators *in vitro* as can be concluded from the fact that there was no responsiveness in the peripheral blood of healthy subjects or of the patients in remission of CD.

In rheumatoid arthritis it has been shown that specific proliferative responsiveness at the inflammation site, i.e., the joint, is only present during the early phase of the disease. Later this response can only be measured in peripheral blood (29). Here again we find significant differences between the responsiveness at the inflammation site, the draining lymph nodes, and the PB.

Because proliferation of all MNC in the preparation is measured, it is not certain whether T cell proliferation accounts for all of the observed results. The lymph node suspensions contain variable numbers of B cells varying from 6 to 78% positive for the CD20 antigen as determined by FACS analysis of all active CD patients (data not presented). However, no correlation was found between CD20 and enhanced proliferation to particular antigens. Therefore, it is unlikely that B cell stimulation contributes to the responses found.

In our study we also found a higher responsiveness to the 65-kDa antigen in the LN of the A-CD group compared to the PB of the same group. The 65-kDa antigen was used as a control bacterial nonpeptidoglycan antigen in this study. According to Res *et al.* (30), the 65-kDa response could reflect a local T cell response against epitopes on self-heat shock proteins, a common feature of chronic inflammation.

In summary, the results do show that immunological responsiveness to bacterial antigens to which we are in continuous close contact can be measured in CD patients and that these antigens are present in the bowel wall. In patients suffering from active disease these responses are higher compared to patients in remission, and at the active inflammation site the responses are even higher. This suggests a trigger role for peptidoglycan–polysaccharide complexes in Crohn's disease.

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