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Circulating antibodies to heat-shock protein 60 in Crohn's disease and ulcerative colitis

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SUMMARY

Heat-shock proteins (HSPs) are highly conserved immunogenic intracellular molecules that are induced by inflammatory mediators and may induce autoimmune phenomena *in vivo*. We have recently demonstrated the increased expression of HSP-60 in the colonocytes of patients with ulcerative colitis. To study further the role of HSP-60 in inflammatory bowel disease, we have now measured antibodies to recombinant mycobacterial HSP-65 (a member of the HSP-60 family) in patients with Crohn's disease, ulcerative colitis, healthy volunteers and, as disease controls, patients with confirmed bacterial diarrhoea. In comparison with healthy controls ($n=20$; median level of 89 ELISA units; range 24–292), serum IgA HSP-60 antibodies were elevated in Crohn's disease ($n=21$; 157; 57–364; $P<0{\cdot}05$) and active ulcerative colitis ($n=16$; 188; 58–373; $P<0{\cdot}01$) but not bacterial diarrhoea ($n=10$; 106; 51–285). Increased IgA HSP-60 antibody levels in patients with inflammatory bowel disease may occur as the result of HSP release from injured gut epithelium; alternatively, increased intestinal permeability could facilitate mucosal access of luminal antigens and the generation of cross-reactive anti-bacterial HSP antibodies.

Keywords Crohn's disease heat-shock protein IgA HSP-60 antibodies ulcerative colitis

INTRODUCTION

Heat-shock proteins (HSPs) are highly conserved, immunogenic molecules whose cellular levels are elevated by heat, inflammatory mediators and other forms of physiological stress [1,2]. Various functions have been ascribed to HSPs which suggest that their role might be to enhance cellular survival under stressful conditions [3]. These include their ability to function as molecular chaperones by facilitating the correct oligomeric assembly of proteins [4].

Because of their high interspecies conservation and antigenicity, HSPs may stimulate autoimmune phenomena *in vivo* [5,6]. Autoantibodies and T cells reactive with HSPs have been detected in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [7–9] and increased expression of HSPs has been reported in the human synovium [10,11]. Furthermore, T lymphocyte clones isolated from rats made arthritic by injection with heat-killed *Mycobacterium tuberculosis* (in mineral oil), react specifically with mycobacterial HSP-65 and can induce arthritis when transferred to irradiated recipients [12].

Using the ML30 antibody (which recognizes members of the 60-kD family of several species), we recently demonstrated the increased expression of heat-shock protein 60 (HSP-60) in the colonocytes of patients with ulcerative colitis (UC; [13]). In order to study further the role of HSPs in inflammatory bowel disease (IBD), we have now measured circulating antibodies to the HSP-60 family, using mycobacterial 65-kD as substrate, in patients with Crohn's disease (CD) and UC, compared their levels with healthy controls and patients with bacterial diarrhoea as an intestinal disease control, and related them to disease activity, extent and treatment.

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PATIENTS AND METHODS

Controls

Twenty healthy blood donors were used as normal controls (Table 1). Ten patients with confirmed bacterial diarrhoea (BD;

Table 1. Demographic data of healthy controls and patients with bacterial diarrhoea (BD), Crohn's disease (CD) and ulcerative colitis (UC)

	n	Sex M/F	Median age (years)	Age range
Controls	20	12/8	36	28–60
BD	10	6/4	35	24–65
CD	21	7/14	35	22–71
UC	30	16/14	44	20–74

three campylobacter jejuni, four salmonella, three shigella) tested before and after recovery, served as disease controls. The age ranges and median age were similar in all groups (Table 1).

Patients with inflammatory bowel disease

Crohn's disease ($n=21$; 10 active, 11 inactive) and UC ($n=30$; 16 active, 14 inactive) were diagnosed by conventional clinical, radiological, endoscopic and histological criteria (Table 1). Disease activity was determined by Harvey's clinical index modified for laboratory and endoscopic variables as described [14].

ELISA for antibodies to HSP-60

Serum samples were coded and stored at −20 C until use. Circulating antibodies to recombinant *M. bovis* BCG/tuberculosis HSP-65 (HSP-60 antibodies) were measured by conventional ELISA based on the method of Tsoulfa *et al.* [7]. Microtitre plates were coated with 100 μl of recombinant mycobacterial HSP-65 (1 μg/ml in 0·05 M carbonate buffer, pH 9·6) overnight at 4 C. After three washes in 0·01 M PBS (pH 7·4) containing 0·05% Tween-20 (PBS-T), 100 μl of PBS-T containing 1% bovine serum albumin (PBS-T/BSA) were added to each well and incubated for 1 h at 37 C to block non-specific binding. The plates were then washed and 100 μl of test sera diluted in PBS-T/BSA (1:50–1:400) added and maintained overnight at 4 C. All sera were assayed in duplicate. In addition, a reference serum was included in duplicate or triplicate on each plate. After three washes in PBS-T, peroxidase-conjugated rabbit anti-human immunoglobulin isotypes (Dako) were added at 1:1000 dilution and left to incubate overnight at 4 C. The plates were then washed three times and developed by the addition of orthophenylene diamine (34 mg/100 ml in 0·1 M citrate phosphate buffer, pH 5·0) supplemented with hydrogen peroxide. Colour development was stopped by the addition of 4 M sulphuric acid and absorbance monitored at a wavelength of 492 nm.

The reference serum was from a normal donor which had an optical density reading at 492 nm (OD^{492}) around the mean of the normal range. Normal OD^{492} readings (±s.d.) varied as follows: IgA 0·036 (0·022), IgG 0·061 (0·040), IgM 0·054 (0·022) where $n=7$. The serum was stored in aliquots at −20 C with the test sera and thawed once, a fresh aliquot being used on each occasion. Since OD^{492} readings can vary greatly between assays, the use of a reference serum (OD^{492} control) allows a more accurate comparison. In this study, inter-assay and intra-assay variability were less than 10% and 5% respectively.

Results were calculated as the ratio [(OD^{492} sample/OD^{492} control) × 100] using serum dilutions of 1:100 for IgA and 1:200 for IgG and IgM. The dilutions were chosen so that the OD^{492} levels lay on the linear portion of the curve.

Statistical analysis

Statistical comparisons were made using the two-tailed Mann–Whitney *U*-test for unpaired data and Wilcoxon's signed rank test for paired data.

RESULTS

Circulating IgA (Fig. 1) but not IgG (Fig. 2) or IgM (Fig. 3) HSP-60 antibodies were significantly elevated in patients with CD (median 157; range 57–364; $P<0·05$) compared with healthy controls (89; 24–292). In UC, median IgA HSP-60 antibody levels (139; 25–373) were similar to controls.

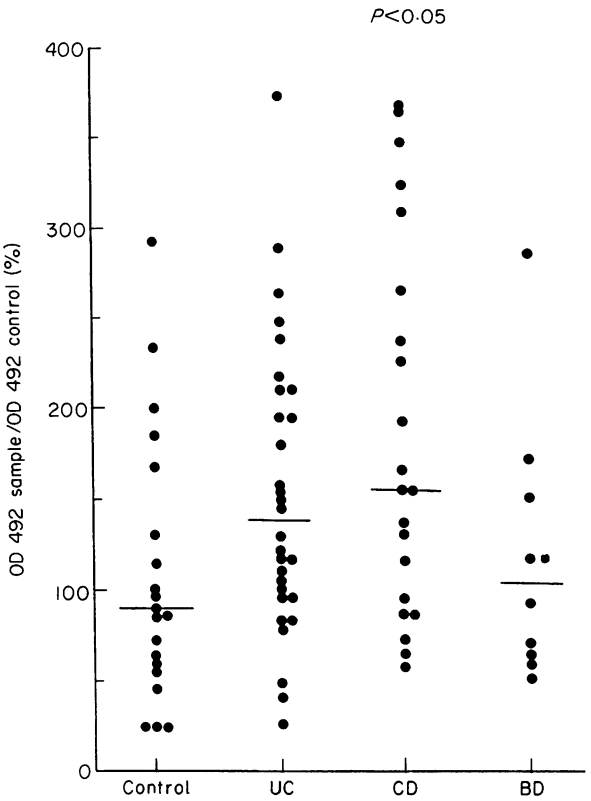

Fig. 1. IgA antibodies to a recombinant mycobacterial 65-kD heat shock protein in patients with ulcerative colitis (UC; $n=30$) and Crohn's disease (CD; $n=21$) and in normal healthy controls or patients with bacterial diarrhoea (BD; $n=10$). Patients with CD had significantly elevated titres ($P<0·05$). The bar represents median levels.

When IBD patients were subclassified according to disease activity, median IgA HSP-60 antibody levels were significantly higher in patients with active UC compared with inactive disease ($P<0·05$) or healthy controls ($P<0·01$) (Table 2). In CD, IgA HSP-60 antibody levels were elevated regardless of disease activity. IgA HSP-60 antibodies were otherwise unrelated to site or extent of disease or treatment (data not shown).

In patients with confirmed BD, median IgA HSP-60 levels were similar to healthy controls but fell significantly on recovery ($P<0·001$) (Table 2).

DISCUSSION

The immunodominant 65-kD mycobacterial heat-shock protein shows high protein sequence homology with the *E. coli Gro*EL stress protein, the common antigen' of all Gram-negative bacteria and the human 58-kD stress protein. All are members of the 60-kD family [1]. Thus, antibodies recognizing the mycobacterial heat-shock protein may cross-react with human and bacterial homologues. We have therefore defined the antibodies directed towards HSP-65 as HSP-60 antibodies.

Our finding of elevated IgA HSP-60 antibody levels in patients with active UC as well as in patients with CD extends the studies of Tsoulfa *et al.* [7] who reported elevated IgA but not IgG or IgM antibodies (using an identical recombinant

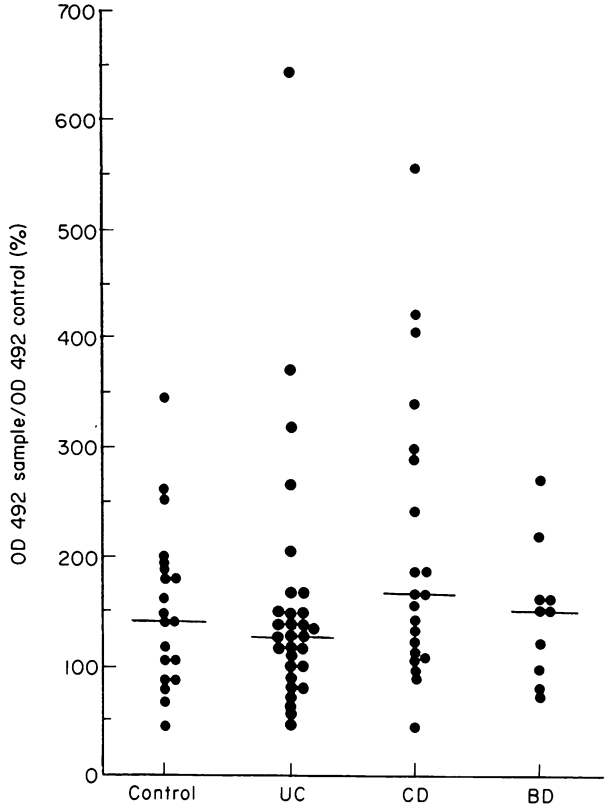

Fig. 2. IgG antibodies to a recombinant mycobacterial 65-kD heat-shock protein in patients with ulcerative colitis (UC; $n = 30$) and Crohn's disease (CD; $n = 21$) and in normal healthy controls or patients with bacterial diarrhoea (BD; $n = 10$). No significant differences were noted between the groups irrespective of whether the patients had active or inactive disease. The bar represents median levels.

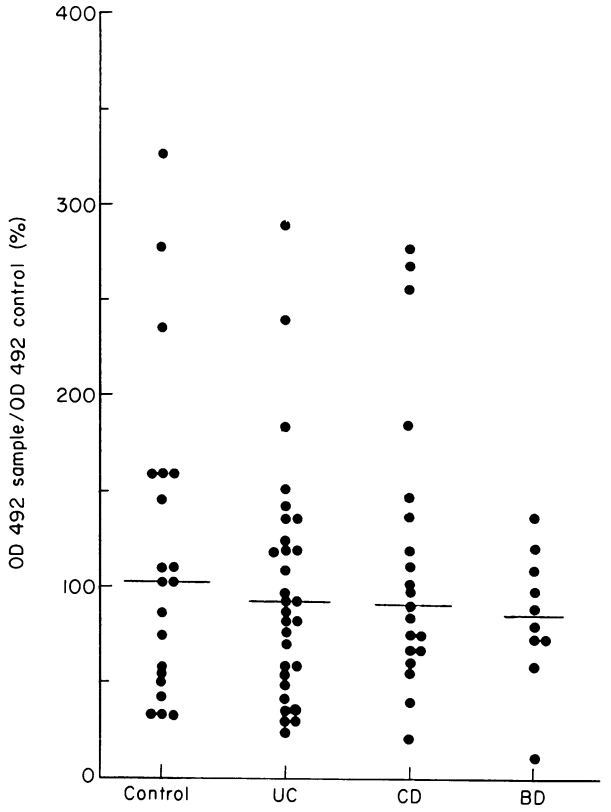

Fig. 3. IgM antibodies to a recombinant mycobacterial 65-kD heat-shock protein in patients with ulcerative colitis (UC; $n = 30$) and Crohn's disease (CD; $n = 20$) and in normal healthy controls or patients with bacterial diarrhoea (BD; $n = 10$). No significant differences were noted between the groups irrespective of whether the patients had active or inactive disease. The bar represents median levels.

HSP-65 from *M. bovis*) in patients with CD. Other workers, using immunoblotting techniques, have confirmed the lack of IgM and IgG HSP-60 antibodies in patients with IBD [15]. Tsoulfa's studies also demonstrated increased IgA HSP-60 antibody levels in patients with SLE and RA. However, the lack of any association between raised IgA antibodies to HSP-60 and arthropathy in our patients argues against the hypothesis that HSP-60 antibodies might induce joint disease in IBD.

The finding that median IgA HSP-60 antibody levels are elevated in patients with active but not quiescent UC may signify fundamental differences in the heat-shock protein response in these patients compared with those with CD. We have recently detected increased expression of HSP-60 in UC colonocytes as indicated by increased binding of ML30 antibody (a broadly cross-reactive antibody raised against *M. leprae* 65-kD heat-shock protein) to sections of colonic mucosa [10,13]. HSP-60 expression was significantly higher than control in mucosal sections from patients with both inactive and active UC, suggesting that these cells either constitutively synthesize HSP-60 or are undergoing continual stress. In the active phase of UC, injury to the colonic mucosa is pronounced and there is substantial epithelial cell destruction. Thus, it is conceivable that elevated IgA HSP-60 antibody levels in active UC are generated as a response to endogenous HSP release from damaged cells. Alternatively, loss of the mucosal barrier in

Table 2. IgA HSP-60 antibody levels in patients with active and inactive ulcerative colitis (UC), Crohn's disease (CD) and bacterial diarrhoea (BD)

	n	Median IgA anti HSP-60 (%)	Range	*P versus* control
Active UC	16	188*	58–373	<0·01
Inactive UC	14	103	25–263	NS
Active CD	10	181	73–364	<0·05
Inactive CD	11	157	57–342	<0·05
Active BD	10	106*	51–285	NS
Recovered BD	10	73	35–242	NS

* IgA HSP-60 antibody levels significantly higher in active than in inactive (recovered BD) disease ($P < 0·05$).

active UC might lead to increased mucosal access by bacterial flora, or their products, with consequent generation of IgA antibodies cross-reactive with luminal bacterial antigens (exogenous HSPs). A similar mechanism has been proposed as an explanation for the raised levels of IgA *Klebsiella* antibodies found in patients with ankylosing spondylitis and IBD [16].

In mucosal biopsies taken from patients with Crohn's colitis, expression of HSP-60 antigen appears to be similar to controls [13]. Although, by analogy with the association between HSP-60 antigen and HSP-60 antibody in UC discussed above, it might therefore seem surprising that circulating IgA HSP-60 antibodies are elevated in CD, it is conceivable that, since this disorder affects all layers of the bowel wall, full thickness operative samples might have shown increased HSP-60 expression deeper in the tissue.

In view of the proposed link between mycobacterial infection and CD [17], it could be argued that the elevated IgA HSP-60 antibodies are directed towards HSPs from mycobacteria. The isolation of mycobacteria in patients with CD, however, has been infrequent and serological responses to common and specific mycobacterial antigens are absent [18,19]. Furthermore, unlike patients with UC or gastro-duodenal ulcer, those with CD do not have significantly elevated humoral immune responses to the mycobacterial 70-kD family of proteins [20].

The increased intestinal permeability of patients with CD [21,22] raises the possibility that HSP-60 antibody responses may be directed towards enteric bacterial antigens. Thus, an epitope-specific assay capable of differentiating between antibodies to human or bacterial HSP-60 is required to test the extent to which the results reported here and elsewhere in Crohn's disease and ulcerative colitis represent serological responses to endogenous or exogenous HSPs.

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