Immunoglobulin G (IgG), IgG1, and IgG2 determinations from endoscopic biopsy specimens in control, Crohn's disease, and ulcerative colitis subjects

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Abstract

Acute exacerbations of chronic inflammatory bowel disease (ulcerative colitis and Crohn's disease) are characterised by an increase in immunoglobulin G (IgG) positive cells in the mucosa, whereas uninfamed mucosa of inflammatory bowel disease patients displays only moderately increased or normal numbers of these cells. Previous data suggest that acute exacerbations of ulcerative colitis and Crohn's disease can be distinguished by different IgG subclass expression of mucosal immunocytes and a different IgG subclass production pattern of lamina propria lymphocytes. A procedure to obtain enough intestinal mononuclear cells from biopsy specimens to measure in vitro IgG and IgG1 production in control subjects and various patient groups has been established. IgG2 could be measured in Crohn's disease and ulcerative colitis only, as the concentrations in control subjects were below the sensitivity of the ELISA method. We found that IgG and IgG1 production correlated with the degree of local inflammation in both diseases, even in slightly inflamed mucosa, compared with control subjects. The proportion of IgG1 subclass was significantly increased in severely inflamed mucosa of both ulcerative colitis and Crohn's disease patients. A major difference between Crohn's disease and ulcerative colitis mucosa is apparent in mild or no inflammation. In Crohn's disease mucosa in remission, the IgG1/IgG ratio is comparable with that in controls, yet ulcerative colitis mucosa still displays significantly increased proportions of IgG1. In addition, the IgG2/IgG ratio is 0.12 in ulcerative colitis and 0.19 in Crohn's disease patients. The results show the dependence of local IgG and IgG1 production on the degree of inflammation and that an increase in subclass IgG1 in ulcerative colitis is present at all stages, including remission. These findings support the hypothesis that different immunoregulatory mechanisms are involved in Crohn's disease and ulcerative colitis. Environmental stimuli or genetic background may be responsible for the observed differences.

are increased in inflamed lamina propria of ulcerative colitis and Crohn's disease patients. With isolated cells, it was also shown that membrane IgG positive membrane cells were increased,⁶ but in two studies^{7,8} in which patients with inflammatory bowel disease in remission were investigated, the number of IgG immunocytes in the mucosa was marginally or not increased. However, IgG synthesis is not merely dependent on the number of plasma cells, but also on the functional state of these cells and factors which control this function. Lamina propria mononuclear cells (LPMC) of macroscopically unaffected mucosa obtained by surgery still produced more IgG than those of normal controls.⁹ The latter finding favours the hypothesis that IgG immunocyte infiltration of the mucosa and IgG production are early events in the inflammatory cascade in ulcerative colitis and Crohn's disease mucosa.

The first aim of our study was to obtain intestinal mononuclear cells (IMNC) from all clinical and histological stages of inflammatory bowel disease, including remission, to measure IgG and IgG subclass production of these cells in vitro, and to compare both disease groups with normal subjects and non-inflammatory bowel disease control subjects with unspecific colitis. To achieve this goal it was essential to establish a technique to isolate enough IMNC from endoscopic biopsy specimens.

Severely inflamed mucosa of ulcerative colitis and Crohn's disease patients shows a different IgG subclass distribution on histological examination.¹⁰ In LPMC obtained by surgical specimen from ulcerative colitis patients the increase in total IgG produced is predominantly IgG1.¹¹ However, in functional studies significant differences in the proportion of IgG subclasses have not been shown. The second aim of the study was to investigate whether there was a significant change in IgG subclass ratios in patients with inflammatory bowel diseases compared with control subjects and how it was related to the degree of inflammation.

Methods

PATIENTS

Seventy eight patients were recruited from the endoscopy units of Medizinische Universitätsklinik Würzburg, Medizinische Universitäts-Poliklinik Würzburg, and the Medical Department at the Juliuspital in Würzburg. Patients were not endoscoped for the purposes of this

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Accepted for publication
July 1991

The aetiology and pathogenesis of ulcerative colitis and Crohn's disease are still unknown, yet several phenomena make it likely that a disturbance of the mucosal immune system plays an important role in both. In this respect immunohistochemical examination has shown that cytoplasmic immunoglobulin G (IgG)¹⁻⁵ positive cells

study. All patients consented to undergo biopsy for this study and the investigation was approved by the ethical committee of the university.

Nineteen patients were referred for endoscopy with a preliminary diagnosis of ulcerative colitis and 29 patients with Crohn's disease. Fifteen samples were excluded from this study because of reasons stated in the results section. All patients underwent total endoscopy and had biopsy specimens taken from the ascending colon or the most proximal part of large bowel if they had a right sided colectomy.

All patients were evaluated using a simplified index of Crohn's disease^{12,13} or a modified form for ulcerative colitis,¹⁴⁻¹⁶ consisting of clinical and subjective variables only. All patients had blood examination of acute phase proteins and erythrocyte sedimentation rate (ESR) (Westergren method). Systemic inflammation¹⁷ was assumed if at least two inflammation indicators were in the abnormal range (ESR above 30 mm/1st hour, C reactive protein above 15 mg/l, orosomucoid above 1400 mg/l). Only patients with no abnormal acute phase parameters were classified as being without systemic inflammation. Macroscopic and histological grading of the colon was done according to Gomes *et al*¹⁴ by addition of six grading areas from caecum to rectum. The colon was graded 0-3 macroscopically (0=normal, 3=severe disease with ulcers and spontaneous bleeding) and 0-4 (0=normal, 3 and 4=destructive crypt abscess and ulceration) histologically at each area.

INTESTINAL CELL ISOLATION

Six to eight biopsy specimens used for cell isolation were immediately transferred into cold RPMI-1640 medium (Biochrom, Berlin, Germany). Single cells were obtained by a modified enzymatic technique.⁶ The specimens were washed several times in Ca/Mg free PBS (GIBCO, Berlin, Germany) and then incubated at 4°C overnight in culture medium (RPMI 1640 medium with 10% fetal calf serum (GIBCO), antibiotics (GIBCO), and fungizone (GIBCO)), containing collagenase CLSPA (Biochrom) and hyaluronidase (Sigma, Deisenhofen, Germany). The next day the cell suspension was incubated at 37°C for 60 minutes with constant stirring to release mononuclear cells. The digested specimens were passed through steel meshes with a pore size of 500 and 300 µm. After thorough washing, the cells were put on a discontinuous Percoll density gradient with 70% Percoll, 30% Percoll and culture medium and centrifuged. IMNC could be harvested from the 70%/30% Percoll interface.

IMNC viability was >85%, as determined by trypan blue exclusion. In several experiments flow cytometry was performed with an aliquot of cells using anti-CD20 (clone L27), anti-CD3 (clone SK7) (Becton-Dickinson, Heidelberg, Germany) and anti-CD22 (clone To15) (Dako, Hamburg, Germany) using a protocol supplied by the companies. Some 400 000 IMNC were seeded into each well of a 96 well microtitre plate and supplemented with 250 µl of culture medium. No stimulants were added. The cell culture was incubated at 37°C in a humidified

atmosphere with 5% CO₂ for 14 days. Supernatants of the cultured cells were collected after centrifugation at 1000 g for five minutes and stored at -20°C until used for analysis.

ELISA TO MEASURE IgG, IgG1 AND IgG2

IgG in the culture supernatants was determined by an ELISA with 96 well flat-bottomed microtitre plates as described previously.¹⁸ For IgG1 determinations we replaced the coating antibody by a monoclonal anti-IgG1 (clone SG16 as processed ascites fluid) (Biomakor, Renner, Dannstadt, Germany). The optical densities were measured by a photometer (SLT-easy reader, Gröding, Austria) and the sample IgG1 concentration was calculated from IgG1 standards (Sigma) by double logarithmic regression.

To assess the cross reactivity of our ELISA procedures, we used a subclass standard (primary standard WHO reference serum 67/97) (Nordic, Bochum, Germany), which contained known quantities of all IgG subclasses. IgG and IgG1 subclass determinations with our procedure reproduced the concentration of this sample (+/- 10%). Double determination of IgG and IgG1 subclass with a commercial IgG subclass assay (Binding Site, Birmingham, UK) and our assay showed less than 7% deviation between each other.

IgG2 determinations were done with a commercial assay (Binding Site, Birmingham, UK) using a similar protocol, that was supplied with the ELISA test. According to the supplier, the specificity of the antibodies used in this assay were determined in a WHO/IUIS collaborative study.¹⁹

STATISTICAL EVALUATION

As IgG and IgG1 data in all groups were not normally distributed we used centiles in all Tables and Figures; median (=50th centile), 90th, and 10th centile are shown. Non-parametric statistical tests were used as indicated (Wilcoxon U test and Spearman signed rank test).

Results

We isolated IMNC from 78 patients. In this study, 29 patients with Crohn's disease, 19 patients with ulcerative colitis, three patients with unspecific colitis, and 12 patients without gastrointestinal disease were evaluated. Patients with inflammation of the ileum (Crohn's disease of the ileum or backwash ileitis in ulcerative colitis) were included in this study, but ileal inflammation was not counted in the disease scores. There were no patients with inflammation of the ileum only.

ISOLATION OF IMNC FROM BIOPSY SPECIMENS

With enzymatic digestion and density gradients we were able to isolate viable IMNC from specimens that were used for functional studies. With this technique we could obtain between 500 000 and 5 million cells from 6 to 8 specimens. Viability was always >85%. The cell number was dependent on the size of the specimens and

TABLE I Immunoglobulin G (IgG) and IgG1 production in the different groups of subjects

	No	Immunoglobulin G			Immunoglobulin G1		
		10%	Median	90%	10%	Median	90%
Controls	12	30	46	101	12	24	65
Crohn's disease	29	56	114*	373	13	48*	243
Ulcerative colitis	19	30	139*	521	13	125*	391
Unspecific colitis	3	35	86	100	11	20	80

*p<0.05 compared with controls using Wilcoxon U test.

TABLE II Wilcoxon U test to determine significance of changes in immunoglobulin G (IgG) and IgG1 production between Crohn's disease or ulcerative colitis patients with and without acute phase reactions in blood examinations

	No	Median (ng/ml)	
		IgG	IgG1
CD with SIR	12	118	110
CD without SIR	9	86 } NS	40 } NS
UC with SIR	10	143	151
UC without SIR	5	80 } *	30 } *

SIR=systemic inflammatory reaction.

the degree of inflammation. Phenotyping of IMNC in several experiments showed that 6–26% were CD20 positive, 5–27% were CD22 positive, and 45–88% were CD3 positive cells.

IGG AND IgG1 PRODUCTION IN THE FOUR GROUPS

In all Tables and Figures median and 10% and 90% centiles are shown. The IgG and IgG1 production in vitro for the different disease groups is shown in Table I. The IgG and IgG1 production of patients with Crohn's disease or ulcerative colitis was significantly raised in comparison with controls (Wilcoxon U test). In mild unspecific colitis we found increased IgG and normal IgG1. Because of the small sample number we could not test unspecific colitis patients against other groups.

IGG AND IgG1 IN RELATION TO CLINICAL INDICES OF DISEASE ACTIVITY

When Crohn's disease and ulcerative colitis

patients were separated into subgroups of patients with and without systemic inflammation we could show increased IgG and IgG1 in supernatants of those with systemic inflammation, yet this only reached significance in ulcerative colitis patients (Table II). There was no significant correlation between IgG or IgG1 production and clinical activity scores such as the simplified CDAI or the similar index for ulcerative colitis (UCAI according to Gomes *et al* modified from Truelove and Witts) (data not shown).

IGG AND IgG1 PRODUCTION IS DEPENDENT ON ENDOSCOPIC DISEASE SEVERITY ASSESSED BY THE GOMES'S INDEX IN CROHN'S DISEASE AND ULCERATIVE COLITIS

As IgG and IgG1 production in vitro is probably influenced by the degree of inflammation we subsequently grouped the patients with Crohn's disease and ulcerative colitis according to the grade of macroscopic inflammation. Using the endoscopic score devised by Gomes⁷ for the entire colon (Fig 1, open bars), we found significant differences in IgG1 production according to the degree of inflammation (Gomes's score > or <5) in Crohn's disease and ulcerative colitis patients. Similar results for IgG are not shown.

Finally we used the macroscopic appearance of the mucosa at the site from which the biopsy specimen was taken to classify Crohn's disease and ulcerative colitis patients into two groups. To emphasise the difference between inflammatory stages we have grouped patients with Gomes grade <2 and Gomes's grade >2 and have omitted patients with intermediate inflammation. We found significant differences in IgG1 concentrations between severely inflamed and slightly inflamed specimens in both diseases (Fig 1, hatched bars). Similar data were obtained when total IgG production was compared.

IGG AND IgG1 PRODUCTION IN RELATION TO HISTOLOGICAL INFLAMMATION OF THE MUCOSA

When Crohn's disease and ulcerative colitis patients were grouped according to histological criteria devised by Gomes into severely and mildly inflamed cases (Gomes's score < or >9), we found a tendency to higher IgG and IgG1 production in the subgroup with severe inflammation. In spite of this trend the difference did not reach significance in either disease (IgG production not shown, IgG1 production shown in Fig 2, open bars). However, when local inflammation in the colonic segment was compared with the IgG and IgG1 production in the same segment, we found a significant difference in ulcerative colitis patients (Fig 2 hatched bars).

IGG AND IgG1 PRODUCTION OF IMNC IN ULCERATIVE COLITIS AND CROHN'S DISEASE PATIENTS WITH SLIGHT INFLAMMATION OF THE MUCOSA

To examine if IgG and IgG1 production is an early event in the inflammatory reaction of the mucosa, we compared local IgG and IgG1 production of Crohn's disease and ulcerative colitis

Figure 1: Immunoglobulin G1 (IgG1) production of intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease. Patients are subdivided according to the macroscopic appearance of the mucosa with a Gomes's score (><5, □) and with a local score (><2, ▨) at the site from which the biopsies were taken.

Figure 2: Immunoglobulin G1 (IgG1) production of intestinal mononuclear cells from patients with ulcerative colitis and Crohn's disease. Patients are subdivided according to the histological Gomes's score (>9, □) and according to a local score (>2, ▨) at the site from which the biopsies were taken.

Figure 3: Ratio (SEM) of in vitro production of immunoglobulin G1 (IgG1) and IgG. Ratios for controls, Crohn's disease, and ulcerative colitis, as well as subgroups of the diseases are shown (according to the macroscopic and histological score at the biopsy site). Severe inflammation is indicated by ▨ and mild inflammation by ▩. * p<0.05 indicates significant differences using Wilcoxon U test, NS indicates no significant difference.

patients with minimal inflammation with that of control subjects. Grade 0 and grade 1 inflammation of the mucosa were grouped together, as grade 0 was only rarely encountered. We found that IgG and IgG1 production in Crohn's disease v control subjects was raised, but just missed significance using Wilcoxon U test (Table III). In ulcerative colitis patients there was a significant difference between patients with mild disease and control subjects using macroscopic criteria (Table III). When histological criteria are used, we did not find significant differences, probably because of the small sample size.

IGG1/IGG RATIOS IN ULCERATIVE COLITIS, CROHN'S DISEASE, AND CONTROL SUBJECTS
The contribution of IgG1 to total IgG in subjects was quite variable in all groups and subgroups so that single ratios were not characteristic for one disease. However, mean IgG subclass ratios of disease groups were different from controls. The IgG1/IgG ratio was significantly different

TABLE III Wilcoxon U test to determine significance of changes in IgG and IgG1 production between controls and ulcerative colitis or Crohn's disease patients with minimal histological inflammation of the mucosa

Group	Significance	Median (ng/ml)
Control (n=12)		IgG 46
CD local macroscopic index <2 (n=14)	p=0.06 IgG	IgG1 24
CD local histological index <2 (n=10)	p=0.06 IgG	IgG1 84
UC local macroscopic index <2 (n=15)	p=0.08 IgG1	IgG1 44
UC local histological index <2 (n=8)	p=0.08 IgG1	IgG1 101
	p<0.05 IgG	IgG1 48
	p<0.05 IgG1	IgG1 123
	p=0.30 IgG	IgG1 121
	p=0.25 IgG1	IgG1 114
		IgG1 104

between ulcerative colitis patients and controls. This difference was also significant when ulcerative colitis patients with low inflammation and only slightly raised total IgG were compared. In contrast Crohn's disease patients showed an inflammation related increase in the proportion of IgG1. Thus, controls and all Crohn's disease patients did not have significantly different IgG1/IgG ratios. Only the subgroup of Crohn's disease patients with severe inflammation (local Gomes's index >2) produce significantly more IgG1, resulting in a significant change in the IgG1/IgG ratio (Wilcoxon U test, Fig 3).

COMPARISON OF IGG, IGG1, AND IGG2 PRODUCTION IN ULCERATIVE COLITIS AND CROHN'S DISEASE PATIENTS

In five control, 14 Crohn's disease, and 8 ulcerative colitis patients IMNC we could test IgG2 production in addition to that of IgG and IgG1. IgG2 could not be measured in all controls because it was below the detection limit of the assay used (standard curve from 10 ng to 125 ng/ml). Median IgG production was 115 ng/ml in ulcerative colitis and 106 ng/ml in Crohn's disease patients. Thus both groups showed similar degrees of inflammation. The proportion of IgG2 in total IgG was higher in Crohn's disease than ulcerative colitis patients, but this did not reach significance. As the IgG1/IgG ratio in Crohn's disease patients with severe

Figure 4: Percentage of immunoglobulin G1 (IgG1) and IgG2 subclasses of total IgG in ulcerative colitis, Crohn's disease, and Crohn's disease subgroups. IgG1/IgG ▨ and IgG1/IgG2 ratios (not shown) are significantly different between ulcerative colitis and Crohn's disease. IgG2/IgG ▨ is not significantly changed between ulcerative colitis and Crohn's disease or Crohn's disease subgroups.

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inflammation was higher, we tested the possibility that Crohn's disease patients with low IgG concentrations might have significantly different IgG2 proportions compared with ulcerative colitis patients. We found a further increase in the IgG2/IgG ratio (Fig 4), but this was not significantly different from ulcerative colitis patients.

Discussion

We have established an isolation procedure for IMNC from biopsy specimens with cell yields high enough to perform functional studies. The use of biopsy specimens means that even inflammatory bowel disease patients who do not undergo surgery can participate in functional research of mucosal immunity.

It has been shown histologically by Soltoft,² Brandtzaeg *et al.*,³ Baklien *et al.*,²⁰ and several other groups,^{7,8,21} that IgG positive immunocytes are increased in inflamed mucosa. Baklien *et al.*²⁰ showed that ileal mucosal inflammation and IgG immunocyte infiltration were correlated and that IgG immunocytes were not increased in slightly inflamed ileum of Crohn's disease patients. Kobayashi *et al.*,²² however, found that IgG immunocytes were still increased in marginally inflamed mucosa from ulcerative colitis patients. Several studies examining inflammatory bowel disease as an entity^{7,8,22} found a correlation between inflammation and the number of IgG containing cells.

In functional studies, it was shown by Aiuti *et al.*,²³ MacDermott *et al.*,²⁴ Danis *et al.*,²⁵ Wu *et al.*,²⁶ and Verspaget *et al.*,⁹ that Crohn's disease and ulcerative colitis IMNC produce increased amounts of IgG. Since Crohn's disease or ulcerative colitis patients in these studies underwent surgery, it seems likely that they had severe inflammation of the mucosa.

The data obtained from healthy controls show that IgG production in vitro can be measured even in normal mucosa. In vitro IgG production of IMNC from biopsy specimens confirms previous results in ulcerative colitis and Crohn's disease mucosa and thus validates our biopsy method. Using this method to evaluate patients with differing degrees of inflammation, we showed that IgG and IgG1 production is not correlated to subjective clinical indices and shows only loose correlation to acute phase parameters of the patients' sera. Yet IgG production in vitro and inflammation of the mucosa – measured endoscopically and histologically – correlate well in Crohn's disease and ulcerative colitis. These functional data supplement histological data from several groups and show that biopsy specimens are sufficient to measure IgG production of the mucosa at different disease stages, including remission. When Crohn's disease and ulcerative colitis patients with slight inflammation of the mucosa were compared with controls, we found increased median IgG and IgG1 production in both diseases. Ulcerative colitis patients with a low macroscopic Gomes's score were significantly different from controls. Our data support the idea that increased IgG production is an early event in the inflammation of Crohn's disease and ulcerative colitis, and that this may

contribute to inflammation by its proinflammatory potency.

IgG subclasses have been examined histologically by Kett *et al.*¹¹ and Iizuka.²⁸ Kett *et al.* describe significant differences between ulcerative colitis and Crohn's disease mucosa in terms of percentages of IgG1 and IgG2 positive immunocytes. They did not examine controls. Iizuka reported that ulcerative colitis patients had increased numbers of all IgG subclass positive cells compared with controls. However, relative changes were only significant for IgG1 subclass. Functional studies by Scott *et al.*¹¹ showed IgG subclass shifting between controls, ulcerative colitis, and Crohn's disease patients. They found a significant increase in IgG and IgG1 subclass production in ulcerative colitis, but the percentages of IgG subclasses were not tested for significance.

Our data show that the percentage of IgG1 is significantly increased in ulcerative colitis compared with controls and Crohn's disease patients. In ulcerative colitis, we also found an intrinsic shift in IgG subclasses towards IgG1 at all stages of inflammation. The relative IgG1 increase in ulcerative colitis is significant, even in patients with slight mucosal inflammation and marginally raised IgG and IgG1. Thus ulcerative colitis patients have a genetically or environmentally induced immunoregulation abnormality at all stages. In contrast, in Crohn's disease patients we found significantly increased proportions of IgG1 in severely inflamed mucosa only. As the IgG1 percentages in ulcerative colitis patients with severe inflammation are also higher than in ulcerative colitis patients with less inflamed mucosa, we suggest that IgG1 production is correlated to inflammation in all diseases of the gut.

Because IgG2 production could not be measured in controls (it was below the sensitivity of our assay) we could only compare Crohn's disease and ulcerative colitis patients. The percentage of IgG2 was increased in Crohn's disease compared with ulcerative colitis patients (19 v 12%) but this difference was not significant. The percentage of IgG1 and IgG2 produced by IMNC in our study and percentage of IgG subclass immunocytes according to Kett *et al.*¹⁰ are very similar, again showing that IgG subclass production and phenotype are correlated.

IgG antibodies in colonic mucosa are considered to be a 'second line' of defence when immune exclusion by IgA antibodies is not sufficient.¹ However, IgG has proinflammatory properties that can lead to aggravation or perpetuation of immune reactions. In this respect it is of interest that IgG1 is more effective in complement activation than IgG2.^{28,29} In addition, IgG1 and activated complement have been shown along the epithelium in ulcerative colitis.³⁰ Thus it seems likely that IgG1 and complement may contribute to the epithelial damage in ulcerative colitis.

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