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Specificity of antibodies secreted by hybridomas generated from activated B cells in the mesenteric lymph nodes of patients with inflammatory bowel disease

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SUMMARY Hybridomas have been prepared from active B cells in lymphoid tissue draining lesions of Crohn's disease (CD) and ulcerative colitis (UC), by fusion of fresh mesenteric lymph node suspensions with the murine JK myeloma. Two hundred and fifty nine immunogloblin secreting hybridomas have been obtained from nine patients. The antibodies have been screened for binding to food antigens, sections of human gut, and bacteria including two unidentified acid fast isolates from CD lymph nodes. Autoantibodies, and antibodies to food antigens implicated by others in the aetiology of CD were rare, comprising 1·2%, and 2·5% respectively. Most donors yielded none of these. Thus neither food antigens nor autoantigens are major antigenic stimuli in nodes draining inflammatory bowel disease. On the other hand between 19% and 83% of supernatants from different donors bound to one or more bacterial genus. The mycobacteria and the CD isolates were amongst the genera to which most antibodies bound, though binding to *E coli* was more frequent. Significantly more CD than UC derived supernatants bound to BCG. As mycobacteria are not thought to be part of the normal bowel flora, the high percentage of hybridomas secreting antibodies which bind to this genus is surprising.

Crohn's disease is an inflammatory disorder of unknown cause often involving the terminal ileum, but also affecting other parts of the gastrointestinal tract, particularly the colon. Ulcerative colitis also involves the colon though its relationship to Crohn's disease remains uncertain. There has been much speculation as to the aetiology of these conditions, but there is no convincing evidence for any of the existing suggestions. Amongst the many hypotheses put forward, the most appealing have been autoimmunity, bacterial infection, and food allergy. Thus some authors consider that an autoimmune mechanism is indicated by the presence in the serum of gut binding antibodies,[1-3] and in the lamina propria, lymph nodes, or peripheral blood of lymphocytes which recognise surface components of gut epithelial cells.[4-7] Other workers, emphasising the presence of tuberculoid granulomata in the lesions of some patients with Crohn's disease, have suggested a mycobacterial aetiology, and have reported the isolation of imperfectly characterised cell wall defective organisms from lymph nodes draining affected gut,[8] or from the gut itself.[9] Sporadic association of conventional mycobacterial species with these culture systems has led to further speculation.[8 9] The evidence for a role for food allergy[10] comes mostly from clinical improvement seen when some patients are put on antigen depleted diets.[11]

We reasoned that the pattern of specificity of antibody-secreting B cells in lymph nodes draining active lesions of Crohn's disease or ulcerative colitis, might indicate whether the major antigenic stimulus

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in these nodes is an intestinal autoantigen, a bacterium of whatever genus, or a food antigen. In order to study this we have prepared human/mouse hybridomas without prior *in vitro* stimulation of the lymph node cells, and investigated the frequency and specificity of the antibodies secreted.

Methods

PATIENTS

Mesenteric lymph nodes draining diseased gut were obtained from nine patients undergoing bowel resection for Crohn's disease (CD) or ulcerative colitis (UC) (Table 1). Usable data were not obtained from mesenteric nodes taken from donors without inflammatory bowel disease, because the frequency of antibody secreting cells was too low.

PRODUCTION OF HUMAN/MOUSE HYBRIDOMAS

The cells were teased from the nodes, and fused immediately with the JK mouse myeloma line (P3-X63-Ag8,653) at a ratio of 5–10 node cells to one myeloma cell. Fusion was promoted with 50% (v/v) polyethylene glycol (BDH no 29575, MW 1500) in RPMI 1640 (Gibco). The fused cells were then seeded into HAT medium in flat bottomed 96 well microtitre trays at $1–3\times10^5$ cells/well in the presence of irradiated (3000 rads) murine peritoneal cells (5×10^4/well). The HAT medium was RPMI 1640 containing 20% fetal calf serum (Gibco), hypoxanthine (10^{-4}M), aminopterin (4×10^{-7}M), thymidine ($1{\cdot}6\times10^{-5}$M), penicillin (100 U/ml), and streptomycin (100 ug/ml). At the cell densities used less than 30% of the wells showed growth, most of which was assumed to be clonal. No further cloning of lines from positive wells was undertaken.

Seventy two hours after the fusion fresh HAT medium was added, and the wells were fed with fresh medium every four to five days for three weeks. At this time supernatants of positive wells were assayed for immunoglobulin, and when this was detected the supernatant was stored, and the relevant cell line was maintained in RPMI 1640 with 20% FCS. The supernatant of these secreting lines was stored for as long as immunoglobulin production continued. Eventually because of the inherent instability of human/mouse hybridomas, immunoglobulin production or growth ceased in all lines.

ENZYME LINKED IMMUNOSORBENT ASSAY OF IMMUNOGLOBULIN PRODUCTION BY THE HYBRIDOMAS, AND OF THE SPECIFICITY OF THE ANTIBODIES SECRETED

The class and approximate content of human immunoglobulin present in the cell supernatants was determined by a conventional immunoglobulin capture assay as described elsewhere,[12] except that 20 μl Terasaki wells (Gibco 1-63118) were used instead of 200 μl microtitre wells, in order to economise on supernatants. Thus the wells were coated for two hours with 20 μl of rabbit antihuman alpha, gamma, or mu chain-specific antibody (DAKO) diluted to 12 μg/ml in 0·2 molar carbonate/bicarbonate buffer, pH 9·6. Subsequently the wells were washed and blocked with 0·5% Tween 20 in phosphate buffered saline, pH 7·2. Then 20 μl of hybridoma supernatant, control culture medium, or serial doubling dilutions of purified human IgA, IgG, or IgM (DAKO) were incubated in appropriate wells for three hours. After further washing in PBS/Tween, peroxidase conjugated rabbit antisera to human IgA, IgG, or IgM (Fc-specific) were added to the relevant wells and incubated for three hours. After a final wash, the binding of the peroxidase conjugate to the wells was revealed using 2,2'-Azino di-(3-ethylbenzthiazoline) sulphonic acid as chromogen as described previously.[12]

Binding of the human immunoglobulin in the hybridoma supernatants to bacterial or food antigens was assayed similarly, except that the wells were coated with the antigens, or control preparations described below. These antigens were always diluted to 12 μg/ml in the carbonate/bicarbonate buffer.

Table 1 *Donors of the mesenteric lymph-nodes used for the generation of hybridomas*

Patient	*Age*	*Sex*	*Diagnosis*	*Duration (years)*	*Site*	*Treatment*
CB (34)*	27	F	CD	6	colon	prednisolone
CE (14)	23	F	CD	2	ileum	—
MdN (17)	27	F	CD	?	ileum and rectum	prednisolone
BW (34)	45	M	CD	17	ileum	prednisolone
MA (20)	63	M	CD	21	rectum	prednisolone azathioprine
MiL (18)	16	F	CD	8	ileum and colon	—
RS (30)	54	M	UC	35	colon	azathioprine
DT (82)	37	M	UC	3	rectum	prednisolone azathioprine
ML (10)	42	F	UC	6 months	ileum and colon	—

*Numbers in parentheses are the numbers of immunoglobulin-secreting hybridomas obtained from each donor.

PREPARATION OF SOLUBLE ANTIGENS FROM BACTERIA AND FOOD

The bacterial strains used to produce soluble antigen preparations are listed in the Tables, and were obtained from our own collections. The mycobacteria were subcultured on Sauton's medium. Other strains were subcultured on blood agar, except for the organisms listed as Baker and Daw. These are unidentified organisms which have produced very limited growth at the meniscus of Robertson's cooked meat broth cultures five years after inoculation with tissue from the lymph nodes of two CD patients. Organisms of this type, the significance of which is quite unknown, have been described elsewhere.[8]

Organisms were harvested, washed twice in PBS (except Baker and Daw of which only microgram quantities were available), resuspended, and disrupted in an MSE ultrasonicator in a water cooled chamber at an amplitude of 7 μ for 15 minutes. Sonicates were then centrifuged at $4{\cdot}5 \times 10^4$ *g* and passed through membrane filters (Schleicher and Schuel, 0·22 μ). The protein concentration was estimated by the method of Folin and Ciocalteau. When the sonicates from Baker and Daw were used in the ELISA, a sample of supernatant from Robertson's cooked meat broth was used in parallel as a control antigen.

The food antigens used were selected from those implicated by some authors[11] in the pathogenesis of CD. Thus removal from the diet of wheat, cabbage, and milk were claimed to be most likely to lead to clinical improvement. These antigens were prepared in the same way as the bacterial ones. Cooked meat broth was also used as a control for the organisms grown in this medium.

SCREENING FOR AUTOANTIBODY ACTIVITY IN THE SUPERNATANTS

Undiluted hybridoma supernatants were screened by routine indirect immunofluorescence for binding to 4 μ cryostat sections of normal human colon and duodenum. The appropriate heavy chain specific fluorescein conjugated rabbit antihuman reagent (DAKO) was used at 1 in 20 as the second layer.

Results

CLASS DISTRIBUTION OF THE ANTIBODY SECRETING HYBRIDOMAS OBTAINED

Two hundred and fifty nine immunoglobulin secreting lines were obtained from the nine donors. There were approximately equal numbers of hybrids secreting each of the three major immunoglobulin classes. In this respect CD and UC donors were similar. More than one class was found in only 20% of the supernatants. When this occurred, the specificity of only the most abundant class was investigated in the subsequent ELISA studies. Supernatants used for these studies contained between 0·3–10 μg/ml of the dominant class.

BINDING OF THE HYBRIDOMA SUPERNATANTS TO BACTERIAL ANTIGENS

As a preliminary screening procedure, the 259 supernatants were tested for binding to: (1) a pool of the five mycobacterial antigens; (2) pooled *Bacteroides fragilis* and *vulgatus*; (3) Daw and Baker; (4) a heterogeneous pool of the remaining organisms.

Table 2 shows the percentage of all supernatants, and the percentage of supernatants of each individual class which bound to each pool. A significantly greater percentage of the CD derived hybridomas bound to bacterial antigens. This was largely because of increased percentages of IgG and IgM hybridomas binding to the mycobacterial pool ($p<0{\cdot}001$, Fisher's exact test), and to the heterogeneous pool of organisms ($p<0{\cdot}001$). There was no difference in the percentage of hybridomas binding to the organisms isolated from CD lymph-nodes (Baker and Daw), and no differences between the IgA antibodies from the two groups.

Table 2 *Preliminary screening of hybridoma supernatants against pooled bacterial antigens*

	Hydrids screened (n)		*Crohn's disease % positive of*				*Ulcerative colitis % positive of*			
Antigen pool	*Crohn's disease*	*Ulcerative colitis*	*total*	*IgA*	*IgG*	*IgM*	*total*	*IgA*	*IgG*	*IgM*
Mycobacteria*	137	122	30‖	10	28	29	13	10	14	7
B fragilis and vulgatus†	109	75	17¶	10	13	7	11	2	8	4
Daw or Baker‡	106	75	21¶	10	18	7	19	8	14	4
Other organisms§	137	122	36‖	23	38	21	17	16	15	9

*A pool of sonicate from *M chelonei*, *M kansasii*, *M gilvum*, *M vaccae* and BCG (Glaxo); †A pool of sonicate from *Bacteroides fragilis* and *Bacteroides vulgatus*; ‡The two isolates from Crohn's disease described in the methods section; §A pool of sonicates from Group A Streptococci, *Corynebacterium diphtheriae* and *C pseudotuberculosis*, *Candida albicans*, and *Escherichia coli*; ‖Significantly greater than for UC derived hybridomas ($p<0{\cdot}001$, Fisher's exact test); ¶Not significantly different from results with UC derived hybridomas.

Table 3 *Binding of hybridoma supernatants to individual bacterial species*

	Number screened		% positive	
Species	*Crohn's disease*	*Ulcerative colitis*	*Crohn's disease*	*Ulcerative colitis*
M chelonei	137	122	19	8
M kansasii	137	122	16	6
M gilvum	137	122	9	7
M vaccae	137	122	4	4
BCG (Glaxo)*	137	122	18	8
B fragilis	109	75	14	7
B vulgatus	109	75	14	11
Daw	106	75	21	13
Baker	106	75	21	19
C diphtheriae	137	122	8	7
C pseudotuberculosis	137	122	15	8
E coli	137	122	27	11
Group A strep	137	122	19	10
Candida albicans	137	122	7	6

*% of CD derived significantly greater than % of UC derived monoclonal antibodies binding to BCG ($p<0.05$, Mann-Whitney U test).

Positive supernatants were then screened for binding to the individual components of the pools. In Table 3 these results are expressed as the percentage of the total number originally tested which bound to each species. The highest percentage of the UC derived monoclonals bound to Daw and Baker, but this was not significantly more than bound to *E coli*, Bacteroides, or group A streptococci. Similarly about 20% of the CD derived monoclonals bound to each of a heterogeneous assortment of organisms, including mycobacteria, *E coli*, Baker, and group A streptococci. The percentage which bound to BCG was significantly greater amongst monoclonals derived from CD than amongst those derived from UC (Mann-Whitney U test, $p<0.05$). No other difference between the two patient groups was significant by this test because of wide variations between individuals.

CROSS REACTIVITY OF THE UC OR CD DERIVED MONOCLONAL ANTIBODIES WHICH BOUND TO THE CD DERIVED ORGANISMS

The binding to other organisms of monoclonals already shown to bind to Baker and Daw could in theory illuminate the taxonomic relationships of the latter. Table 4 shows that this group of monoclonals (22 from CD, 14 from UC) showed very variable cross reactivity with all the species tested except *M vaccae*.

It was noted that 27% (CD) and 43% (UC) of the monoclonals binding to Baker did not bind to any other organisms tested. Table 5 shows, however, that these figures were only marginally higher than the corresponding values for *E coli* binding monoclonals, and do not therefore suggest a special relationship between Baker and inflammatory bowel disease.

Table 4 *Percentage of hybridoma supernatants known to bind to the Crohn's disease isolate Baker, which also bound to the organism indicated*

	% binding to the cross reacting antigen	
Cross reacting antigen	*Crohn's disease (n=22)*	*Ulcerative colitis (n=14)*
M chelonei	27	14
M kansasii	23	7
M gilvum	14	14
M vaccae	0	0
BCG (Glaxo)	18	29
B fragilis	50	14
B vulgatus	45	29
C diphtheriae	14	21
C pseudotuberculosis	27	14
E coli	32	14
Group A strep	9	7
Candida albicans	9	21

BINDING OF CD AND UC DERIVED MONOCLONAL ANTIBODIES TO FOOD ANTIGENS, AND TO SECTIONS OF NORMAL GUT

Monoclonals binding to these antigens were rare. Only three of 121 hybridoma supernatants (69 from CD, 52 from UC) contained autoantibodies. One bound to the duodenal brush border, one gave cytoplasmic staining of duodenal and colonic tissue, and one gave nuclear staining on duodenal tissue. These three antibodies failed to bind to any of the organisms, even when concentrated five fold.

Antibodies to food antigens were equally rare. Three IgG antibodies from one patient (MdN) bound to wheat. The same three antibodies also bound to the two bacteroides strains.

Discussion

Most experimental approaches to inflammatory bowel disease are hampered by the fact that we

Table 5 *Hybridomas which bound to only one of the bacterial species tested*

	Supernatants binding to the indicated organism, which bound to no other organism tested (n and %)	
Organism	*Crohn's disease*	*Ulcerative colitis*
Baker	6/22 (27)	6/14 (43)
E coli	11/37 (30)	3/13 (23)
Group A strep	2/26 (8)	4/12 (33)
M kansasii	2/22 (9)	0/7 (0)

cannot distinguish reliably between the causes and the consequences of disease. To some extent the present study avoids this problem. The data indicate that on average 42% of all the hybridomas secreted antibody which bound to bacteria (range 19%–83%), whereas only three of 259 (1·2%) bound to any of the food antigens. These three antibodies all came from one CD donor, and they also bound to the bacteroides strains. Thus it seems unlikely that food antigens, which may be as abundant in the gut as bacterial ones, are dominant immunogens in the draining nodes of these patients.

The same argument applies to the autoantibodies as only three were found (2·5% of those tested). These antigens must be abundant in the diseased gut tissue, and yet there is clearly little response to them relative to the response to bacteria. It was interesting that even when concentrated the three autoantibodies failed to bind to any of the bacteria studied, suggesting that they may not be evoked by cross reactive bacterial antigens.

Ideally these figures should have been compared with similar data obtained with lymph nodes draining normal bowel. Unfortunately this is not possible because in our experience such nodes contain too few activated B cells and yield only two or three hybridomas per node. Only certain actively secreting B cells will form secreting hybridomas. We deliberately avoided activating the B cells *in vitro* by preincubation with a polyclonal B cell activator such as endotoxin or pokeweed mitogen because it is known that this results in production of a high proportion of autoantibodies, and would have rendered the data meaningless. Because we were unable to obtain sufficient hybridomas from control nodes, we cannot rule out the possibilities that the preponderance of bacterium binding hybridomas is entirely normal, or that it is a result of trapping of irrelevant B cells in activated nodes, though we do not consider either of these mechanisms to be probable.

Our data therefore suggest that mesenteric nodes from IBD patients show intense antibacterial responses, probably much more than in normal nodes, whereas there is little response to autoantigens or to food antigens. Numerous authors have reported raised serum antibody concentrations to a wide variety of bacterial genera.[13-20] We have attempted to analyse the specificity of this antibacterial response at the clonal level. The data indicate that there was binding to a broad range of genera. This could arise either through non-specific absorption of antigen from the bowel flora, or, in view of the considerable cross reactivity between all bacterial strains, it could point to a specific bacterial aetiology if the antigen preparations used did not include a close relative of the putative pathogen. We clearly cannot distinguish between these possibilities. There were, however, three aspects of the results which were unexpected. First, the percentage of the hybridomas which bound to mycobacteria was as high as the percentage binding to *E coli* or to the bacteroides, although mycobacteria are not thought to be part of the normal bowel flora, so exposure to their antigens may depend on the contamination of water supplies. It seems improbable that this constitutes an antigenic challenge comparable with that of the commonest bowel flora. Second, significantly more CD derived than UC derived hybridomas secreted antibody binding to BCG according to the non-parametric Mann-Whitney U test, which takes account of differences between individuals within the groups. (Using Fisher's exact test the difference is even more significant). Third, the two unidentified acid-fast CD derived organisms were also amongst those to which most hybridomas supernatants bound. Moreover precisely the same supernatants bound to both organisms, suggesting that they are similar or identical.

In conclusion, the data presented here are compatible with a mycobacterium like aetiological agent, or with an excessive response to a common bacterial component, but even this analysis of the antibody response of patients with CD and UC at the clonal level, which to the authors' knowledge is unique, has failed to point clearly at a particular bacterial genus.

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