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Serum agglutinins to *Eubacterium* and *Peptostreptococcus* species in Crohn's and other diseases

By F. WENSINCK and J. P. VAN DE MERWE

Department of Medical Microbiology, Erasmus University, Rotterdam, The Netherlands

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SUMMARY

Sera from patients suffering from Crohn's and other diseases and from healthy subjects were tested for agglutinins to anaerobic, gram-positive coccoid rods belonging to species of *Eubacterium* and *Peptostreptococcus*. Four strains labelled *Eubacterium contortum* (two strains), *Eubacterium rectale* and *Peptostreptococcus productus* were agglutinated by a higher percentage of sera from patients with Crohn's disease than from healthy subjects and from patients with liver and intestinal diseases (including ulcerative colitis), ankylosing spondylitis, granulomatous diseases, diseases of immunity and malignancies.

The agglutinins were of the IgG and IgM classes and strain-specific; the titres were low.

The results obtained with sera from patients with Crohn's disease and healthy people were subjected to discriminant analysis to estimate the probability, based on the combined results with the four strains, that a patient suffers from Crohn's disease. When sera giving an *a posteriori* probability $\geqslant 0{\cdot}95$ (*a priori* probability $= 0{\cdot}5$) were considered positive, the test with four strains had a sensitivity of 54 % and a specificity of nearly 100 %. The results with sera submitted for diagnosis showed that positive reactions in patients with a diagnosis apparently incompatible with Crohn's disease were within acceptable limits.

INTRODUCTION

The faecal flora of patients with Crohn's disease of the terminal ileum contains a higher number of anaerobic gram-negative and gram-positive coccoid rods than the flora of healthy subjects. Neither duration of illness nor ileocaecal resection had an effect on flora composition (Wensinck *et al.* 1981).

In a preliminary study it was found that sera from patients with Crohn's disease agglutinate a strain of *Eubacterium contortum* more frequently than sera from patients with other diseases and sera from healthy subjects (Wensinck, 1975, 1976). Twenty-five isolates belonging to species of *Eubacterium* and *Peptostreptococcus* have been tested and, except for a few strains that were not agglutinated, most were agglutinated by a higher percentage of sera from patients with Crohn's disease than from healthy subjects. Four strains were selected for their capacity to discriminate between sera from patients with Crohn's disease and sera from

healthy subjects. The agglutination of these strains by sera from healthy subjects and from patients with Crohn's and other diseases is reported here. The results have been used to estimate the probability that a patient suffers from Crohn's disease by means of an interpretation based on discriminant analysis (Van de Merwe, Schmitz & Wensinck, 1981). Subsequently, sera submitted for the establishment of the diagnosis of Crohn's disease have also been interpreted in this way.

MATERIALS AND METHODS

Patients

Sera were provided by physicians from various hospitals (see acknowledgements). Requests for serum included specifications of certain clinical features and laboratory findings. The patients suffering from Crohn's disease and other diseases (called 'control' diseases) were considered by their physicians to represent the classical diseases. Patients with Crohn's disease were under treatment at the Departments of Internal Medicine II and Surgery, University Hospital Dijkzigt, Erasmus University, Rotterdam, The Netherlands. The healthy subjects were blood donors (Red Cross Transfusion Service, Rotterdam). The 'control' diseases are listed in Table 1 and include diseases of the intestinal tract and liver, ankylosing spondylitis, diseases of immunity (atopy, rheumatoid arthritis), granulomatous diseases (leprosy, tuberculosis, sarcoidosis) and malignant disease. Some relevant clinical data are given in the table.

The diagnosis was uncertain in many patients from whom serum was submitted for the establishment of the diagnosis of Crohn's disease. Detailed clinical information was requested in all cases with positive serological results.

Bacteria

Three strains of gram-positive coccoid anaerobes (code numbers ME_{44}, ME_{46} and ME_{47}) were isolated from the faeces of patients with Crohn's disease as described by Wensinck *et al.* (1981). One strain (C_{18}), labelled *Peptostreptococcus productus*, was received from Dr W. E. C. Moore (Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA). Some characteristics of the strains are given in Table 2. Two strains (ME_{44} and ME_{47}) were identified as *Eubacterium contortum*, one (ME_{46}) as *Eubacterium rectale* and one (C_{18}) as *P. productus*.

Bacterial suspensions

Suspensions were prepared from cultures in anaerobic broth (Wensinck *et al.* 1981) grown for 24–48 h at 37 °C. Formaldehyde solution was added to a final concentration of 0·5% and, after incubation for 18 h at 37 °C, the bacteria were washed twice and resuspended in 0·85% (w/v) saline with 0·01% (w/v) sodium ethylmercurithiosalicylate. Cell density was adjusted to 5×10^8 bacteria per ml and pH to 6·8–7·0; the pH of freshly prepared suspensions had to be re-adjusted after 3–4 days and the suspensions were checked monthly for contamination.

Table 1. *'Control' diseases*

Intestinal tract and liver

Acute appendicitis: post-operative course uneventful
Carcinoma of large bowel
Chronic active liver disease: all patients HBsAg positive
Chronic diarrhoea: in children, median age 5 years (1–15), origin of diarrhoea unknown
Cirrhosis of liver: due to alcohol abuse in most of the patients
Coeliac disease: treated as well as untreated patients
*Irritable bowel syndrome**
Primary biliary cirrhosis: increased serum alkaline phosphatase, antimitochondrial antibodies, normal cholangiogram and liver biopsy compatible with diagnosis in all patients
Schistosomiasis: Schistosoma mansoni, patients from The Netherlands and Ethiopia. Control sera from Ethiopians not suffering from schistosomiasis
Ulcerative colitis

Other

Ankylosing spondylitis: all patients HLA-B27 positive and not suffering from inflammatory bowel disease
*Atopy**
Rheumatoid arthritis: rheumatoid factor present in all patients
Leprosy: all patients under medical treatment
*Pulmonary tuberculosis:** all patients under medical treatment
*Sarcoidosis**
Bronchial carcinoma
Haematological malignancies: Hodgkin's disease and lymphosarcoma with equal number of patients

* Studied with strains ME_{44} (*Eubacterium contortum*) and C_{18} (*Peptostreptococcus productus*) only.

Agglutination test

Sera were tested within three days after collection. Two drops of serum and one drop of bacterial suspension were thoroughly mixed with a platinum loop on a slide that was shaken (150 rev./min) on the platform of a rotary mixer (Mini-Shaker, Kühner A.G., Basel, Switzerland). Results were scored after 5 min as negative (0) or positive (1, 2 or 3 according to strength of agglutination). With strongly positive sera (score 3), the agglutinate consisted of a clump and the surrounding fluid was clear. Inactivation of complement (30 min at 56 °C) or storage at −40 °C had no effect on agglutination titres.

Titres were determined with doubling dilutions of serum in 0·85 % saline. For agglutinin absorption, the bacterial sediment of 1 ml of suspension was resuspended in 1 ml of serum; after 1 h at room temperature, the supernatant was withdrawn after centrifugation.

Antisera

Antisera were prepared in New Zealand White female rabbits by intramuscular injection of 1 ml of bacterial suspension twice weekly for 3 weeks.

Immunoglobulin class of agglutinins

The sera from patients with Crohn's disease were applied to a Sephadex G-200 column, according to Thompson & Stokes (1977). The column was eluted with Tris-HCl buffer 0·4 M, pH 8·2 and the effluents of the individual protein peaks in

Table 2. *Characteristics of the anaerobic gram-positive coccoid rods*

	Strain			
	ME_{44}	ME_{47}	ME_{46}	C_{18}
Arabinose	+	+	+	+
Xylose	+	+	+	+
Fructose	+	+	+	+
Glucose	+	+	+	+
Mannose	−	−	−	+
Cellobiose	−	+	−	+
Lactose	−	+	+	+
Maltose	+	+	+	+
Sucrose	+	+	+	+
Trehalose	−	−	−	+
Melezitose	−	−	−	+
Raffinose	+	+	+	+
Starch	−	−	−	−
Amygdalin	−	−	−	−
Esculin (hydrolysis)	+	+	−	+
Salicin	+	+	−	+
Erythritol	−	−	−	−
Mannitol	−	−	−	−
Sorbitol	−	−	−	+
Gas	+	+	+	−
Final pH	4·7	5·4	5·0	4·3
Fermentation products	E, A, F	E, A	nB, L	A, F

+ = acid produced; − = no acid produced; A = acetic acid; nB = normal butyric acid; E = ethanol; F = formic acid; L = lactic acid.

the chromatogram were pooled and concentrated by selective membrane filtration with cut-off point MW 15000 daltons (Amicon B 15, Amicon Corp., Lexington, Mass., USA). Immunoglobulin levels in the concentrated fractions were determined with radial immunodiffusion as described by Milford-Ward (1977) using commercially available plates and reference serum (Behringwerke A.G., Marburg, Federal Republic of Germany).

Statistical methods

Results from different groups were compared using the χ^2 test with Yates' correction for continuity. The strength of agglutination should be considered when the tests with four strains were used to obtain one combined result. This was achieved by using discriminant analysis to estimate the probability that a patient suffers from Crohn's disease (Van de Merwe, Schmitz & Wensinck, 1981). In the present report, sera with agglutination reactions giving an *a posteriori* probability $\geqslant 0{\cdot}95$ were considered positive.

RESULTS

Results are presented under three headings: (1) patients with Crohn's disease and healthy subjects; data on titres, absorption and immunoglobulin class of agglutinins are also given in this section; (2) 'control' disease and (3) sera submitted for diagnosis.

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Table 3. *Agglutination of* Eubacterium contortum (*strain* ME_{44})

	Percentage positive*	No. of patients
Intestinal tract and liver		
Acute appendicitis	5	21
Carcinoma of large bowel	19	52
Chronic active liver disease	19	27
Chronic diarrhoea (in children)	5	20
Cirrhosis of liver	21	38
Coeliac disease	23	44
Irritable bowel syndrome	10	20
Primary biliary cirrhosis	5	20
Schistosomiasis	26	68
(Ethiopian control sera)	(30)	(23)
Ulcerative colitis	15	48
Other		
Ankylosing spondylitis	21	75
Diseases of immunity		
Atopy	27	29
Rheumatoid arthritis	6	51
Granulomatous diseases		
Leprosy	25	85
Pulmonary tuberculosis	21	42
Sarcoidosis	23	80
Malignant diseases		
Bronchial carcinoma	16	58
Haematological malignancies	10	80
Crohn's disease	62 (< 0·001)	125
Healthy subjects	15	100

* *P*-value of difference with healthy subjects in parentheses; values not given when $P > 0{\cdot}05$.

Patients with Crohn's disease and healthy subjects

The data in Tables 3–6 show that the four strains were agglutinated more frequently by sera from patients with Crohn's disease than by sera from healthy subjects. Fifty-four per cent of the patients with Crohn's disease were positive (*a posteriori* probability $\geqslant 0{\cdot}95$) whereas all healthy subjects were negative (Table 7).

The agglutination titres of strongly positive Crohn's sera (score 3) were low (1/16 – 1/32).

Rabbit immune sera agglutinated the strains to titres of 1/32–1/64 and showed no cross-reactivity. Accordingly, the agglutinins were absorbed specifically from Crohn's sera as shown in Table 8.

In most sera, the agglutinins were present in the IgG or IgG + IgM fractions (Table 9).

'Control' diseases

Table 3 shows that strain ME_{44} (*Eubacterium contortum*) was agglutinated by sera from 'control' patients with percentages not significantly different from the value in healthy subjects.

The other strain of *E. contortum* (ME_{47}) was agglutinated more frequently by sera from patients with large bowel carcinoma but percentages of other 'control' diseases were not significantly different from that in healthy subjects (Table 4).

Strain ME_{46} (*E. rectale*) was also agglutinated more frequently by patients with

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Table 4. *Agglutination of* Eubacterium contortum (*strain* ME_{47})

	Percentage positive*	No. of patients
Intestinal tract and liver		
Acute appendicitis	14	21
Carcinoma of large bowel	15 (= 0·05)	39
Chronic active liver disease	15	27
Chronic diarrhoea (in children)	5	20
Cirrhosis of liver	10	21
Coeliac disease	11	37
Irritable bowel syndrome	—	—
Primary biliary cirrhosis	5	20
Schistosomiasis	—	—
Ulcerative colitis	4	48
Other		
Ankylosing spondylitis	8	24
Diseases of immunity		
Atopy	—	—
Rheumatoid arthritis	0	19
Granulomatous diseases		
Leprosy	14	28
Pulmonary tuberculosis	—	—
Sarcoidosis	—	—
Malignant diseases		
Bronchial carcinoma	5	20
Haematological malignancies	5	22
Crohn's disease	37 (< 0·001)	118
Healthy subjects	4	100

* *P*-value difference with healthy subjects in parentheses; values not given when $P > 0{\cdot}05$.
— indicates not tested.

colonic carcinoma. It also showed a higher than normal percentage in ulcerative colitis patients; the percentage was however much lower than in patients with Crohn's disease (Table 5).

Sera from patients with various 'control' diseases agglutinated *P. productus* (strain C_{18}) more frequently than did sera from healthy subjects (Table 6) but the percentages were significantly lower than in Crohn's disease.

The percentages of positive sera as calculated from the combined results with four strains are shown in Table 7. They were higher than normal in cirrhosis of liver, coeliac disease and ulcerative colitis but significantly lower than in Crohn's disease.

Sera submitted for diagnosis

Of a sample representing about one-quarter of the sera received for diagnostic purposes in 1978, 21 % was positive (Table 10). In about 60 % of the positive cases the diagnosis of Crohn's disease had been established. In 27 % of the patients, symptoms suggested Crohn's colitis or ulcerative colitis and 8 % showed presenting symptoms of Crohn's disease.

Serological results in these patients cannot be interpreted satisfactorily until the course of the disease allows a firm diagnosis. In 3 % of the positive cases, the clinical histories appeared incompatible with the diagnosis of Crohn's disease. This

Table 5. *Agglutination of* Eubacterium rectale (*strain* ME_{46})

	Percentage positive*	No. of patients
Intestinal tract and liver		
Acute appendicitis	5	21
Carcinoma of large bowel	10 (< 0·05)	41
Chronic active liver disease	7	27
Chronic diarrhoea (in children)	5	20
Cirrhosis of liver	8	25
Coeliac disease	8	38
Irritable bowel syndrome	—	—
Primary biliary cirrhosis	10	20
Schistosomiasis	4	48
Ulcerative colitis	17 (< 0·001)	48
Other		
Ankylosing spondylitis	8	24
Diseases of immunity		
Atopy	—	—
Rheumatoid arthritis	7	30
Granulomatous diseases		
Leprosy	6	31
Pulmonary tuberculosis	—	—
Sarcoidosis	—	—
Malignant diseases		
Bronchial carcinoma	0	20
Haematological malignancies	0	22
Crohn's disease	49 (< 0·001)	124
Healthy subjects	1	100

* *P*-value of difference with healthy subjects in parentheses; values not given when $P > 0{\cdot}05$.
— indicates not tested.

percentage appears acceptable, because sera were considered positive when the probability of Crohn's disease was $\geqslant 0{\cdot}95$, implying that 5 % of positive results may occur in patients without Crohn's disease.

DISCUSSION

The finding that intestinal coccoid anaerobes are agglutinated more frequently by sera from patients with Crohn's disease than from healthy subjects is compatible with data showing that these patients produce antibodies to a wide range of food and microbial antigens like bovine serum albumin (Falchuk & Isselbacher, 1976), gluten (Eterman & Feltkamp, 1978), maize (Davidson *et al.* 1979), *Bacteroides fragilis* (Brown & Lee, 1974), *Bacteroides vulgatus* (Helphingstine *et al.* 1979), *Escherichia coli* (Tabaqchali, O'Donoghue & Bettelheim, 1978) and viruses like rotavirus (DeGroote *et al.* 1977). This may be explained by assuming that conditions for antibody production are favourable in the inflamed mucosa with dense infiltrates of immunocompetent cells. In the case of *Bacteroides* and coccoid anaerobes, the high numbers present in the intestinal microflora (Wensinck *et al.* 1981) may promote antibody production.

In the present study, sera from 54 % of the patients with Crohn's disease were positive with an *a posteriori* probability of $\geqslant 0{\cdot}95$. In a study of correlations

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Table 6. *Agglutination of* Peptostreptococcus productus (*strain* C_{18})

	Percentage positive*	No. of patients
Intestinal tract and liver		
Acute appendicitis	10	21
Carcinoma of large bowel	12	52
Chronic active liver disease	7	27
Chronic diarrhoea (in children)	0	20
Cirrhosis of liver	26 (< 0·001)	38
Coeliac disease	9	44
Irritable bowel syndrome	5	20
Primary biliary cirrhosis	5	20
Schistosomiasis	41 (< 0·001)	68
(Ethiopian control sera)	(13)	(23)
Ulcerative colitis	21 (< 0·01)	48
Other		
Ankylosing spondylitis	39 (< 0·001)	40
Diseases of immunity		
Atopy	34 (< 0·001)	29
Rheumatoid arthritis	6	34
Granulomatous diseases		
Leprosy	29 (< 0·001)	59
Pulmonary tuberculosis	19 (< 0·05)	42
Sarcoidosis	7	29
Malignant diseases		
Bronchial carcinoma	2	49
Haematological malignancies	3	30
Crohn's disease	55 (< 0·001)	125
Healthy subjects	5	100

* *P*-value of difference with healthy subjects in parentheses; values not given when $P > 0{\cdot}05$.

between agglutination reactions and clinical data of Crohn's and ulcerative colitis patients we found that agglutination was positively correlated with localization of Crohn's disease in the colon, with the presence of fistulae and with levels of serum immunoglobulins (Van de Merwe, 1980).

There is one striking difference between the studies on antibody production mentioned and our results. Antibodies to the various food and microbial antigens were present in both Crohn's and ulcerative colitis patients but, with the coccoid anaerobes, results in these diseases were significantly different. From the group of 48 ulcerative colitis patients, 12% were positive ($P \geqslant 0{\cdot}95$) and from the Crohn's patients 54%. Moreover, the percentages of sera that were negative with all strains were 79% in healthy subjects, 65% in ulcerative colitis and 17% in Crohn's patients; the differences between Crohn's patients and the two other groups were highly significant. In view of the well-known difficulties with the differential diagnosis of these conditions, reviewed by Kirsner (1975), we feel that misdiagnosis should be considered when serological reactions are positive in patients presumed to suffer from ulcerative colitis.

From the 'control' diseases, coeliac disease and cirrhosis of liver showed slightly higher percentages of positive sera than normal. Cirrhosis, schistosomiasis, ankylosing spondylitis, atopy and two granulomatous diseases (leprosy, tuberculosis) had relatively high percentages of C_{18} positive sera. Because agglutination of strain

Table 7. *Positive sera in patients with Crohn's and 'control' diseases and healthy subjects*

	Percentage positive*	No. of patients
Intestinal tract and liver		
Acute appendicitis	5	21
Carcinoma of large bowel	5	41
Chronic active liver disease	7	27
Chronic diarrhoea (in children)	5	20
Cirrhosis of liver	8 (= 0·05)	25
Coeliac disease	8 (< 0·05)	38
Irritable bowel syndrome	—	—
Primary biliary cirrhosis	0	20
Schistosomiasis	—	—
Ulcerative colitis	12 (< 0·001)	48
Other		
Ankylosing spondylitis	8	26
Diseases of immunity		
Atopy	—	—
Rheumatoid arthritis	7	28
Granulomatous diseases		
Leprosy	6	31
Pulmonary tuberculosis	—	—
Sarcoidosis	—	—
Malignant diseases		
Bronchial carcinoma	0	20
Haematological malignancies	5	22
Crohn's disease	54 (< 0·001)	118
Healthy subjects	0	100

* Positive: *a posteriori* probability ⩾ 0·95 with *a priori* probability = 0·5. *P*-value of difference with healthy subjects in parentheses; values not given when $P > 0·05$.
— indicates not tested with four strains.

Table 8. *Agglutination of strains ME_{44}, ME_{47}, ME_{46} and C_{18} by two Crohn's sera before and after absorption*

	Agglutination*	
	Serum 2701	Serum 2719
Whole serum	3 2 3 3	3 2 3 3
Serum absorbed with strain		
ME_{44} (*Eubacterium contortum*)	0 2 3 3	0 2 3 3
ME_{47} (*E. contortum*)	3 0 3 3	3 0 3 3
ME_{46} (*E. rectale*)	3 2 0 3	3 2 0 3
C_{18} (*Peptostreptococcus productus*)	2 2 3 0	3 2 3 0

* Strain ME_{44}, ME_{47}, ME_{46} and C_{18} respectively, according to strength of agglutination.

C_{18} was correlated with IgG levels in Crohn's patients, the frequent agglutination of C_{18} in 'control' diseases may also be due to hyperglobulinaemia which occurs in a rather high number of patients suffering from liver diseases (Triger & Wright, 1973), schistosomiasis (Camus *et al.* 1977) and leprosy (Bullock, 1971).

Ideally, the interpretation of a diagnostic test should be based on the results from samples of the patient population and a similar population differing only by

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Table 9. *Agglutination reactions in whole Crohn's sera and corresponding IgM and IgG fractions*

	Whole serum		
Code number	Agglutination*	IgM fraction Agglutination*	IgG fraction Agglutination*
2593	3232	0100	3033
2627	3333	2301	2033
2628	3033	3030	3033
2629	3333	2333	3033
2657	3030	0000	3030
2660	3033	0001	2030
2669	3121	0101	3003
2677	3203	2000	2302
2702	3333	0000	2100
2728	2133	0002	0023
2752	1233	0200	0223
2831	2133	0123	2003
2832	3133	3203	3133

* Agglutination of strain ME_{44}, ME_{47}, ME_{46} and C_{18} respectively, according to strength of agglutination.

Table 10. *Sera submitted for diagnosis* (*June, July and August* 1978)

Total number	517
Number positive*	110
Clinical diagnosis of positives	
Crohn's disease (established)	68 (62%)
Crohn's disease (probable)	16 (15%)
Colitis	13 (12%)
Presenting symptoms of Crohn's disease (anal fistula 4, diarrhoea 5)	9 (8%)
Others (coeliac disease 1; carcinoma 1; growth retardation 1; uncertain 1)	4 (3%)

* *A posteriori* probability $\geqslant$ 0·95 (*a priori* probability = 0·5).

the absence of the particular disease. For obvious reasons this is difficult when Crohn's disease is studied. The clinical value of the diagnostic test with the four strains combined was therefore evaluated from results in patients with Crohn's disease and healthy subjects. As the average period of time between onset of symptoms and diagnosis of Crohn's disease is 3–4 years (Dyer & Dawson, 1970; Brandes & Eulenburg, 1976) it is evident that a number of sera submitted for diagnostic purposes are from patients with symptoms suggesting the disease but in which the diagnosis has not yet been established. At present, the lapse of time after the serological test is too short to interpret positive reactions in these patients conclusively. The low percentage of positive reactions in patients with a diagnosis which appears to be incompatible with Crohn's disease is considered acceptable.

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