serum antibodies to anaerobic coccoid rods in crohn's disease

PROEFSCHRIFT

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PROMOTOREN : PROF. DR F. WENSINCK
R. VAN STRIK
CO-REFERENTEN: PROF. DR M. FRENKEL
J.H.P. WILSON

The photograph on the cover is of *Eubacterium rectale* (strain Me 46)

voor

Selma: "..onder het proefschrift heb ik niet geleden.."

Leontine (5): "mogen we dan hakschoenen ?"

Marieke (5): "heb je dan een feestje ?"

Martine (2½): ".. wikke nie ..."

SERUM ANTIBODIES TO ANAEROBIC COCCOID RODS IN CROHN'S DISEASE

CONTENTS

chapter 1

INTRODUCTION

The faecal flora of patients with Crohn's disease of the ileum has been found to differ from that of healthy subjects (Wensinck, 1975; 1976). Patients with Crohn's disease have a higher number of anaerobic gram-negative bacteria and about 10% of the flora are anaerobic, gram-positive coccoid rods that are not usually found in the faeces of healthy subjects. Wensinck found no evidence that the flora abnormality was influenced by the duration of the inflammation or by ileocaecal resection. Serum agglutinins were found in a considerable percentage of patients with Crohn's disease, directed against some strains of the coccoid rods, later identified as *Eubacterium* and *Peptostreptococcus* species. In patients suffering from other diseases and in healthy subjects, antibodies were found less frequently. The interpretation of these findings is the subject of this thesis in which particular attention is paid to the significance of the agglutinins in Crohn's disease and the use of the agglutination reactions as a diagnostic test.
In Chapter 2 recent studies on microbial agents in tissues of patients with Crohn's disease and the presence of antibodies to viruses, bacteria and tissue components are reviewed.
Chapter 3 deals with the presence of serum agglutinins to coccoid anaerobes in patients with Crohn's and other diseases.
Relationships between agglutination reactions and clinical and laboratory features in patients with Crohn's disease and ulcerative colitis are the subject of Chapter 4.
In Chapter 5 the results are reported of studies on the nature and properties of the antibodies, in particular their effector functions.
In Chapter 6 logical interpretation of laboratory results is discussed

and this chapter serves as an introduction to Chapter 7, in which the use of the agglutination reactions as a diagnostic tool is described. Chapter 8 is a general discussion on the relevance of the findings for the understanding of the aetiology of Crohn's disease.

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chapter 2

REVIEW OF RECENT LITERATURE ON THE PRESENCE OF MICROBIAL AGENTS AND ANTIBODIES IN PATIENTS WITH CROHN'S DISEASE

VIRUSES

Transmissible agents. In 1970 Mitchell and Rees described an experiment in which homogenates from Crohn gut or lymph-node tissue were inoculated into the footpads of normal and immunologically deficient mice. In both groups of mice, epithelioid and giant-cell granulomas were seen in the footpads, in 63% a month and in 33% 169 to 500 days after inoculation. One of the 58 mice given non-Crohn's lymph-node homogenates showed these characteristics after one month and none of the 48 surviving mice after 169-500 days. These experiments were continued by Cave *et al.* (1973). Six rabbits were inoculated intra-ileally with tissue homogenates from 2 patients with Crohn's disease. The control group consisted of 3 rabbits. Tissues for histological assessment were obtained at 3, 6 and 9 months. All animals were killed after 10 months. Results were inconsistent, but most rabbits inoculated with Crohn's tissue homogenates showed a granulomatous response after various intervals in at least one of the biopsies. Bolton *et al.* (1973) inoculated homogenates, fresh and sterilized, of diseased and normal bowel from 7 patients with Crohn's disease into 96 rats, 69 guinea-pigs and 130 mice. Histological examination was performed 6-9 weeks and 6-8 months after inoculation. No sarcoid-like granulomas were found in any biopsy, whether taken early or late. In 1975 Cave *et al.* reported mucosal ulceration, ileal thickening, abscess formation and granulomatous changes in rabbits, inoculated intramurally into the

intestine with 0.2 or 100 nm filtrates of homogenate of fresh ileum or colon from each of 6 patients with Crohn's disease. Moreover, a successful first passage was achieved from 6 of 11 rabbits. This was followed by completely negative results reported by Heatley *et al.* (1975) in 17 mice, 67 rats and 7 rabbits, inoculated with tissue homogenates from 17 patients with Crohn's disease. Taub *et al.* (1976) inoculated footpads of 13 mice with tissue homogenates from patients with Crohn's disease. Twelve showed epithelioid granulomas at the injection site 25 days later and these were most numerous in mice injected with actively inflamed granulomatous material. Homogenates taken from patients with ulcerative colitis and from control patients undergoing resections for unrelated diseases produced granulomas in 4 of 4 and 4 of 9 instances, respectively. In mice inoculated with Crohn's disease tissue homogenates, granulomas were no longer identifiable 150 days after injection. Donnelly *et al.* (1977) reported experiments in which rabbits were inoculated with homogenates of normal and Crohn's affected human bowel tissues. Crohn's like changes were found in 11 of 27 rabbits after 6 months, but 12 months after injection the rabbit bowel had reverted to normal. Moreover, the addition of ampicillin to the homogenates prevented the appearance of the Crohn's like changes. Contradictory reports continue to appear. Simonowitz *et al.* (1977) only observed changes in the bowel wall of rabbits receiving Crohn's disease inoculum. However, the characteristic features of Crohn's disease were not reproduced. Bergstrand and Holmström (1978) reported negative results with 0.2 nm filtrates in rats but Cave *et al.* (1978) again reported positive results in normal and immunodeficient mice with crude and cell free filtrates of Crohn's tissues. The same authors (Cave *et al.*, 1979) recently had to admit, however, that two figures in their latest paper showed a leiomyosarcoma of uterine origin and a lymphoma of aging mice rather than granulomatous reactions and they emphasized the difficulties inherent in interpreting histologic experiences in long-term animal studies.

Electron microscopy. Gitnick and Rosen (1976) characterized viral particles isolated from ileal filtrates of patients with Crohn's disease; control filtrates showed no evidence of viral agents. The mean diameter of the particles was 30 nm. The physical chemical properties and the electron microscopic appearance were consistent with those of a picornavirus. Riemann (1977) found virus-like particles, with diameters ranging from 30-70

nm, in biopsies of 7 of 9 patients with Crohn's disease of the colon.
Tissue culture and immunofluorescence methods. Farmer *et al.* (1973) investigated cytopathogenic effects in tissue cultures of tissue specimens from 4 patients with Crohn's disease and 6 with ulcerative colitis. Tissues were also investigated with indirect fluorescent antibody tests and electron microscopy. In tissues from one of the patients with Crohn's disease and 3 of those with ulcerative colitis, cytomegalovirus was demonstrated. Whorwell *et al.* (1977) found cytopathogenic effects of 220 nm filtrates of intestinal resections from 6 of 10 patients with Crohn's disease, whereas control filtrates had no effect.The agent appeared to be a RNA virus, 55-60 nm in diameter, probably belonging to the reovirus group. Indirect immunofluorescence for antigens of rotavirus in Crohn's disease tissue yielded positive results with tissues of 5 from 9 patients. Since fluorescence could not be blocked with specific calf antibody, the authors concluded that a reaction with viral antigen in the tissue was unlikely and that the observed phenomenon was not specific.
Viral antibodies. In sera of patients with Crohn's disease, normal titres of antibodies to Epstein-Barr virus were found (Grotsky *et al.*, 1970; Kane & Nye, 1971). Farmer *et al.* (1973) found normal titres of antibody to cytomegalovirus in patients with Crohn's disease, but a higher frequency of antibody and higher titres were found in ulcerative colitis patients. DeGroote *et al.* (1977) found a higher frequency (90%) of antibodies to rotavirus in patients with Crohn's disease than in those with ulcerative colitis (68%) and the control group (75%). No differences were seen between patients with Crohn's disease and controls in antibody titres to coxsackievirus B4 and poliovirus 2. Greenberg *et al.* (1978) found comparable antibody titres in patients with Crohn's disease and controls to rotavirus and norwalkvirus. Korsmeyer *et al.* (1976) demonstrated antibodies to double-stranded RNA in 23% of the patients with Crohn's disease and 12% of those with ulcerative colitis. DeHoratius *et al.* (1978) observed that serum from patients with inflammatory bowel disease and their unaffected spouses bound significantly more synthetic single and double-stranded RNA than did serum from age-matched controls and their spouses. The authors interpreted these findings as indirect support for the presence of RNA viruses in patients with inflammatory bowel disease and the transmission of such agent to close personal contacts.

Electron microscopy. In 1971 Aluhiware described results of an electron microscopic study of colonic tissues from 16 patients with Crohn's disease. In 6, intramural clusters of bacteria were seen in the deeper layers of the colon with intact epithelium and minimal inflammatory changes. The bacteria were not identified, the appearance varying from degenerating bacilli to coccoid organisms. The organisms were not seen in any of the normal colons or in any of those with ulcerative colitis and intact epithelium.

Cultural methods and immunofluorescence methods. Parent and Mitchell (1978) isolated cell wall-defective variants of pseudomonas-like bacteria from filtrates of homogenized bowel tissues and lymph-nodes, obtained from 8 patients with Crohn's disease. Positive results were obtained only if the filtrates were cultured in hypertonic media. Conventional bacteriological cultures and all cultures from 9 patients with ulcerative colitis and 20 patients without inflammatory bowel disease yielded negative results. Burnham *et al.* (1978) cultured mesenteric lymph-nodes from patients with inflammatory bowel disease and controls on mycobacterial media. A node from one out of 27 patients with Crohn's disease yielded a strain of *Mycobacterium kansasii*. Cultures from 22 other patients with Crohn's disease, 7 out of 13 with ulcerative colitis and one out of 11 control subjects yielded pleomorphic organisms with the electron microscopic appearance of cell wall-deficient organisms. These organisms could not be further characterized. Whorwell *et al.* (1978), however, found no evidence by indirect immunofluorescence methods, for the presence of *Pseudomonas maltophilia* and *Mycobacterium kansasii* in tissues from 9 patients with Crohn's disease and 4 with ulcerative colitis.

Bacterial antibodies. Thayer *et al.* (1969) studied the incidence of antibodies to 2 strains of *Escherichia coli*, O:14 and O:119. An indirect haemagglutinating antibody titre of $\geq$16 was considered positive. Positive results for *E. coli* O:14 occurred with sera of 39% of the patients with Crohn's disease, 33% of the patients with ulcerative colitis and 3% of the sera of patients with various diseases. There was no correlation between site of the disease process and the incidence of positive titres. Studies with *E. coli* O:119 were negative in Crohn's disease. Brown and Lee (1971) using a radioimmunological method found normal anti-

body titres against an unidentified *E. coli* strain in patients with Crohn's disease. In 1974 the same authors found slightly increased titres to enterococci in patients with Crohn's disease as well as in those with ulcerative colitis. Marked elevations of antibody titres to *Bacteroides fragilis* were found in both diseases and the titres correlated with the severity of disease and the presence of pyogenic complications.
Tabaqchali *et al.* (1978) studied the incidence of agglutinating antibodies to 159 strains of *E. coli* in 16 patients with Crohn's disease and 14 with ulcerative colitis. Titres of $\geq$200 were considered positive. In the sera of patients with Crohn's disease, antibodies were demonstrated to 0-30 strains (median 10) and in those from patients with ulcerative colitis to 0-26 strains (median 5). Antibody titres in both diseases were similar. The highest titres were found in the IgM fractions, but IgG fractions yielded positive reactions as well. No specific O-serotypes were associated with either disease and no correlations were seen between the number of agglutinins to *E. coli* and the site and severity of the disease or type of therapy. These results are different from those of Thayer *et al.* (1969), who found an increased incidence of antibodies to *E. coli* O:14 in patients with inflammatory bowel disease.
Parent *et al.* (1978) investigated with an indirect immunofluorescence technique the incidence of antibodies to pseudomonas-like cell wall deficient bacteria, previously cultured from tissues of patients with Crohn's disease (Parent & Mitchell, 1978). In patients with Crohn's disease, 15 out of 17 had titres of $\geq$80. In 7 patients with ulcerative colitis and 10 healthy controls titres were found up to 40. White *et al.* (1978) found with indirect immunofluorescence in 9 out of 11 patients with Crohn's disease and 8 out of 10 with ulcerative colitis antibody titres of $\geq$20 to *Mycobacterium kansasii*. The tests were negative in 22 control subjects. Helphingstine *et al.* (1979) found by counterimmunoelectrophoresis in 12 out of 12 sera from patients with Crohn's disease antibodies to a heat-extracted antigen of *Bacteroides vulgatus*. In a mixed group of patients with inflammatory bowel disease, 59% positive reactions were seen compared to 32% in a healthy control group. The antibodies occurred in low titres of about 4 and were predominantly of the IgM class. With antigens from 6 other *Bacteroides* strains no differences were found between the groups.

CHLAMYDIA

Schuller *et al.*(1979) detected antibodies to *Chlamydia trachomatis* of predominantly LGV type 2 in 69% of sera from patients with Crohn's disease, in 10% of those with other gastrointestinal diseases and in 2% of healthy control sera. These results were not confirmed by Taylor-Robinson *et al.*(1979) who found antibodies to *Chlamydia trachomatis* in patients with Crohn's disease and ulcerative colitis with incidences of 14.5 and 21.7%, respectively, resembling the incidence in a healthy, non-venereal-disease population. In no case was antibody directed specifically to the LGV types. Swarbrick *et al.*(1979) found antibodies to *Chlamydia trachomatis* types A-K or 207 in only 7% of patients with Crohn's disease, in 12% of those with ulcerative colitis and in 14% of patients with other digestive diseases. Again, none had antibodies to LGV types or *Chlamydia psittaci*.
The results obtained by Taylor-Robinson *et al.*(1979) and Swarbrick *et al.* (1979) suggest that antibodies to *Chlamydia trachomatis*, LGV types included, and *Chlamydia psittaci* do not occur more commonly in patients with inflammatory bowel disease than in control populations.

ANTIBODIES TO TISSUES, CELLS AND DIETARY PROTEINS

Antibodies to homologous tissues. In 6-25% of the sera from patients with Crohn's disease, antibodies to connective tissue and reticulin were demonstrated (Alp & Wright, 1971; Magalhaes *et al.*, 1974; Eterman & Feltkamp, 1978). Results obtained in other diseases suggest that these antibodies were secondary to tissue damage or absorption of dietary antigens. Normal incidences were found for antibodies to human colon, smooth muscle, mitochondria, thyroglobulin, thyroid cytoplasma, gastric parietal cells and the nuclei of cells (Perrett *et al.*, 1971). Walker (1978) studied sera of patients with inflammatory bowel disease and controls for the presence of antibodies to buccal mucosa with an immunofluorescence technique. Direct staining with polyvalent antiserum showed no deposit of antibody in normal, Crohn's disease and ulcerative colitis buccal mucosa. When the mucosae were incubated with homologous serum, a perinuclear fluorescence was seen only when both mucosa and serum were obtained from patients with Crohn's disease. These studies were extended

by Matthews *et al.* (1979) who found positive results in only 2 out of 9 patients with Crohn's disease with autologous sera and 4 out of 11 with the use of heterologous sera. These antibodies also reacted with normal epithelial tissue and also occurred in one out of 4 patients with ulcerative colitis.

Antibodies to lymphocytes. Antibodies to lymphocytes have been demonstrated in a wide variety of human disease states, including connective tissue diseases and viral and bacterial infections. They also occur in healthy subjects, during pregnancy and following vaccinations (see references of Strickland *et al.* , 1975). They were detected in 40% of the patients with Crohn's disease and ulcerative colitis (Strickland *et al.* , 1975). The antibodies were reactive with determinants of both T- and B-lymphocytes. An increased prevalence of these antibodies was seen in family members and household contacts of patients with inflammatory bowel disease (Korsmeyer *et al.* , 1975). The relevance of these lymphocytotoxic antibodies in patients with inflammatory bowel disease remains a matter of speculation. The authors suggest that they may indicate an exposure of probands and their family members to a common environmental agent.

Antibodies to heterologous tissues. Carlsson *et al.* (1977) found that 61% of their patients with Crohn's disease had haemagglutinating antibodies in titres of $\geq$16 to colon antigen from germ-free rats. These antibodies also occurred in increased frequencies (47 to 69%) in other diseases like ulcerative colitis, salmonellosis, polyposis coli and their family members, gastroenteritis, irritable colon, liver cirrhosis and urinary tract infections. In other, non-gastrointestinal diseases and healthy controls the percentages of positive reactions were 35% and 13%, respectively. The results do not allow simple interpretation. The colon antigen probably contains several antigenic determinants, some of which in common with *E. coli* 0:14 (Perlmann *et al.* , 1967).

Antibodies to dietary proteins. Taylor *et al.* (1964) found normal frequencies of antibodies to a fraction of gluten. Eterman and Feltkamp (1978), however, demonstrated antibodies to gluten in 52% of patients with Crohn's disease. Highest frequencies were found in children with untreated coeliac disease (100%), and in half of the adults with untreated disease. In patients on a gluten-free diet these percentages decreased to 87 and 32, respectively. High percentages were also found in cys-

tic fibrosis (42%), recurrent diarrhoea (37%), dermatitis herpetiformis (18%) and ulcerative colitis (18%). These results were confirmed by Davidson *et al.* (1979) who also found that maize antibodies occurred in 33% of patients with Crohn's disease as well as in those with ulcerative colitis (50%), coeliac disease (44%), and healthy subjects (14%). Falchuk and Isselbacher (1976) found antibodies to bovine serum albumin in 86% of patients with Crohn's disease, in 93% of those with ulcerative colitis, in 5 out of 5 with untreated coeliac disease and in healthy subjects (43%). The most likely explanation of these findings is that increased frequencies of dietary antibodies result from an increased absorption of dietary proteins as a consequence of damage of the intestinal mucosa.

CONCLUSIONS

The results of experiments on the transmissibility of Crohn's disease are contradictory and no general conclusion can be drawn. It appears likely, however, that the capacity to induce a granulomatous inflammatory response is not specific for Crohn's disease or otherwise inflamed tissues. The relevance of these findings to the aetiology of Crohn's disease is therefore doubtful.

With electron microscopy, cultural methods and indirect immunofluorescence, the presence of a variety of viruses has been demonstrated in tissues of patients with inflammatory bowel disease. None of these investigations, however, provide strong evidence for a viral aetiology of Crohn's disease.

Studies on the presence of bacteria in Crohn's disease tissues have also yielded contradictory results. Neither from results obtained with cultural methods, nor from serological studies, does a bacterial aetiology seem likely. Results of studies in which a wide variety of tissue and dietary antibodies were demonstrated suggest that these antibodies are secondary to tissue damage, increased absorption or cross reactions. From the many studies mentioned it can be concluded that in patients with Crohn's disease as well as in other gastrointestinal diseases, antibodies to a wide variety of antigens, especially those present in the intestine, are found frequently.

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chapter 3

SERUM ANTIBODIES TO EUBACTERIUM *AND* PEPTOSTREPTOCOCCUS *SPECIES IN CROHN'S AND OTHER DISEASES**

INTRODUCTION

The faeces of patients with Crohn's disease of the ileum has been shown to contain higher numbers of anaerobic gram-negative rods and of gram-positive coccoid anaerobes than that of healthy subjects. Duration of illness and ileocaecal resection had no effect on flora composition and this suggests that the flora may be abnormal before the disease becomes manifest (Wensinck *et al.*, 1980).
In a preliminary study it has been found that Crohn's sera agglutinate a strain of *Eubacterium contortum* more frequently than sera from patients suffering from other diseases and healthy subjects (Wensinck, 1975; 1976). Since then, some 25 isolates belonging to species of *Eubacterium* and *Peptostreptococcus* have been tested and, except for a few strains that were not agglutinated at all, they were agglutinated by a higher percentage of sera from patients with Crohn's disease than from healthy subjects. Four strains were eventually selected on the basis of their capacity to discriminate between patients with Crohn's disease and healthy subjects by means of agglutination reactions.
In this chapter the results are presented of these agglutination reactions. From these results the *a posteriori* probability of Crohn's disease (see Chapter 7) is calculated in patients with Crohn's disease, ulcerative colitis, various other diseases and healthy subjects. Subsequently, sera submitted for diagnosis have been interpreted in the

*) This chapter forms part of a paper by Wensinck and Van De Merwe, submitted for publication.

same way and the results with these sera are also given.

MATERIALS AND METHODS

Sera were provided by physicians from various hospitals (see acknowledgements). Requests for serum included specifications as to certain clinical features or laboratory parameters. Control patients suffering from other diseases (Table 1), called "control diseases", were considered by the physicians to have the classical disease. The diagnosis was uncertain

Table 1. Diseases of control patients, with certain characteristics

INTESTINAL TRACT AND LIVER
acute appendicitis: uneventfull postoperative course
carcinoma of large bowel
chronic diarrhoea: of unknown origin, in children with median age of 5 yrs (1-15)
coeliac disease: treated and untreated patients
irritable bowel syndrome *
ulcerative colitis: group similar to that of Chapter 4
chronic active liver disease: all patients HBsAg positive
cirrhosis of liver: mostly due to alcohol abuse
primary biliary cirrhosis: antimitochondrial antibodies in all patients
schistosomiasis: patients from The Netherlands and Ethiopia
OTHER
ankylosing spondylitis: HLA-27 positive; no suspicion of bowel disease
atopy*: out-patients and students with a history of atopy
rheumatoid arthritis: rheumatoid factor positive
leprosy: all patients under medical treatment
pulmonary tuberculosis*: all patients under medical treatment
sarcoidosis*: groups I-IV present
bronchial carcinoma
haematological malignancies: nearly equal numbers of patients with Hodgkin's disease and lymphosarcoma

*) Studied with strains Me 44 and C 18 only.

in many patients from whom serum was submitted for diagnostic purposes and detailed information was requested in all cases with positive serological results.

Crohn's disease and healthy subjects. All patients (n = 125) were under treatment at the Depts. of Internal Medicine II or Surgery, University Hospital Dijkzigt, Erasmus University, Rotterdam. Healthy subjects (n = 100) were blood donors (Red Cross Transfusion Service, Rotterdam). The groups of patients and healthy subjects are similar to those in Chapters 4 and 7.

Sera submitted for diagnosis. This group consisted of all sera received during June, July and August, 1978.

Bacteria. From the four strains tested, three were isolated from the faeces of patients with Crohn's disease as described by Wensinck *et al.* (1980), code numbers Me 44, Me 46 and Me 47. One strain (C 18) was received from Dr Moore (The Virginia Polytechnic Institute and State University, Anaerobe Laboratory, Blacksburg, 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 *Peptostreptococcus productus*.

Agglutinating suspensions. Suspensions were prepared from cultures in anaerobic broth (see Wensinck *et al.*, 1980) 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 0.9% (w/v) saline with 0.01% (w/v) sodium ethylmercurithiosalicylate. Cell density was adjusted to about 5 x 10^8 bacteria per ml and pH to 6.8 - 7.0; pH of freshly prepared suspensions had to be readjusted after 3-4 days and the suspensions were checked monthly for contamination.

Agglutination test. Sera were tested within 3 days after collection. Two drops of serum and one drop of suspension were thoroughly mixed with a platinum loop and the slide was shaken (150 rpm) on the platform of a rotary mixer (Mini-Shaker, Kühner AG, Basel, Switzerland). Results were scored after 5 min as negative (0) or positive (1, 2 or 3, according to strength). With strongly positive sera, 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 the test.

Statistical methods. Results were compared using χ^2-tests with Yates' cor-

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

E = ethanol

A = acetic acid

L = lactic acid

nB= normal butyric acid

F = formic acid

rection. Because it was evident that strength of agglutination should be considered when the four strains were used as a diagnostic set, an interpretation of test results based on the weight of each strain has been worked out. The result with a given serum (scored as 0, 1, 2 or 3 for each strain) was used to estimate the *a posteriori* probability that the patient suffers from Crohn's disease. Details of the method are described in Chapter 7. In the present chapter, sera with test results giving an *a posteriori* probability ≥ 0.95 were considered positive.

RESULTS

Results are presented under three headings: (1) patients with Crohn's disease and healthy subjects; (2) "control diseases" and (3) sera submitted for diagnosis.

Crohn's disease and healthy subjects. From the data in Tables 3-6 it is evident that the four strains were agglutinated more frequently by sera from patients with Crohn's disease than by those from healthy subjects. Fifty-four percent of sera from patients with Crohn's disease were positive (*a posteriori* probability ≥ 0.95) and all sera from healthy subjects negative (Table 7). The test with four strains, thus has a sensitivity of 54% and a specificity of nearly 100%.

"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 normal value. The other strain of *Eubacterium 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 *(Eubacterium rectale)* was also agglutinated more frequently by sera from patients with colonic carcinoma. It also showed a higher than normal percentage in ulcerative colitis patients (Table 5); the percentage was however much lower than in patients with Crohn's disease ($P < 0.001$). Sera from patients with various "control diseases" agglutinated *Peptostreptococcus productus* (strain C 18) more frequently than sera from healthy subjects (Table 6) but in all cases the percentages were significantly lower than in Crohn's disease. It should be noted that results in Ethiopian controls were normal and that sera from atopic out-patients agglutinated C 18 more frequently than normal but those

Table 3. Agglutination of Eubacterium contortum *(strain Me 44)*

	% positive*	no. of patients
INTESTINAL TRACT AND LIVER		
acute appendicitis	5	21
carcinoma of large bowel	19	52
chronic diarrhoea (in children)	5	20
coeliac disease	23	44
irritable bowel syndrome	10	20
ulcerative colitis	15	48
chronic active liver disease	19	27
cirrhosis of liver	21	38
primary biliary cirrhosis	5	20
schistosomiasis	26	68
(Ethiopian control sera)	(30)	(23)
OTHER		
ankylosing spondylitis	21	75
atopy		
out-patients	28	29
students	21	34
rheumatoid arthritis	6	51
leprosy	25	85
pulmonary tuberculosis	21	42
sarcoidosis	23	80
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; P-values only given when ≤ 0.05.

Table 4. Agglutination of Eubacterium contortum *(strain Me 47)*

	% positive*	no. of patients
INTESTINAL TRACT AND LIVER		
acute appendicitis	14	21
carcinoma of large bowel	15(=0.05)	39
chronic diarrhoea (in children)	5	20
coeliac disease	11	37
irritable bowel syndrome	-	-
ulcerative colitis	4	48
chronic active liver disease	15	27
cirrhosis of liver	10	21
primary biliary cirrhosis	5	20
schistosomiasis	-	-
OTHER		
ankylosing spondylitis	8	24
atopy	-	-
rheumatoid arthritis	0	19
leprosy	14	28
pulmonary tuberculosis	-	-
sarcoidosis	-	-
bronchial carcinoma	5	20
haematological malignancies	5	22
Crohn's disease	37(<0.001)	118
healthy subjects	4	100

*) P-value of difference with healthy subjects in parentheses; P-values only given when ≤ 0.05.
-: not tested.

Table 5. Agglutination of Eubacterium rectale *(strain Me 46)*

	% positive*	no. of patients
INTESTINAL TRACT AND LIVER		
acute appendicitis	5	21
carcinoma of large bowel	10(<0.05)	41
chronic diarrhoea (in children)	5	20
coeliac disease	8	38
irritable bowel syndrome	-	-
ulcerative colitis	17(<0.001)	48
chronic active liver disease	7	27
cirrhosis of liver	8	25
primary biliary cirrhosis	10	20
schistosomiasis	4	48
OTHER		
ankylosing spondylitis	8	24
atopy	-	-
rheumatoid arthritis	7	30
leprosy	6	31
pulmonary tuberculosis	-	-
sarcoidosis	-	-
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; P-values only given when ≤ 0.05.
-: not tested.

Table 6. Agglutination of Peptostreptococcus productus *(strain C 18)*

	% positive*	no. of patients
INTESTINAL TRACT AND LIVER		
acute appendicitis	10	21
carcinoma of large bowel	12	52
chronic diarrhoea (in children)	0	20
coeliac disease	9	44
irritable bowel syndrome	5	20
ulcerative colitis	21(<0.01)	48
chronic active liver disease	7	27
cirrhosis of liver	26(<0.001)	38
primary biliary cirrhosis	5	20
schistosomiasis	41(<0.001)	68
(Ethiopian control sera)	(13)	(23)
OTHER		
ankylosing spondylitis	39(<0.001)	40
atopy		
out-patients	34(<0.001)	29
students	3	34
rheumatoid arthritis	6	34
leprosy	29(<0.001)	59
pulmonary tuberculosis	19(<0.05)	42
sarcoidosis	7	29
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; P-values only given when ≤ 0.05.

Table 7. Percentage of positive sera with combined test

	% positive*	no. of patients
INTESTINAL TRACT AND LIVER		
acute appendicitis	5	21
carcinoma of large bowel	5	41
chronic diarrhoea (in children)	5	20
coeliac disease	8(<0.05)	38
irritable bowel syndrome	-	-
ulcerative colitis	12(<0.001)	48
chronic active liver disease	7	27
cirrhosis of liver	8(=0.05)	25
primary biliary cirrhosis	0	20
schistosomiasis	-	-
OTHER		
ankylosing spondylitis	8	26
atopy	-	-
rheumatoid arthritis	7	28
leprosy	6	31
pulmonary tuberculosis	-	-
sarcoidosis	-	-
bronchial carcinoma	0	20
haematological malignancies	5	22
Crohn's disease	54(<0.001)	118
healthy subjects	0	100

*) Positive: posterior probability ≥ 0.95 with prior probability = 0.5. P-value of difference with healthy subjects in parentheses; P-values only given when ≤ 0.05.

-: not tested with four strains.

Table 8. Sera submitted for diagnosis

Period	: June, July and August, 1978
Total number of sera	: 517
Positive*	: 110
Clinical diagnosis	
Crohn's disease	: 68 (62%)
probable Crohn's disease	: 16 (15%)
colitis (undefined)	: 13 (12%)
presenting symptoms:	: 9 (8%)
anal fistulae	: 4
diarrhoea	: 5
other	: 4 (3%)
coeliac disease	: 1
carcinoma	: 1
growth retardation	: 1
uncertain	: 1

*) Posterior probability $\geq$0.95 (prior probability = 0.5)

from atopic students did not.
The percentages of positive sera as calculated from the results with four strains are shown in Table 7 . The percentage was higher than normal in cirrhosis of liver, coeliac disease and ulcerative colitis but significantly lower than in Crohn's disease.
Sera submitted for diagnosis. From the sample representing about one quarter of the sera received in 1978, 21% was positive (Table 8). In 62 % of the positive cases, the diagnosis Crohn's disease had been established, in 15% Crohn's disease was likely, in 12% colitis was undefined and in 8% anal fistulae or diarrhoea suggested inflammatory bowel disease. In 3% of the positive cases, clinical data appeared incompatible with the diagnosis of Crohn's disease. This percentage is acceptable as sera were considered positive when the (*a posteriori*) probability of Crohn's disease was $\geq$ 0.95, 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 in line with data showing that these patients produce antibodies to a wide range of food and microbial antigens (see Chapter 2). This may be explained by assuming that conditions for antibody production are favourable in the inflamed mucosa with dense infiltrates of lymphocytes. In the case of coccoid anaerobes, the high numbers present in the intestinal microflora (Wensinck *et al.*, 1980) may promote antibody production.
In the present study, 17% of patients with Crohn's disease did not agglutinate any of the four coccoid strains and 54% were positive with a *a posteriori* probability of $\geq$ 0.95. In a study of correlations between agglutination reactions and nosographic characteristics of patients with Crohn's disease and ulcerative colitis (Chapter 4) it was found that agglutination was positively correlated with colonic localization of Crohn's disease, the presence of fistulae and with levels of serum immunoglobulins.
There is one striking difference between the studies on antibody production (Chapter 2) 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 groups of patients with Crohn's disease, 54% were positive and from the patients with ulcerative colitis 12%. Moreover, the percentage of sera that were negative with all strains was only 17% in Crohn's disease, compared to 65% in ulcerative colitis and 79% in healthy subjects. In view of the well-known difficulties with the differential diagnosis of Crohn's disease of the colon and ulcerative colitis (Glotzer *et al.*, 1970; Dyer & Dawson, 1970; Kirsner, 1975; Brandes & Eulenburg, 1976), we feel that misdiagnosis should be considered when serological reactions turn out to be 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 healthy subjects. Cirrhosis of liver, schistosomiasis, ankylosing spondylitis, atopy (out-patients), leprosy and pulmonary tuberculosis had more C 18 positive sera than healthy subjects. The agglutination of strain C 18 was correlated with IgG levels in patients with Crohn's disease and, therefore, the frequent agglutination of C 18 in these "control diseases" may be due to the hyperimmunoglobulinaemia which occurs in a rather high number of patients suffering from schistosomiasis (Camus *et al.*, 1977), leprosy (see Bullock, 1971) and hepatic diseases (Osserman & Takatsuki, 1963; Zawadzki & Edwards, 1970; Triger & Wright, 1973). C 18 reactivity was increased in out-patients but not in atopic students, who were not under medical treatment at the time of blood collection. The increase of IgG levels, known to occur during hyposensitization (Devey *et al.*, 1976), may be responsible for the different results in atopic subjects. Antibody production to the other strains was less (Me 44) or not (Me 46 and Me 47) correlated with IgG levels in patients with Crohn's disease, but with a more specific characteristic like fistulae.

We conclude that antibodies to anaerobic coccoid rods are found much more frequently in Crohn's disease than in ulcerative colitis and other diseases. Using the interpretation of agglutination reactions as described in Chapter 7, the percentage of false positive results of sera submitted for diagnosis, was found to be satisfactorily low.

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chapter 4

ANTIBODIES TO EUBACTERIUM *AND* PEPTOSTREPTOCOCCUS, *CLINICAL FEATURES AND LABORATORY PARAMETERS IN PATIENTS WITH CROHN'S DISEASE AND ULCERATIVE COLITIS*

INTRODUCTION

In Chapter 3 it was shown that coccoid *Eubacterium* and *Peptostreptococcus* strains are agglutinated by many, but not all sera from patients with Crohn's disease, whereas about 20% of the patients are negative with all strains on repeated testing.
It is likely that the presence of the coccoid anaerobes in the faecal flora is a necessary determinant for the production of agglutinins, but other factors are also necessary or contributory. Therefore, the relationship between the presence of agglutinins, and clinical and laboratory parameters (nosographic characteristics) was studied.

MATERIALS AND METHODS

Patients. During the period from October 1, 1975, to May 1, 1978, all consecutive patients with inflammatory bowel disease visiting the departments of internal medicine and surgery were studied. The data and blood obtained at the first presentation of the patient during the period mentioned were used. The diagnoses of Crohn's disease and ulcerative colitis*

*) Diagnoses were established by M. VAN BLANKENSTEIN and J. DEES, Department of Internal Medicine II (Prof. Dr M. FRENKEL), University Hospital Dijkzigt, Erasmus University, Rotterdam

were based on generally accepted criteria (Lennard-Jones *et al.*, 1968; Kirsner, 1975). One hundred-and-seventeen patients with Crohn's disease were studied, 75 women and 42 men, median age 32 years (range 14-74). Fourty-four had ileal disease, 26 ileocolonic and 47 colonic disease. Fifty-four patients had undergone intestinal resections. Twenty patients were receiving salicylazosulphapyridine (SASP), 10 were on corticosteroids and 8 on corticosteroid enemas. Fourty-six patients with ulcerative colitis were studied, 23 women and 23 men, median age 32 years (range 18-83). Eleven patients had undergone partial or total colonic resections. Twenty patients were receiving SASP, 2 were on corticosteroids and 2 on corticosteroid enemas. The group of healthy subjects consisted of 100 volunteers of the Red Cross Transfusion Service, Rotterdam, 57 men and 43 women. The median age was 35 years (range 23-64).

Data. The following data were collected: age, sex, duration and localization of disease, frequency of diarrhoea and the presence of macroscopically visible blood, abdominal pain, weight loss, arthritis, erythema nodosum, fever (temperature >38^{o}C), fistulae and anal lesions. The following determinations were performed in serum or blood: α_1-acid glycoprotein as an index of disease activity (Van De Merwe & Mol, 1977), IgA, IgG and IgM (see Chapter 5), albumin, haematocrit and agglutination reactions.

Statistical methods. To compare results, non-parametric statistical methods were used, *viz.* the χ^2-test with Yates' correction for continuity, Wilcoxon's two-sample rank sum test, Spearman's rank correlation test and the Yates-Cochran test. Probability values (P) were derived from two-tailed tests. Some data were dichotomized for the calculations: agglutination reactions were considered either negative (0) or positive (1, 2 or 3), diarrhoea was considered to be present when the patient had watery or at least twice daily loose stools and low haematocrit values were defined as <0.47 for men and <0.42 for women (Best *et al.*, 1976). *A posteriori* probabilities of Crohn's disease were calculated as described in Chapter 7 and the Crohn's disease activity index (CDAI) was calculated according to Best *et al.*, 1976.

The number of statistical tests performed was 344 for Crohn's disease and 293 for ulcerative colitis. For both diseases the numbers of spuriously significant results to be expected by coincidence and the numbers observed are recorded in Table 1. From this table it is seen that in Crohn's disease the number of significant results is much higher than expected by

Table 1. Numbers of expected and observed tests showing statistical significance in patients with Crohn's disease and ulcerative colitis

	NUMBER OF TESTS WITH STATISTICAL SIGNIFICANCE			
	CROHN'S DISEASE		ULCERATIVE COLITIS	
Significance level	expected	observed	expected	observed
$P < 0.05$	17.2	99	14.7	18
$P < 0.025$	8.6	74	7.3	11
$P < 0.01$	3.4	54	2.9	7
$P < 0.005$	1.7	45	1.5	4
$P < 0.0025$	0.9	41	0.7	4
$P < 0.001$	0.3	34	0.3	2
$P < 0.0005$	0.17	26	0.15	1
$P < 0.00025$	0.09	26	0.07	0
$P < 0.0001$	0.03	21	0.03	0
Total numbers of tests	344		293	

chance alone. In ulcerative colitis, on the other hand, the number of expected and observed results for $P \geq 0.0025$ were of the same order of magnitude. Therefore, for Crohn's disease $P < 0.05$ and for ulcerative colitis $P < 0.0025$ are considered critical significance levels.

RESULTS

Relationships were studied between all nosographic characteristics. In the tables only statistically significant correlations are presented. Results with agglutination reactions will be given for Crohn's disease (A), for ulcerative colitis (B) and for healthy subjects (C). The following groups were compared: (D) Crohn's disease and healthy subjects (E) ulcerative colitis and healthy subjects (F) Crohn's disease and ulcerative colitis and (G) Crohn's disease of the colon and ulcerative colitis. A number of nosographic characteristics and their interrelations are only tabulated (see Appendix I) and will not be discussed. Frequency distributions of agglutination reactions in patients with Crohn's disease, ulcerative colitis and healthy subjects, and in Crohn's

Table 2. Frequency distribution of agglutination reactions in patients with Crohn's disease, ulcerative colitis and healthy subjects

STRAIN	RESULT OF AGGLUTINATION	NUMBER OF SERA[1]		
		CROHN'S DISEASE (n=117)	ULCERATIVE COLITIS (n=46)	HEALTHY SUBJECTS (n=100)
Me 44	0	43 (37)	38 (83)	85 (85)
	1	12 (10)	2 (4)	5 (5)
	2	16 (14)	2 (4)	7 (7)
	3	46 (39)	4 (9)	3 (3)
C 18	0	51 (44)	37 (80)	95 (95)
	1	15 (13)	4 (9)	3 (3)
	2	11 (9)	2 (4)	1 (1)
	3	40 (34)	3 (7)	1 (1)
Me 46	0	59 (50)	39 (85)	99 (99)
	1	7 (6)	3 (7)	1 (1)
	2	7 (6)	1 (2)	0 (0)
	3	44 (38)	3 (7)	0 (0)
Me 47[2]	0	74 (64)	44 (96)	96 (96)
	1	9 (8)	1 (2)	2 (2)
	2	22 (19)	0 (0)	2 (2)
	3	10 (9)	1 (2)	0 (0)

1) Percentages in parentheses.
2) 115 Patients with Crohn's disease.

disease according to site of disease are given in Tables 2 and 3.

(A) Crohn's disease. Tables 4 - 8 show that correlations were found between agglutination reactions and the presence of fistulae (strain C 18, Me 46 and Me 47), colonic or ileocolonic disease (Me 44) and serum IgG and IgM (Me 44 and C 18). Mutual correlations were found between the agglutination reactions, except between Me 46 and Me 47. Agglutination of C 18 was weakly correlated with the presence of arthritis or erythema nodosum, whereas apparently contradicting negative correlations were seen between agglutination of Me 46 and both bloody stools and haematocrit value. Agglutination reactions were not correlated with any index

Table 3. Frequency distribution of agglutination reactions in patients with Crohn's disease according to localization of disease

STRAIN	RESULT OF AGGLUTINATION	NUMBER OF SERA[1]		
		LOCALIZATION OF CROHN'S DISEASE		
		ileum (n=44)	ileocolon (n=26)	colon (n=47)
Me 44	0	22 (50)	7 (27)	14 (30)
	1	2 (5)	5 (19)	5 (11)
	2	7 (16)	4 (15)	5 (11)
	3	13 (30)	10 (38)	23 (49)
C 18	0	23 (52)	11 (42)	17 (36)
	1	6 (14)	4 (15)	5 (11)
	2	4 (9)	1 (4)	6 (13)
	3	11 (25)	10 (38)	19 (40)
Me 46	0	27 (61)	13 (50)	19 (40)
	1	5 (11)	0 (0)	2 (4)
	2	2 (5)	1 (4)	4 (9)
	3	10 (23)	12 (46)	22 (47)
Me 47	0	26 (62)[2]	13 (50)	35 (74)
	1	4 (10)	2 (8)	3 (6)
	2	9 (21)	8 (31)	5 (11)
	3	3 (7)	3 (12)	4 (9)

1) Percentages in parentheses.
2) 42 Patients with ileal disease.

of disease activity, like CDAI, α_1-acid glycoprotein or serum albumin level.

(B) Ulcerative colitis. A positive correlation was found between duration of disease and reactivity with strain Me 44 ($P < 0.0005$).

(C) Healthy subjects. In the group of healthy subjects only a few determinations were performed in serum: α_1-acid glycoprotein, IgA, IgG and IgM and the agglutination reactions. Out of 100 healthy subjects, 20 showed at least one positive agglutination reaction. These subjects tended to have higher immunoglobulin levels, but this trend did not reach

Table 4. Nosographic characteristics correlated with agglutination of Me 44 in Crohn's disease

	CORRELATION WITH AGGLUTINATION OF ME 44×
positive:	•IgM
	IgG
	•agglutination of C 18
	•agglutination of Me 46
	agglutination of Me 47
negative:	disease restricted to ileum

×: $P < 0.05$; •: $P < 0.01$

Table 5. Nosographic characteristics correlated with agglutination of C 18 in Crohn's disease

	CORRELATION WITH AGGLUTINATION OF C 18×
positive:	•fistulae
	arthritis/erythema nodosum
	IgM
	•IgG
	•agglutination of Me 44
	•agglutination of Me 46
	•agglutination of Me 47

×: $P < 0.05$; •: $P < 0.01$

Table 6. Nosographic characteristics correlated with agglutination of Me 46 in Crohn's disease

	CORRELATION WITH AGGLUTINATION OF ME 46×
positive:	fistulae
	•agglutination of Me 44
	•agglutination of C 18
negative:	bloody stools
	haematocrit

×: $P < 0.05$; •: $P < 0.01$

Table 7. Nosographic characteristics correlated with agglutination of Me 47 in Crohn's disease

	CORRELATION WITH AGGLUTINATION OF ME 47 ×
positive:	fistulae
	agglutination of Me 44
	agglutination of C 18

×: $P < 0.05$

Table 8. Nosographic characteristics correlated with posterior probability in Crohn's disease

	CORRELATION WITH POSTERIOR PROBABILITY ×
positive:	•fistulae
	fever
	IgA
	IgM
	•IgG
negative:	bloody stools
	haematocrit
	disease restricted to ileum

×: $P < 0.05$; •: $P < 0.01$

statistical significance.

(D) Crohn's disease *versus* healthy subjects. Patients with Crohn's disease had more positive reactions with all strains ($P < 0.0001$) and, consequently, higher *a posteriori* probabilities than healthy subjects.

(E) Ulcerative colitis *versus* healthy subjects. Patients with ulcerative colitis showed more positive agglutination reactions with strains C 18 ($P < 0.025$) and Me 46 ($P < 0.0025$) than healthy subjects, resulting in higher *a posteriori* probabilities of Crohn's disease ($P < 0.01$).

(F) Crohn's disease *versus* ulcerative colitis. Patients with Crohn's disease had more positive agglutination reactions with all strains than patients with ulcerative colitis (Me 44 and C 18: $P < 0.0001$; Me 46 and Me 47: $P < 0.00025$).

(G) Crohn's disease of the colon *versus* ulcerative colitis. Comparison of patients with ulcerative colitis and Crohn's disease may result in

deceptive conclusions due to the heterogeneity of the Crohn group with respect to site of disease. Therefore, ulcerative colitis was also compared with Crohn's disease of the colon.Similar results were obtained as under (F), the only relevant difference being a decrease of the significance of the correlation between Crohn's disease and agglutination with strain Me 47 from $P < 0.00025$ to $P < 0.01$, due to the low reactivity of strain Me 47 in colonic Crohn's disease.

DISCUSSION

In Crohn's disease no correlation was found between antibodies to coccoid rods and any index of disease activity, age, duration of disease and bowel resection. On the other hand, antibodies were found more frequently when the disease was localized in the colon, when fistulae were present and when immunoglobulin levels were relatively high.

Agglutination with strain Me 46 was less frequently positive in patients with bloody stools. The negative effect of bloody stools must be strong as it was seen despite the high frequency of agglutination with Me 46 in colonic Crohn's disease and the positive correlation between colonic disease and bloody stools.

The data suggest that a limited number of features acts upon the production of antibodies to the coccoid rods. We suppose that the presence of the anaerobic coccoid rods in the faecal flora is a necessary but not the only determinant for the production of antibodies to levels that allow their demonstration with agglutination reactions.

The first contributory determinant probably is the high number of coccoid rods in the faecal flora, which, in patients with Crohn's disease, is about fourty times higher than in healthy subjects. The negative effect of bloody stools on some agglutination reactions might be due to a decrease of the number of anaerobic bacteria.

A second contributory determinant probably is colonic disease. In the colon, bacterial antigen concentration is certainly higher than elsewhere in the intestine and conditions for antibody production are optimal when an inflammatory process is localized there. The correlation between agglutination reactions and fistulae may also be explained in this way, although it may be supposed that certain strains cause both antibody production and fistulae.

A third contributory determinant seems to be a high level of serum immunoglobulins. It is unlikely that antibodies to coccoid rods only are responsible for higher levels. Both immunoglobulin levels and titres of antibody to the coccoid rods are probably determined by the strength of the general humoral responsiveness. This response shows marked individual variations in healthy subjects and in patients, as is evident from the wide range of immunoglobulin levels. This mechanism may be responsible for positive agglutination reactions in diseases with hyperimmunoglobulinaemia, like cirrhosis of liver, schistosomiasis, leprosy and ankylosing spondylitis (see Chapter 3).
Summarizing, it is concluded that for antibody production to the coccoid rods in patients with inflammatory bowel disease the high number of coccoid anaerobes probably is a necessary determinant. Contributory determinants probably are colonic disease, fistulae and the strength of the humoral immune response.

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chapter 5

ANTIBODY NATURE AND BIOLOGICAL PROPERTIES OF AGGLUTININS AGAINST EUBACTERIUM *AND* PEPTOSTREPTOCOCCUS

INTRODUCTION

In Chapters 3 and 4 it has been shown that a high percentage of sera from patients with Crohn's disease agglutinate isolates belonging to *Eubacterium* and *Peptostreptococcus*. It was most likely that agglutination was due to immunoglobulins. Their physical-chemical properties and biological effects like activation of complement and opsonization were studied and the results are described in this chapter.

MATERIALS AND METHODS

Sera were obtained from patients with definite Crohn's disease (see Chapter 4) and selected on positive agglutination reactions. Agglutination reactions were performed as described in Chapter 3. Agglutinating titres were determined with sera diluted with 0.9% saline. Sera were absorbed by incubating 0.5 ml under continuous rotation for 1 h at 20°C with the sedimented bacteria of 1.5 ml of the agglutination suspension (Chapter 3). Immunoglobulin class was determined by application of the sera to a Sephadex G-200 column, according to Thompson and Stokes (1977). The effluents obtained after gel filtration were pooled as far as they belonged to the same protein peaks in the chromatogram and were concentrated to 1 ml by selective membrane filtration (Amicon B 15, Amicon Corp., Lexington, USA). Immunoglobulin levels in the concentrated fractions were determined with radial immunodiffusion as described by Milford-Ward (1977) with commercially available plates and reference serum (Behringwerke AG,

Marburg, Federal Republic of Germany).
The accuracy and precision of radial immunodiffusion was established with 12 determinations on different plates and occasions of the concentrations of IgA, IgG and IgM in the reference serum batch.
From Table 1 it is seen that coefficients of variation were 4.8, 3.2

Table 1. Immunoglobulin levels of reference serum

	IMMUNOGLOBULIN (mg/100 ml serum)		
	IgA	IgM	IgG
reference value*:	242	108	1260
number of determination			
1	244	88	1195
2	267	86	1303
3	244	99	1278
4	245	69	1210
5	244	92	1181
6	234	79	1198
7	220	93	1238
8	250	106	1216
9	255	88	1231
10	233	90	1254
11	244	96	1169
12	240	81	1216
mean value:	243	89	1224
standard deviation:	11.7	9.7	39.3
coefficient of variation(%):	4.8	10.9	3.2
deviation from reference value(%):	+0.4	-21.4	-2.9

*) Serum batch no. 1001 obtained from and reference values given by Behringwerke AG, Marburg, Federal Republic of Germany.

and 10.9%, respectively and the precision therefore is within acceptable limits. Accuracy was good for IgA (deviation 0.4%) and IgG (deviation 2.9%). IgM was found to be about 20% lower than the value given by Behringwerke.

Table 2 records the results of immunoglobulin determinations with radial

Table 2. Immunoglobulin levels of IgM and IgG fractions of sera from patients with Crohn's disease

CODE NO. OF SERUM	IG LEVELS OF IGM FRACTIONS*			IG LEVELS OF IGG FRACTIONS*		
	IgA	IgM	IgG	IgA	IgM	IgG
2593	21	68	2	45	2	66
2627	27	68	2	42	7	78
2628	30	65	3	29	0	55
2629	34	61	3	44	5	78
2660	29	76	3	39	11	57
2702	32	48	2	21	1	45
2728	32	76	3	51	11	63
2752	10	34	10	34	0	37
2740	4	58	1	18	2	56

*) Percentages of whole-serum levels.

immunodiffusion in the pooled and concentrated effluents after application of sera from 9 patients with Crohn's disease to Sephadex G-200 columns. The "IgM fraction" predominantly contained IgM and only slight amounts of IgG and the "IgG fraction" *vice versa*. Both fractions, however, contained IgA.

Complement fixation by agglutinin-antigen complexes was tested by incubation during 16 h at 4° C with guinea-pig complement.

Residual complement was determined by the degree of lysis of the haemolytic system. Preserved guinea-pig serum (Wellcome, Beckenham, England) was used as a source of complement. Sheep red cells in Alsever's solution were obtained from the Rijks Instituut voor de Volksgezondheid, Bilthoven, washed and diluted in complement fixation test diluent (Oxoid Ltd., London, England). Rabbit haemolytic serum was obtained from Wellcome.

Verification. All four sensitized strains incubated with anti-human immunoglobulin antibodies (RAHu/Fc, Nordic, Tilburg) bound complement and the anticomplementary titres were much higher than of controls in which unsensitized bacteria were incubated with anti-human immunoglobulin antibodies only (Table 7). It is clear, therefore, that complement fixation by antigen-antibody complexes was demonstrated with this assay.
Control experiments in saline showed that three of the bacterial strains fixed complement. The method of Fine *et al.* (1972) was used to see whether complement was activated by the alternative pathway. This method depends upon the fact that, whereas the classical pathway requires both calcium and magnesium, the alternative pathway requires only magnesium. Calcium was chelated with the magnesium salt of ethyleneglycoltetraacetic acid (Mg EGTA) as described by Bryan (1974) and Fine (1977). After incubation with chelated fresh human control serum, bacteria were sedimented at 10,000 *g*. The supernatant was withdrawn and 0.1 ml of 100 mM $CaCl_2$ per ml of serum was added before addition of the haemolytic system. Tests were performed with serum from two healthy subjects.
Verification. Treatment of fresh human control serum with Mg EGTA completely inhibited lysis of the haemolytic system, but haemolysis occurred after saturation with calcium, indicating that the classical pathway had indeed been inhibited by Mg EGTA.
Phagocytosis. Neutrophils were isolated according to Böyum (1968) with modifications by Steffelaar *et al.* (1976). Blood was obtained from healthy laboratory volunteers and heparinized with 10 IE/ml of Thromboliquine (Organon, Oss). After harvesting, cells were suspended in concentrations of 2 x 10^6 cells/ml in "Hijmans" fluid (see Steffelaar *et al.*, 1976). To 0.2 ml of this suspension, 0.2 ml "Hijmans" fluid and 0.2 ml of bacterial suspension (see below) were added and the mixture was incubated for 30 min at 37°C under continuous rotation. The tubes were centrifuged for 10 min at 90 *g*, the pellet was resuspended in saline and slides were prepared. After Gram staining, 200 cells were counted per slide and the percentage of cells containing bacteria in vacuoles was calculated from duplicate counts. All tests were performed in duplicate. The coefficient of variation was 13%.
Preparation of sensitized bacteria. To 0.125 ml of a washed bacterial suspension, 0.25 ml of antibody-containing samples was added. After incubation under continuous rotation for 1 h at 20°C and subsequent

sedimentation, the bacteria were washed and resuspended in 0.9% saline. Whole Crohn's serum, specifically absorbed serum and the IgG and IgM fractions obtained after gel filtration were used as sources of antibodies in 4 dilutions (1/1, 1/2, 1/4 and 1/8) and heated for 30 min at 56°C to inactivate complement. IgG and IgM concentrations were expressed as percentage of Ig in undiluted serum.

RESULTS

Agglutination titres. In Table 3 results are presented of agglutination

Table 3. Titres of serum agglutinins to coccoid rods in patients with Crohn's disease

CODE NO. OF SERUM	AGGLUTINATION*	TITRES TO STRAINS: ME 44	C 18	ME 46	ME 47
2593	3 2 3 2	4	4	2	2
2627	3 3 3 3	1	8	8	4
2628	3 3 3 0	8	16	32	-
2629	3 3 3 3	4	4	4	2
2702	3 3 3 3	1	1	2	4
2728	2 3 3 1	1	4	2	1
2740	3 3 3 1	8	16	8	1
2752	1 3 3 2	2	2	8	4

*) Results for strains Me 44, C 18, Me 46 and Me 47 according to strength of agglutination (0, 1, 2 or 3).

reactions with diluted sera from 8 patients with Crohn's disease. This table shows that the titres were usually low.

Absorption of agglutinins. In Table 4 results are given of agglutination reactions before and after absorption of two sera from patients with Crohn's disease with each of the 4 strains. From this table it is evident that agglutinins were absorbed specifically.

Immunoglobulin class of agglutinins. The agglutination reactions of sera from 13 patients with Crohn's disease are given in Table 5. From this table it is seen that the agglutinins in the IgM and IgG fractions were responsible for agglutination by whole serum. The data are grouped

Table 4. Absorption of agglutinins from 2 Crohn sera by coccoid rods

	AGGLUTINATION*	
	Crohn serum no.	
	2701	2719
whole serum	3 3 3 2	3 3 3 2
after incubation with strain:		
Me 44	0 3 3 2	0 3 3 2
C 18	2 0 3 2	3 0 3 2
Me 46	3 3 0 2	3 3 0 2
Me 47	3 3 3 0	3 3 3 0

*) Results for strains Me 44, C 18, Me 46 and Me 47 according to strength of agglutination.

Table 5. Agglutination of coccoid rods by Crohn sera, IgM and IgG fractions

WHOLE SERUM		IGM FRACTION	IGG FRACTION
code no.	agglutination*	agglutination*	agglutination*
2593	3 2 3 2	0 0 0 1	3 3 3 0
2627	3 3 3 3	2 1 0 3	2 3 3 0
2628	3 3 3 0	3 0 3 0	3 3 3 0
2629	3 3 3 3	2 3 3 3	3 3 3 0
2657	3 0 3 0	0 0 0 0	3 0 3 0
2660	3 3 3 0	0 1 0 0	2 0 3 0
2669	3 1 2 1	0 1 0 1	3 3 0 0
2677	3 3 0 2	2 0 0 0	2 2 0 3
2702	3 3 3 3	0 0 0 0	2 0 0 1
2728	2 3 3 1	0 2 0 0	0 3 2 0
2752	1 3 3 2	0 0 0 2	0 3 2 2
2831	2 3 3 1	0 3 2 1	2 3 0 0
2832	3 3 3 1	3 3 0 2	3 3 3 1

*) Results of strains Me 44, C 18, Me 46 and Me 47 according to strength of agglutination.

in Table 6, which shows that agglutinins to Me 44 were usually present in the IgG fraction and less frequently in the IgM fraction. Agglutinins to C 18 were found in both fractions, whereas those to Me 46 were mostly IgG and to Me 47 IgM.

Table 6. Agglutinins in IgM and IgG fractions of sera from 13 patients with Crohn's disease

STRAIN	NO. OF AGGLUTINATING SERA	NO. OF AGGLUTINATING FRACTIONS		
		IgM	IgG	IgM and IgG
Me 44	13	0	6	5
C 18	12	1	4	6
Me 46	12	1	7	2
Me 47	10	5	2	2

Complement activation. *Unsensitized bacteria.* Strain Me 44 showed no anticomplementary activity at all. The median titre was 8 for C 18 and 32 for Me 46 and Me 47 (Table 7). Complement activation by the alternative pathway of the bacterial strains was tested by incubation with fresh, Mg EGTA chelated control serum. No lysis of the haemolytic system was observed after saturation with calcium of the supernatant chelated sera incubated with strains C 18, Me 46 and Me 47. In contrast, chelated serum incubated with Me 44, as expected, induced lysis. From the results it is concluded that strain Me 44 does not activate complement, whereas C 18, Me 46 and Me 47 activate complement by the alternative pathway. Some classical pathway activation by the latter strains is not excluded by this experiment.

Sensitized bacteria. As can also been seen from Table 7, sensitized bacteria had anticomplementary titres equal to or lower than those found with unsensitized bacteria. Whereas higher titres would have indicated complement fixation by specific antibodies, equal or lower titres could also be caused by the bacteria themthelves. Therefore, the amount of antibody was varied by incubation of bacteria with serum dilutions ranging from 1/1 to 1/1024. Table 7 shows that anticomplementary activity of antibody-coated C 18 diminished when higher dilutions of serum were applied, indicating that it was (partly) caused by antibodies. The anticomplementary activity found with Me 46 and Me 47 did not diminish at

Table 7. Anticomplementary activity of coccoid rods

PRETREATMENT[1] OF BACTERIA:	ANTICOMPLEMENTARY TITRE[2] WITH STRAIN:			
	Me 44	C 18	Me 46	Me 47
Saline				
test 1	0	8	64	64
2	0	4	32	32
3	0	8	32	32
4	0	4	32	32
Crohn serum				
2783	0	4	-	-
2735	-	-	8	32
2831	0	8	8	16
2832	0	8	4	8
2835				
1/1	0	32	16	32
1/2 - 1/4	0	8	16	32
1/8 - 1/1024	0	4	16	32
2831				
+ anti-Ig	64	64	64	64
2832				
+ anti-Ig	64	64	64	64
Anti-Ig	0	8	32	16

1) Pretreatment during 1 h at 20°C.
2) -: not tested

higher serum dilutions and it is concluded that antibodies to these strains, like those to Me 44, lacked complement fixing properties.

Phagocytosis. Results of phagocytosis in control experiments with bacteria in 0.9% saline are given in Table 8, from which it is seen that phagocytosis of strains Me 44 and C 18 was slight in contrast to strains Me 46 and Me 47 which were taken up by 45-61% of the neutrophils.

Table 8. Phagocytosis of coccoid rods by neutrophils

STRAIN[1]	% OF NEUTROPHILS CONTAINING COCCOID RODS[2]
Me 44	19 (14 - 28)
C 18	10 (5 - 19)
Me 46	61 (54 - 63)
Me 47	45 (24 - 48)

1) Coccoid rods suspended in 0.9% saline.
2) Median values of 4 determinations; range in parentheses.

Sensitized bacteria. Strain Me 44. From Fig. 1 it is evident that marked phagocytosis of Me 44, related to the amount of antibody, was induced by whole serum and the IgG fraction. After absorption of the serum with Me 44, phagocytosis was negligible. Fig. 2 shows that the IgG fraction and whole serum were active, whereas the IgM fraction and absorbed serum showed no opsonic effect. From these results it is concluded that IgG agglutinins to Me 44 had opsonic properties, whereas no such effects could be demonstrated of IgM agglutinins.

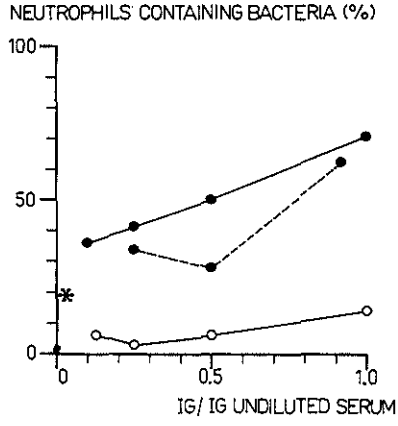

*Fig.1. Phagocytosis of Me 44 after incubation with serum (●—●), the IgG fraction (●--●) and absorbed serum (○—○). * Control in 0.9% saline. Crohn serum no. 2831, agglutinins only of IgG class*

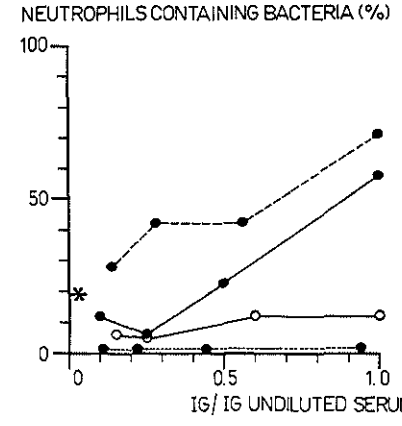

*Fig.2. Phagocytosis of Me 44 after incubation with serum (●—●), the IgG fraction (●--●), the IgM fraction (●···●) and absorbed serum (○—○). * Control in 0.9% saline. Crohn serum no. 2832, agglutinins of IgG and IgM class*

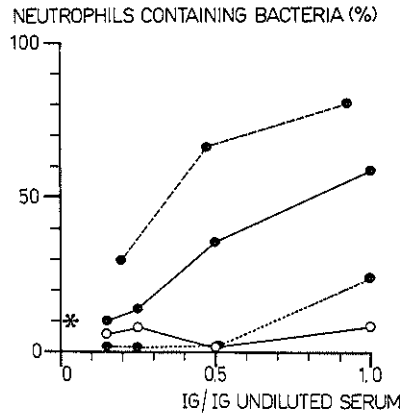

Fig.3. Phagocytosis of C 18 after incubation with serum (●—●), the IgG fraction (●---●), the IgM fraction (●····●) and absorbed serum (○—○). ✱ Control in 0.9% saline. Crohn serum no. 2831, agglutinins of IgG and IgM class

Strain C 18. From Fig. 3 it is seen that opsonization of C 18 by specific antibodies was similar to that of Me 44.

Strain Me 46. Fig. 4 shows that phagocytosis of Me 46, preincubated with whole serum, the IgM fraction and absorbed serum was lower than in the

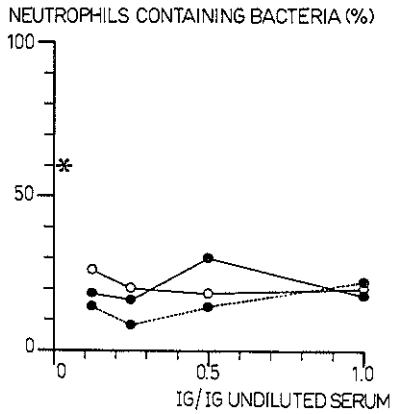

Fig.4. Phagocytosis of Me 46 after incubation with serum (●—●), the IgM fraction (●····●) and absorbed serum (○—○). ✱ Control in 0.9% saline. Crohn serum no. 2831, agglutinins only of IgM class

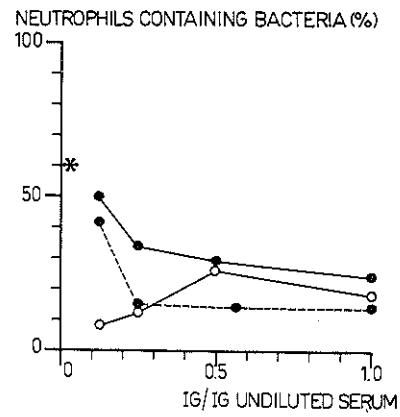

Fig.5. Phagocytosis of Me 46 after incubation with serum (●—●), the IgG fraction (●---●) and absorbed serum (○—○). ✱ Control in 0.9% saline. Crohn serum no. 2832, agglutinins only of IgG class

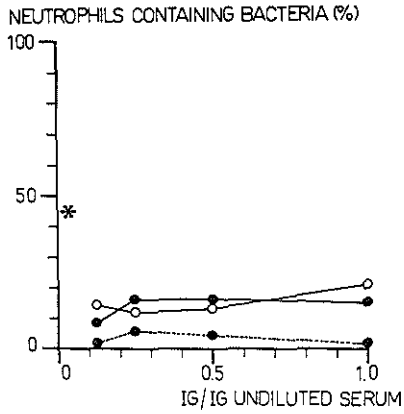

Fig.6. Phagocytosis of Me 47 after incubation with serum (●—●), the IgM fraction (●···●) and absorbed serum (○—○). ✱Control in 0.9% saline . Crohn serum no. 2831, agglutinins only of IgM class

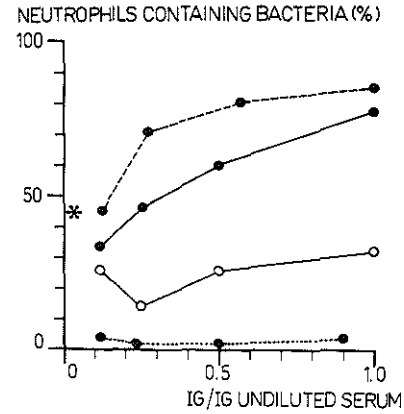

Fig.7. Phagocytosis of Me 47 after incubation with serum (●—●), the IgG fraction (●--●), the IgM fraction (●···●) and absorbed serum (○—○). ✱Control in 0.9% saline. Crohn serum no. 2832, agglutinins of IgG and IgM class

Table 9. Summary of results on complement fixation and phagocytosis

	STRAIN			
	Me 44	C 18	Me 46	Me 47
anticomplementary activity	0	+	++	++
activation of complement by alternative pathway	0	+	+	+
fixation of complement by specific antibodies	0	+	0	0
phagocytosis unsensitized bacteria	±	±	++	++
bacteria sensitized with: positive Crohn sera	++	++	0	++
negative Crohn sera	n.t.	n.t.	0	n.t.
in the presence of complement	n.t.	n.t.	+++	n.t.

n.t. : not tested

control. Similar results were obtained with serum with only IgG agglutinins and the IgG fraction (Fig. 5). This differs markedly from the results obtained with the other strains. The inhibitory effect of whole serum and absorbed serum was confirmed with three other positive Crohn sera. Sera from three Crohn's patients without Me 46 agglutinins and serum from a healthy subject also inhibited phagocytosis. It is evident that results with Me 46 differ in two respects from those with Me 44 and C 18:

(1) the IgG agglutinins do not promote phagocytosis;
(2) phagocytosis is inhibited by serum, IgG and IgM fractions and also by absorbed serum.

Obviously, inhibition of phagocytosis is not mediated by the agglutinins but by another, unknown factor present in serum and absorbed serum. So far, results were obtained with serum samples in which complement had been inactivated. To see whether complement could promote phagocytosis despite the presence of inhibitory serum or non-opsonizing antibody, phagocytosis was investigated with bacteria preincubated with fresh serum. In these cases, 90-100% of neutrophils contained bacteria and phagocytosis was therefore higher than with the controls in saline.

Strain Me 47. From Fig. 6 and 7 it is seen that IgG agglutinins to Me 47, like those to Me 44 and C 18, were opsonins. Absorbed serum, as in the case with Me 46, also inhibited phagocytosis but the effect was not strong enough to counteract the opsonic effect of IgG agglutinins.

Conclusions. IgG agglutinins to Me 44, C 18 and Me 47 induced phagocytosis, despite the presence of specific IgM antibodies or (inhibiting) serum. IgG antibodies to Me 46 from patients with Crohn's disease, on the other hand, lacked opsonizing properties.

Results of complement fixation and phagocytosis are summarized in Table 9.

DISCUSSION

Bacteria. Unexpectedly, it was found that strains C 18, Me 46 and Me 47 activate complement. As this occurred in the absence of antibodies, it was most likely to proceed by the alternative pathway and this was confirmed experimentally. Activation of complement usually results in the

elimination of invading microorganisms like *Haemophilus influenzae* (Quinn *et al.*, 1977), group B *Streptococcus* (Shigeoka *et al.*, 1978), *Neisseria gonorrhoeae* (Ingwer *et al.*, 1978) and *Pseudomonas aeruginosa* (Peterson *et al.*, 1978), but has also been supposed to initiate inflammatory reactions. *Propionibacterium acnes* probably causes acne vulgaris in this way (Webster *et al.*, 1978; Dahl *et al.*, 1979).
Under physiological conditions, little if any complement is present in the external secretions of the gastrointestinal tract but once the mucosa is inflamed, complement could be brought into play, giving rise to submucosal reactions (Thompson, 1979). Feinstein *et al.* (1976) found abnormalities in the alternative pathway activation in Crohn's patients which could not be demonstrated during remissions (Lake *et al.*, 1979).
Bacteria of the resident intestinal flora are obviously not eliminated by complement activation under these circumstances. Complement cleavage and its effects (histamine release, kininlike activity, chemotactic activity, lysosomal enzyme release) may be expected to occur continuously and to contribute to perpetuation of the inflammatory process.
Antibodies. The occurrence of the agglutinins in the IgG and IgM fraction, the specific binding to antigen and the opsonic effect are compatible with their immunoglobulin nature.
Antibodies to *Peptostreptococcus productus* strain C 18, fixed complement and had a marked opsonic effect. This suggests that a normal immune defence mechanism against infection by *Peptostreptococcus productus* is likely to occur when the organism enters the tissues.
The antibodies to the *Eubacterium contortum* strains Me 44 and Me 47 had also an opsonic effect, but in contrast to antibodies to *Peptostreptococcus productus* lacked complement fixing properties.
Antibodies to the *Eubacterium rectale* strain Me 46, however, lacked both opsonizing and complement fixing properties. This may be important as the strain itself activated complement. Phagocytosis of strain Me 46 could be induced by complement but complement also is consumed by the bacteria by the alternative pathway. It is impossible to predict the biological consequences of the interactions between strain Me 46, specific antibodies, complement and neutrophils *in vivo* in inflamed bowel in conditions of limited complement availability. Complement may act as the limiting factor for both phagocytosis of the bacteria and activation of complement by these bacteria. These phenomena may thus be competitive

and represent a state of unstable equilibrium.
Both properties of strain Me 46, *viz.* activation of complement and resistence to phagocytosis despite the presence of specific antibodies may thus be of pathogenetic importance for Crohn's disease.
The absence of complement fixing and opsonizing properties of anti-Me 46 antibodies in patients with Crohn's disease suggests that they belong to the IgG4 subclass. Specific IgG4 antibodies are rather unusual and only reported as auto-antibodies to clotting factors (Anderson & Terry, 1968) and as antibodies to grass pollen (Van Der Giessen *et al.*, 1976; Devey *et al.*, 1976). The capacity to produce antibodies to one or another IgG subclass to particular antigens is under genetic control (Natvig & Kunkel, 1973) and shows marked geographic variations (Fudenberg *et al.*, 1978). The production of antibodies of this "non-phagocytic" subclass to strain Me 46 in patients with Crohn's disease may thus be a reflection of a genetic predisposition. Other diseases exist in which associations with IgG subclasses or genetic markers on IgG have been found. Examples are typhoid, leprosy, auto-immune haemolytic anaemia, cystic fibrosis and atopic diseases (Van Der Giessen *et al.*, 1976; Devey *et al.*, 1976; Shakib *et al.*, 1976; Shanfield, 1978).

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chapter 6

INTRODUCTION TO A LOGICAL INTERPRETATION OF DIAGNOSTIC LABORATORY TESTS

The concepts of normal limits, sensitivity, specificity, *a priori* and *a posteriori* probability of disease have received some attention in recent medical literature (Price & Vlahcevic, 1971; Holland & Whitehead, 1974; McNeil *et al.*, 1975; Wulff, 1976; Gorry *et al.*, 1978; Burke, 1978; Ransohoff & Feinstein, 1978; Diamond & Forrester, 1979) but are not familiar to many of those who interpret test results. The interpretation of test results, however, largely depends on these concepts and their importance for clinical decisions, therefore, is discussed.
An ideal test would always be positive in the presence of disease and negative in its absence and the interpretation of the test should be simple. Usually, however, tests yield quantitative results with overlap between diseased and non-diseased subjects. At least two different procedures can be followed to interpret individual test results. Results may be classified as normal or abnormal on the basis of a selected cutoff point. Alternatively, the numerical value of the test result may be interpreted in terms of *a posteriori* probability of the disease concerned.

APPLICATION OF A CUTOFF POINT

The problem is where to draw the line separating normal from abnormal values. In Fig. 1 several possible cutoff points are given and when point A was chosen, about 95% of non-diseased subjects would have normal results, compared to a third of diseased subjects. If all diseased subjects should be detected, the line should be drawn at a lower value, say point B. As a consequence, more non-diseased subjects will have abnormal test results than at point A. If it is desired, on the

other hand, that abnormal test results certainly indicate the presence of disease, a higher value will be chosen (point C), but this results in a higher number of undetected diseased subjects than at points A or B.

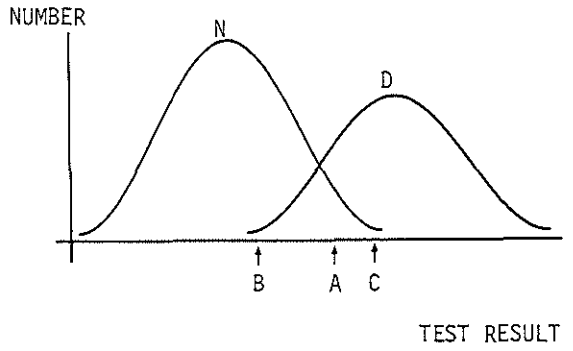

Fig. 1. Frequency distribution of the results x of a hypothetical test performed in patients (curve D) and non-diseased subjects (curve N). Points A, B and C refer to several cutoff points separating normal from abnormal values

Once a cutoff point is selected, sensitivity and specificity of the test can be calculated. Sensitivity is the percentage of abnormal tests in subjects who do have the disease. Specificity, on the other hand, is the percentage of normal test results in subjects without that particular disease. Sensitivity and specificity thus indicate the probability of a test result for classified subjects. The clinician, however, wishes to determine the probability of disease on the basis of the test result, *i.e.* the predictive value of a positive or negative test. The predictive value, or *a posteriori* probability of disease, not merely depends on sensitivity and specificity of the test, but is also largely influenced by the prevalence of the disease (*a priori* probability) in the population tested. This is illustrated by the following (fictitious) example:

A test is used of which the results may vary from 0 to 1. A cutoff point was chosen that resulted in a sensitivity of the test of 75% and a specificity of 94%. Consequences of application of this test are illustrated when used in a population with a prevalence of disease of 50% and in a

population with a prevalence of 56 per 10^5 inhabitants[1].

A) Prevalence = 50%. When 1000 subjects are tested, 500 have the disease of which 0.75 x 500 = 375 will show a positive and 125 a negative test. Of the 500 non-diseased subjects, 500 x 0.94 = 470 will have a negative and 30 a positive test. The total number of positive results is 375 + 30 = 405 of which 375 were obtained from diseased subjects. The probability of disease for a positive[2] test thus is 375/405 = 0.93 (see Fig. 2). The total number of negative results is 125 + 470 = 595 of which 125 were derived from diseased subjects. Consequently, the probability of disease for a negative test[2] is 125/595 = 0.21 (Fig. 2). From these data it is evident that a positive result strongly supports the presence of disease but a negative test does not exclude disease. The total number of misclassifications is 125 (negative results in diseased subjects) + 30 (positive results in non-diseased subjects) = 155 and the percentage of misclassifications of all tests is 155/1000 x100% = 15.5% (Fig. 3).

B) Prevalence = 0.056%. In an analogous way as under (A) it can be calculated that with the same test, but used in a population with lower prevalence, the probability of disease for a positive test is 0.007 and for a negative test 0.0001. The percentage of misclassifications is 6%. From the results it is concluded that in this population a positive test does not indicate disease. The interpretations of positive and negative tests are thus identical and consequently, the test should not be done in this population.

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[1]) This is the prevalence of Crohn's disease in the City of Cardiff in 1977 (Mayberry *et al.*, in press).

[2]) The results may also be obtained by application of Bayes' theorem:

$$P(D+|T+) = \frac{P(T+|D+)\,P(D)}{P(T+|D+)\,P(D) + \{1 - P(T-|D-)\}\,\{1 - P(D)\}}$$

$$P(D+|T-) = 1 - \frac{P(T-|D-)\,\{1 - P(D)\}}{P(T-|D-)\,\{1 - P(D)\} + \{1 - P(T+|D+)\}\,P(D)}$$

with: $P(D+|T+)$: *a posteriori* probability of disease for a positive test
$P(D+|T-)$: *a posteriori* probability of disease for a negative test
$P(D)$: *a priori* probability of disease
$P(T+|D+)$: sensitivity of the test
$P(T-|D-)$: specificity of the test
} expressed as numbers between 0 and 1

In Fig. 2 the relationship between prevalence of disease and *a posteriori* probabilities of disease for positive and negative tests are shown. From this figure it is seen that probabilities of disease increase at increasing prevalence of disease but may theoretically vary from 0 to 1. In Fig. 3 the percentage of misclassifications is given for all tests in this

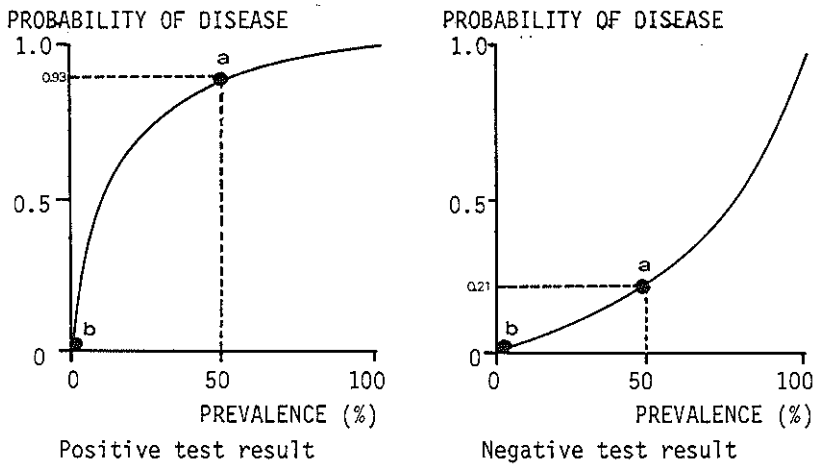

Fig. 2. A posteriori *probabilities of disease for positive and negative test results in relation to the prevalence of disease. Points a and b correspond to the examples with prevalence of 50% (a) and 0.056% (b), respectively. Figure applies to a test with sensitivity of 75% and specificity of 94%.*

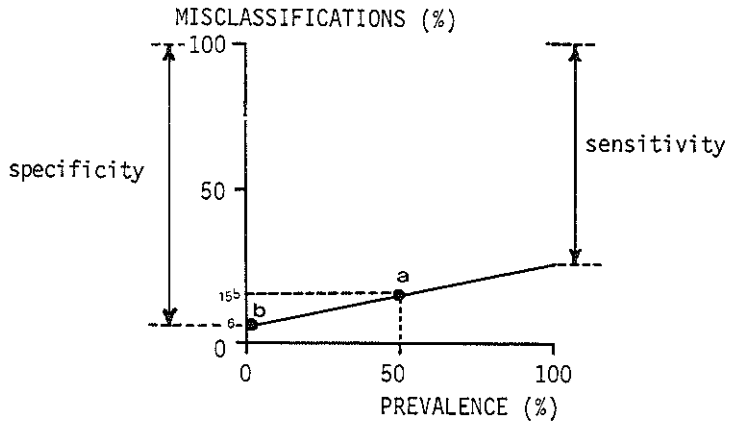

Fig. 3. Misclassifications (%) for all test results in relation to the prevalence of disease. Points a and b correspond to the examples with prevalence of 50% (a) and 0.056% (b), respectively. Figure applies to a test with sensitivity of 75% and specificity of 94%.

example as a function of the prevalence of disease. From this figure it is seen that the chance of misclassification increases at increasing prevalence. For a test with a sensitivity higher than its specificity, the chance of misclassification undergoes changes in an opposite direction at increasing prevalence.
Depending on the reasons for performing a test, the relevance of misclassification at negative and positive results in not the same. Statistical methods are available to select an appropriate cutoff point depending on the relevance of false negative and false positive results and the prevalence of disease in the population tested (Metz *et al.*, 1973).
A major disadvantage of interpretation of test results on the basis of a cutoff point is that the test subsequently only has two possible outcomes. For many tests, the numerical value of the result is much more relevant than the conclusion that the result is simply higher or lower than a chosen cutoff point. The way in which tests may be interpreted without application of a cutoff point is discussed in the next paragraph.

INTERPRETATION OF TESTS IN TERMS OF PROBABILITIES OF DISEASE

In Fig. 4 the frequency distribution is given of the numerical outcomes of the same hypothetical test as in Fig. 1. The probability that a subject with test result x belongs to either population, is proportional

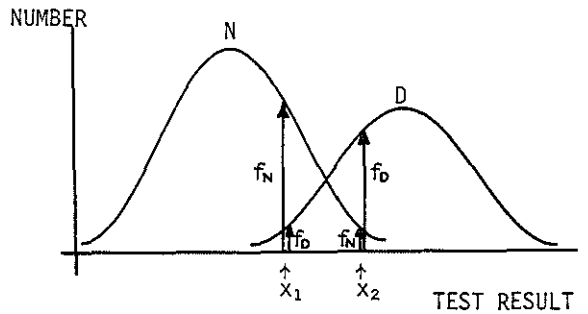

Fig. 4. Frequency distribution of the same hypothetical test results as in Fig. 1. The number of subjects with test result x is indicated by f_N for the non-diseased subjects and by f_D for the diseased subjects

to the frequencies of that particular value x in the populations concerned (f_N and f_D) and can thus be written as:

$$P(D+|x) = \frac{f_D}{f_N + f_D}; \qquad P(D-|x) = \frac{f_N}{f_N + f_D}$$

with:

$P(D+|x)$: *a posteriori* probability of disease for test result x
$P(D-|x)$: *a posteriori* probability of non-disease for test result x
f_D : frequency of test result x in the diseased group D
f_N : frequency of test result x in the non-diseased group N

These *a posteriori* probabilities are based on prevalences (or *a priori* probabilities) of disease and non-disease proportional to the sizes of the populations N and D. In Fig. 4 two x values are indicated, x_1 and x_2. If test result x_1 is obtained, the probability of the individual tested having the disease decreases whereas with result x_2 this probability increases, both compared to the *a priori* probability of disease.

INFORMATION FROM TEST RESULTS

Tests are performed to obtain information about diagnoses and this information depends on three parameters: sensitivity and specificity of the test and the prevalence of disease in the population tested (Metz *et al.*, 1973). The information can also be defined as a function of *a priori* and *a posteriori* probabilities and essentially is the difference between them. In Fig. 5 the relationship between information[1] obtained by a test[2] and the prevalence of disease in the population tested is shown. From this figure it is seen that the information reaches maximum values at a prevalence of disease of about 50% (*a priori* probability = 0.5), thus in situations with maximal diagnostic uncertainty. The information decreases at lower and higher prevalences or, in other words, the more certainty on the diagnosis is available, the less information is to be expected from test results.

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1) Calculated according to Metz *et al.* (1973).
2) The same hypothetical test as used before with sensitivity of 75% and specificity of 94%.

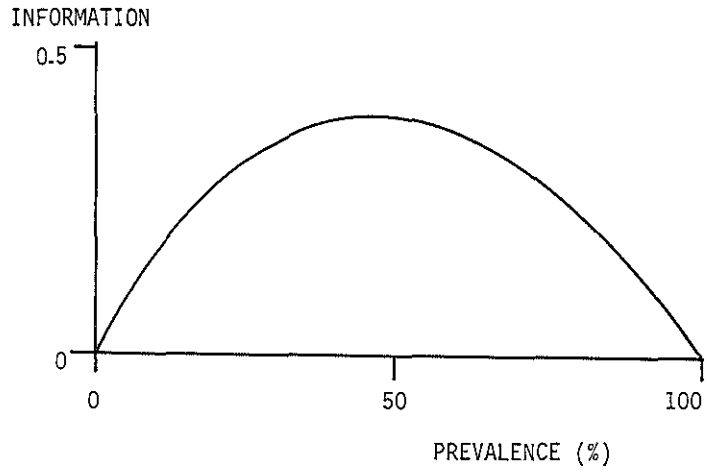

Fig. 5. Information obtained by the same hypothetical test as used in figures 2 and 3, in relation to the prevalence of disease in the population tested

INTERPRETATION OF MORE THAN ONE TEST RESULT

Sometimes, several diagnostic tests are available for a single disease and for the interpretation, the results should be combined. This may be accomplished by discriminant analysis with the following additional advantages:

- there is no need to select a cutoff point for interpretation of the test result
- test results are weighed for diagnostic relevance

For test results with a normal distribution, linear discriminant analysis and for test results with discontinuous distributions, logistic discriminant analysis may be used. In Chapter 7, application of logistic discriminant analysis is described to interpret the results of the four agglutination reactions.

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chapter 7

ANTIBODIES TO EUBACTERIUM *AND* PEPTOSTREPTOCOCCUS *AND THE ESTIMATED PROBABILITY OF CROHN'S DISEASE* *

INTRODUCTION

There is great variation in the duration of symptoms before the diagnosis of Crohn's disease (CD) is established, the average delay being 3 to 4 years (Dyer & Dawson, 1970; Brandes & Eulenburg, 1976; Mekhjian *et al.*, 1979). The diagnosis can be made only after thorough evaluation has excluded other causes of ileal or colonic disease. Unfortunately, there are up to now no laboratory tests to confirm the diagnosis. As has been described in previous chapters, antibodies to *Eubacterium* and *Peptostreptococcus* strains are found in a considerable percentage of sera of patients with CD. The antibody titres change only slightly or not at all during the disease. In healthy subjects and many other diseases these antibodies are found less frequently.
Apart from other implications of these findings, it is evident that the antibodies can serve diagnostic purposes. Four strains of the coccoid anaerobes seemed appropriate for discrimination between CD and non-CD. With each strain, agglutination can be negative (0) or positive, scored as 1, 2 or 3 according to strength. Because four strains are used as a set, 256 test results are possible.
In this chapter, discriminant analysis is applied to the results of agglutination with the four strains.

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*) This chapter is based on a paper by Van De Merwe and Schmitz, submitted for publication.

Agglutination reactions. See Chapter 3.

Patients and controls. Between October 1, 1975, and February 1, 1978, 114 patients with CD and 95 healthy subjects were studied. Patients with CD, healthy subjects and sera were similar to those described in Chapter 4. The agglutination reactions of patients and controls are given in Appendix II (p. 100).

Statistical methods. Logistic discriminant analysis (Anderson, 1972) was used for discrimination between patients with CD and healthy subjects (HS). Details of the method are presented in Appendix II.

Allocation rules and interpretation. After calculation of the estimated *a posteriori* probability of CD, $P(CD|x)$, several allocation rules may be used. It could be stated, for instance, that a subject with agglutination reactions x is classified as:

- non-CD if $0 \leq P(CD|x) < 0.5$
- CD if $0.5 \leq P(CD|x) \leq 1$ (1)

When we classify as "CD" only when the *a posteriori* probability exceeds a certain value, say 0.8, and classify as "non-CD" when the probability is below another value, say 0.2, we have an allocation rule with a region of doubt. Thus:

- non-CD if $0 \leq P(CD|x) \leq 0.2$
- no decision (doubt) if $0.2 < P(CD|x) < 0.8$
- CD if $0.8 \leq P(CD|x) \leq 1$ (2)

On the basis of the results of the analysis (see Results), we divided the possible outcomes of *a posteriori* probabilities in four classes with the following interpretation:

- no support for CD if $0 \leq P(CD|x) < 0.8$
- suspected CD if $0.8 \leq P(CD|x) < 0.95$
- probable CD if $0.95 \leq P(CD|x) < 0.99$
- definite CD if $0.99 \leq P(CD|x) \leq 1$ (3)

RESULTS

The calculated *a posteriori* probabilities for the patients and controls are given in Table 1. When allocation rule (1) is used, classifications are obtained as summarized in Table 2. If allocation rule (2) were used, a classification is obtained as given in Table 3. From this table it is

Table 1. Frequency distribution of posterior probabilities P(CD|x) of CD at prior probability of 0.5 in patients with CD and healthy subjects

P(CD|x)	CROHN'S DISEASE	HEALTHY SUBJECTS
0 - <0.10	0	0
0.10 - <0.20	21	75
0.20 - <0.30	3	4
0.30 - <0.40	1	8
0.40 - <0.50	3	2
0.50 - <0.60	2	0
0.60 - <0.70	4	2
0.70 - <0.80	5	3
0.80 - <0.90	11	1
0.90 - <0.95	7	0
0.95 - <0.99	9	0
0.99 - 1	48	0
Total	114	95

Table 2. Classification matrix for allocation rule (1)

		POPULATION OF ORIGIN	
		CD	HS
POPULATION OF ALLOCATION	CD	86	6
	HS	28	89
	Total	114	95

Table 3. Classification matrix for allocation rule (2)

		POPULATION OF ORIGIN	
		CD	HS
POPULATION OF ALLOCATION	CD	75	1
	doubt	18	19
	HS	21	75
	Total	114	95

evident that about 20% of the elements in both samples are not classified. With the use of the allocation rule (3) *a posteriori* probabilities are obviously interpreted more realistically (Table 4). In this situation, 42% of the patients with CD are classified as "definite CD", 8% as "probable CD", 16% as "suspected CD" and 34% as "no support for CD". Of the healthy subjects, 99% are classified in this latter category, whereas only 1% is classified as "suspected CD" and none as "probable" or "definite CD".

Table 4. Frequency distribution of a posteriori *probabilities P(CD|x) in four classes for allocation rule (3) in patients with CD and healthy subjects (HS)*

P(CD|x)	INTERPRETATION	NUMBER (%) OF CD	NUMBER (%) OF HS
0 - <0.80	no support for CD	39 (34)	94 (99)
0.80 - <0.95	suspected CD	18 (16)	1 (1)
0.95 - <0.99	probable CD	9 (8)	0 (0)
0.99 - 1	definite CD	48 (42)	0 (0)
	Total	114 (100)	95 (100)

Calculation of *a posteriori* probabilities at *a priori* probability $\neq 0.5$.

All estimations of probabilities of CD and the corresponding interpretations in this chapter have been based on *a priori* probabilities of 0.5. For applications at other *a priori* probabilities for all individuals, the formula (see Appendix II) as well as the interpretations of the

a posteriori probabilities have to be adjusted. For situations with considerable individual variations of *a priori* probabilities, the formula cannot be adjusted individually because a reliable interpretation of the *a posteriori* probabilities is only possible on the basis of an evaluation of the results. With the present material, this is not possible.

DISCUSSION

With the system described, 50% of patients with known CD could be recognized with only the agglutination reactions as "definite" or "probable CD". None of the healthy subjects were classified in these categories. Moreover, 16% of the patients with CD were classified as "suspected CD" compared to only 1% of healthy subjects. About one-third of the patients with CD were classified as "no support for CD" compared to 99% of healthy subjects. From these results we conclude that application of the agglutination reactions in combination with the interpretation given, yields an improvement in discrimination of individuals into groups of patients with CD and healthy subjects. If this system is used for diagnostic purposes, it should discriminate between "CD" and "non-CD". The extrapolation from the group "healthy subjects" to "non-CD" is only allowed when there are no relevant differences in frequencies and combinations of agglutination reactions between these groups. In Chapter 3 it is shown that this is true for a large number of "control diseases", but in a few diseases like cirrhosis of liver and coeliac disease, the results have to be interpreted otherwise.
An objection is that the test is evaluated in the same samples as those with which the coefficients of the discriminant function were estimated. This is partly overcome by the verification as described in Appendix II (p. 98), but, ideally, the clinical value of a diagnostic test should be evaluated with samples of the patient population and a similar population that only differs by the absence of the particular disease. The classification of patients as "CD" or "non-CD", however, requires long-term studies (4 years or more) and even then it is doubtful whether all subjects could be classified.

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chapter 8

GENERAL DISCUSSION

In patients with Crohn's disease, conventional bacteriological and virological investigations have not revealed specific causative agents, but the number of investigations was small and the methods were rather conventional. Moreover, epidemiological data are not suggestive for an infectious nature of Crohn's disease, but this is not a *conditio sine qua non* (*cf.* tetanus and actinomycosis).
Like many other idiopathic diseases, Crohn's disease has been the subject of immunological studies. Reports in which deficient cellular immunity was found, have not appeared to be tenable as minor changes of cellular immunologic phenomena are probably secondary to active disease. More recently, immune complexes were found in 50% of patients with Crohn's disease, but the complexes demonstrated probably were heat-aggregated immunoglobulins (Soltis *et al.*, 1979a; 1979b).
A wide variety of antibodies to bacteria, viruses and dietary proteins have been demonstrated, none of which appears likely to be of primary importance.
So far, data don't support the hypothesis that Crohn's disease is caused by infectious agents or primary immunologic dysfunction.

Concept of disease

We have to consider the possibility that Crohn's disease will only become manifest after fulfilment of several conditions (McConnell, 1972; Ward, 1977). Even a typical infectious disease like tuberculosis is determined by other factors than only the causative agent. Sequelae of infection may vary from subclinical events to serious, sometimes lethal disease. In this multiconditional approach of disease, *Mycobacterium*

tuberculosis is not said to be the cause of tuberculosis, but a necessary determinant. The clinical picture further depends on other necessary or contributory determinants, some of which are genetically determined (Harvald & Hauge, 1965).

Implications for Crohn's disease

The following data have to be considered when aetiological factors of Crohn's disease are discussed:

a) a genetic predisposition for Crohn's disease is likely (Kirsner, 1973; Lewkonia, 1973), but genetic markers have not been found;
b) Crohn's disease probably is a multiconditional disease; McConnell (1972) stated: "the environmental factor causing Crohn's disease is affecting large sections of the population, but only those with a genetic susceptibility develop the disease".
c) patients with Crohn's disease have an abnormal faecal flora of probably permanent character; numbers of gram-negative anaerobes and of anaerobic, gram-positive coccoid rods are much higher than in healthy subjects (Wensinck, 1975; 1976).
d) complement activation by the alternative pathway probably occurs in patients with Crohn's disease (Lake *et al.*, 1979);
e) Crohn's disease is a granulomatous disease; granuloma development is considered to represent a second line of defense, particularly when the evoking agent persists, has a particulate form and when antigen-antibody complexes are formed in antibody excess (Spector & Heeson, 1969; Spector, 1969; Adams, 1976)

The following points, based on results in this thesis should be added:

f) several bacteria of the resident faecal flora (C 18, Me 46 and Me 47) activate complement by the alternative pathway, obviously without their subsequent elimination (Chapter 5).
g) if condition (c) is fulfilled, intestinal damage results in the production of antibodies to the coccoid rods (Chapter 4).
h) specific IgG antibodies fail to promote phagocytosis of strain Me 46 in patients with Crohn's disease: these antibodies probably belong to a genetically determined subclass of IgG (Chapter 5).

On the basis of these data the following hypothesis is proposed and illustrated with strain Me 46. The genetic predisposition of Crohn's disease consists of:

(1) the presence of strain Me 46 in the resident faecal flora;
(2) the property to produce, if other conditions are fulfilled, anti-Me 46 non-opsonizing antibodies.

Crohn's disease will only develop if some intestinal injury (ulcerative colitis, diverticulitis, enteritis caused by *Yersinia, Salmonella, Campylobacter,* clostridial toxins ?) occurs but fails to induce opsonizing anti-Me 46 antibodies. The subsequent resistance to phagocytosis enhances the virulence of Me 46, mediated by activation of complement by the alternative pathway which unfortunately cannot result in elimination of Me 46 from the resident intestinal flora. This activation of complement liberates major mediators of inflammation and results in intestinal damage. This secondary damage closes the circle. Particulate antigen-antibody complexes persist and evoke a granulomatous inflammatory response, a second line of defence.
If only the first predisposition is present, intestinal damage will result in the production of anti-Me 46 antibodies of another (opsonizing) IgG subclass and the inflammation will subside.
It should be noted here that agglutinating antibodies to Me 46 do not occur in all patients with Crohn's disease. Non-agglutinating antibodies may exist, however, but the occurrence of other strains and antibodies with similar biological effects in Crohn's disease is not excluded.
In this hypothesis, Crohn's disease could be treated by the induction of antibodies to Me 46 of another IgG subclass, with complement fixing and opsonizing properties. Competition of these biologically active antibodies with inactive antibodies would restore the (unstable) equilibrium that was present before Crohn's disease developed.

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SUMMARY

The faecal flora of patients with Crohn's disease has been found to differ from that of healthy subjects in that the numbers of anaerobic gram-negative rods and of gram-positive coccoid anaerobes, belonging to species of *Eubacterium* and *Peptostreptococcus* were higher.The flora composition was independent of duration of illness and was not influenced by ileocaecal resection. Serum agglutinins against some strains of coccoid rods were found in a considerable percentage of patients with Crohn's disease, whereas percentages of positive sera were much lower in healthy subjects and in patients with various diseases. The interpretation of these data established by Wensinck and the use of the agglutination reactions as a diagnostic test are the subject of this thesis.
In Chapter 2 recent microbiological and immunological findings in patients with Crohn's disease are reviewed. They show that in Crohn's disease as well as in other intestinal diseases, like ulcerative colitis, antibodies to dietary and microbial antigens are found frequently.
In Chapter 3 results are presented of investigations on the prevalence of agglutinins to four strains of anaerobic coccoid rods in patients with Crohn's disease, ulcerative colitis, a number of other diseases and in healthy subjects. Antibodies to coccoid rods were found much more frequently in Crohn's disease than in ulcerative colitis and other diseases. Using the interpretation of agglutination reactions as described in Chapter 7, the percentage of false positive results of sera submitted for diagnosis was found to be satisfactorily low.
The data in Chapter 4 show that the presence of antibodies to the coccoid rods in patients with Crohn's disease is correlated with colonic disease, the presence of fistulae and with serum immunoglobulin levels. No correlation was found between antibodies and any index of disease activity.
In Chapter 5 it is demonstrated that the agglutinins were predominantly IgG and less frequently IgM antibodies. Complement fixation only occurred with antibodies directed against strain C 18. Strains C 18, Me 46 and Me 47 activated complement by the alternative pathway. Antibodies to the strains Me 44, C 18 and Me 47 were opsonins, in contrast to those against strain Me 46. These results suggest that the presence of strain Me 46 in the resident intestinal flora is more relevant than that of the

other strains. Both properties of strain Me 46, activation of complement and resistance to phagocytosis, may be of pathogenetic importance in Crohn's disease. The absence of complement fixing and opsonic properties of antibodies to strain Me 46 suggest that they belong to the IgG4 subclass and possibly are an expression of the genetic predisposition to Crohn's disease.

Logical interpretation of laboratory test results is introduced in Chapter 6 and it is explained why logistic discriminant analysis was applied to the interpretation of the agglutination reactions (Chapter 7). By application of this technique, 50% of patients with Crohn's disease were classified as "probable" or "definite Crohn's disease" compared to none of the control subjects. It is concluded, therefore, that the agglutination reactions can be used as a diagnostic test for Crohn's disease.

Chapter 8 is a general discussion on the relevance of the findings for the understanding of the aetiology of Crohn's disease.

Door Wensinck werd gevonden dat de fecale flora van patiënten met de ziekte van Crohn van het ileum verschilde van die van gezonden. Het aantal anaërobe gram-negatieve bacteriën was hoger en ongeveer 10% van de darmflora bestond uit anaërobe, gram-positieve coccoïde staafjes behorende tot soorten van de geslachten *Eubacterium* en *Peptostreptococcus*, die bij gezonden niet of slechts in lage percentages voorkwamen. In een hoog percentage van sera van patiënten met de ziekte van Crohn kwamen agglutininen voor tegen stammen van de coccoïde staafjes, in tegenstelling tot de sera van gezonden en patiënten met andere ziekten.
Het doel van het in dit proefschrift beschreven onderzoek was, inzicht te verkrijgen in de betekenis van deze waarnemingen en een methode te ontwikkelen waarmee de agglutinatie-reakties met 4 van de coccoïde stammen als diagnostische test konden worden gebruikt.
In Hoofdstuk 2 wordt een overzicht gegeven van recente literatuur over microbiologische en immunologische bevindingen bij de ziekte van Crohn, met name het voorkomen van antistoffen tegen virussen, bacteriën en weefselbestanddelen. Hieruit kon worden geconcludeerd dat zowel bij de ziekte van Crohn als bij andere darmziekten, met name colitis ulcerosa, hogere percentages antistoffen voorkomen tegen voedings- en microbiële antigenen uit de darm dan bij gezonden.
In Hoofdstuk 3 worden de resultaten beschreven van het onderzoek naar het voorkomen van de antistoffen tegen de 4 anaërobe coccoïde staafjes bij patiënten met de ziekte van Crohn, colitis ulcerosa, andere ziekten en gezonde controle-personen. Hieruit bleek dat de antistoffen tegen de coccoïde staafjes bij de ziekte van Crohn veel vaker voorkwamen dan bij colitis ulcerosa en andere ziekten. Bij toepassing van de in Hoofdstuk 7 beschreven interpretatie van de agglutinatie-reakties, was het percentage vals-positieve resultaten bij de voor diagnostische doeleinden onderzochte sera bevredigend laag.
Uit Hoofdstuk 4 blijkt dat het voorkomen van de antistoffen tegen de coccoïde staafjes bij de ziekte van Crohn samenhangt met een dikkedarmlokalisatie van het ziekteproces, de aanwezigheid van fistels en de immuunglobuline-spiegels in het serum. Een verband met enige parameter voor de ernst van de ziekte bestond niet.
In Hoofdstuk 5 wordt aangetoond dat de agglutininen voornamelijk IgG en

soms IgM antistoffen zijn. Met uitzondering van de antistoffen tegen stam C 18 bleken ze geen complement-bindende effectorfunctie te bezitten. De stammen C 18, Me 46 en Me 47 bleken echter zelf complement te aktiveren via de alternatieve weg. De antistoffen tegen de stammen Me 44, C 18 en Me 47 hadden opsoniserende eigenschappen, in tegenstelling tot die tegen stam Me 46. Deze waarnemingen doen vermoeden dat de aanwezigheid van stam Me 46 in de residente darmflora van patiënten met de ziekte van Crohn een grotere betekenis heeft dan die van de andere stammen. De combinatie van eigenschappen, complement te aktiveren en resistent te zijn tegen fagocytose, kan van pathogenetische betekenis zijn voor de ziekte van Crohn. Het ontbreken van complement-bindende en opsoniserende eigenschappen van de antistoffen tegen Me 46 maakt aannemelijk dat ze tot de IgG4 subklasse behoren en mogelijk een expressie zijn van de genetische predispositie voor de ziekte van Crohn.
In Hoofdstuk 6 wordt een inleiding gegeven tot een logische interpretatie van laboratorium-gegevens en duidelijk gemaakt waarom in Hoofdstuk 7 logistische discriminant analyse wordt toegepast voor de interpretatie van de agglutinatie-reakties. Met behulp van deze techniek bleek het mogelijk,50% van de patiënten met de ziekte van Crohn te classificeren als "zekere" of "waarschijnlijke ziekte van Crohn"; bij gezonden was dit percentage 0. Uit deze gegevens wordt geconcludeerd dat de agglutinatie-reakties bruikbaar zijn als diagnostische test voor de ziekte van Crohn.
Hoofdstuk 8 is een algemene discussie over de gegevens, waarbij wordt ingegaan op de mogelijke plaats van deze waarnemingen voor het begrip van de etiologie van de ziekte van Crohn.

APPENDIX I

Data are given in the following tables:

1. patients with Crohn's disease
2. patients with ulcerative colitis
3. abnormal findings in Crohn's disease and ulcerative colitis

Nosographic characteristics in Crohn's disease correlated with:

4. disease restricted to the ileum
5. disease restricted to the colon
6. female sex
7. age
8. bowel resection
9. duration of Crohn's disease
10. abdominal pain
11. diarrhoea
12. weight loss
13. fistulae
14. bloody stools
15. fever
16. Crohn's disease activity index
17. arthritis or erythema nodosum
18. α_1-acid glycoprotein
19. serum albumin
20. haematocrit
21. serum IgA
22. serum IgM
23. serum IgG
24. treatment with corticosteroids
25. treatment with salicylazosulfapyridine

Nosographic characteristics:

26. mutually correlated in patients with ulcerative colitis
27. different frequencies in Crohn's disease and healthy subjects
28. different frequencies in ulcerative colitis and healthy subjects
29. different frequencies in ulcerative colitis and Crohn's disease of the colon

Explanation of abbreviations in Tables 1 and 2

number: code for the individual patient

age : in years at date of entry in the study

m/f : male or female

yrs : duration of disease at date of entry in the study

loc. : location of disease at date of entry in the study:
i = ileum; ic = ileum and colon; c = colon; r = rectum

CDAI : Crohn's disease activity index

α_1*-AGP:* α_1-acid glycoprotein level in serum (g/l)

IgA : immunoglobulin A level in serum (mg/100 ml)

IgM : immunoglobulin M level in serum (mg/100 ml)

IgG : immunoglobulin G level in serum (mg/100 ml)

Aggl. : results of agglutination reactions with strains Me 44, C 18, Me 46 and Me 47, respectively, according to strength (0, 1, 2 or 3)

alb. : serum albumin level (g/l)

symptoms/signs at the date of entry in the study:

b = bloody stools
d = diarrhoea
p = abdominal pain
w = weight loss
ht= low haematocrit
a = arthritis
en= erythema nodosum
py= pyoderma gangraenosum
fe= fever
fi= fistula
an= anal lesion
st= stenosis

Table 1. Data of patients with Crohn's disease

Code no.	age	m/f	yrs	loc.	CDAI	α_1-Agp	IgA	IgM	IgG	Aggl.	alb.	symptoms/signs
1002	22	f	5	ic	323	1.9	524	417	1994	3330	42	d,p,w,ht,fe
1003	29	f	5	ic	164	1.7	268	194	1939	3333	46	ht,fi,st
1004	48	m	3	c	106	0.8	403	160	1431	3002	41	b,d,ht
1005	51	m	>9	ic	44	1.0	224	100	627	0000	31	d
1006	56	f	>9	c	55	0.7	536	42	1939	0132	50	p,fi
1007	29	f	4	ic	228	1.7	278	107	1946	2001	39	b,d,ht,fi,st
1008	38	f	8	i	270	2.1	549	417	1884	3333	44	d,p,w,ht,a,en,fe
1009	26	m	5	ic	329	1.6	243	121	1564	0301	45	b,p,ht,fi,st
1010	25	f	1	ic	293	3.8	561	190	2253	3130	39	p,w,ht,a
1011	66	m	3	i	248	2.0	442	31	1236	0000	41	b,ht
1012	25	f	>9	i	175	1.6	433	194	1844	0000	39	d,w,ht,st
1013	21	f	1	c	447	3.6	114	72	580	0020	35	d,p,w,ht,en,fe,fi
1014	28	f	1	i	154	0.9	425	385	1047	3301	51	d,p,w,fi
1015	27	m	6	ic	14	1.2	305	137	1304	3102	55	d
1016	33	m	>9	ic	12	0.6	295	185	1228	2130	49	-
1017	44	f	1	i	112	1.1	269	54	1658	0301	41	d
1018	17	f	3	i	193	1.5	209	57	982	0210	41	d,p
1019	21	f	<1	c	423	1.7	155	122	1318	3203	45	b,d,p,w
1020	22	m	<1	i	205	1.6	216	122	1228	0031	62	d,p
1021	54	m	>9	c	205	0.8	181	322	1595	3330	49	d
1022	43	f	5	i	20	0.8	404	285	2159	1322	42	a
1023	35	m	1	c	238	1.6	151	107	719	0000	35	b,d
1024	32	m	4	i	351	1.2	403	244	943	1030	28	d,p,ht,fe,st
1025	25	m	>9	i	66	0.6	118	139	1123	3100	39	p,fi,st
1026	33	f	5	c	534	2.3	322	385	784	1000	27	b,d,p,w
1027	43	f	>9	i	78	1.2	520	82	1085	3010	47	d
1028	23	f	2	i	55	1.0	263	216	1165	0100	49	b,d,p
1029	41	f	7	i	32	0.9	280	160	1165	0032	48	d
1030	29	m	4	i	348	2.3	717	119	1749	3330	29	d,p,w,ht,fi,st
1031	21	f	1	i	217	2.5	241	220	1019	0010	40	d
1032	26	f	1	c	188	1.2	238	87	1513	2230	36	d,w,ht,a,en,py
1033	39	m	7	i	30	1.3	238	60	1085	2200	49	an
1034	20	m	3	c	54	1.2	362	206	1424	3300	44	d
1035	52	m	5	i	105	1.3	238	106	1513	3030	49	d,ht
1036	35	f	8	ic	241	1.8	194	137	904	0030	29	d,p,w,ht
1037	32	m	1	i	44	1.2	235	164	1088	0000	54	d
1038	26	f	2	i	69	0.7	211	307	1088	2002	53	b
1039	49	m	7	ic	126	1.0	269	425	1700	3332	43	d,w,ht,fi
1040	47	f	>9	i	121	1.5	81	218	1378	0000	47	p
1041	19	f	1	c	210	1.7	252	369	1129	3331	42	p,w,a,en,st
1042	18	m	1	i	54	0.9	393	157	1353	0030	42	ht
1043	17	f	4	c	54	0.8	362	297	1217	1310	43	d
1044	42	f	>9	ic	26	0.8	195	231	1305	3303	49	d
1045	28	f	>9	c	31	0.7	491	198	1761	3330	46	-
1046	25	m	>9	ic	17	0.8	322	284	1337	3332	53	-

Table 1. Data of patients with Crohn's disease (cont'd)

Code no.	age	m/f	yrs.	loc.	CDAI	α_1-Agp	IgA	IgM	IgG	Aggl.	alb.	symptoms/signs
1047	45	m	2	c	95	0.9	287	134	2887	3202	44	d,a
1048	35	f	>9	ic	39	1.3	226	134	1047	1000	49	d
1049	19	f	3	c	368	1.8	252	366	1608	1300	36	b,d,p,w,en,py,fe
1050	36	m	>9	i	34	1.2	186	39	1378	0310	48	d,st
1051	33	f	7	c	242	1.3	339	212	1490	3133	52	d,p,w,fi
1052	20	m	6	c	218	0.8	342	121	1327	3330	49	d,fi,st
1053	46	f	4	i	93	0.7	373	121	958	3300	47	d,p
1054	27	f	7	c	287	2.6	307	509	1282	3332	32	d,w,ht,a,fi
1055	38	m	1	ic	204	1.7	415	54	996	0000	33	d,p,w,ht
1056	19	f	1	c	396	2.4	342	220	1458	0300	36	b,d,p,w,ht,en
1057	42	m	>9	ic	42	1.3	191	54	883	3133	35	-
1058	51	f	1	c	372	2.4	342	211	1505	3332	27	b,d,w,ht,a,en,fi
1059	61	m	1	i	154	1.9	448	54	845	0000	27	d,w
1060	22	m	6	ic	240	1.5	223	73	1156	0000	36	d
1061	41	f	3	i	12	1.0	299	93	1842	3330	46	d
1062	39	f	5	i	138	0.6	142	246	1569	2000	50	b,d,p
1063	53	f	4	i	162	1.7	186	75	1460	2200	45	d,w
1064	37	f	6	i	14	1.6	226	155	1077	0000	42	-
1065	42	f	5	i	38	1.4	226	375	1033	2332	48	fi
1066	45	m	1	i	188	1.4	234	125	1250	0003	50	d,p,w,st
1067	60	m	5	c	204	1.7	229	103	904	3333	43	d,fi,st
1068	25	f	3	c	160	1.0	218	190	1623	0030	42	b,d,p
1069	24	f	2	c	212	1.6	409	131	1077	3230	42	d,p,w,st
1071	33	m	1	i	276	1.2	203	40	1033	2010	38	b,d,p,w,fi
1072	26	f	8	i	213	1.2	182	163	1077	3102	32	p,ht,a,fi
1073	22	m	1	i	94	1.0	373	93	1475	0302	47	d
1074	29	f	5	c	30	0.9	356	200	1844	2000	58	-
1075	28	f	6	c	145	0.8	234	206	1614	3010	39	b,d,st
1076	71	f	5	c	226	2.0	234	300	1382	2301	33	b,d,ht
1077	30	f	>9	i	75	1.5	167	263	1029	3123	43	d,p
1078	30	f	>9	i	30	0.6	43	200	693	0000	48	p,fi
1082	29	f	>9	ic	340	4.1	235	218	897	1232	30	d,p,ht,fe,fi
1083	25	f	1	i	224	1.9	233	255	1216	0102	45	d,p,st
1084	34	m	<1	c	476	5.1	314	255	1864	3120	28	b,d,p,w,ht,fe,fi
1085	21	f	>9	ic	110	1.1	280	200	1216	1332	40	d,p,w,fi
1087	55	f	>9	ic	119	0.6	495	255	1419	2002	43	d,p
1088	38	m	1	i	146	1.1	187	135	620	0002	35	d,p,w,st
1089	27	f	3	c	415	2.2	253	220	1027	3330	28	d,p,w,ht,fe,fi
1090	29	f	<1	ic	288	4.4	257	425	1221	1302	39	b,d,p,w,fe,fi,an
1091	33	f	>9	c	125	1.3	229	229	1430	0000	42	b,d,p
1092	25	f	1	c	10	1.0	214	124	1308	0000	49	-
1093	32	m	>9	i	10	1.5	490	282	1522	0200	45	-
1094	32	f	7	i	48	0.8	164	193	947	3300	40	d,fi
1095	14	m	2	c	265	3.2	690	139	947	0330	30	d,p,w,ht,fi
1096	20	f	2	c	80	0.8	188	131	1108	2230	46	d,w

Table 1. Data of patients with Crohn's disease (cont'd)

Code no.	age	m/f	yrs.	loc.	CDAI	α_1-Agp	IgA	IgM	IgG	Aggl.	alb.	symptoms/signs
1097	55	f	8	i	97	0.8	386	247	1065	2132	42	d,p,fi,st
1098	40	f	6	i	105	1.0	83	104	632	3001	54	d,p
1099	34	f	<1	c	129	1.2	231	193	1740	3330	37	d,w
1100	27	f	5	c	327	3.7	161	237	875	3130	27	b,d,ht,a,en,py,fe
1101	62	f	>9	c	60	1.1	303	66	1221	0000	48	d,a
1104	40	f	3	i	38	0.6	191	202	1430	3000	48	d,ht
1105	45	m	8	c	30	0.8	196	103	1470	1331	52	-
1107	18	m	1	c	242	1.5	312	185	1462	1330	52	d,p,w,ht
1108	44	f	>9	ic	127	1.1	247	376	1740	1300	40	d,p,fi
1109	32	f	7	c	42	0.7	199	724	1619	3323	40	d
1110	38	f	>9	i	50	0.7	332	283	999	0000	52	p
1111	74	f	1	c	203	1.8	83	724	1186	3030	35	b,d,p,w,ht,fi
1112	25	f	5	ic	84	0.9	329	168	1366	0030	50	p,fi
1113	68	m	1	c	70	0.7	724	100	1423	3000	46	b,d,w
1114	18	f	3	ic	10	0.6	214	276	1683	3000	42	-
1117	22	m	4	c	27	0.8	194	211	913	0000	52	-
1118	25	f	1	c	282	1.8	180	131	1164	0000	30	b,d,p,w
1119	28	m	2	ic	106	2.0	390	346	2133	3332	46	d,ht,an
1120	50	f	2	c	241	1.7	194	26	626	0000	28	b,d,p,w,ht
1121	18	m	4	c	0	1.7	338	246	1491	0000	52	-
1122	44	f	4	i	150	0.5	192	229	1303	0000	44	d,p,w,st
1123	61	m	<1	c	35	1.2	366	227	1005	2030	50	b,p
1124	18	f	<1	c	70	0.6	137	267	2206	0230	40	b,d,p
1125	26	f	<1	ic	240	2.5	199	210	1552	0000	38	b,d,p,w
1126	22	f	5	ic	50	0.6	338	103	1533	2020	42	-
1127	29	m	5	c	30	1.0	563	220	1398	3120	49	d
1128	30	f	2	c	166	1.8	37	235	932	3300	29	b,d,p,w,ht,fe

Table 2. Data of patients with ulcerative colitis

Code no.	age	m/f	yrs.	loc.	CDAI	α1-Agp	IgA	IgM	IgG	Aggl.	alb.	symptoms/signs
2001	27	m	3	c	100	0.9	197	87	984	0000	52	b
2004	47	m	2	c	18	0.9	252	112	1345	0000	49	b
2005	25	m	7	c	84	1.1	474	125	2151	1330	47	ht
2006	67	m	5	c	134	1.0	147	99	910	0000	57	d
2007	63	f	2	c	345	1.7	578	93	1345	0000	44	b,d,w,ht
2008	53	m	3	r	203	1.6	103	208	910	0000	52	d
2009	37	f	2	r	240	1.4	295	335	1345	0100	43	b,d
2010	28	f	3	c	0	0.8	147	268	1098	0100	52	d
2011	48	m	>9	c	100	0.9	282	161	1252	0000	51	b,d
2013	18	m	2	c	0	0.8	22	238	1950	0000	57	d
2014	64	m	<1	c	352	1.6	366	182	1971	0000	30	b,d,w,ht,fe,fi
2015	29	m	>9	c	18	0.8	155	186	2003	0000	54	b
2016	36	f	3	c	120	0.9	285	174	1335	0000	51	b,d,a
2017	26	f	1	c	219	2.4	262	61	1123	0010	28	b,ht
2018	34	f	4	c	130	0.7	162	211	1047	0000	46	d
2019	63	m	5	c	70	1.1	257	154	2055	0000	44	b,d
2020	32	f	>9	c	56	1.0	440	220	1426	2020	41	-
2021	38	f	>9	c	210	1.4	342	214	2323	0100	45	b,d
2022	29	m	8	c	12	0.7	218	356	1047	3000	54	-
2023	28	f	6	c	130	0.7	230	130	1058	0030	49	d
2024	56	m	2	c	161	1.0	289	144	1565	0000	49	b,d,w,ht
2025	35	f	3	c	79	0.5	214	40	984	0000	56	-
2026	68	m	>9	c	30	0.2	246	123	1752	3300	51	d
2027	33	m	1	c	345	1.7	298	151	1201	0000	43	b,d,ht
2028	35	f	>9	c	165	0.7	170	130	1345	2000	43	d,p
2029	83	m	1	r	24	1.0	324	151	1474	0000	49	-
2030	27	f	1	r	56	0.6	315	90	873	0000	45	d
2031	18	f	>9	c	6	0.9	192	174	1021	0000	52	-
2032	24	f	4	r	120	1.2	230	197	984	0000	47	b,p
2033	47	m	2	r	54	1.0	124	90	1165	0000	43	d,ht
2034	25	f	1	c	0	0.8	112	194	1603	0000	51	b,d
2035	25	m	3	c	124	0.6	238	147	1501	0100	50	b,d
2036	32	f	6	c	0	0.5	238	170	2212	0000	52	-
2037	23	f	4	r	175	1.0	83	124	1031	0000	51	b,p
2038	64	m	6	c	56	1.1	294	90	2323	3310	45	ht
2040	45	f	7	r	0	0.6	185	117	902	0200	51	-
2041	29	m	3	c	91	1.1	138	159	1335	0000	46	b
2042	20	f	4	c	42	0.8	94	457	1164	0010	49	-
2043	24	m	7	r	60	0.6	72	178	1164	0000	57	-
2045	18	f	6	r	148	0.8	315	178	1759	0000	49	b,d
2046	27	f	8	c	0	0.9	481	209	1738	3233	44	-
2047	30	f	6	c	338	2.6	133	68	1016	0000	35	b,d,p,w
2048	53	m	8	c	18	0.5	157	144	1236	0000	48	-
2049	28	m	2	r	30	1.0	147	103	766	0000	49	b,d
2050	35	m	>9	r	132	0.7	320	175	1194	1000	48	b,d,p,ht,a,en
2051	54	f	3	c	10	0.7	283	140	1191	0001	48	-

Table 3. Nosographic characteristics in patients with Crohn's disease and ulcerative colitis

	PREVALENCE OF ABNORMAL FINDINGS (%)			
	Crohn's disease of			Ulcerative colitis
	ileum (n=46)	ileocolon (n=26)	colon (n=47)	(n=46)
abdominal pain	50	46	45	11
bloody stools	11	15	43	48
diarrhoea	75	65	81	52
weight loss	23	31	47	9
fever	5	12	13	2
fistulae	20	31	23	2
arthritis/erythema nodosum	2	8	19	4
bowel resection	50	65	32	24
CDAI > 150	34	42	55	24
α_1-acid glycoprotein >1.1 g/l	55	58	60	20
low haematocrit[1]	20	38	32	20
albumin < 35 g/l	9	15	23	4
IgA < 72 mg/100 ml[2]	2	0	2	2
IgA > 455 mg/100 ml	9	12	11	7
IgM < 52 mg/100 ml	7	0	4	2
IgM > 355 mg/100 ml	7	15	13	4
IgG < 600 mg/100 ml	0	0	2	0
IgG > 1767 mg/100 ml	9	19	11	17

[1]) men: <0.47; women: <0.42.

[2]) Cutoff values of immunoglobulin levels represent 2.5 (lower limits) and 97.5 (upper limits) percentiles of values in healthy subjects.

Table 4. Nosographic characteristics correlated with Crohn's disease restricted to ileum

	CORRELATION WITH ILEAL DISEASE ×
positive:	serum albumin
negative:	bloody stools
	IgG
	agglutination of Me 44
	posterior probability

×: $P < 0.05$

Table 5. Nosographic characteristics correlated with Crohn's disease restricted to colon

	CORRELATION WITH COLONIC DISEASE ×
positive:	weight loss
	•bloody stools
	arthritis/erythema nodosum
	CDAI
negative:	•duration of disease
	bowel resection

×: $P < 0.05$; •: $P < 0.01$

Table 6. Nosographic characteristics correlated with female sex of patients with Crohn's disease

	CORRELATION WITH FEMALE SEX ×
positive:	abdominal pain
	arthritis/erythema nodosum
	•IgM

×: $P < 0.05$; •: $P < 0.01$

Table 7. Nosographic characteristics correlated with age of patients with Crohn's disease

	CORRELATION WITH AGE×
positive:	duration of disease
negative:	CDAI
	•IgM

×: $P < 0.05$

Table 8. Nosographic characteristics correlated with bowel resection of patients with Crohn's disease

	CORRELATION WITH BOWEL RESECTION×
positive:	•duration of disease
	•serum albumin
	•haematocrit
negative:	abdominal pain
	•weight loss
	bloody stools
	•CDAI
	•α_1-acid glycoprotein
	disease restricted to colon
	•treatment with corticosteroids
	treatment with SASP

×: $P < 0.05$; •: $P < 0.01$

Table 9. Nosographic characteristics correlated with duration of Crohn's disease

	CORRELATION WITH DURATION OF DISEASE ×
positive:	age
	•bowel resection
negative:	•weight loss
	•bloody stools
	•CDAI
	•α_1-acid glycoprotein
	•disease restricted to colon

×: P < 0.05; •: P < 0.01

Table 10. Nosographic characteristics correlated with abdominal pain in patients with Crohn's disease

	CORRELATION WITH ABDOMINAL PAIN ×
positive:	female sex
	•weight loss
	•fever
	bloody stools
	•fistulae
	•CDAI
	α_1-acid glycoprotein
negative:	bowel resection
	serum albumin
	IgG

×: P < 0.05; •: P < 0.01

Table 11. Nosographic characteristics correlated with diarrhoea in patients with Crohn's disease

	CORRELATION WITH DIARRHOEA[×]
positive:	•weight loss
	•CDAI
	α_1-acid glycoprotein
negative:	•serum albumin

×: $P < 0.05$; • $P < 0.01$

Table 12. Nosographic characteristics correlated with weight loss in patients with Crohn's disease

	CORRELATION WITH WEIGHT LOSS[×]
positive:	•abdominal pain
	•diarrhoea
	•fever
	arthritis/erythema nodosum
	disease restricted to colon
	•CDAI
	•α_1-acid glycoprotein
	•treatment with corticosteroids
negative:	•duration of disease
	•bowel resection
	•serum albumin
	•haematocrit

×: $P < 0.05$; • $P < 0.01$

Table 13. Nosographic characteristics correlated with fistulae in patients with Crohn's disease

	CORRELATION WITH FISTULAE[×]
positive:	•abdominal pain
	•CDAI
	•agglutination of C 18
	agglutination of Me 46
	agglutination of Me 47
	•posterior probability
negative:	serum albumin
	haematocrit

×: $P < 0.05$; •: $P < 0.01$

Table 14. Nosographic characteristics correlated with bloody stools in patients with Crohn's disease

	CORRELATION WITH BLOODY STOOLS[×]
positive:	abdominal pain
	•CDAI
	α_1-acid glycoprotein
	•disease restricted to colon
negative:	•duration of disease
	bowel resection
	disease restricted to ileum
	•serum albumin
	agglutination of Me 46
	posterior probability

×: $P < 0.05$; •: $P < 0.01$

Table 15. Nosographic characteristics correlated with fever in patients with Crohn's disease

	CORRELATION WITH FEVER ×
positive:	•abdominal pain
	•weight loss
	bowel resection
	arthritis/erythema nodosum
	•CDAI
	•α_1-acid glycoprotein
	•IgM
	posterior probability
negative:	•serum albumin
	•haematocrit

×: $P < 0.05$; •: $P < 0.01$

Table 16. Nosographic characteristics correlated with the Crohn's disease activity index (CDAI) in patients with Crohn's disease

	CORRELATION WITH CDAI ×
positive:	•bloody stools
	disease restricted to colon
	•α_1-acid glycoprotein
	•treatment with corticosteroids
negative:	age
	•duration of disease
	•bowel resection
	•serum albumin

×: $P < 0.05$; •: $P < 0.01$

Table 17. Nosographic characteristics correlated with arthritis/erythema nodosum in patients with Crohn's disease

CORRELATION WITH ARTHRITIS/ERYTHEMA NODOSUM ×	
positive:	weight loss
	fever
	•CDAI
	•α_1-acid glycoprotein
	agglutination of C 18
	female sex
	disease restricted to colon
negative:	serum albumin
	•haematocrit

×: $P < 0.05$; •: $P < 0.01$

Table 18. Nosographic characteristics correlated with α_1-acid glycoprotein in patients with Crohn's disease

CORRELATION WITH α_1-ACID GLYCOPROTEIN ×	
positive:	abdominal pain
	•weight loss
	diarrhoea
	•fever
	bloody stools
	•arthritis/erythema nodosum
	•CDAI
	•treatment with corticosteroids
negative:	•duration of disease
	•bowel resection
	•serum albumin
	•haematocrit

×: $P < 0.05$; •: $P < 0.01$

Table 19. Nosographic characteristics correlated with serum albumin in patients with Crohn's disease

	CORRELATION WITH SERUM ALBUMIN ×
positive:	•bowel resection
	disease restricted to ileum
	•haematocrit
negative:	abdominal pain
	•weight loss
	•diarrhoea
	•fever
	•bloody stools
	fistulae
	arthritis/erythema nodosum
	•CDAI
	•α_1-acid glycoprotein
	treatment with corticosteroids

×: $P < 0.05$; •: $P < 0.01$

Table 20. Nosographic characteristics correlated with haematocrit value in patients with Crohn's disease

	CORRELATION WITH HAEMATOCRIT ×
positive:	•serum albumin
	•bowel resection
negative:	•weight loss
	•fever
	fistulae
	•arthritis/erythema nodosum
	•CDAI
	•α_1-acid glycoprotein
	agglutination of Me 46
	posterior probability

×: $P < 0.05$; •: $P < 0.01$

Table 21. Nosographic characteristics correlated with serum IgA in patients with Crohn's disease

	CORRELATION WITH IGA ×
positive:	posterior probability

×: $P < 0.05$

Table 22. Nosographic characteristics correlated with serum IgM in patients with Crohn's disease

	CORRELATION WITH IGM ×
positive:	•fever
	•female sex
	IgG
	•agglutination of Me 44
	agglutination of C 18
	posterior probability
negative:	•age

×: $P < 0.05$; •: $P < 0.01$

Table 23. Nosographic characteristics correlated with serum IgG in patients with Crohn's disease

	CORRELATION WITH IGG ×
positive:	IgM
	agglutination of Me 44
	•agglutination of C 18
	•posterior probability
negative:	abdominal pain
	disease restricted to ileum

×: $P < 0.05$; •: $P < 0.01$

Table 24. Nosographic characteristics correlated with treatment with corticosteroids of patients with Crohn's disease

	CORRELATION WITH CORTICOSTEROIDS×
positive:	•weight loss
	•CDAI
	•α_1-acid glycoprotein
negative:	serum albumin
	•bowel resection

×: $P < 0.05$; •: $P < 0.01$

Table 25. Nosographic characteristics correlated with treatment with salicylazosulfapyridine (SASP) of patients with Crohn's disease

	CORRELATION WITH SASP×
negative:	bowel resection

×: $P < 0.05$

Table 26. Nosographic characteristics mutually correlated in patients with ulcerative colitis

CHARACTERISTICS MUTUALLY CORRELATED ×
weight loss and low haematocrit
weight loss and short duration of disease
•weight loss and CDAI
weight loss and bloody stools
CDAI and low haematocrit
CDAI and bloody stools
•CDAI and low serum albumin
•CDAI and α_1-acid glycoprotein
α_1-acid glycoprotein and low haematocrit
α_1-acid glycoprotein and low serum albumin
serum albumin and haematocrit
IgA and low haematocrit
duration of disease and posterior probability
IgA and IgG
IgA and age
•duration of disease and agglutination of Me 44
bloody stools and no agglutination of Me 44
bloody stools and low posterior probability

×: $P < 0.05$; •: $P < 0.0025$

Table 27. Nosographic characteristics with higher frequency in patients with Crohn's disease than in healthy subjects

CHARACTERISTICS WITH HIGHER FREQUENCY IN CROHN'S DISEASE •
•α_1-acid glycoprotein
•IgG (higher level)
•agglutination of Me 44
•agglutination of C 18
•agglutination of Me 46
•agglutination of Me 47
•posterior probability (higher value)

• : $P < 0.0001$

Table 28. Nosographic characteristics with higher frequency in patients with ulcerative colitis than in healthy subjects

CHARACTERISTICS WITH HIGHER FREQUENCY IN ULCERATIVE COLITIS×
•α_1-acid glycoprotein (higher level)
IgG (higher level)
agglutination of C 18
•agglutination of Me 46
•posterior probability (higher value)

×: $P < 0.05$; •: $P < 0.01$

Table 29. Nosographic characteristics with higher frequency in patients with Crohn's disease of the colon than in patients with ulcerative colitis

CHARACTERISTICS WITH HIGHER FREQUENCY IN COLONIC CROHN'S DISEASE×
•diarrhoea
•abdominal pain
•weight loss
arthritis/erythema nodosum
•fistulae
•CDAI (higher value)
α_1-acid glycoprotein(higher level)
•IgM (higher level)
•agglutination of Me 44
•agglutination of C 18
•agglutination of Me 46
•agglutination of Me 47
•posterior probability (higher value)
•low serum albumin

×: $P < 0.05$; •: $P < 0.01$

Logistic discriminant analysis (Anderson, 1972) was used to discriminate between patients with Crohn's disease (CD) and healthy subjects(HS) on the basis of the agglutination reactions. The *a posteriori* probability of CD can be written as:

$$\Pi\ (CD|x_1,x_2,x_3,x_4) = \frac{1}{1 + \exp\ (\beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4)} \qquad (1)$$

In this formula, x_1-x_4 are the results of the agglutination reactions with strains Me 44, C 18, Me 46 and Me 47, respectively. The expression is known as the multivariate logistic function. The coefficients β_0-β_4 were estimated with the maximum likelihood method (Cox, 1970) and the estimates are denoted by b_0-b_4, respectively. The estimated *a posteriori* probability of CD can thus be written as:

$$P\ (CD|x_1,x_2,x_3,x_4) = \frac{1}{1 + \exp\ (b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4)} \qquad (2)$$

The estimates b_0-b_4 are obtained from the reference samples (n_1 patients with CD and n_2 healthy subjects) with *a priori* probabilities P(CD) and P(HS) proportional to the sample sizes n_1 and n_2:

$$P(CD) = \frac{n_1}{n_1 + n_2}; \qquad P(HS) = \frac{n_2}{n_1 + n_2} \qquad (3)$$

If *a posteriori* probabilities have to be calculated from other *a priori* probabilities Q(CD) and Q(HS) = 1 - Q(CD), b_0 is replaced by d_0:

$$d_0 = b_0 + \ln\frac{n_1}{n_2} + \ln\frac{Q(HS)}{Q(CD)} \qquad (4)$$

Results. With the results of the agglutination reactions x_1(Me 44), x_2 (C 18), x_3 (Me 46) and x_4 (Me 47) of 114 patients with CD and 95 healthy subjects, the coefficients β_0 - β_4 in (1) were estimated:

$$b_0 = 1.33 \qquad (5)$$

$b_1 = -0.45$
$b_2 = -0.95$
$b_3 = -2.32$
$b_4 = -1.04$ (6)

The coefficient b_0 is connected with *a priori* probabilities:

$P(CD) = 114/209 = 0.54$; $P(HS) = 0.46$

For equal *a priori* probabilities $Q(CD) = Q(HS) = 0.5$, coefficient b_0 is replaced by d_0 according to (4), thus:

$d_0 = 1.51$ (7)

The other coefficients remain unchanged. The 95% confidence intervals for these coefficients are:

$-0.86 < \beta_1 < -0.05$
$-1.51 < \beta_2 < -0.38$
$-4.23 < \beta_3 < -0.41$
$-1.77 < \beta_4 < -0.31$ (8)

From (8) it is concluded that coefficients $\beta_1 - \beta_4$ differ significantly from 0 ($\alpha = 0.05$). With the estimated coefficients (6) and (7) the *a posteriori* probabilities were calculated for the agglutination reactions of the patients with Crohn's disease and of healthy subjects (Table 1), as well as for all possible agglutination reactions (Table 2).

Verification. Up to here, the results of allocation are mentioned for the same elements as those with which the coefficients of the logistic function were estimated (see Chapter 7). It would be better to evaluate the classification in new samples from the patients with Crohn's disease and healthy subjects. As these samples were not available, a split-sample method was used. The sample consisting of 114 patients with CD has been randomly divided into two groups of 57 subjects. The sample consisting of 95 healthy subjects has been divided in a group of 48 and one of 47 subjects. With the agglutination results of the first group of 57 patients with CD and the group of 48 healthy subjects, new coefficients were estimated. The results, for equal *a priori* probabilities are:

$$d_0 = 1.75$$
$$b_1 = -0.56$$
$$b_2 = -0.94$$
$$b_3 = -2.01$$
$$b_4 = -1.66 \qquad (9)$$

The *a posteriori* probabilities for the elements of both groups, as determined with these coefficients, are summarized in Table 3. From this table it is seen that the results of both groups are similar. It is concluded, therefore, that the coefficients (6) and (7) obtained with the original samples may be used (see Chapter 7).

REFERENCES

Anderson, J.A. (1972) Separate logistic discrimination. *Biometrika 59*, 19-35

Cox, D.R. (1970) The analysis of binary data. *Methuen & Co Ltd., London*

Table 1. Frequency distribution of agglutination reactions in 114 patients with Crohn's disease (CD) and 95 healthy subjects (HS)

AGGLUTINATION*	CD	HS	AGGLUTINATION*	CD	HS
0 0 0 0	21	75	2 0 0 0	1	5
0 0 0 1	0	1	2 0 0 1	1	0
0 0 0 2	2	1	2 0 0 2	2	0
0 0 0 3	1	0	2 0 1 0	1	0
0 0 1 0	1	0	2 0 3 0	1	0
0 0 2 0	1	0	2 1 3 0	1	0
0 0 3 0	3	0	2 1 3 2	1	0
0 0 3 1	1	0	2 2 0 0	3	1
0 0 3 2	1	0	2 2 3 0	2	0
0 1 0 0	0	2	2 3 0 1	1	0
0 1 0 1	0	1	2 3 3 2	1	0
0 1 0 2	1	0	3 0 0 0	3	2
0 1 1 0	1	0	3 0 0 1	1	0
0 1 3 2	1	0	3 0 0 2	1	0
0 2 0 0	2	0	3 0 1 0	2	1
0 2 3 0	1	0	3 0 3 0	2	0
0 3 0 0	1	1	3 1 0 2	2	0
0 3 0 1	2	0	3 1 2 0	1	0
0 3 0 2	1	0	3 1 2 3	1	0
0 3 1 0	1	0	3 1 3 0	2	0
0 3 3 0	1	0	3 1 3 3	2	0
1 0 0 0	3	4	3 2 0 2	1	0
1 0 0 2	0	1	3 2 0 3	1	0
1 0 2 0	1	0	3 2 3 0	1	0
1 2 3 2	1	0	3 3 0 0	3	0
1 3 0 0	2	0	3 3 0 1	1	0
1 3 0 2	1	0	3 3 0 3	1	0
1 3 1 0	1	0	3 3 2 3	1	0
1 3 2 2	1	0	3 3 3 0	9	0
1 3 3 0	1	0	3 3 3 1	1	0
1 3 3 1	1	0	3 3 3 2	4	0
1 3 3 2	1	0	3 3 3 3	3	0

*) Agglutination reactions with strains Me 44, C 18, Me 46 and Me 47, respectively, according to strength.

Table 2. A posteriori *probabilities P(CD|x) of Crohn's disease at* a priori *probability of 0.5 for all agglutination reactions with strains Me 44, C 18, Me 46 and Me 46, respectively*

AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)
0000	0.18	0231	1	1122	1	2013	0.99
0001	0.38	0232	1	1123	1	2020	0.98
0002	0.64	0233	1	1130	1	2021	0.99
0003	0.83	0300	0.79	1131	1	2022	1
0010	0.69	0301	0.92	1132	1	2023	1
0011	0.86	0302	0.97	1133	1	2030	1
0012	0.95	0303	0.99	1200	0.70	2031	1
0013	0.98	0310	0.97	1201	0.87	2032	1
0020	0.96	0311	0.99	1202	0.95	2033	1
0021	0.98	0312	1	1203	0.98	2100	0.58
0022	0.99	0313	1	1210	0.96	2101	0.80
0023	1	0320	1	1211	0.99	2102	0.92
0030	1	0321	1	1212	0.99	2103	0.97
0031	1	0322	1	1213	1	2110	0.93
0032	1	0323	1	1220	1	2111	0.98
0033	1	0330	1	1221	1	2112	0.99
0100	0.36	0331	1	1222	1	2113	1
0101	0.62	0332	1	1223	1	2120	0.99
0102	0.82	0333	1	1230	1	2121	1
0103	0.93	1000	0.26	1231	1	2122	1
0110	0.85	1001	0.50	1232	1	2123	1
0111	0.94	1002	0.73	1233	1	2130	1
0112	0.98	1003	0.89	1300	0.86	2131	1
0113	0.99	1010	0.78	1301	0.94	2132	1
0120	0.98	1011	0.91	1302	0.98	2133	1
0121	0.99	1012	0.97	1303	0.99	2200	0.78
0122	1	1013	0.99	1310	0.98	2201	0.91
0123	1	1020	0.97	1311	0.99	2202	0.97
0130	1	1021	0.99	1312	1	2203	0.99
0131	1	1022	1	1313	1	2210	0.97
0132	1	1023	1	1320	1	2211	0.99
0133	1	1030	1	1321	1	2212	1
0200	0.60	1031	1	1322	1	2213	1
0201	0.81	1032	1	1323	1	2220	1
0202	0.92	1033	1	1330	1	2221	1
0203	0.97	1100	0.47	1331	1	2222	1
0210	0.94	1101	0.72	1332	1	2223	1
0211	0.98	1102	0.88	1333	1	2230	1
0212	0.99	1103	0.95	2000	0.35	2231	1
0213	1	1110	0.90	2001	0.61	2232	1
0220	0.99	1111	0.96	2002	0.81	2233	1
0221	1	1112	0.99	2003	0.92	2300	0.90
0222	1	1113	1	2010	0.85	2301	0.96
0223	1	1120	0.99	2011	0.94	2302	0.99
0230	1	1121	1	2012	0.98	2303	1

cont'd on next page

Table 2. cont'd

AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)	AGGLUT.	P(CD|x)
2310	0.99	3020	0.99	3130	1	3300	0.94
2311	1	3021	1	3131	1	3301	0.98
2312	1	3022	1	3132	1	3302	0.99
2313	1	3023	1	3133	1	3303	1
2320	1	3030	1	3200	0.85	3310	0.99
2321	1	3031	1	3201	0.94	3311	1
2322	1	3032	1	3202	0.98	3312	1
2323	1	3033	1	3203	0.99	3313	1
2330	1	3100	0.69	3210	0.98	3320	1
2331	1	3101	0.86	3211	0.99	3321	1
2332	1	3102	0.95	3212	1	3322	1
2333	1	3103	0.98	3213	1	3323	1
3000	0.46	3110	0.96	3220	1	3330	1
3001	0.71	3111	0.98	3221	1	3331	1
3002	0.87	3112	0.99	3222	1	3332	1
3003	0.95	3113	1	3223	1	3333	1
3010	0.90	3120	1	3230	1		
3011	0.96	3121	1	3231	1		
3012	0.99	3122	1	3232	1		
3013	0.99	3123	1	3233	1		

Table 3. Frequency distribution of a posteriori *probabilities of Crohn's disease P(CD|x) for the elements from the first sub-sample of 57 patients with Crohn's disease (CD) and 48 healthy subjects (HS) and the second sub-sample of 57 patients with CD and 47 HS, with use of coefficients (9)*

P(CD|x)	SUB-SAMPLE 1		SUB-SAMPLE 2	
	CD	HS	CD	HS
0 - <0.10	0	0	0	0
0.10 - <0.20	9	38	12	37
0.20 - <0.30	0	3	3	1
0.30 - <0.40	1	3	0	4
0.40 - <0.50	1	1	2	2
0.50 - <0.60	2	0	1	0
0.60 - <0.70	0	0	0	0
0.70 - <0.80	4	2	3	1
0.80 - <0.85	4	0	1	1
0.85 - <0.90	1	1	1	1
0.90 - <0.95	5	0	5	0
0.95 - <0.99	6	0	6	0
0.99 - 1	24	0	23	0
Total	57	48	57	47

Voor het totstandkomen van dit proefschrift was de medewerking van velen een noodzakelijke of bijdragende faktor. Ik wil hen allen hartelijk bedanken en enkelen met name noemen:

Prof. Dr F. Wensinck, mijn promotor, bood mij de gelegenheid aan het onderzoek mede te werken. Behalve door de ontelbare gesprekken met hem over het onderzoek, is zijn stimulerende invloed terug te vinden in de wijze waarop de resultaten zijn beschreven: met veel geduld heeft hij mij telkens weer duidelijk gemaakt dat een zekere "liefde voor de lezer" ook noodzakelijk was.
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Jan Dees en *Mark van Blankenstein* dragen de verantwoordelijkheid voor de diagnose van de meeste patiënten. Zonder hun welwillende medewerking bij het verkrijgen van klinische gegevens over en bloed van de patiënten, was het onderzoek niet mogelijk geweest.
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Paul Schmitz heeft op prettige wijze ervoor gezorgd dat een groot aantal patiëntengegevens met de computer kon worden geanalyseerd. In de vele gesprekken die ik met hem had, zijn mij vele mogelijkheden en onmogelijkheden van de toegepaste statistiek duidelijker geworden.
Gesprekken met *Jaap van der Sluis* leidden tot een versneld uitkristalli-

seren van conclusies uit enkele immunologische valstrikken.
Zonder de hulp van de medewerkers van de poliklinieken Inwendige Geneeskunde II en Heelkunde, met name *Jeanine Boender*, *Elleke Schalekamp* en *Liesbeth Benckhuysen-Bongers* zou het onderzoek aanzienlijk moeizamer zijn verlopen.
Tenslotte dank ik *Paula Delfos* voor haar hulp bij het maken van de omslagfoto.

CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1945 geboren. In 1963 behaalde hij het diploma HBS-B aan het Groen van Prinsterer-lyceum te Vlaardingen. De studie in de geneeskunde vond plaats aan de Rijksuniversiteit te Leiden. Na het doctoraalexamen in 1968 werden de co-assistentschappen gevolgd aan de Medische Faculteit te Rotterdam. Het artsexamen werd afgelegd in 1971. Na het vervullen van de militaire dienstplicht volgde specialisatie in de inwendige geneeskunde op de afdeling Interne Geneeskunde II (Hoofd: Prof. Dr M. Frenkel) van het Academisch Ziekenhuis Dijkzigt te Rotterdam en werd hij in 1977 in het specialistenregister ingeschreven. Sedert 1975 is de schrijver verbonden aan de afdeling Medische Microbiologie (Hoofd: Prof. Dr F. Wensinck) van de Erasmus Universiteit te Rotterdam.

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