The immune response to ϕ X 174 in man

III Evidence for an association between hyposplenism and immunodeficiency in patients with coeliac disease¹

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SUMMARY The ability of patients with coeliac disease to produce primary and secondary antibody in response to a new antigen has been tested. Eight patients with coeliac disease were injected intravenously with the coliphage ϕ X 174. Antibody levels were measured throughout the primary response. On day 28 a second injection was given and the secondary response was also studied in detail. The production of antibody in the primary response is lower than in a group of normal subjects. The secondary response is severely reduced and the difference between the coeliac group and controls is highly significant. The secondary response in the coeliac patients contains a much higher proportion of IgM antibody than normal. These abnormalities correlate approximately with clinical status and with hyposplenism. It is concluded that in patients with coeliac disease the ability to produce antibody is impaired, with a defect in switching from IgM to IgG antibody.

Levels of serum and secretory immunoglobulins are frequently abnormal in patients with adult coeliac disease (Asquith, Thompson, and Cooke, 1969; Wallington, Ajdukiewicz, Read, and Jones, 1972). Studies of antibody production, however, have given conflicting results. Pettingale (1970, 1971) suggested that the response to intramuscular tetanus toxoid was impaired, while Beale, Parish, Douglas, and Hobbs (1971) found a normal response to tetanus toxoid, but diminished antibody production after oral poliomyelitis vaccine. Since there is evidence of hyposplenism in patients with coeliac disease (Engel, 1939; Martin and Bell, 1965; McCarthy, Fraser, Evans, and Read, 1966; Ferguson, Hutton, Maxwell, and Murray, 1970), any disturbance of antibody production would be likely to affect the response to intravenous antigens more than to those administered subcutaneously or intramuscularly.

The bacteriophage ϕ X 174 is a coliphage, which can be used intravenously as a test for antibody producing capacity in man (Peacock, Jones, and

Gough, 1973). Very few normal subjects have pre-existing antibodies, and the first injection produces a clear-cut primary response. The method for measuring antibody is sensitive and accurate, and the range of primary and secondary responses has been defined. The antigen has now been widely used in normal and diseased subjects, and no side effects have been observed from its administration. We have used it to study antibody production in eight patients with adult coeliac disease, and have found for the first time a highly significant depression of the secondary response in six out of eight patients, together with moderate impairment of the primary response. The antibody produced in the secondary response is predominantly IgM, suggesting a partial failure of the mechanism for switching from IgM to IgG antibody production.

Patients

Eight adult patients with coeliac disease were studied. Details of the patients are given in table I. In all cases the diagnosis was based on histological changes of subtotal villous atrophy in a jejunal biopsy. Six cases showed clinical and morphological improvement following withdrawal of dietary gluten. Two cases (5, 2) showed no morphological improvement on gluten withdrawal. Case 2 showed

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Patient	Sex	Age (yr)	Spleen Area ¹ (cm ²)	C3 ² (mg/ 100 ml)	IgA ³ (mg/ 100 ml)	IgG ⁴ (mg/ 100 ml)	IgM ⁵ (mg/ 100 ml)	Gluten-free Diet Status at Time of Study	Xylose Excretion ⁶ (%)		Clinical History
									Before Gluten- free Diet	After Gluten- free Diet	
1	F	31	18	70	192	108	108	2 mth diet (occasional lapses)	20	26	Childhood coeliac disease, biopsy 1973 on routine admission, occasional diar- rhoea
2	M	59	21	74	755	1203	82	2 yr diet (strict adherence)	13	25	1971 weight loss, anaemia, clinical improvement on gluten-free diet, re- lapsed after gluten exposure 1974
3	M	58	20	58	332	1579	197	6 yr diet (strict adherence)	4	36	1963 steatorrhoea, subtotal villous atrophy, 1967 gluten-free diet
4	F	27	36	95	184	1347	108	Normal diet	30	32	Coeliac disease aged 3, gluten-free diet aged 3 to 13, 1967, 1973 subtotal villous atrophy. On normal diet
5	M	81	21	66	531	866	101	2 mth diet (strict adherence)			1962 steatorrhoea, subtotal villous atrophy, 1973 gluten-free diet: no improvement, responded to cortico- steroids
6	F	33	26	58	212	1062	163	5 yr diet (occasional lapses)	21	35	Coeliac disease infancy, gluten-free diet—5 years, 1968 relapse in preg- nancy, subtotal villous atrophy, re- sponded to gluten-free diet
7	F	35	42	79	250	805	77	Normal diet	30	32	1964 macrocytic anaemia, treated B ₁₂ , 1973 subtotal villous atrophy, symptom free, morphological responses to gluten-free diet
8	M	48	25	74	198	930	55	1 mth diet (strict adherence)	21	28	1973 diarrhoea, subtotal villous atrophy, good responses to gluten-free diet

Table I Clinical details of patients with coeliac disease

¹Normal value 27-103 cm², ²normal value 80-140 mg/100 ml, ³normal value 140-420 mg/100 ml, ⁴normal value 800-1700 mg/100 ml, ⁵normal value 50-190 mg/100 ml, ⁶normal value 0-5 hr > 23% dose excreted in urine

clinical and functional improvement following gluten withdrawal and relapsed when gluten was re-introduced. Case 5 continued to deteriorate on a gluten-free diet but eventually responded to treatment with corticosteroids. No patients had received corticosteroids before immunological testing. All patients were volunteers who gave informed consent after the nature of the investigation had been explained to them. The project was approved by the Ethical Committee of the United Bristol Hospitals.

Materials and Methods

The method of preparing the bacteriophage has been described previously (Peacock *et al*, 1973). Batches were tested for sterility and pyrogenicity by standard methods before injection. Patients were injected intravenously with 2.1×10^9 plaque-forming units (PFU) of ϕ X 174. Blood samples were taken before immunization and at intervals for 28 days. The blood was allowed to clot at room temperature, the serum was removed and stored at -20°C , and was inactivated for 30 minutes at 56°C before use. Patients were given a second intravenous dose of 2.1×10^9 PFU on day 28. Blood samples were taken for a further month.

Antibody was measured by inactivating bacteriophage with a series of dilutions of serum, and calculating the 50% bacteriophage neutralization titre (SD₅₀) (Peacock *et al*, 1973). Serum was subjected to rate zonal centrifugation on a sucrose density gradient to separate IgG and IgM antibodies. Antibody was measured in the fractions as previously described and IgM- and IgG-containing fractions were identified by immunodiffusion against commercial antisera to IgM and IgG (Peacock *et al*, 1973).

The levels of antibody in the secondary response were compared with those of the control normal subjects already published (Peacock *et al*, 1973) and also with a group of 10 patients with Crohn's disease (Bucknall, Jones, and Peacock, 1975).

In all patients the spleen size was estimated by measuring the splenic scan following an intravenous injection of 2 to 3 microcuries of Technetium-99 (Larson, Tuell, Moores, and Nelp, 1971). Immunoglobulin concentrations and C3 levels were estimated using Hoechst Tripartigen immunodiffusion plates. Serum and red cell folate were estimated by the *Lactobacillus casei* method. Xylose excretion was measured in a five-hour urinary collection following a 5 g dose of xylose. Faecal fat was

estimated on three-day samples of stools. Jejunal biopsies were taken from the region adjacent to the ligament of Treitz under radiological control using a Crosby capsule. Gluten intake was assessed at interview by a physician and a dietician. The gluten-free diet was based on the recommendations of the Coeliac Society *List of gluten-free foods 1972-3* (Colman, 1972). All the patients in this study were skin-tested with a battery of delayed hypersensitivity antigens, (Baker, Read, and Jones, 1975).

Results

Before studying the effects of immunization we tested 61 samples of serum from 40 patients with coeliac disease for preimmunization antibody to ϕ X 174. Only one subject (case 6) had antibody at a titre of 10.

PRIMARY RESPONSE

The peak levels of antibody production are set out in table II and the patterns of response to the first injection are shown in figure 1. Three cases (3, 5, 8) showed gross impairment of the primary response with a peak antibody level of less than 20. The remaining five fell within the range of normal primary responses. Case 6, who had a preexisting antibody titre of 10, developed a primary response which was in the middle of the range. Those patients who produced antibody showed no distinct variation from normal in the time of appearance of antibody, rate of increase of antibody titre, height and time of peak response, or rate of decline of antibody.

Patient	Primary Response		Secondary Response	
	Day of Peak Response	Greatest Titre	Day of Peak Response	Greatest Titre
1	18	1044	6	3583
2	9	570	13	2468
3	28	18	9	2006
4	14	999	14	3258
5	—	10	7	865
6	14	952	9	5888
7	18	4800	9	17824
8	7	16	9	15318

Table II Antibody production

SECONDARY RESPONSE

All eight patients were given a second injection of 2.1×10^9 PFU of bacteriophage on day 28 of the primary response. Antibody titres are shown in figure 2. There is a striking impairment in the amount of antibody produced in the secondary response compared with that in normal subjects. Two cases (7, 8) developed antibody titres which fall within the

Fig 1 Antibody levels following primary injection.

range for 30 normal subjects. The remainder are outside the normal range.

The patients with coeliac disease include some individuals who are older than our normal controls. Since there is a suggestion that the immune response may decline with age, we have also taken into account a group of 10 patients with Crohn's disease. The ages of this group range from 22 to 58, making them comparable to the patients with coeliac disease. Their secondary antibody production, however, was entirely normal (Bucknall *et al*, 1975). For statistical purposes, we pooled the titres of secondary antibody in normals and Crohn's patients, and compared them with the coeliac group, so that the two groups are homogeneous in age distribution.

Fig 2 Secondary response in coeliac disease. Antibody levels following secondary injection 28 days after first injection. (The shaded area is mean response ± 1 SD for 30 normal subjects.)

Tests for significance at seven, nine, 14, and 20 days, using Student's t test, show that the difference between the coeliac group and the controls is highly significant ($P < 0.01$, $P < 0.0005$, $P < 0.0005$, $P < 0.0005$).

In general, there was a correlation between the severity of the disease and the depression of antibody production. The two cases with the highest secondary response (7, 8) were excreting only 2 and 3 g of fat daily, and both had spleens of normal size. The lowest secondary response was produced by case 5, who failed to respond clinically and morphologically to gluten withdrawal, and eventually required treatment with corticosteroids.

SPLEEN SIZE AND ANTIBODY PRODUCTION

In five patients, the area of the spleen determined from the Technetium-99 scan was below the normal range. When those with spleens of 25 cm² and above were compared with those below 25 cm² there was no separation of peak primary responses. Peak secondary responses, however, were clearly separated (fig 3), showing that those with small spleens were particularly likely to have an impaired secondary response.

IMMUNOGLOBULIN CLASS OF ANTIBODY

Serum from the secondary response of cases 5, 6, and 7 was separated by centrifugation on a sucrose density gradient. The antibody titre recovered in the IgM and IgG containing fractions is shown in table III and the recovery profile in case 7 appears in figure 4. A striking feature was the large amount of antibody found in the IgM-containing fractions. This is in complete contrast to all of our normal

Patient	Secondary Response		
	Summed Titres of Antibody Recovered from Sucrose Density Gradient		
	IgM	IgG	IgG ¹ (%)
5	101	24	19
6	1186	786	40
7	2697	1402	34

Table III Immunoglobulin class of antibody

¹Values for six normal subjects range between 80 and 95%.

subjects (Peacock *et al*, 1973) and patients with Crohn's disease (Bucknall *et al*, 1975), where antibody response to a second injection of antigen is almost entirely confined to the IgG peak.

Fig 4 Serum from peak secondary response of patient 7. IgG and IgM separated by sucrose density gradient ultracentrifugation. Antibody appears mainly in IgM fractions. (—) Absorbance.

Discussion

Our results show a clear impairment of the secondary response to intravenous ϕX 174 in patients with adult coeliac disease. On days when it was possible to compare the coeliac group directly with controls the differences are highly significant. The primary response in normal subjects is extremely variable. Three of the coeliac cases (3, 5, 8) produced a primary antibody titre of less than 20, while only one out of 20 normals and one out of 10 patients with Crohn's disease had a primary response at this level. It seems very likely, therefore, that the primary response is also impaired. It is striking that the two patients (7, 8) who were among the least affected clinically had the best antibody response, while case 5, who had the lowest levels of antibody, was the only patient who continued to deteriorate on a gluten-free diet and required treatment with corticosteroids. The three cases (2, 3, 5) with the highest

Fig 3 Peak levels of primary and secondary antibody in those with small (< 25 cm²) and normal (≥ 25 cm²) spleens (normal range 27-103 cm²).

levels of serum IgA were also the three poorest producers of antibody. With the exception of case 5 all subjects were clinically well at the time of testing. None had evidence of malnutrition, and all except case 5 had weights which were steady within the expected range for their age and height. In a study of patients with Crohn's disease (Bucknall *et al*, 1975), we found no evidence of impairment in the response to ϕ X 174, despite the fact that several patients were ill and wasted at the time of study. The impairment of antibody production in the coeliac group is therefore a significant feature of this disease, rather than a complication due to illness or weight loss.

Other workers have investigated the immune response of patients with adult coeliac disease to oral or intramuscular antigens (Pettingale, 1970; Beale *et al*, 1971). Pettingale (1970) found that both primary and secondary antibody production to intramuscular tetanus toxoid was impaired, and suggested that the deficiency was especially marked in the IgM response. Beale *et al* (1971), on the other hand, found a normal response to intramuscular tetanus toxoid but impaired antibody production to oral poliomyelitis vaccine.

Other studies have shown evidence of hypoplasia in adult coeliac disease (Engel, 1939; Martin and Bell, 1965; McCarthy *et al*, 1966; Ferguson *et al*, 1970). McCarthy *et al* (1966) also found prolonged survival of antibody-damaged erythrocytes in patients with adult coeliac disease. The spleen plays an important role in processing intravenous antigens (Geier, Trigg, and Merrill, 1973) and it is therefore of interest to find such clear evidence of an impaired antibody response to intravenous antigen in our patients.

ANTIBODY CLASS

Serum from the peak secondary response of three patients was fractionated on a sucrose density gradient, and all showed a large proportion of antibody in the IgM peak. In case 5 the response to the second injection of antigen was entirely IgM, and since there was no detectable response to the first injection of antigen, this can be regarded as a 'delayed primary' response. Case 6 showed a reasonable primary response and a rather low secondary response, with secondary antibody distributed evenly between IgM and IgG. Case 7 showed primary and secondary responses which fell within the normal limits, and it is therefore of particular interest that even at the peak of the secondary response there is a marked predominance of IgM antibody. Taken together the results from these three cases suggest a significant defect in switching

from IgM to IgG antibody production. This abnormality may be related to the finding (Douglas, Crabbé, and Hobbs, 1970) that there are increased numbers of intestinal plasma cells secreting IgM in patients with coeliac disease.

Our studies throw no light on the problem of whether the splenic atrophy in coeliac disease is primary or secondary to the intestinal disease, but they do provide evidence for the importance of this feature in the immune response and show that the ability to produce a secondary antibody response to a new antigen is significantly impaired in patients with coeliac disease.

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