BRITISH MEDICAL JOURNAL

LONDON SATURDAY DECEMBER 30 1961

AN IMMUNOLOGICAL STUDY OF COELIAC DISEASE AND IDIOPATHIC STEATORRHOEA

SEROLOGICAL REACTIONS TO GLUTEN AND MILK PROTEINS

BY

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The mechanism of the harmful action of wheat and rye gluten in coeliac disease and idiopathic steatorrhoea remains uncertain. That these proteins are responsible for the continued disability in the great majority of cases of coeliac disease was first shown by Dicke (1950). This observation was extended by Weijers and van de Kamer (1950) and first confirmed in this country by Anderson *et al.* (1952). The present theories of pathogenesis have been reviewed recently by Weijers and van de Kamer (1960) and by Frazer (1960). The more popular theory postulates that there is a congenital enzyme deficiency, with resulting incomplete digestion of gluten. Consequently a glutamine-containing polypeptide accumulates sufficiently to damage the intestinal mucosa and to cause harmful systemic effects after absorption. The second theory holds that gluten itself, or a breakdown product, acts through an immunological mechanism. The two theories are not mutually exclusive, because the postulated injurious polypeptide might behave antigenically.

French *et al.* (1957) reviewed earlier work and showed that adults with idiopathic steatorrhoea also responded to a gluten-free diet. Many patients with idiopathic steatorrhoea have histories suggesting that the disease started in childhood, and the term "adult coeliac disease" has been proposed as a better one than idiopathic steatorrhoea or non-tropical sprue (Cooke, 1958).

An obstacle to the acceptance of the immunological theory of causation has been the lack of satisfactory demonstration of antibodies to the protein concerned. We have therefore made a systematic search for antibodies to a breakdown product of gluten in the sera of patients with coeliac disease, idiopathic steatorrhoea, and other types of steatorrhoea.

As well as testing for the presence of antibodies to gluten, we have also studied the antibodies to purified milk proteins, for two reasons. First, there have been reports of clinical remissions in patients with coeliac disease after the withdrawal of milk from the diet (Kunstadter and Shultz, 1953; Breton *et al.*, 1959). Secondly, we have recently found that, compared with healthy subjects, patients with ulcerative colitis are much more likely to have antibodies at high titre to milk proteins (Taylor and Truelove, 1961).

Materials and Methods

Sera.—These were obtained from children with coeliac disease and adults suffering from various types

of steatorrhoea. Control sera were taken from children at operation for tonsillectomy or circumcision, under general anaesthesia, and from healthy adults, either the staff of the Nuffield Department of Clinical Medicine or visitors to patients in a general medical ward. All sera were stored at -20° C. until use.

Antigens.—The antigen used to test for antibodies to gluten was a peptic-tryptic digest of wheat gluten (fraction III) as described by Frazer *et al.* (1959). Gluten itself is insoluble in water and the usual salt solutions used in immunological procedures. Fraction III has been shown to cause clinical and biochemical relapse when fed to children with coeliac disease who have been treated with a gluten-free diet (Frazer *et al.*, 1959). Freshly prepared fraction III was used in a 1/5 dilution in phosphate-saline buffer at pH 6.4. It was not previously freeze-dried. The chief proteins of milk, casein, α -lactalbumin and β -lactoglobulin were used at a concentration of 0.5% in phosphate-saline buffer at pH 6.4. These proteins were supplied to us in a highly purified form by Dr. R. Aschaffenburg, of the National Institute for Research in Dairying, who has described the methods of preparation (Gunther *et al.*, 1960).

Tests Performed.—Sera were examined for precipitating antibodies by the gel-diffusion method of Ouchterlony (1948), using undiluted sera against fraction III. A standard complement-fixation test (Mayer *et al.*, 1948; Price, 1950) for complement-fixing antibodies to fraction III was performed. The complement-fixation test as described by Berger (1958), using alcoholic extracts of gluten and gliadin, was also attempted. The coated tanned-red-cell test (Boyden, 1951) modified by Gunther *et al.* (1960) was used with fraction III and each of the three milk proteins. The tests were read at 2 and 24 hours and the results were scored as described previously (Taylor and Truelove, 1961). The sera were tested in batches, each batch being composed of experimental and control sera. Finally all the sera from both groups were tested on a single day against the preparation of fraction III, and the results showed close agreement with the earlier batch results.

Results

Gel-diffusion Tests.—Repeated tests have been made with the sera of all the classes of patients and control subjects studied against fraction III, and the results have been uniformly negative.

Complement-fixation Tests.—No evidence of complement-fixing antibodies to fraction III has been found.

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Since positive complement fixation with alcoholic extracts of wheat gluten and gliadin by the sera of patients with coeliac disease and normal children have been reported by Berger (1958), we have repeated these tests, using a constant serum dilution of 1/4 and diluting the antigen. The results have been uniformly negative.

Coated Tanned Red Cells.—Positive results were obtained with many of the sera, which were tested in dilutions of 1/20, 1/200, 1/2,000, and 1/20,000.

Results in Children

The reactions in 24 children with coeliac disease have been compared with those in 50 children not suffering from this disease. The subjects with coeliac disease were all under the age of 15 years and consisted of children who, with one exception, had developed the symptoms of coeliac disease during the first three years of life. All suffered, or had suffered, from frank steatorrhoea as judged by biochemical tests. None had evidence of other diseases which cause steatorrhoea, such as fibrocystic disease (mucoviscidosis). In addition, all the subjects either had biopsy evidence of partial or subtotal villous atrophy of the small-intestinal mucosa (Doniach and Shiner, 1957) or had shown a favourable response to a gluten-free diet.

Reactions to Gluten Fraction III

Table I and Fig. 1 show that the spectrum of results obtained with fraction III is different in coeliac disease from that found in health, with fewer coeliac sera giving negative reactions and more giving positive reactions at higher titres (1/2,000 and 1/20,000). Although there is considerable overlap between the results in the normals and those with coeliac disease, the distributions are sharply different, with more than twice as many positive results at the higher titres in coeliac disease. In essence, therefore, patients with coeliac disease are much more likely than normal children to have circulating antibodies at high titre to fraction III.

In view of the fact that most patients diagnosed as suffering from coeliac disease are to-day treated with a

FIG. 1.—Serological reactions in coeliac disease (coated tanned-red-cell test).

TABLE I.—Serological Reactions to Gluten Fraction III in Coeliac Disease

	Detailed Results						Compressed Version				Significance of Difference
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. or 1/20	1/200	1/2,000 or 1/20,000	Total	
Control children ..	25	15	5	4	1	50	40 (80%)	5 (10%)	5 (10%)	50 (100%)	$\chi^2 = 13.4$ $n = 2$ $P < 0.01$
Coeliac disease ..	5	4	9	4	2	24	9 (37.5%)	9 (37.5%)	6 (25%)	24 (100%)	

gluten-free diet, which in itself might alter the serum reactions, we have analysed our material with this point in mind (Table II). Unfortunately our numbers are

TABLE II.—Serological Reactions to Gluten Fraction III in Coeliac Disease in Relation to Gluten-free Diet

Diet	Neg. or 1/20	1/200 and Over
Normal	0	3
Gluten-free for <1 year ..	2	3
„ „ >1 „ ..	6	7

small, but none of the three patients on normal diet had negative or low-titre reactions (1/20) to fraction III. This is an issue which deserves further study.

Likewise, our present small numbers make it impossible to draw any firm conclusions when the serological reactions are related to the biopsy findings (Table III).

TABLE III.—Serological Reactions to Gluten Fraction III in Coeliac Disease in Relation to Biopsy Findings

Histological Changes in Biopsy Specimen	Neg. or 1/20	1/200 or Higher
Normal	2	0
Villous atrophy ..	3	9

Among the 14 patients who had biopsy performed, the two with negative biopsy findings were both on a gluten-free diet which may permit reversion of the mucosa towards normality (Anderson, 1960). Those with positive biopsy findings but negative or low-titre serological reactions to fraction III had all been on a gluten-free diet for several years.

Reactions to Milk Proteins

Table IV and Fig. 1 show the results obtained with the purified individual cow's milk proteins. Once again the sera from patients with coeliac disease show a relative scarcity of negative reactions and a relative excess of positive reactions at high titre. For each milk protein, the differences between coeliac sera and normal sera are significant statistically, although the differences are least pronounced in the case of lactoglobulin.

Independence of Reactions to Gluten Fraction III and to Milk Proteins

In view of the fact that the sera of patients with coeliac disease show more strongly positive reactions to both fraction III and milk proteins, it is of interest to know whether these reactions are related or independent of one another. Table V sets out the association between the results for fraction III and the individual milk proteins in the form of simple contingency tables. It can be seen that there is no obvious correlation, either positive or negative.

Results in Adults

The reactions in 60 cases of steatorrhoea in adults have been compared with those in 64 healthy adult subjects. The steatorrhoea patients comprised 45 examples of idiopathic steatorrhoea, seven with pancreatic steatorrhoea, and eight with post-gastrectomy steatorrhoea.

Reactions to Gluten Fraction III

Table VI shows the results obtained with fraction III. It can be seen that the patients with idiopathic steatorrhoea have a much higher incidence of positive reactions than the normal subjects. For example, the reactions at high titre (1/2,000 or 1/20,000) are more than 10 times as frequent in idiopathic steatorrhoea as in health. The adult patients with idiopathic steatorrhoea (Fig. 2) therefore show essentially the same difference from their normal controls as do children with coeliac disease. By contrast, the patients with pancreatic steatorrhoea and post-gastrectomy steatorrhoea give results which appear to conform with those of healthy adults, although a more extensive study will be necessary to settle this point.

Reactions to Milk Proteins

When the results obtained with milk proteins are examined (Table VII) it can be seen that, as with fraction III, the patients with idiopathic steatorrhoea frequently have strongly positive reactions to these proteins and that the differences from the normal subjects are considerable and reach very high levels of statistical significance. In effect, the adult patients with idiopathic steatorrhoea resemble the children with coeliac disease in showing an excessive number of strongly positive reactions to milk proteins as well as to fraction III. By contrast, the patients with pancreatic or post-gastrectomy steatorrhoea resemble normal subjects in their reactions to milk proteins as well as to fraction III.

It should be mentioned here that the number of high-titre reactions to casein with normal adult sera is lower than that reported in a previous communication (Taylor and Truelove, 1961). This difference appears to be due

to a change in technique, the use of rabbit-serum-saline as the serum diluent making the system with this antigen more stable and reducing the number of 1/2,000 and 1/20,000 reactions. The overall difference between normal and ulcerative colitis sera remains unchanged. It is interesting that this technical modification does not affect the results whether using lactalbumin or lactoglobulin.

Independence of the Reactions to Gluten Fraction III and to Milk Proteins

As in the children, the reactions to fraction III and to the milk proteins appear to be essentially independent, as can be seen from Table VIII.

FIG. 2.—Serological reactions in idiopathic steatorrhoea in adults (coated tanned-red-cell test).

TABLE IV.—Serological Reactions to Cow's Milk Proteins in Coeliac Disease

Antigen	Subjects	Detailed Results						Compressed Version				Significance of Difference
		Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. or 1/20	1/200	1/2,000 or 1/20,000	Total	
Casein	Control children ..	6	37	7	0	0	50	43 (86%)	7 (14%)	0 (0%)	50 (100%)	$\chi^2 = 29.6$ $n = 2$ $P < 0.001$
	Coeliac disease ..	0	3	11	3	0	17	3 (17.6%)	11 (64.7%)	3 (17.6%)	17 (100%)	
α -Lactalbumin	Control children ..	8	13	19	8	2	50	21 (42%)	19 (38%)	10 (20%)	50 (100%)	$\chi^2 = 29.5$ $n = 2$ $P < 0.001$
	Coeliac disease ..	0	0	1	8	8	17	0 (0%)	1 (5.9%)	16 (94.1%)	17 (100%)	
β -Lactoglobulin	Control children ..	26	11	8	4	1	50	37 (74%)	8 (16%)	5 (10%)	50 (100%)	$\chi^2 = 7.4$ $n = 2$ $P < 0.05$
	Coeliac disease ..	2	5	4	6	0	17	7 (41.2%)	4 (23.6%)	6 (35.2%)	17 (100%)	

TABLE V.—Showing Lack of any Definite Association Between Serological Reactions in Coeliac Disease to Gluten Fraction III and to Individual Milk Proteins

Gluten Fraction III	Casein				α -Lactalbumin				β -Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Neg. and 1/20 ..	2	3	1	6	0	1	5	6	2	2	2	6
1/200 ..	1	3	2	6	0	0	6	6	4	1	1	6
1/2,000 and 1/20,000 ..	0	5	0	5	0	0	5	5	1	1	3	5
Totals ..	3	11	3	17	0	1	16	17	7	4	6	17

TABLE VI.—Serological Reactions to Gluten Fraction III in Steatorrhoea

	Detailed Results						Compressed Version				Significance of Difference
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	
Adult controls ..	45	14	4	1	0	64	59 (92.2%)	4 (6.2%)	1 (1.6%)	64 (100%)	$\chi^2 = 21.0$ $n = 2$ $P < 0.001$
Idiopathic steatorrhoea ..	14	11	10	9	1	45	25 (55.6%)	10 (22.2%)	10 (22.2%)	45 (100%)	
Pancreatic ..	3	3	1	0	0	7	6	1	0	7	
Post-gastrectomy ..	4	2	1	1	0	8	6	1	1	8	

Reactions to Gluten Fraction III in Relation to the Clinical Picture in Idiopathic Steatorrhoea

Age at Onset of Disease.—The patients with idiopathic steatorrhoea included a number in whom the symptoms began during childhood, a group which conforms to the conception of adult coeliac disease (Cooke, 1958). It is of interest to compare the immunological reactions of this group of cases with those of the remainder in whom idiopathic steatorrhoea appeared to develop during mature life. Table IX shows that there is no significant difference between these two groups of cases that comprise the total group of idiopathic steatorrhoea.

Small-intestinal Biopsy Findings.—Of the 45 patients with idiopathic steatorrhoea, the results of small-intestinal biopsy were available for 25. Among these 25, all except one showed villous atrophy of the mucosa. The immunological reactions in this group of patients were similar to those in the remainder in whom biopsy had not been employed as one of the diagnostic criteria.

Anaemia.—Among the 44 patients with idiopathic steatorrhoea, 34 had had anaemia as a prominent feature of their illness. These 34 subjects were more liable to show high-titre reactions to fraction III than the remainder; but the difference was not statistically significant, and more extensive observations will be necessary to decide the issue.

Gluten-free Diet.—Among the patients with idiopathic steatorrhoea there appeared to be little difference in the serological reactions of those being treated on a gluten-free diet and those on an unrestricted diet, although precise details of the diet were sometimes uncertain, so that further study is needed.

Discussion

The present study shows beyond doubt that circulating antibodies to fraction III are much more often present in high titre in patients with coeliac disease and idiopathic steatorrhoea than in healthy subjects. In addition, patients with these diseases frequently possess a high titre of antibodies to the proteins of cow's milk. Do these findings imply that gluten exerts its harmful action in these diseases by an immunological mechanism? If so, do milk proteins similarly have an aetiological role in these diseases, at any rate in some patients?

It has long been known from animal experiments that wheat gluten and gliadin can behave as antigens (Wells and Osborne, 1913). The mechanism by which, in man, ingested protein results in the formation of circulating antibodies is unknown. Anderson *et al.* (1926) have produced some evidence that infants absorb protein from cow's milk and eggs, but concluded that this absorption ceased after a few months of life. The fact that some of our control subjects had high titres of circulating antibodies to fraction III is sufficient evidence

TABLE VII.—*Serological Reactions to Cow's Milk Proteins in Steatorrhoea*

		Detailed Results						Compressed Version				Significance of Difference
		Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	
Casein	Adult controls ..	13	29	5	0	0	47	42 (89.4%)	5 (10.6%)	0 (0%)	47 (100%)	$\chi^2 = 14.3$ $n = 2$ $P < 0.001$
	Idiopathic steatorrhoea ..	4	19	15	4	0	42	23 (54.8%)	15 (35.7%)	4 (9.5%)	42 (100%)	
	Pancreatic steatorrhoea ..	6	0	1	0	0	7	6	1	0	7	
	Post-gastrectomy steatorrhoea ..	4	2	1	0	0	7	6	1	0	7	
α -Lactalbumin	Adult controls ..	10	13	21	3	0	47	23 (48.9%)	21 (44.7%)	3 (6.4%)	47 (100%)	$\chi^2 = 28.3$ $n = 2$ $P > 0.001$
	Idiopathic steatorrhoea ..	1	3	16	13	9	42	4 (9.5%)	16 (38.1%)	22 (52.4%)	42 (100%)	
	Pancreatic steatorrhoea ..	5	0	2	0	0	7	5	2	0	7	
	Post-gastrectomy steatorrhoea ..	1	2	4	0	0	7	3	4	0	7	
β -Lactoglobulin	Adult controls ..	26	18	3	0	0	47	44 (93.6%)	3 (6.4%)	0 (0%)	47 (100%)	$\chi^2 = 23.9$ $n = 2$ $P < 0.001$
	Idiopathic steatorrhoea ..	10	10	14	7	1	42	20 (47.6%)	14 (33.3%)	8 (19.1%)	42 (100%)	
	Pancreatic steatorrhoea ..	6	0	1	0	0	7	6	1	0	7	
	Post-gastrectomy steatorrhoea ..	6	0	1	0	0	7	6	1	0	7	

TABLE VIII.—*Showing Lack of any Definite Association Between Serological Reactions in Idiopathic Steatorrhoea to Gluten Fraction III and to Individual Milk Proteins*

Gluten Fraction III	Casein				α -Lactalbumin				β -Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Neg. and 1/20 ..	16	6	1	23	3	11	9	23	15	3	5	23
1/200 ..	2	6	2	10	0	2	8	10	2	6	2	10
1/2,000 and 1/20,000 ..	5	3	1	9	1	3	5	9	3	5	1	9
Totals ..	23	15	4	42	4	16	22	42	20	14	8	42

TABLE IX.—*Similarity of Serological Reactions in Idiopathic Steatorrhoea Beginning in Childhood (Adult Coeliac Disease) and in Idiopathic Steatorrhoea Beginning in Mature Life*

	Fraction III				Casein				α -Lactalbumin				β -Lactoglobulin			
	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Adult coeliac disease ..	9	5	3	17	9	5	2	16	1	6	9	16	10	4	2	16
Other idiopathic steatorrhoea ..	16	5	7	28	14	10	2	26	4	9	13	26	10	10	6	26

TABLE X.—Serological Reactions to Gluten Fraction III in Ulcerative Colitis

	Detailed Results						Compressed Version			
	Neg.	1/20	1/200	1/2,000	1/20,000	Total	Neg. and 1/20	1/200	1/2,000 and 1/20,000	Total
Adult controls	45	14	4	1	0	64	59 (92.2%)	4 (6.2%)	1 (1.6%)	64 (100%)
Ulcerative colitis	25	22	3	0	0	50	47 (94%)	3 (6%)	0 (0%)	50 (100%)

that dietary gluten can act as an antigen in apparently healthy subjects. However, we do not know whether these control subjects with high titres did not have some abnormality of the small intestinal mucosa either at the time of collection of serum or at some time in the past.

The greatly increased titres to fraction III exhibited by steatorrhoea sera might be merely an indication of enhanced absorption of unchanged, antigenic protein when the small-intestinal mucosa is diseased. However, the factor which most militates against this is that serum reactions to fraction III and milk proteins are not related in individual patients.

A further observation is that in ulcerative colitis the serological reactions to fraction III resemble those in healthy subjects (Table X), although this disease resembles idiopathic steatorrhoea in showing abnormally high numbers of high-titre reactions to milk proteins (Taylor and Truelove, 1961). The discrepancy between the behaviour in the two diseases can be best illustrated by summarizing the data as follows, in which a significant surplus of high-titre reactions compared with normal proportions is indicated by a + sign.

	Idiopathic Steatorrhoea	Ulcerative Colitis
Fraction III	+	—
Casein	+	—
Lactalbumin	+	—
Lactoglobulin	+	+

The one proviso that should be made about regarding this discrepancy as ruling out abnormal absorption as the explanation for a surplus of high-titre reactions is that proteins may vary in the ease with which they are digested, and therefore the site of mucosal damage within the gastro-intestinal tract may have an important bearing.

At least the possibility exists that the high-titre reactions in patients with steatorrhoea represent an abnormal degree of specific response to one or more dietary proteins associated with hypersensitivity of the small-intestinal mucosa to these same proteins. Testing this hypothesis requires quantitative measurement of hypersensitivity of the small-intestinal mucosa itself. Rubin *et al.* (1960) have introduced gluten into the small intestine of patients with idiopathic steatorrhoea maintained in remission on a gluten-free diet and have shown that lesions typical of the disease appeared in the previously histologically normal mucosa of the proximal ileum. This elegant study may provide a valuable technique for future investigations of this problem.

Studies of circulating antibodies to dietary proteins may also prove to be of importance in the diagnosis and treatment of various enteropathies. For example, it is well known that some patients with idiopathic steatorrhoea fail to show any beneficial response when gluten is excluded from the diet. We have recently seen one such patient whose jejunal biopsy showed subtotal villous atrophy. Reaction of her serum to fraction III was negative. In such subjects it would be of interest to determine levels of circulating antibodies to other food

proteins, such as those of milk, and then to determine by exclusion diets whether any favourable result obtained was related to the level of circulating antibodies.

ADDENDUM.—The serum of the patient just mentioned was found to give high-titre reactions to cow's milk proteins. She was treated with a milk-free diet (but one containing gluten) and has shown an excellent clinical response during the two months that have elapsed. More prolonged observation of this patient, as well as studies of other patients, will be necessary before it is possible to conclude with certainty that milk can play a decisive part in some patients with idiopathic steatorrhoea or coeliac disease.

Summary

Sera of patients with coeliac disease and idiopathic steatorrhoea contain antibodies to a proteolysed fraction of wheat gluten in significantly higher incidence and titre than the sera of control subjects. This is also true for purified cow's milk, casein, α -lactalbumin, and β -lactoglobulin. These findings are discussed in relation to aetiology and treatment of coeliac disease and idiopathic steatorrhoea.

We are indebted to Dr. R. Aschaffenberg, of the National Institute for Research in Dairying, for supplying us with the purified milk proteins. We are most grateful to the following physicians who assisted us by collecting sera from their patients with coeliac disease and idiopathic steatorrhoea and supplying us with clinical data: Dr. Elizabeth Pringle, Dr. Margot Shiner, Dr. Winifred Young, Dr. W. T. Cooke, Dr. Clifford Hawkins, and Dr. John Badenoch. Our anaesthetist colleague, Dr. J. V. Mitchell, kindly collected blood under anaesthesia from children undergoing minor surgical operations. We thank Miss Marjory Alcock, Miss Ruby Gearing, and Mrs. Jean Jeffs for technical assistance. The expenses of the study were partly met by a grant to one of us (S. C. Truelove) from the Medical Research Council, and it is a pleasure to acknowledge this.

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