Circulating antibodies to cow’s milk proteins in ulcerative colitis

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SUMMARY  Sera from patients with ulcerative colitis (51), Crohn’s disease (30), hypolactasia (13), untreated adult coeliac disease (11), irritable colon syndrome (24), and sera from 38 healthy control subjects were tested for antibodies to the principal cow’s milk proteins—casein, α-lactalbumin, and β-lactoglobulin. The red-cell-linked antigen-antiglobulin reaction was used to determine the titres of direct agglutinating antibodies and IgA and IgG incomplete antibodies.

Apart from patients with coeliac disease, direct agglutinating antibodies were found infrequently and then in low titres. Approximately 50% of subjects had low titres of IgA and IgG antibodies. However, the titres found in sera from patients with ulcerative colitis did not differ from those found in the control subjects or in patients with Crohn’s disease, hypolactasia, or irritable colon syndrome. Patients with untreated coeliac disease frequently had high antibody titres to the milk proteins. In all subjects tested, incomplete antibodies of IgA or IgG immunoglobulin class occurred with equal frequency.

The frequent occurrence in adults of low titres of antibodies to the milk proteins may be due to continued absorption of minute amounts of protein. Absorption of allergens may be facilitated by mucosal damage, such as that of coeliac disease, with stimulation of antibody production. At the present time, however, there is little evidence to suggest that milk allergy is a factor in the aetiology of ulcerative colitis.

The finding of raised circulating antibody titres to the cow’s milk proteins in patients with ulcerative colitis (Taylor and Truelove, 1961; Taylor, Truelove, and Wright, 1964) supported the earlier clinical observations that an allergy to milk proteins might be involved in the aetiology of the disease (Andresen, 1942; Rowe, 1942; Mackie, 1942; Sarles, Deck, Chalvet, and Ambrosi, 1959; Truelove, 1961). A controlled therapeutic trial of a milk-free diet showed that about 20% of patients with ulcerative colitis would benefit from milk withdrawal (Wright and Truelove, 1965a). However, when serial observations were made during the trial period on the antibody titres to the milk proteins, it was concluded that the response to a milk-free diet in an individual patient could not be predicted from the antibody titres (Wright and Truelove, 1965b). Patients with high titres to whole cow’s milk were especially liable to suffer from multiple relapses but, apart from this, no correlation was found between the height of the titre and any of the important clinical features of the disease.

The test used in these earlier studies was the tanned-red-cell haemagglutination technique. Since tannic acid alters the red cell membrane in such a way that proteins combine with the red cell non-specifically, an antiglobulin reaction cannot be employed. The red-cell-linked antigen-antiglobulin reaction (Steele and Coombs, 1964) has therefore been used in the present study in order to detect incomplete antibodies to milk proteins in the serum of patients with ulcerative colitis. The use of specific anti-immunoglobulin antisera has allowed the immunoglobulin class of these antibodies to be determined.

Methods

Patients’ sera
Sera from patients with ulcerative colitis was stored in 1 ml aliquots at −20°C. Sera were tested within two weeks of collection and were not thawed and refrozen more than three times. They were not heat-inactivated. Sera from patients with Crohn’s disease, hypolactasia, coeliac disease, and irritable colon syndrome and from healthy subjects were also
tested. The healthy controls had no history of milk intolerance.

**Milk Proteins**

Purified casein, \(\alpha\)-lactalbumin, and \(\beta\)-lactoglobulin were supplied by Dr R. Lyster of the National Institute for Research in Dairying. They had been prepared by the method of Gunther, Aschaffenburg, Matthews, Parish, and Coombs (1960).

**Antiserum**

Antiserum to casein, \(\alpha\)-lactalbumin, and \(\beta\)-lactoglobulin were prepared in rabbits by injecting intramuscularly 1 mg of allergen in complete Freund’s adjuvant, followed by weekly injections of allergen alone. An anti-rabbit-globulin antiserum was similarly prepared in a goat. Antisera were heat inactivated at 56°C for 30 min and 1 ml aliquots were absorbed twice for 15 minutes each with an equal volume of fresh human group O red cells which had been washed six times in phosphate-buffered saline, \(pH\) 7.2.

Antisera specific for IgA and IgG were obtained commercially (Hyland).

Antiserum to human group O red cells was made in rabbits. Fresh red cells were washed three times in phosphate-buffered saline. Then 2.5 ml of a 10% suspension was injected intraperitoneally on alternate days for five doses. The animal was bled 14 days later and the serum obtained was inactivated at 56°C for 30 min. The globulin fraction of the antiserum was precipitated with 50% ammonium sulphate, washed twice in 50% ammonium sulphate solution, and finally resuspended in phosphate-buffered saline. Excess ammonium sulphate was removed by dialysis against phosphate-buffered saline. The protein content of the antiserum was estimated by the method of Lowry, Rosebrough, Farr, and Randall (1951). A protein concentration of approximately 5 mg/ml was obtained by ultracentrifugation.

**Photo-oxidization**

The individual milk proteins were coupled to the antihuman group O red cell antiserum by the process of photo-oxidization (Steele and Coombs, 1964). Two ml of antiserum and 2 ml of a solution of allergen, containing about 5 mg of protein/ml, were placed in a 50 ml conical flask: 0.2 ml of Rose Bengal (1%) was added and the flasks were gently shaken on a mechanical shaker under bright illumination. The process was continued until conjugation had taken place, as determined serologically, and this usually occurred between 13 and 16 hours. To test for adequate coupling, the conjugates were serially diluted to 1 in 512 in 0.2 ml volumes. Five drops of 4% fresh human group O red cells (washed three times in phosphate-buffered saline) were added to each tube and allowed to incubate in the dark for one hour. The sensitized cells were washed twice and then added to (1) normal rabbit serum (absorbed twice with group O red cells, heat inactivated) 1/500; (2) anti-allergen antiserum 1 in 100 and 1 in 500; (3) goat anti-rabbit-globulin antiserum 1/200. Satisfactory conjugation of the allergen to the anti-red-cell antibody was judged by (a) the absence of direct agglutinating activity of the anti-red-cell serum and (b) agglutination to a high titre with anti-allergen and anti-rabbit globulin antibody (Table 1).

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<th>RED-CELL-LINKED, ANTIGEN-ANTIGLOBULIN REACTION</th>
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| The conjugates of the milk proteins, prepared by photo-oxidization, were used at a 1 in 10 dilution. Two ml of each (0-2 ml conjugate + 2-0 ml phosphate-buffered saline) was incubated at room temperature for one hour with 2 ml of 4% fresh human group O red cells (washed three times in phosphate-buffered saline). The sensitized cells were washed twice in phosphate-buffered saline and resuspended to 2%. Five drops of sensitized cells were then added to 0.2 ml of patient’s sera, which had been serially diluted to 1 in 512, and incubated for a further hour. The cells were washed twice in normal rabbit serum (heat inactivated, absorbed twice with group O red cells and used at a dilution of 1/500). One drop of resuspended cells was then added to 1 drop of: (1) normal rabbit serum 1/500, (2) anti-IgA antiserum, 1/100, (3) anti-IgG antiserum, 1/500. Agglutination was read after one to one and a half hours. The presence of direct agglutinating antibody in patient’s sera was shown by agglutina-

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| Table 1 Serological testing for adequate conjugation of \(\alpha\)-lactalbumin to anti-red cell antibody by photo-oxidation for 15 hours |
tion observed in normal rabbit serum, whereas incomplete IgA or IgG antibodies were detected by agglutination in the presence of the anti-immunoglobulin antisera.

Results

Sera from 51 patients with ulcerative colitis were compared with healthy control subjects (38) and with patients suffering from Crohn's disease (30), hypolactasia (13), irritable colon syndrome (24), and coeliac disease (11). The age and sex distribution of these patients is shown in Tables II and III. Duplicate observations were not routinely made on each serum but, when they were made, the results did not differ by more than one titre.

The antibody titres obtained in each diagnostic category are represented as histograms shown in Figures 1-3.

Direct agglutinating antibodies to casein, α-lactalbumin, and β-lactoglobulin were found in only a small minority of patients and then in low titres. This was so in all the groups of patients studied except for patients with coeliac disease who frequently had direct agglutinating antibodies to each of the three proteins, often in high titres.

Sera from patients with coeliac disease also frequently contained high titres of IgA and IgG anti-
Fig. 2  Circulating antibody to α-lactalbumin

Fig. 3  Circulating antibody titres to β-lactoglobulin
bodies to each of the three proteins. In the other
diagnostic groups, low titres were present in approxi-
mately 50% of subjects but there were no striking
differences between them. Patients with ulcerative
colitis did not differ from the control groups either
in the frequency with which antibodies occurred or
in the height of the titres demonstrated. Low titres
of both IgA and IgG antibodies were especially
frequent in patients with hypolactasia. Patients with
Crohn's disease tended to have somewhat higher
titres to α-lactalbumin and β-lactoglobulin than the
control subjects.

The antibody titres obtained in sera from patients
with ulcerative colitis or Crohn's disease were
analysed in relation to the clinical features of the two
diseases. No correlation was found between the anti-
body titre and the length of history, severity or
extent of the disease, or corticosteroid therapy.

The height of the titres of direct or indirect IgA
or IgG antibodies to the individual milk proteins
was also analysed in relation to the results obtained
with intradermal testing (Jewell and Truelove, 1972).

The other correlation found was that patients with
positive skin tests to casein were more likely to have
direct agglutinating antibodies in their sera.

In each of the diagnostic categories, the frequency
of antibodies of IgA immunoglobulin class was
completely similar to that of IgG antibodies. This applied
to each of the three milk proteins.

Three patients with irritable colon syndrome had
high IgG antibody titres (1 in 512) to casein. One
patient had relief of symptoms with a milk-free diet
and relapsed when challenged with either pure
casein or whole milk. The second had no improve-
ment with a milk-free diet and the third, similarly,
had no improvement in bowel symptoms but found
that aphthous ulcers rapidly healed. The aphthous
ulceration recurred when milk was re-introduced into
her diet but healed again on withdrawing milk. All
three patients had strongly positive skin tests to
casein.

Discussion

No difference has been found between the sera from
patients with ulcerative colitis and sera from healthy
control subjects with respect to antibody titres to the
three milk proteins. This applies to the direct
agglutinating antibodies as well as to the incomplete
IgA and IgG antibodies. This confirms the negative
results of some earlier reports (Sewell, Cooke, Cox,
and Meynell, 1963; Dudek, Spiro, and Thayer,
1965) but is in contrast to the raised titres found
in ulcerative colitis by Taylor and Truelove (1961)
and Taylor et al (1964). When the titres found in
sera from patients with ulcerative colitis or Crohn's
disease in the present study were analysed in relation
to length of history, extent and severity of disease,
and corticosteroid therapy, no correlation was found.

The discrepancy between the results of Taylor and
his colleagues and the present results may be due to
differences in method. The earlier studies employed
tanned-red-cell haemagglutination as opposed to the
red-cell-linked antigen-antiglobulin reaction. No
attempt was made to compare the two methods, but
Steele and Coombs (1964) have found that there is a
poor correlation between the two haemagglutination
tests. The linked antigen test often gave significant
titres when the tanned cell test failed to demonstrate
antibody. This is probably due to the fact that
incomplete antibodies are hardly detected, if at all,
by the tanned cell test. Similarly the tanned cell test
often gave high titres when the direct agglutination
titre of the linked antigen test was lower. Three
possible explanations were advanced, namely,
(1) the tanned cell may be coated with more antigen
per unit surface area; (2) the tanned cell is less stable
as it is very 'sticky' and therefore very sensitive; (3) in
the linked antigen test, the antigen may be slightly
denatured by photo-oxidization.

Another possible explanation for the discrepant
results depends on the frequency of liver disease in
the patients studied. Triger, Alp, and Wright (1972)
have shown that patients with liver disease have
higher antibody titres to dietary allergens than
patients without liver disease. The proportion of
patients with ulcerative colitis or Crohn's disease
who had liver disease in the present study was very
small and it is therefore possible that the results of
Taylor and his colleagues may have been biased by a
higher proportion of patients with liver disease
included in their series.

The frequency with which circulating antibodies
are found in serum probably reflects the absorption
of whole protein molecules from the intestine. As
far as milk proteins are concerned the absorption of
minute amounts of unaltered protein has been shown
to occur in children (Ratner and Gruehl, 1934;
Lippard, Schloss, and Johnson, 1936). It has also
been shown that antibody titres to cow's milk
proteins are higher in infants than in adults, es-
specially in infants who have received cow's milk
within the first week or two of life (Gunther et al,
1960; Gunther, Cheek, Matthews, and Coombs,
1962). Another indication that healthy individuals
are commonly sensitized to the milk proteins is the
high frequency of positive skin reactions to these
proteins observed in normal subjects (Jewell and
Truelove, 1972), although it is presumed that ana-
phylaxis is prevented by other mechanisms such as
the presence of blocking antibody.

When the small-intestinal mucosa is damaged, as
Circulating antibodies to cow’s milk proteins in ulcerative colitis

in coeliac disease, it is possible that absorption of protein molecules may be facilitated. This might provide an explanation for the finding of high titres of antibodies to the milk proteins in patients with coeliac disease both in this study and in other reported series (Sewell et al, 1963; Taylor et al, 1964; Kenrick and Walker-Smith, 1970; Ferguson and Carswell, 1972). As coeliac patients have antibodies to a variety of dietary antigens, it seems likely that these occur secondary to mucosal damage with increased absorption and are not of prime aetiological significance.

There are at least two possible reasons why the absorption of whole protein molecules may be increased in Crohn’s disease and ulcerative colitis: (1) minor villous abnormalities of the jejunal mucosa are common in both these diseases (Salem, Truelove, and Richards, 1964); (2) an appreciable proportion of digested dietary protein is undigested by the terminal ileum is reached (Borgström, Dahlqvist, Lundh, and Sjövall, 1957) and it is therefore liable to come into contact with the inflamed mucosa.

There is, therefore, little evidence to support the hypothesis that an allergy to milk proteins is an aetiological factor in ulcerative colitis. No evidence of a reaginic response to milk has been obtained (Jewell and Truelove, 1972) and the present results show that circulating IgA, IgG, or direct agglutinating antibodies are found no more frequently in patients with ulcerative colitis than in control subjects. Using a delayed skin reaction as an indicator of a cellular immune response, no evidence has been found for a cell-mediated hypersensitivity to the milk proteins (Jewell, 1972).

The clinical benefits of a milk-free diet in ulcerative colitis may depend on factors other than immunological ones. Cady, Rhodes, Littman, and Crane (1967) found an increased frequency of hypolactasia in ulcerative colitis but a detailed study by Pena and Truelove (1972) showed that only patients with a severe attack of the disease had a significantly higher frequency of hypolactasia when compared with controls. In the majority of patients, hypolactasia persisted following treatment of the attack and it was postulated that patients with ulcerative colitis who also have an isolated lactase deficiency are more prone than the other patients with the disease to suffer from attacks and for these attacks to become severe.

References

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