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Cell-mediated immunity to gluten fraction III in adult coeliac disease

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SUMMARY

Peripheral blood lymphocytes were obtained from twenty-seven healthy control subjects, twenty-one coeliac patients on a gluten-free diet and fourteen patients on a normal diet. When the cells were cultured *in vitro* in the presence of 2 and 4 mg of gluten fraction III, there were significant increases in the mean ratios of response for lymphocytes from gluten-free coeliacs compared to healthy controls after 4, 5 and 6 days of culture, but for those on a normal diet significant increases were found only when using 4 mg of gluten on the 4th and 5th days of culture. When three further patients were changed from a normal to a gluten-free diet, the ratios of response for their lymphocytes increased. The results suggest that certain coeliacs may exhibit a weak delayed hypersensitivity reaction to gluten. Its more ready demonstration in patients on a gluten-free diet could be explained on the release of sensitized lymphocytes from the intestinal mucosa into the peripheral circulation after gluten withdrawal.

INTRODUCTION

The mechanism of the damaging action of gluten in adult coeliac disease is unknown. Whereas a specific enzyme deficiency in the jejunal mucosa has not so far been demonstrated (Douglas & Booth, 1970), there is evidence suggesting that gluten toxicity may have an immunological explanation, such as the occurrence of 'gliadin shock' (Krainick *et al.*, 1958), the clinical (Aldersberg, Colcher & Drachman, 1951; Cooke, 1953) and histological response to steroids (Wall *et al.*, 1970), splenic atrophy (McCarthy *et al.*, 1966) and the infiltration of the jejunal mucosa with lymphocytes and plasma cells (Ferguson & Murray, 1971; Holmes *et al.*, 1974).

More direct evidence has come from studies of both humoral and cellular immunity in coeliac disease. For example, changes in the levels of serum and intestinal juice immunoglobulins and abnormal densities of immunoglobulin-containing cells in the jejunal mucosa occur (Hobbs & Hepner, 1968; Asquith, Thompson & Cooke, 1969, 1970; Douglas, Crabbe & Hobbs, 1970). Patients often show serum and intestinal juice antibodies to wheat and gluten extracts (Heiner *et al.*, 1962; Katz, Kantor & Herskovic, 1968), but in addition have antibodies to foodstuffs to which they are not sensitive clinically (Kenrick & Walker-Smith, 1970; Ferguson & Carswell, 1972). Serum antibodies to reticulin (Seah *et al.*, 1971) and basement membrane (Amman & Hong, 1971) are more likely to be found in patients on a normal diet than in those on a gluten-free diet and they also appear to correlate with antibodies to gluten fraction III (Alp & Wright, 1971) and cross-react with this material (Seah *et al.*, 1972).

In spite of disturbances in humoral immunity, it is still not clear whether such changes are any more than secondary manifestations of the disorder. With respect to changes in cellular immunity in coeliac disease of possible relevance to gluten toxicity, some coeliacs show evidence of a cell-mediated response to gluten fraction III, (Housley, Asquith & Cooke, 1969; Asquith, Housley & Cooke, 1970), although other authors have not confirmed this observation (Morganroth, Watson & French, 1972).

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Cell-mediated responses to a potential antigen such as gluten can be examined *in vitro* by measuring the degree of stimulation of lymphocytes cultured in the presence of the antigen (Oppenheim, 1968). Using this technique, we have re-investigated the *in vitro* responses to gluten fraction III of peripheral blood lymphocytes from healthy individuals and from patients with coeliac disease.

PATIENTS AND METHODS

Patients. Thirty-five patients with coeliac disease were studied, the diagnosis having been confirmed by a jejunal biopsy in each case. Twenty-one of the patients had been on a gluten-free diet (GFD) for at least 3 months, while fourteen were taking a normal diet (ND) at the time of the study. Three additional patients were studied on a normal diet and then re-studied between 3 and 11 months after gluten withdrawal. Twenty-seven healthy individuals formed the control group.

Methods. 50 ml of peripheral venous blood was defibrinated and the red cells sedimented in a freshly made 3% solution of gelatin in normal saline for approximately 1 hr at 37°C (one volume of defibrinated blood to two volumes of gelatin). The buffy coat and supernatant plasma were aspirated and the leucocytes concentrated by centrifugation at 750 rev/min for 5 min. The cell button was gently agitated and washed once with 5 ml of Eagle's medium. Following re-suspension in a similar volume of the medium, a drop was removed and the lymphocytes counted. The remaining leucocyte suspension was then added to a further quantity of Eagle's medium supplemented with a 20% serum gelatin mixture so that the final lymphocyte concentration was 1×10^6 per ml. The serum in the serum gelatin mixture was from a single pool obtained from the control group. Duplicate 1-ml cultures were set up containing either 0·1 ml phosphate-buffered saline (PBS) which acted as the control culture, or 0·1 ml PBS containing 2 or 4 mg of gluten fraction III sterilized by filtration. Gluten fraction III was prepared by the method of Frazer *et al.* (1959) using Australian gluten (Scientific Food Ltd). No further purification of the material was undertaken.

Preliminary experiments were performed using lymphocytes from four healthy controls and nine coeliac patients. Cultures were set up with increasing amounts of gluten fraction III, i.e. 7, 15, 30, 62·5, 125, 250, 500 and 750 μg and 1, 2, 4, 6 and 8 mg of the material. Since a maximal response was obtained with 2 and 4 mg, these amounts were used throughout the remainder of the study; quantities in excess of 4 mg were found to be toxic to lymphocytes.

Cultures were incubated at 37°C for 4, 5 and 6 days in an atmosphere of 5% CO_2 in air. Twenty-four hours before the end of the culture period 0·5 μCi of tritiated thymidine of specific activity 150 mCi/mM (Radiochemical Centre, Amersham) was added. The cultures were subsequently harvested by the method of Ling & Holt (1967) except that the precipitate was finally dissolved in 0·1 ml N NaOH. The amount of thymidine incorporation (which is an index of DNA synthesis) was measured by counting the radioactivity of the harvested cultures in a liquid scintillation counter and the results expressed in disintegrations per minute (d/min). The degree of stimulation of lymphocytes by gluten fraction III was calculated from the mean d/min in the two gluten-containing cultures and the mean d/min in the two control cultures. The results are expressed as a net proliferative response or as a ratio of response calculated as follows:

$$\text{Net proliferative response} = \text{d/min in treated culture} - \text{d/min in untreated culture.}$$

$$\text{Ratio of response} = \frac{\text{d/min in treated culture}}{\text{d/min in untreated culture}}.$$

RESULTS

Unstimulated cultures

The mean proliferative responses expressed as disintegrations per minute (d/min) for the groups studied are shown in Table 1. Cells obtained from coeliac patients taking either a normal or a gluten-free

TABLE 1. Mean d/min values for control and coeliac lymphocytes grown in unstimulated cultures for 4, 5 and 6 days

	Mean d/min		
Group studied	Day 4	Day 5	Day 6
Controls	3171·6	4540·5	4726·2
Coeliacs (ND)	1841·8	1247·0	1360·8
Coeliacs (GFD)	1326·7	1092·3	1808·0
Total coeliacs	1212·7	1154·2	1623·9

TABLE 2. *t*-Test analysis of the results shown in Table 1

Group studied	Day 4	Day 5	Day 6
Controls *v* coeliacs (ND)	< 0·05	< 0·05	< 0·01
Controls *v* coeliacs (GFD)	< 0·01	< 0·01	< 0·01
Controls *v* total coeliacs	< 0·001	< 0·001	< 0·001
Coeliacs (ND) *v* coeliacs (GFD)	n.s.	n.s.	n.s.

TABLE 3. Mean d/min values for control and coeliac lymphocytes grown for 4, 5 and 6 days in cultures containing either 2 mg or 4 mg of gluten fraction III

	2 mg			4 mg		
Group studied	Day 4	Day 5	Day 6	Day 4	Day 5	Day 6
Controls	−14·3	1140·5	1495·9	−124·0	555·5	969·1
Coeliacs (ND)	519·2	631·9	529·2	372·9	689·0	1003·8
Coeliacs (GFD)	741·0	2164·1	2356·3	823·8	2201·9	2522·5
Total coeliacs	652·3	1551·2	1604·0	649·3	1616·3	1915·0

diet incorporated thymidine significantly less well than cells from controls on all harvest days. However, when the two groups of coeliac patients were compared no differences emerged (Table 2).

Stimulated cultures

Net proliferative responses. The mean net proliferative response obtained when control and coeliac lymphocytes were cultured in the presence of 2 mg or 4 mg of gluten fraction III are shown in Table 3. There were no statistically significant differences between the controls and the patients or between the two groups of patients when they were compared on any of the days of harvest.

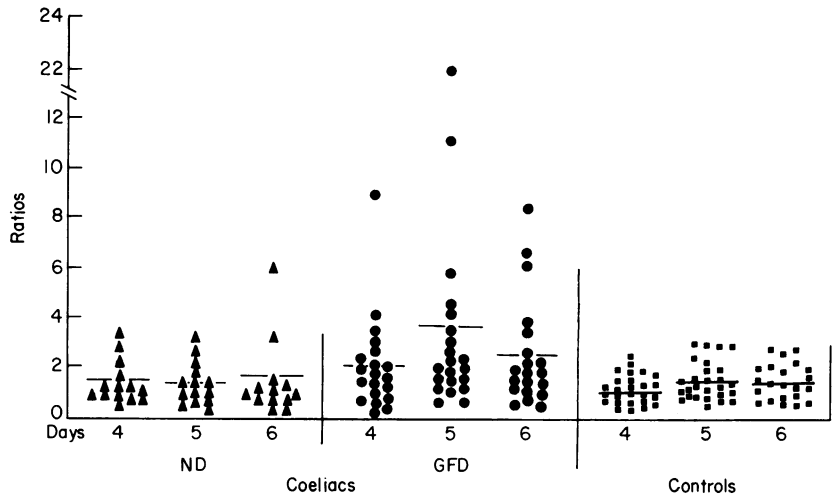

FIG. 1. The ratios of response of lymphocytes obtained from normal diet coeliacs (ND) gluten-free coeliacs (GFD) and healthy controls cultured for 4, 5 and 6 days in the presence of 2 mg of gluten fraction III. The mean ratios of response are shown for each group of individuals.

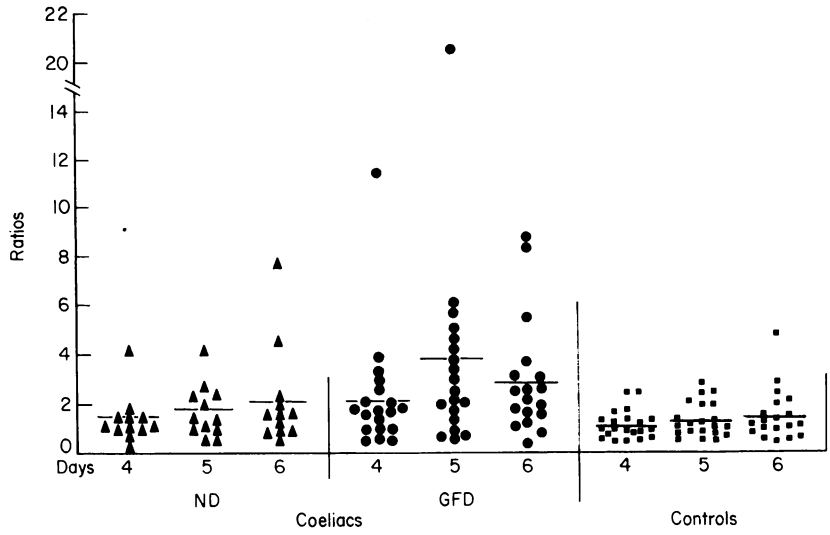

FIG. 2. Legend as for Fig. 1, but using 4 mg of gluten fraction III.

Ratios of response. The means ± 2 s.d. range of the ratios for the control group, the gluten-free group and the normal diet group were calculated and these groups were compared statistically using Student's '*t*'-test.

Figs 1 and 2 show the ratios of response of lymphocytes obtained from coeliacs on a normal diet, those on a gluten-free diet and from healthy controls, cultured for 4, 5 or 6 days in the presence of 2 mg and 4 mg of gluten fraction III respectively. There was no significant increase in the mean response of lymphocytes from healthy controls when the culture period was extended from 4 to 6 days. The mean response of gluten-free coeliacs was significantly greater than for healthy controls on each of the three days of harvest ($P<0{\cdot}05$) for both 2 mg and 4 mg of gluten. On the other hand, when the mean response of coeliacs on a normal diet was compared with that of the controls, there was no significant difference for 2 mg, though with 4 mg of gluten the difference became significant ($P<0{\cdot}05$) on the 4th and 5th day of harvest.

The percentage of patients on a gluten-free diet and on a normal diet with ratios greater than 2 s.d. of the mean for the control group are shown in Table 4. For each day of harvest a greater percentage of gluten-free coeliacs had increased ratios compared with those taking a normal diet for both 2 mg and

TABLE 4. Number and percentage of coeliac patients with ratios of response for their lymphocytes > 2 s.d. of the mean of the control group, after 4, 5 or 6 days in cultures containing 2 mg or 4 mg of gluten fraction III (the numbers studied in each group are shown in parentheses). The number of patients with at least one abnormal result irrespective of day of harvest or amount of gluten fraction III used is also set out

		> 2 s.d. of mean of control group				No. of patients with at least one abnormal result on days 4, 5 or 6 for 2 mg or 4 mg of gluten fraction III	
		2 mg gluten fraction III		4 mg gluten fraction III			
Group studied	Day of harvest	No.	Percentage	No.	Percentage		
Coeliacs (ND)	4	3 (14)	21	2 (12)	17	4 (14)	29%
	5	1 (14)	7	2 (12)	17		
	6	2 (14)	14	2 (12)	17		
Coeliacs (GFD)	4	6 (20)	30	7 (19)	37	11 (21)	52%
	5	7 (20)	35	9 (19)	47		
	6	5 (19)	26	6 (18)	33		

TABLE 5. Effect of gluten withdrawal on *in vitro* response of lymphocytes from three patients with adult coeliac disease

	2 mg*			4 mg		
	Day 4†	Day 5	Day 6	Day 4	Day 5	Day 6
Patient 1						
Normal diet	0·7	1·0	0·5	0·3	0·5	0·5
Gluten-free diet (11 months)	2·2	4·1	2·8	2·2	3·3	2·4
Patient 2						
Normal diet	0·7	2·0	1·4	—	—	—
Gluten-free diet (9 months)	1·7	3·8	3·7	5·6	3·7	4·2
Gluten-free diet (11 months)	—‡	5·0	—	—	4·3	—
Patient 3						
Normal diet	1·2	0·9	—	0·8	0·6	—
Gluten-free diet (3 months)	1·7	1·7	1·8	1·5	1·8	2·2

* Amount of gluten fraction III used.
† Day of harvest.
‡ Not studied.

4 mg of gluten fraction III. In addition the percentage of patients having a gluten-free diet with at least one abnormal result on day 4, 5 or 6 irrespective of the amount of gluten fraction III used in the cultures was 52% (11/21) compared with 29% (4/14) for normal diet subjects. Table 5 shows the results in the three patients studied individually both before and following gluten withdrawal. The available results show an increased ratio of response on the 4th, 5th and 6th day of harvest using both 2 mg and 4 mg of gluten during the gluten-free period.

DISCUSSION

When lymphocytes from coeliac patients and normal controls are exposed to gluten fraction III there are difficulties in comparing the results for there are marked differences in reactivity when these cells are grown in untreated cultures. The decreased thymidine incorporation by coeliac lymphocytes in untreated culture as compared with normal lymphocytes could be due either to a reduced number of dividing lymphocytes in these cultures or to a generalized depression of lymphocyte reactivity. Hence, when gluten fraction III is added to the cultures it could be argued that lymphocytes from coeliac patients make a greater response than do normal cells since they begin at a lower base line. The results have therefore also been assessed in terms of ratios of response to illustrate and emphasize this effect, which is not evident if only net proliferative responses are considered.

Housley *et al.* (1969), using peripheral blood and mesenteric node lymphocytes of coeliac patients, found that *in vitro* responses to gluten fraction III tended to occur with only the node lymphocytes. However, further experiments on peripheral blood and peripheral node lymphocytes (Asquith *et al.*, 1970; Asquith, 1970) showed that in some patients the response could be more generalized. This present independent study confirmed the response in peripheral blood lymphocytes, and in addition showed that 52% of the coeliacs on a gluten-free diet and 29% of coeliacs on a normal diet had ratios of response significantly different from the control group. These differences seem independent of serum factors for the lymphocytes were washed free of autologous serum and the same normal serum–gelatin pool was used throughout the study. This suggests that there is an abnormality in cell-mediated immunity in coeliac disease. A possible explanation is that certain patients possess a subpopulation of lymphocytes sensitized to gluten or a subfraction of the material.

It seems somewhat anomalous that peripheral blood lymphocytes from patients on a gluten-free diet should show greater reactivity to gluten than such cells from normal diet subjects. This phenomenon

could be explained by postulating that a change in the distribution of sensitized cells occurs in coeliac patients depending upon the type of diet ingested, whether gluten-containing or gluten-free. Thus intraluminal gluten in those patients on a normal diet might provide an antigenic stimulus to the mucosa of the small intestine with the consequent accumulation of sensitized lymphocytes in the epithelial cell layer and lamina propria. In such circumstances the peripheral blood would contain smaller numbers of sensitized cells and hence *in vitro* lymphocyte stimulation by gluten fraction III might be difficult to demonstrate. In contrast, when gluten is removed from the diet, the antigenic stimulus is absent and lymphocytes capable of reacting to gluten by increased DNA synthesis are released into the peripheral circulation. Such a hypothesis is in keeping with the observation that more patients on a gluten-free diet exhibit lymphocyte stimulation than those on a normal diet. Furthermore, the increased ratios seen in the three coeliac patients following gluten withdrawal adds further evidence that the difference between the two groups is real and could well result from the redistribution of sensitized lymphocytes.

The amount of gluten fraction III (2 mg and 4 mg) employed in these experiments was much greater than that used by Morganroth *et al.* (1972) who used approximately 30 μg per 10^6 lymphocytes and this difference in technique may account for the different results. The large quantities needed to produce stimulation in the present experiments may also be significant, suggesting that the amount of the actual mitogenic component of gluten fraction III is small. This would be in keeping with the minute amounts of antigen required for other specific stimuli, e.g. 10 μg of tuberculin necessary to produce lymphocyte transformation.

A question which arises is whether cells sensitized to gluten play any part in the pathogenesis of the flat mucosa in coeliac disease. If such cells do accumulate in the mucosa of patients taking a normal diet as suggested above, then it is possible that damage may occur at this site as these cells interact with gluten. Ferguson (1974) has provided evidence from animal studies which suggests that cell-mediated immune reactions may damage the small intestine and produce histological appearances of the mucosa somewhat similar to those seen in coeliac disease. However, if cell-mediated mechanisms are important in producing mucosal damage in coeliac disease, it would still need to be explained why sensitized lymphocytes occur in the first instance. The answer to this question might be more relevant to the aetiology of coeliac disease

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