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Activation of the alternative pathway by gluten

A POSSIBLE AETIOLOGICAL FACTOR IN DERMATITIS HERPETIFORMIS

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Summary. Gluten fractions are shown to activate the alternative pathway of complement when added to normal human serum. Breakdown of C3 and Factor B occur in a manner analogous to that when activated by zymosan, in the presence of MgEGTA and in serum devoid of classical pathway activity. The suggestion is made that bypass activation may be the primary event when gluten enters the serum across a damaged gut mucosa. Immune complexes containing non-complement fixing IgA antigluten antibody are carried to the skin where it is proposed that complexed gluten activates C3 and initiates an inflammatory reaction.

INTRODUCTION

Coeliac disease (CD) and dermatitis herpetiformis (DH) have long been linked as diseases arising from gluten sensitivity. There is much discussion as to whether an immunological or toxic mechanism is causative. It has recently been reported that abnormalities of the jejunal mucosa were seen at microscopic level in biopsies of thirty-seven out of thirty-eight patients with DH defined by strict criteria (Seah, Fry, Kearney, Campbell, Mowbray, Stewart & Hoffbrand, 1976). In jejunal fluid, levels of IgA are high (Lancaster-Smith, Kumar, Marks, Clark & Dawson, 1974) and levels of IgG- and IgM-producing cells are increased in the gut mucosa of CD and DH patients (Mowbray, Hoffbrand, Holborow, Seah & Fry, 1973). All levels fall on withdrawal of gluten from the diet. In the serum, IgG antibody to reticulin is reported in 17% DH and 45% CD patients, and within this group 64% have circulating IgA antibody as well (Lancaster-Smith, Kumar, Clark, Marks & Johnson, 1975). Absorption of two such sera from DH patients with gluten fraction III (Frazer, Fletcher, Ross, Shaw, Sammons & Schneider, 1959) completely removed the antireticulin antibody, and extensive crossreaction between gluten and reticulin antibodies was inferred (Seah, Fry, Stewart, Chapman, Hoffbrand & Holborow, 1972). Circulating immune complexes were found in the sera of all of a group of fifty-nine DH patients, and half this group have low C3 (Mohammed, Holborow, Fry, Thompson, Hoffbrand & Stewart, 1976).

A diagnostic feature of DH is the deposition of IgA on reticulin of the dermal papillae of affected and unaffected skin (Seah *et al.*, 1972). Further investigation of thirty-four patients with DH showed C3 deposition in sixteen, IgM in nine, IgG in three, and Clq in three associated with either IgG or IgM (Seah, Fry, Mazaheri, Mowbray, Hoffbrand &

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Holborow, 1973). The presence of C3 but not C1q may indicate activation of the alternative pathway as causal of the skin lesions.

Here we provide experimental evidence that direct activation of the alternative pathway by gluten in the serum may account for the pathogenesis of the lesions of DH and CD subsequent to gluten-induced damage to the jejunum.

MATERIALS AND METHODS

C3, Factor B and their respective breakdown products were measured in a two-way, antigen-antibody crossed immunoelectrophoresis adapted from the method of Laurell (1973). Antisera were raised in rabbits to human C3 and Factor B, and the appearance of the respective fragments C3b (β) and Bb (γ) was shown by alternative pathway activation by zymosan in normal human serum. The gels (200 × 110 mm) were of 1% agarose in 0·025 M EDTA pH 8.6. Antibody layers contained 1·2% αC3 or 10% αBf antiserum. Electrophoresis was carried out in 0·05 M EDTA pH 8·6 at 70 mA for 70 min in the long direction and 50 V overnight in the second, shorter direction. A cooling plate (Shandon) was used for both directions.

Wheat gluten (Fraction III, soluble) was prepared by peptic/tryptic digest (Frazer *et al.*, 1959) by Dr P. P. Seah and used as a solution of 50 mg/ml in barbitone-buffered saline. It was boiled to remove proteolytic activity and autoclaved to destroy endotoxin. A preparation of insoluble gluten was used at 50 mg/ml in barbitone-buffered saline. It was boiled, and autoclaved after sonication. Buffers were made to pH 7·6 and autoclaved.

Incubations were set up in a final volume of 50 μl, containing 40 μl serum with 5 μl gluten or zymosan solution, and 2 μl 0·1 M EDTA or 0·1 M MgEGTA where appropriate. Final concentrations in the incubations: gluten or zymosan, 5 mg/ml, EDTA or MgEGTA, 4 mM. Volumes were made up in barbitone-buffered saline, and incubated at 37° for 30 min. One electrophoresis plate comprises six wells, and this represents a single experiment.

Conversion was calculated on the assumption that the peak heights are proportional to concentration, and C3b levels expressed as per cent of total C3 + C3b.

Normal human serum was obtained from laboratory volunteers. Serum from a patient totally deficient in C2 by immunochemical and functional criteria was used as a source lacking classical pathway activity. This serum, however, has only a 50% level of Factor B. A further serum used in this study is from a subject with normal classical pathway activity but with Factor B levels 50% of normal.

RESULTS

C3 Conversion in normal serum

By the incubation procedure described, there was usually a low level of 'spontaneous' conversion. This background level was higher in sera with normal than in those with reduced levels of Factor B activity. The background was abolished by the presence of EDTA in the incubation mixture. It will be seen (Table 1, Fig. 1) that gluten will initiate conversion of C3 in the presence of Mg^{2+} ions but not in EDTA. The conversion does not require Ca^{2+} ions. Conversion produced by gluten is always quantitatively less than that produced by zymosan (Table 1, Fig. 2). The values represent the maximum amount of C3 conversion attained in the system. No further increase in C3b generation was seen when higher concentrations of zymosan or gluten were employed. In a single experiment, the conversion by gluten was always enhanced in the presence of MgEGTA (4 mM) but for the group of experiments tabulated here the difference is not significant.

Table 1. C3 conversion in normal human serum on incubation at 37° with gluten fractions or zymosan. For relevant concentrations see the Materials and Methods section

	Per cent of conversion of C3	
	Single expt	Mean of five expts
Serum control	15·6 (Fig. 1)	16·5±9·8
+Gluten (soluble)	45·6 (Fig. 1)	43·2±4·5
+Gluten (soluble)+EDTA	0 (Fig. 1)	0
+Gluten (soluble)+MgEGTA	47·5 (Fig. 1)	45·2±4·7
+Zymosan+MgEGTA	56·9 (Fig. 2)	60·3±3·8
+Gluten (insoluble)+MgEGTA	35·6 (Fig. 2)	42·2±4·8

C3 Conversion in two sera with half normal factor B levels

Spontaneous conversion is low or absent in these

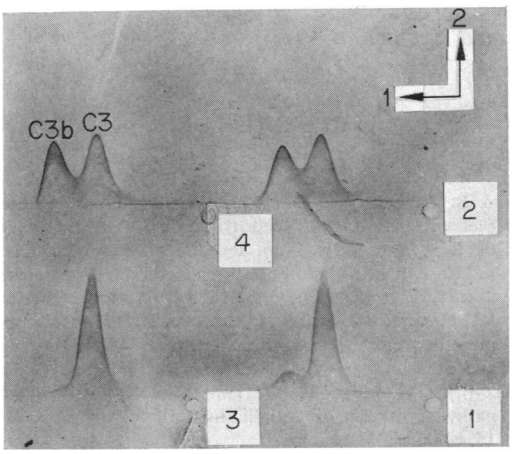

Figure 1. Two-way electrophoresis of C3 and C3b peaks in 1% agarose gel containing C3 antiserum. Well 1: normal human serum; slight spontaneous conversion. Well 2: serum incubated with gluten (5 mg/ml); almost half the C3 is converted. Well 3: serum incubated with gluten (5 mg/ml) and EDTA (4 mM); no conversion in the absence of Mg^{2+}. Well 4: serum incubated with gluten (5 mg/ml) and MgEGTA (4 mM).

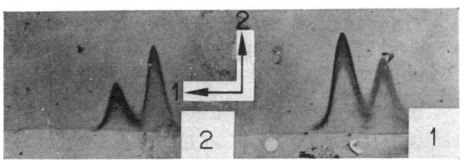

Figure 2. A comparison of the conversion of C3 to C3b by two activators of the alternate pathway. Well 1: serum+ zymosan (5 mg/ml). Well 2: serum+gluten (insoluble) (5 mg/ml). Both incubations contained MgEGTA (4 mM).

Table 2. C3 conversion in sera with half normal Factor B levels

	Per cent conversion of C3	
	C2 deficient	C2 normal
Serum control	0	0
+Gluten+MgEGTA	28	25·5
+Zymosan+MgEGTA	44	36

sera. The maximum amount of conversion seen is also reduced (Table 2).

Factor B breakdown in normal serum

The breakdown value is expressed as the per cent total of Factor B appearing as the fragment with γ mobility. The spontaneous conversion was 6%. When the serum was activated, the conversion of Factor B was 56% and 33% for zymosan and gluten respectively.

C3 Conversion in the presence of Mg^{2+} ions

Serum was incubated with MgEGTA or $MgCl_2$ through the concentration range used in the above experiments. Some conversion was seen over and above the spontaneous level, although the maximum level reached was low. The presence of Mg^{2+} ions may enhance the activation by gluten. Results from a single experiment are given in Table 3.

Table 3. C3 conversion in the presence of added Mg^{2+} ions

	Per cent conversion of C3
Serum control	8·3
+MgEGTA, 4 mM	18·5
+MgEGTA, 8 mM	10·5
+$MgCl_2$, 2 mM	14·7
+$MgCl_2$, 4 mM	13·5
+$MgCl_2$, 8 mM	15·6

DISCUSSION

The ability of gluten to activate the alternative pathway as demonstrated here may provide a new understanding of the pathogenetic mechanism of gluten sensitive diseases.

The characteristic deposition of both IgA and C3 in the affected areas of skin in DH may be explained by suggesting that gluten is complexed with non-complement fixing IgA antibody and that C3 is activated by the antigen and not the immunoglobulin of the complex. Unaffected skin in disease or after gluten withdrawal may show IgA antibody on reticulin, not in a granular pattern. The evidence that antigluten antibody crossreacts with reticulin is consistent with these findings. Lesions are only produced when the complexes are granular, which occurs when they are associated with C3. An inflammation mediated by cleavage of C3 caused by the gluten carried as an IgA complex to the site would seem to be the simplest explanation for the pathogenesis of DH. It would explain why the antibody alone is not associated with skin lesions,

and the requirement for exposure to gluten to manifest disease. The low C3 levels found in half of a group of DH patients (Mohammed *et al.*, 1976) may be explained as consumption by alternative pathway activation by gluten. Low C4 levels reported in 20% of the DH group in the same complement study may be due to classical pathway activation by complement fixing antigluten antibodies, which have been shown to be present in sera from some patients with active DH.

The evidence presented here suggests that gluten acts initially via the alternative pathway. Our evidence, taken in conjunction with recently ascribed 'lectin-like' properties of gluten (Weiser & Douglas, 1976), points to its consideration as a potential B-cell triggering agent, as characterized by Dukor, Schumann, Gisler, Dierich, König, Hadding & Bitter-Suerman (1974b). Their postulate is that cleavage of C3 is a prerequisite to antibody production, and that B-cell mitogens (T cell-independent antigens, of carbohydrate structure) are all activators of the alternative pathway, although the converse is not necessarily true. There is, however, evidence that gluten will not cause proliferation of populations of lymphocytes from patients with the enteropathy (Dukor, Dietrich, Gisler, Schumann & Bitter-Suerman, 1974a). B cells bear surface immunoglobulin specific for antigen, and if gluten has the ability to bind as antigen with concomitant generation of C3b, then production of anti-gluten/anti-reticulin antibody is predicted from this model.

Formation of immune complexes would be a subsequent step, but one that allows localization of the gluten in a manner that causes tissue damage. With regard to CD, studies of patients on gluten-free diets allow investigation of events in intact mucosal tissue. In treated CD without clinical DH, gluten challenge raises the concentration of IgA and IgM antibodies specific for gliadin in jejunal fluid (Falchuk & Strober, 1974) and IgA complexes appear in the gut basement membrane (Shiner & Ballard, 1972). The alternative pathway can then be activated by gluten reacting locally with complement in the mucosa.

For both DH and CD, the first requirement must be the entry of the gluten into the mucosal region, and direct toxicity of gliadin fractions in the presence of serum has been reported (Hudson, Connell, Purdham & Rolles, 1976). This may account for local damage to the integrity of the mucosa, but other factors must also be responsible for disease in order to explain the strong HLA association observed (Seah *et al.*, 1976).

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