Immune Response to Gluten in Adult Coeliac Disease

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Summary: Cultures of mesenteric node lymphocytes obtained from two adult coeliac disease patients were stimulated by gluten fraction III. No stimulation was observed in cultures of axillary node lymphocytes from one of these patients, of mesenteric node lymphocytes from the two patients with other diseases or of peripheral blood lymphocytes from adult coeliacs and normal subjects. Peripheral blood lymphocytes of two of the six adult coeliac patients responded poorly to phytohaemagglutin alone, but this was probably owing to technical factors. In a further six adult coeliacs skin tests to gluten fraction III were negative. It is suggested that delayed hypersensitivity to gluten is likely to have a secondary pathogenic role in adult coeliac disease.

Introduction

Since the initial demonstration that patients with coeliac disease improve after gluten withdrawal (Dicke, Weijers, and Van De Kamer, 1953), attempts have been made to determine the mechanism of the toxic effect. Impaired enzymatic digestion of gluten peptides has been described (Pittman and Pollitt, 1966), but appears to be secondary to the mucosal abnormality (Douglas and Booth, 1968). The finding of serum antibodies to gluten fractions has prompted the suggestion that the disease is the result of an abnormal immune response to gluten, but it seems unlikely that the reaction of these antibodies with ingested gluten produces the gut lesion (Taylor, 1965).

On the other hand, a pathogenic immune response to gluten could be cell-mediated—that is, of a delayed hypersensitivity type. Skin tests with gluten have been negative (Alvey, Anderson, and Freeman, 1957), but the disease might still be explained by a delayed hypersensitivity response that was either localized to the intestine or generalized but too weak to be elicited except by prolonged exposure to antigen (as would occur in the gut).

Delayed hypersensitivity may also be detected by stimulation (blast transformation and increased deoxyribonucleic acid (D.N.A.) synthesis) of lymphocytes cultured in vitro in the presence of antigen (see review by Oppenheim, 1968). We have therefore investigated the reaction of peripheral blood and lymph node lymphocytes of patients with adult coeliac disease to gluten fraction III—a soluble partial digest of gluten which retains the toxic properties (Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider, 1959). In addition, the reaction of lymphocytes to phytohaemagglutinin was measured in view of the suggestion that the peripheral blood response may be depressed in adult coeliac disease (Winter, McCarthy, Read, and Yoffey, 1967).

Materials and Methods

Patients and Normal Subjects for In-vitro Lymphocyte Studies.—Six patients with fully documented adult coeliac disease were investigated; five were on a gluten-free diet with definite improvement and the sixth (Case 6) took a normal diet. Controls were six normal subjects, one patient with gastric carcinoma, and one with duodenal ulcer.

Patients for Skin Tests.—Skin tests were carried out in six further adult coeliac patients, all of whom were in remission on a gluten-free diet.

Gluten fraction III (G.F. III) (kindly provided by Dr. R. F. Schneider) was dissolved in phosphate-buffered saline and sterilized by filtration. The protein content was measured by the AutoAnalyzer N14a method.

Phytohaemagglutinin (P.H.A.) was purchased from Burroughs Wellcome. The same batch was used throughout, the contents of each ampoule being dissolved in 5 ml. of phosphate-buffered saline; 0·033 ml. was added per culture, this quantity having previously been established as about five times the minimum amount necessary to produce maximum lymphocyte stimulation, but not sufficient to be toxic.

Purified protein derivative (kindly provided by the Ministry of Agriculture, Fisheries and Food, Weybridge) was added, 10 μg. per culture.

Tritiated thymidine ($TdRH^3$) (Radiochemical Centre, Amersham) was diluted with cold thymidine to 150 mCi/mmole.

Peripheral blood lymphocytes were obtained from defibrinated blood by sedimentation with gelatin (Coulson and Chalmers, 1964), followed in some cases by filtration through cotton-wool (Cooper and Rubin, 1965). The cells were spun down and resuspended in Eagle's medium; 75% or more were lymphocytes.

Lymph Node Lymphocytes.—Histologically normal mesenteric nodes were obtained from one adult coeliac patient (during cholecystectomy) and from two control subjects (during laparotomies for gastric carcinoma and duodenal ulcer respectively). Mesenteric nodes obtained from a second adult coeliac patient during resection of a lymphoma of the small intestine proved to be infiltrated with malignant cells, but were nevertheless cultured; histologically normal axillary nodes from this patient were also cultured. Lymph nodes were teased out in Eagle's medium and the cells pipetted through a 60-mesh stainless steel sieve, spun down, and resuspended in medium. Ninety-eight per cent. or more of the cells from the normal lymph nodes were lymphocytes; 10% of the cells from the lymphomatous nodes were malignant. From 70 to 80% were viable as judged by eosin dye exclusion (Black and Berenbaum, 1964).

Lymphocyte Cultures.—The lymphocyte suspensions were diluted to $1{\cdot}5 \times 10^6$ cells/ml. with Eagle's medium supplemented by 25% of the serum-gelatin mixture left after preparation of peripheral blood lymphocytes. In view of the known variation in the phytohaemagglutinin response with individual sera (Ling and Holt, 1967) and the possible presence of antibodies to G.F. III in the adult coeliac sera (Taylor, Truelove, and Wright, 1964) serum-gelatin from a single normal individual was used routinely. In two adult coeliac patients (Cases 2 and 3) additional phytohaemagglutinin-stimulated cultures were set up with autologous serum-gelatin. Triplicate 2-ml. cultures (3×10^6 cells) were set up in 5-ml. screw-capped phials (Stayne Laboratories Ltd., High Wycombe) containing no stimulant, phytohaemagglutinin, purified protein derivative, or amounts of G.F. III varying from 125 μg. to 12 mg. The cultures were incubated at 37° C. for four days in the case of phytohaemagglutinin, and for five days with purified protein derivative and G.F. III.

Harvesting.—Sixteen hours before the end of the culture period 1 μCi of $TdRH^3$ was added. When subsequently harvested, two of each set of three cultures were processed for D.N.A. synthesis as described by Ling and Holt (1967), except that the precipitate was finally dissolved in N NaOH. The radioactivity was counted in a Packard Tricarb Scintillation Counter, corrected to 100% efficiency by the channel ratio method, and D.N.A. synthesis expressed as mean disintegrations per minute (d.p.m.) for the two cultures. A Jenner–Giemsa-stained smear was made from the third culture of each set.

Skin Tests.—G.F. III 100 μg. was injected intradermally and the result read at 48 hours. Preliminary tests in normal subjects had shown no significant reaction after this time interval.

Results

Skin tests were negative in all six adult coeliac patients.

Lymphocyte Response to Phytohaemagglutinin.—In the normal subjects D.N.A. synthesis varied from 163,000 to

482,000 d.p.m., and the percentage of blasts from 80 to 96%. Three of the adult coeliac patients were within this range, but one (Case 4) was slightly low (124,307 d.p.m.) and two others (Cases 5 and 6) were very low at 58,000 and 64,500 d.p.m. respectively. However, the initial cell suspensions from these two patients had unusually low proportions of lymphocytes (25% for Case 5 and 32% for Case 6). The peripheral blood cells in Cases 2 and 3 cultured in autologous serum-gelatin responded comparably to the cultures in normal serum-gelatin (Case 2: 235,972 d.p.m. in normal and 203,750 d.p.m. in autologous; Case 3: 149,000 d.p.m. in normal and 230,000 d.p.m. in autologous). The response of mesenteric node lymphocytes in Cases 1 and 2, and that of axillary node lymphocytes in Case 1 was similar to that of their peripheral blood. The results (except for the autologous serum cultures) are set out in Table I.

TABLE I.—*Response of Blood and Lymph Node Lymphocytes to Phytohaemagglutinin*

Case No.	Culture	Peripheral Blood		Subject	Culture	Peripheral Blood	
		D.N.A. Synthesis (d.p.m.)	% Blasts			D.N.A. Synthesis (d.p.m.)	% Blasts
	Adult Coeliac Patients				*Normal Subjects*		
1	0	370	0	A	0	2,733	0
	P.H.A.	170,200	94		P.H.A.	163,640	95
	0	1,200	0	B	0	1,348	0
	P.H.A.	235,972	88		P.H.A.	322,860	80
3	0	—	—	C	0	2,057	0
	P.H.A.	149,000	95		P.H.A.	271,853	94
4	0	610	0	D	0	465	0
	P.H.A.	124,307	—		P.H.A.	210,550	93
5	0	700	0	E	0	777	0
	P.H.A.	58,000	70		P.H.A.	321,338	80
6	0	262	0	F	0	475	0
	P.H.A.	64,500	69		P.H.A.	482,500	96

Case No.	Mesenteric Node		Axillary Node	
	D.N.A. Synthesis (d.p.m.)	% Blasts	D.N.A. Synthesis (d.p.m.)	% Blasts
1	1,095	5	477	0
	235,040	85	235,000	95
2	1,495	0	—	—
	233,150	90	—	—

Lymphocyte Response to G.F. III.—D.N.A. synthesis was somewhat greater in peripheral blood cultures containing G.F. III in Cases 4 and 5 and in the two normal subjects (Table II), but this was not significant because at the low levels observed (highest D.N.A. synthesis 2,575 d.p.m.) the variability was proportionately large, and, furthermore, there was no concomitant increase in blast transformation. Peripheral blood cultures from the other four adult coeliac patients showed no evidence of stimulation by G.F. III. The mesenteric node lymphocytes obtained from Cases 1 and 2 responded to G.F. III. In Case 2 cultures containing 2 mg. of G.F. III showed D.N.A. synthesis of 17,450 d.p.m. and blast response of 14% compared with 2,559 d.p.m. and 2% blasts in unstimulated control cultures. The cultures containing 500 and 125 μg. showed proportionately smaller responses of 9,100 and 4,720 d.p.m. respectively. In Case 1 D.N.A. synthesis and blast transformation in unstimulated mesenteric node cultures were high (7,750 d.p.m. and 4% blasts), presumably owing to proliferation of malignant cells, but despite this D.N.A. synthesis in cultures containing 500 μg., 2 mg., and 6 mg. of G.F. III was about doubled (13,750, 15,650, and 13,400 d.p.m.) with generally higher proportions of blasts (10·5%, 4%, and 7%). There was no response to G.F. III by axillary node lymphocytes in Case 1, nor was any stimulation found in mesenteric node cultures of the two control patients (Table II).

Lymphocyte Response to Purified Protein Derivative.—Mesenteric node lymphocytes of the two adult coeliac patients responded briskly to purified protein derivative, as did the peripheral blood cells; the response of axillary node lymphocytes in Case 1 was definite but less marked (Table III).

TABLE II.—*Response of Blood and Lymph Node Lymphocytes to Gluten Fraction III*

Case No.	Culture	Peripheral Blood		Subject	Culture	Peripheral Blood	
		D.N.A. Synthesis (d.p.m.)	% Blasts			D.N.A. Synthesis (d.p.m.)	% Blasts
	Adult Coeliac Patients				*Normal Subjects*		
1	0	120	0	A. B.	0	1,400	1
	G.F. III 500 μg.	125	0		G.F. III 125 μg.	2,450	0
	G.F. III 2 mg.	215	0		G.F. III 500 μg.	1,650	0
	G.F. III 6 mg.	150	0		G.F. III 2 mg.	2,575	1·5
2	0	1,230	2	C. D.	0	775	0
	G.F. III 125 μg.	1,748	0·5		G.F. III 500 μg.	735	0
	G.F. III 500 μg.	—	—		G.F. III 2 mg.	1,276	0
	G.F. III 2 mg.	1,420	1		*Control Patients*		
3	0	—	—	Subject	Culture	D.N.A. Synthesis (d.p.m.)	% Blasts
	G.F. III 500 μg.	340	0				
	G.F. III 2 mg.	660	0	I	0	276	0
4	0	362	0		G.F. III 500 μg.	80	0
	G.F. III 125 μg.	770	0		G.F. III 2 mg.	180	0
	G.F. III 500 μg.	417	0	II	0	344	—
	G.F. III 2 mg.	616	0		G.F. III 2 mg.	400	—
5	0	934	0				
	G.F. III 2 mg.	1,250	0				
	G.F. III 6 mg.	1,490	1				
	G.F. III 12 mg.	1,650	0				
6	0	307	0·5				
	G.F. III 500 μg.	200	0				
	G.F. III 2 mg.	266	0				
	G.F. III 6 mg.	228	0				
	G.F. III 12 mg.	189	0·5				

Case No.	Mesenteric Node		Axillary Node	
	D.N.A. Synthesis (d.p.m.)	% Blasts	D.N.A. Synthesis (d.p.m.)	% Blasts
1	7,750	4	125	0
	13,750	10·5	210	0
	15,650	4	290	0
	13,400	7	365	0
2	2,559	2	—	—
	4,720	2·5	—	—
	9,100	—	—	—
	17,450	14	—	—

TABLE III.—*Response of Blood and Lymph Node Lymphocytes to Purified Protein Derivative*

Case No.	Culture	Peripheral Blood		Mesenteric Node		Axillary Node	
		D.N.A. Synthesis (d.p.m.)	% Blasts	D.N.A. Synthesis (d.p.m.)	% Blasts	D.N.A. Synthesis (d.p.m.)	% Blasts
1	0	122	0	7,775	4	135	0
	P.P.D.	29,500	9	154,100	20	6,842	14
2	0	1,230	2	2,559	2	—	—
	P.P.D.	41,804	19	30,426	17	—	—

DISCUSSION

The poor response to phytohaemagglutinin of peripheral blood lymphocytes in three of the adult coeliac patients might suggest a cellular immunological defect, as the results of Winter *et al.* (1967) indicate. But, as previously mentioned, the two very low results in our series were in technically unsatisfactory cultures, and this could explain the low D.N.A. synthesis. Though Winter *et al.* (1967) produced some evidence that the poor lymphocyte response in their experiments could be due to serum factors, in our studies in two patients the response to phytohaemagglutinin was substantially the same whether the cells were cultured in autologous or normal serum.

Immunospecific in-vitro stimulation of human or animal peripheral blood or lymph node lymphocytes is well established and is believed to represent a secondary immune response (Dutton, 1967). Therefore the response of mesenteric node lymphocytes to G.F. III in our patients might indicate an immune response to gluten or its derivatives, and the negative reaction of peripheral blood and axillary nodes and the negative skin tests suggest that this response may be localized to the intestine and its draining lymph nodes.

Proliferation of cells involved in a secondary immune response is to be expected even when only antibody production takes place (Dutton, 1967). It is thus possible that proliferation of precursors of antibody-forming cells can account for

antigen-induced D.N.A. synthesis in in-vitro cell cultures. This is indeed suggested by the experiments of Dutton and Eady (1964), who showed in-vitro antigenic stimulation of spleen cells of rabbits immunized intravenously to heterologous serum proteins and therefore likely to have produced an antibody response but little or no delayed hypersensitivity. Stimulation of mesenteric node lymphocytes by G.F. III in adult coeliac patients could then reflect merely the presence of precursors of antibody-forming cells ; moreover, the presence of such cells might be expected because antibodies to G.F. III are known to be present in some patients (Taylor *et al.*, 1964).

On the other hand, the weight of evidence is that stimulation of lymphocytes by antigens in vitro correlates much more closely with the cell-mediated immune response than with the antibody response (Oppenheim, 1968), and our results could alternatively be interpreted as showing localized delayed hypersensitivity in the intestine.

So far as is known, the cell-mediated immune response in man is always generalized (as was shown for purified protein derivative in our experiments when peripheral blood, axillary node, and mesenteric node lymphocytes all responded to antigen in vitro). But some degree of localization may be found in experimentally sensitized guinea-pigs (Oppenheim, Wolstencroft, and Gell, 1967 ; Dineen, Ronai, and Wagland, 1968 ; Oppenheim, 1968), and it is possible that this also may occur in man.

If adult coeliac patients do have delayed hypersensitivity to gluten, manifested only or predominantly in the intestine, it still seems unlikely that this could be the primary aetiological factor. It is difficult to believe that such a response in the gut could initiate the disease in childhood and yet remain localized during adult life. Furthermore, the evidence that relapse may be induced by autoclaved G.F. II (Frazer *et al.*, 1959) and by ultrafiltrates of gluten digests (Krainick and Mohn, 1959), which are unlikely to be antigenic, suggests that the primary mechanism of the toxicity of gluten is probably not immunological. Delayed hypersensitivity to gluten is more likely to be a secondary phenomenon, arising as a consequence of a primary mucosal lesion allowing abnormal access of antigenic gluten fragments to immunologically competent cells in the gut wall ; a suggestion that could also explain the production of antibodies. Nevertheless, a chronic delayed hypersensitivity reaction thus established could perhaps aggravate the primary mucosal lesion.

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Medical Memoranda

Termination of a Tachyarrhythmia by Paired Pacing

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The technique of using paired impulse stimulation of the heart was initially reported by Lopez, Edelist, and Katz (1963). They showed that the leading and trailing edges of a long-duration impulse caused electrical depolarization of the heart of dogs. When the duration of the electrical impulse was of critical length, the second depolarization occurred when the heart was incapable of mechanically responding, though it could still respond electrically. As a result of this it was found that the refractory period of the heart could be prolonged and therefore the ventricular rate decreased.

Later it was found that this long-duration impulse could equally well be replaced by a pair of very short-duration impulses separated by a critical interval (Pickers, 1969). The critical interval between the first and second impulses varies according to the ventricular rate of the heart to be paced and is best found by experiment. An ideal method of finding this critical time interval is to monitor the right ventricular pressure via a hollow electrode catheter. This is not always possible, however, but very close observation of the electrocardiogram on an oscilloscope provides a reasonable compromise.

If the time interval between the first and second impulse is too short, the second impulse will arrive during the absolute refractory period and so will have no effect. If the time interval is too long, both impulses will cause ventricular contractions and serve only to increase the tachycardia.

The technique of paired pacing need not only be confined to pacing the ventricles. In the presence of normal A–V conduction paired pacing can be used to stimulate the atria and so reduce the ventricular rate. Nevertheless, as the A–V node is still refractory from the first impulse, the second of each pair of atrial depolarizations fails to pass through it. Because of this, the decrease in ventricular rate is unaccompanied by electroaugmentation. This situation is in effect an electrical 2 : 1 A–V block. The advantage of this technique is that during paired pacing of the atria the atrial contribution to ventricular filling is maintained.

A disadvantage to the technique of this kind is that the second of each pair must be delivered at about the time of the