Clin. exp. Immunol. (1981) **45**, 234–239.

IgA-containing circulating immune complexes in gluten-sensitive enteropathy

R. P. HALL, W. STROBER, * S. I. KATZ & T. J. LAWLEY *Dermatology and * Metabolism Branches, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA*

(Accepted for publication 6 March 1981)

SUMMARY

Since mucosal immune response involving IgA may be particularly important in the pathogenesis of gluten-sensitive enteropathy (GSE), we examined the sera of 22 patients with GSE for IgA-containing circulating immune complexes using a sensitive radioimmunoassay, the Raji cell assay for IgA-containing circulating immune complexes. The Raji cell assay for IgG-containing circulating immune complexes and the ^{125}I-C1q-binding assay were also used to measure IgG- or IgM-containing circulating immune complexes in these patients. Ten of 22 (45%) patients had IgA-containing circulating immune complexes, while 11 of 22 (50%) had IgG- or IgM-containing circulating immune complexes. Thirteen of 22 (59%) patients had circulating immune complexes detected by at least one of the assays used. Neither the presence nor level of immune complexes correlated with disease activity in any of the patients studied. Five patients, whose disease was well controlled on a gluten-free diet, were studied serially during dietary challenge with gluten. It was found that IgA-containing circulating immune complexes did not develop or increase in amount in the serum of these patients despite the induction of gastrointestinal symptoms. In addition, no significant change in IgG- or IgM- containing circulating immune complexes occurred in any of the challenged patients. No significant abnormalities of serum complement levels (C3, C4, factor B) were detected in any of the patients including those challenged with gluten. Sucrose density-gradient ultracentrifugation studies revealed that the IgA-containing circulating immune complexes had sedimentation characteristics between 9S and 13S. The presence of circulating immune complexes in only 59% of patients with GSE, their lack of correlation with disease activity, and their failure to change during dietary gluten challenge suggests that circulating immune complexes do not play a primary role in the pathogenesis of GSE.

INTRODUCTION

Circulating immune complexes (CIC) have been detected in a majority of patients with gluten-sensitive enteropathy (coeliac disease; GSE); however, their role in the pathogenesis of this disease is controversial (Doe, Booth & Brown, 1973; Kavai *et al.*, 1977; Mohammed *et al.*, 1976). Initially it was thought that these CIC were present in larger amounts in patients on a normal diet than in those on a gluten-free diet (Doe *et al.*, 1973) but this has not been confirmed (Mohammed *et al.*, 1976). One possible explanation for this lack of correlation between the presence of CIC and disease activity in GSE may be that previously all immune complex assays used were able to detect only IgG- or IgM-containing CIC. Since IgA has been shown to be produced by jejunal biopsy specimens of patients with GSE after exposure to dietary gluten and this IgA consists, at least in

Correspondence: Russell P. Hall, MD, Dermatology Branch, Bldg 10, Rm 12N238, National Institutes of Health, Bethesda, Md 20205, USA.

0099-9104/81/0800-0234$02.00

part, of antibodies with anti-gliadin specificity (Loeb *et al.*, 1971; Falchuk & Strober, 1974), it is possible that IgA-containing immune complexes rather than IgG- or IgM-containing CIC play a role in the pathogenesis of GSE. We have recently developed a sensitive, specific Raji cell radioimmunoassay for the detection of IgA-containing CIC (Hall *et al.*, 1980). Utilizing this assay, as well as the conventional Raji cell radioimmunoassay for IgG-containing immune complexes, and the ^{125}I-C1q-binding assay for IgG- or IgM-containing CIC, we have examined the sera of patients with GSE for circulating immune complexes. In addition, the presence and amount of CIC were determined serially in patients during dietary challenge with gluten.

PATIENTS AND METHODS

Patients. Twenty-two patients with gluten-sensitive enteropathy were studied (17 female, five male) ranging in age from 16 to 75 years old (mean age 40). The diagnosis of GSE was established in each case by a positive history of malabsorption syndrome, flattening of the intestinal villi and infiltration of the submucosa by plasma cells and lymphocytes on jejunal biopsy, improvement of clinical symptoms with a gluten-free diet, and worsening of clinical symptoms with dietary gluten challenge. The sera of five patients were also studied serially during dietary challenge with gluten until gastrointestinal symptoms developed.

Seven patients with other bowel diseases were also studied (five inflammatory bowel disease, one ulcerative colitis, one diarrhoea of unknown aetiology). Serum was obtained from 23 normal volunteers in the Clinical Center, National Institutes of Health, for determination of normal values.

Raji cell assays. Raji cells were obtained as a gift from Dr Dean Mann (Immunology Branch, National Cancer Institute, Bethesda, Maryland). Characterization of their surface immunoglobulin, Fc IgG receptors and complement receptors was performed as described by Theofilopoulos, Dixon & Bokisch (1974). Surface immunoglobulins were not detected and receptors for complement were present. Raji cells were grown at 37°C in RPMI 1640 (NIH Media Unit) supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biologic Company, Grand Island, New York), 4 mM glutamine (NIH Media Unit) and penicillin–streptomycin–amphotericin antibiotic (Grand Island Biologic Company, Grand Island, New York).

Raji cell assay for IgA-containing immune complexes. Raji cells (2×10^6) in 50 μl of RPMI 1640 were placed in a 1·5 ml microfuge tube (Beckman Instruments Inc., Mountainside, New Jersey) along with 25 μl of the test sera diluted 1 : 4 with pH 7·4 phosphate-buffered saline (PBS) (NIH Media Unit) and incubated in a 37°C water bath for 45 min with gentle agitation every 20 min. All test sera were analysed in duplicate. The cells were then washed three times with RPMI 1640 and resuspended in 18 μg of the IgG fraction of a heavy chain-specific, ^{125}I-goat anti-human IgA (Atlantic Antibodies, Westbrook, Maine) in 50 μl of RPMI 1640 with 1% human serum albumin (Sigma Chemical Company, St Louis, Missouri). The goat anti-human IgA produced a single precipitin line in double diffusion in gel against normal human sera and against a purified human IgA myeloma protein (gift of Dr Richard Wistar, Naval Medical Research Institute, Bethesda, Maryland). The goat anti-human IgA was radiolabelled using Bolton–Hunter reagent (New England Nuclear, Boston, Massachusetts) as described previously (Lawley *et al.*, 1979). The cells were then incubated at 4°C for 30 min with gentle agitation. Finally, the cells were washed three times with cold RPMI 1640 with 1% HSA, and cell-bound radioactivity was counted.

Analysis of Raji cell assay for IgA-containing immune complexes. Statistical analysis was done using a modification of the method of Woodroffe *et al.* (1977), in collaboration with Dr David Alling (Special Assistant for Biometry, National Institute of Allergy and Infectious Diseases), as previously described (Hall *et al.*, 1980). Briefly, individual serum samples from 13 normal subjects were assayed in duplicate on 9 different days. Components of variance were calculated from these data to estimate variability among persons (σ_P^2), among days (σ_D^2) and residual variation (σ_R^2). These estimates, expressed as standard deviations, were $\sigma_P = 1{,}005$ c.p.m., $\sigma_D = 1{,}020$ c.p.m. and $\sigma_R = 1{,}122$ c.p.m.; the overall mean c.p.m. was 6,595. Four subjects with representative mean values reflecting the range of the total control panel were chosen and daily mean c.p.m. calculated. Differences among these means were taken to represent day-to-day variability so that the

contribution σ_D^2 could be dropped from the overall estimate of variation. Using the remaining contribution (σ_P^2 and σ_R^2) and the appropriate daily mean (Ci), an upper 95% confidence limit (Ui) was constructed for the mean of duplicate determinations on a test serum assayed on the *i*th day, namely:

$$Ui = Ci + 1{\cdot}86\sqrt{5/8\ \sigma_R^2 + 5/4\ \sigma_P^2} = Ci + 2{,}664.$$

The quantity under the square root sign represents the sum of the variances of the mean of the four control sera, and of the mean of the duplicate test serum values, and 1·86 is the upper 0·95 point of the distribution of Student's *t* with 8 degrees of freedom. The mean of the duplicate values of the test sera (Ti) was then divided by Ui. This value (Ti/Ui) represents the fractional relationship of the test serum to the upper 95% confidence level of the control population. If Ti/Ui exceeds 1·0, the value of the test serum exceeds the upper 95% confidence limit and is judged abnormal.

Raji cell assay for IgG-containing immune complexes. The Raji cell assay for IgG-containing immune complexes was performed as described previously by Theofilopoulos, Wilson & Dixon (1976). Raji cells and sera were treated as for the Raji IgA assay (above) except that 18 μg of the IgG fraction of a heavy chain-specific, ^{125}I-goat anti-human IgG antiserum (Atlantic Antibodies, Westbrook, Main) was substituted for the anti-IgA.

Analysis of Raji cell assay for IgG-containing immune complexes. Components of variance were calculated as done for the Raji IgA assay. The estimates of variability for the Raji IgG assay, expressed as standard deviations, were $\sigma_P = 5{,}122$ c.p.m., $\sigma_D = 4{,}564$ c.p.m. and $\sigma_R = 2{,}782$ c.p.m.; the overall mean c.p.m. of the control population was 21,343. Ui for the Raji IgG assay was calculated as in the Raji IgA assay, namely:

$$Ui = Ci + 1{\cdot}86\sqrt{5/8\ \sigma_R^2 + 5/4\sigma_P^2} = Ci + 11{,}410.$$

The mean of the duplicate values of the test sera (Ti) was then divided by Ui. If Ti/Ui was greater than 1, the serum was judged abnormal. The upper 95% confidence limit (Ui), was found to correspond to 30 μg equivalents of heat-aggregated human gammaglobulin per ml of serum.

^{125}I-C1q-binding assay. The ^{125}I-C1q-binding assay (^{125}I-C1qBA) was performed as described previously (Lawley *et al.*, 1979). The upper 95% confidence limit of C1q-binding activity (C1qBA) in normal human serum was determined to be 10%.

Other serological tests. Serum C3, C4, factor B, and immunoglobulin levels were quantitated with commercially available radial immunodiffusion plates (Behring Diagnostics).

Density-gradient ultracentrifugation. Density-gradient ultracentrifugation was performed by layering 0·25 ml of serum diluted 1:5 in PBS, pH 7·4, over a 10–40% continuous sucrose density gradient in 6 ml Beckman cellulose nitrate tubes (Beckman Instruments, Mountainside, New Jersey). The gradients were then centrifuged at 178,000 ***g*** for 17·5 hr at 4°C in an L2-65B Beckman ultracentrifuge equipped with an SW65 rotor. The centrifuged tubes were punctured and 11 fractions collected from the bottom. Fractions thus obtained were tested for IgA CIC, IgG, IgM and IgA by the methods described above.

RESULTS

Immune complex assays

IgA-containing CIC were detected in 10 of 22 (45%) patients with GSE (mean fractional increase of upper 95% confidence limit, Ti/Ui = 1·8) (Fig. 1). Eight of 22 patients had IgG-containing CIC by the Raji cell IgG assay, while only four had evidence of CIC detected using the ^{125}I-C1q-binding assay (Fig. 1). Six of the eight patients who had IgG-containing CIC detected using the Raji cell IgG assay also had IgA-containing CIC, whereas two of the four patients with CIC detected using the ^{125}I-C1q-binding assay also had IgA-containing CIC. In all, 13 of 22 (59%) patients had evidence of CIC by at least one of the three assays used. There was a high rate of concordance (75%) between the Raji IgA and Raji IgG assays and in addition, Spearman rank order analysis showed a correlation between these two assays ($r = 0{\cdot}44$ $P < 0{\cdot}05$). There was no correlation between the Raji IgA assay

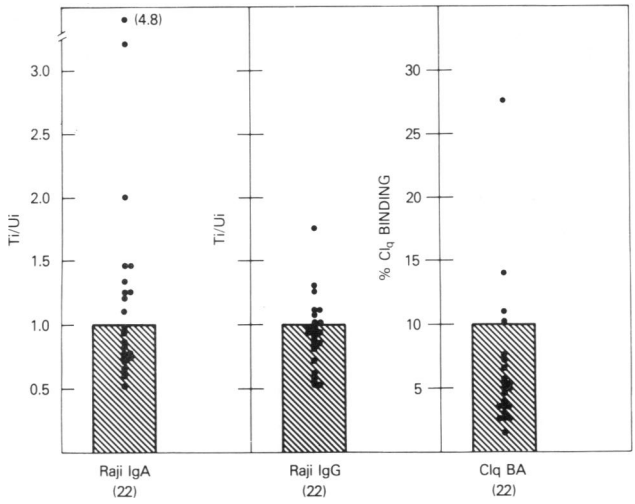

Fig. 1. Comparison of levels of circulating immune complexes in patients with gluten-sensitive enteropathy using the Raji IgA, Raji IgG and ^{125}I-C1q-binding assays. Cross-hatched areas represent the normal ranges. Ti/Ui represents the fractional increase of the serum sample over the upper 95% confidence level.

and the ^{125}I-C1q-binding assay but there was a correlation between the Raji cell IgG assay and ^{125}I-C1q-binding assay using Spearman rank order analysis ($r = 0{\cdot}68$, $P < 0{\cdot}025$). A weak correlation was found between the Raji cell IgA assay and serum IgA levels ($r = 0{\cdot}39$, $P < 0{\cdot}05$). Finally, there was no correlation between the presence or level of IgA-, IgG- or IgM-containing CIC and the duration of disease, symptomatology at the time of study, or whether or not the patient was on a gluten-free diet.

None of the patients with other gastrointestinal diseases (five inflammatory bowel disease, one ulcerative colitis, one diarrhoea of unknown aetiology) had evidence of IgG-containing CIC, while one patient with inflammatory bowel disease had IgA-containing CIC (Ti/Ui = 1·12). Four of seven patients (two with inflammatory bowel disease, one with ulcerative colitis, one with diarrhoea of unknown aetiology) had elevated C1q-binding activity (mean positive C1qBA = 22%).

Dietary gluten challenge

Five patients were studied serially during challenge with a gluten-containing diet. All patients developed gastrointestinal symptoms after a mean duration of challenge of 5·4 days. Two of the five patients studied had no evidence of IgA-containing CIC before gluten challenge. In addition, neither of these patients had IgG-containing CIC detected using the Raji cell IgG assay, while one had low levels of CIC detected using the ^{125}I-C1q-binding assay (C1qBA = 12%). During gluten challenge neither of these two patients developed IgA-containing CIC, despite the development of gastrointestinal symptoms. One patient did develop low levels of IgG-containing CIC using the Raji cell IgG assay (Ti/Ui = 1·1), and the sera of the patient with elevated C1qBA returned to the normal range. The remaining three patients studied serially on gluten challenge had high levels of IgA-containing CIC before institution of gluten challenge (mean Ti/Ui = 3·0). Two of these patients also had IgG-containing CIC detected using the Raji IgG assay (Ti/Ui = 1·35, 1·08) and one had elevated C1qBA (C1qBA = 13%). In none of these patients was a change in immune complex level or type noted despite the development of gastrointestinal symptoms.

Other serological tests

Twenty-one of 22 sera of the patients studied had normal levels of C3, while in one patient's serum the level was slightly elevated (C3 = 140 mg%; normal level = 55–120 mg%). One patient's serum C4 level was low (C4 = 19 mg%; normal level = 20–50 mg%). This patient also had IgA- and IgG-containing CIC. Factor B (FB) levels were abnormally low in three patients (FB = 10·5, 10·8,

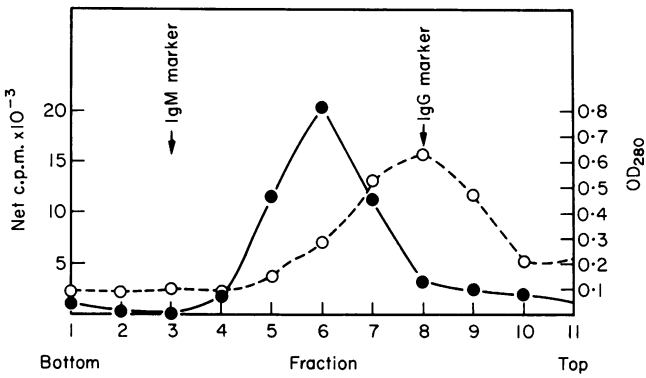

Fig. 2. Results of sucrose density-gradient ultracentrifugation of serum of patient with gluten-sensitive enteropathy and IgA-containing circulating immune complexes. (——) Level of IgA-containing circulating immune complexes detected using the IgA Raji cell radioimmunoassay. (- - - -) OD_{280}. IgA-containing immune complexes are detected in the 9–13S regions.

11·1 mg%; normal level = 12–30 mg%). Two of these patients also had IgA-containing immune complexes, while one patient had IgG-containing immune complexes. Serum IgA levels were low in one patient (76 mg%; normal level = 90–450 mg%), and high in one patient (500 mg%). None of the patients challenged with a gluten-containing diet had significant changes in serum complement component levels (C3, C4, FB) or serum IgA levels.

Density-gradient ultracentrifugation

The sera of two patients with IgA-containing CIC were studied using sucrose-gradient ultracentrifugation. On 10–40% continuous sucrose gradients, IgA-containing immune complexes, as detected by the Raji cell radioimmunoassay, were found to sediment predominantly as 9–13S material (Fig. 2).

DISCUSSION

We have developed a sensitive and specific radioimmunoassay for IgA-containing CIC utilizing the Raji cell and have previously demonstrated that 25% of patients with dermatitis herpetiformis (DH), a skin disease characterized by blisters, cutaneous IgA deposits and an asymptomatic gluten-sensitive enteropathy, have IgA-containing CIC (Hall *et al.*, 1980; Katz & Strober, 1978). Since patients with ordinary GSE have a similar but more severe abnormality of their gastrointestinal tract, and since prior studies of these patients did not assay for immune complexes containing IgA (the predominant immunoglobulin produced by intestinal mucosal cells), it was of interest to determine if IgA-containing CIC were also present in patients with ordinary GSE.

We have detected IgA-containing CIC in 45% of 22 patients with GSE using the Raji cell IgA assay and detected IgG- or IgM-containing CIC in 50% of this patient group using the Raji cell IgG assay or the ^{125}I-C1q-binding assay. In all, 59% of patients were positive by one of the three assays for CIC used. In none of our patients did the presence, level or type of CIC correlate with the presence of disease symptoms, or status of gluten restriction. Indeed, dietary gluten challenge did not result in the induction of CIC or changes in the levels of CIC, despite the development of clinical symptoms. Finally, all but a few patients studied showed no alteration of serum C3, C4 or factor B levels, either on a gluten-free diet or during symptomatic periods on a gluten-containing diet.

Analysis of some of the physicochemical characteristics of the IgA-containing CIC found in GSE using sucrose density-gradient ultracentrifugation showed that the majority of IgA-containing CIC had sedimentation characteristics between 9S and 13S. The high degree of concordance between the Raji IgA assay and Raji IgG assay, as well as the significant correlation between these two assays on Spearman rank order analysis, suggests that IgA and IgG may coexist in a common

immune complex matrix. Alternatively, the IgA CIC present in GSE may have similar physicochemical characteristics to those containing IgG.

These data can be interpreted in several ways. First, it may be that IgA-containing CIC are present in all patients with GSE but in some patients the IgA cannot fix complement in the fluid phase. Such IgA-containing CIC would not be detected in our assay, which relies on the binding of the IgA CIC to the Raji cell via the C3 receptor. This possibility seems unlikely, however, in that aggregated IgA has been shown to be capable of fixing complement (via the alternative pathway) (Götze & Müller-Eberhard, 1971) and would thus allow detection with our assay system. Second, it may be that IgA-containing CIC are present in all patients with GSE but are more rapidly cleared from the circulation in some. This seems unlikely since if this were the case one might expect to see some increase in the level of IgA-containing CIC following dietary gluten challenge. Third, it may be that the occurrence of IgA-containing CIC is related to the nature and intensity of the small intestinal lesion present in GSE. This concept is suggested by the fact that IgA CIC are found both in patients with DH and patients with ordinary GSE, but not in patients with other gastrointestinal or inflammatory skin diseases (Hall *et al.*, 1980). In addition it is supported by the fact that IgA-containing CIC occur less frequently in the patients with the less severe GSE associated with DH than in patients with ordinary GSE. Finally, although IgA CIC were not related to the presence of symptomatology in our patient group, there was an indication that IgA CIC levels were higher in some patients with ongoing morphological gastrointestinal changes not associated with overt malabsorption, since in several instances asymptomatic patients with elevated IgA CIC levels were biopsied and found to have 'silent' villous atrophy. Whichever of these possibilities are ultimately proven true, we believe that the failure to detect any change in the level of IgA, IgG or IgM CIC following dietary gluten challenge suggests that circulating immune complexes are not pathogenetically important in the development of the intestinal lesion of GSE, since disease symptoms can occur in the absence of CIC and without changes in CIC levels.

REFERENCES

Doe, W.F., Booth, C.C. & Brown, D.L. (1973) Evidence for complement-binding immune complexes in adult coeliac disease, Crohn's disease, and ulcerative colitis. *Lancet*, **ii,** 402.

Falchuk, Z.M. & Strober, W. (1974) Gluten-sensitive enteropathy: synthesis of antigliadin antibody *in vitro*. *Gut*, **15,** 947.

Götze, O. & Müller-Eberhard, H.J. (1971) The C3-activator system: an alternate pathway of complement activation. *J. exp. Med.* **134,** 90s.

Hall, R.P., Lawley, T.J., Heck, J.A. & Katz, S.I. (1980) IgA containing circulating immune complexes in dermatitis herpetiformis, Henoch–Schönlein purpura, systemic lupus erythematosus and other diseases. *Clin. exp. Immunol.* **40,** 431.

Katz, S.I. & Strober, W. (1978) The pathogenesis of dermatitis herpetiformis. *J. Invest. Dermatol.* **70,** 63.

Kavai, M., Csorba, S., Szabolcs, M. & Jezerniczky, J. (1977) Circulating immune complexes in coeliac disease. *Lancet*, **i,** 1263.

Lawley, T.J., Moutsopoulos, H.M., Katz, S.I., Theofilopoulos, A.N., Chused, T.M. & Frank, M.M. (1979) Demonstration of circulating immune complexes in Sjögren's syndrome. *J. Immunol.* **123,** 1382.

Loeb, P.M., Strober, W., Falchuk, Z.M. & Laster, L. (1971) Incorporation of L-leucine-^{14}C into immunoglobulins by jejunal biopsies of patients with celiac sprue and other gastrointestinal diseases. *J. clin. Invest.* **50,** 559.

Mohammed, I., Holborow, E.J., Fry, L., Thompson, B.R., Hoffbrand, A.V. & Stewart, J.S. (1976) Multiple immune complexes and hypocomplementaemia in dermatitis herpetiformis and coeliac disease. *Lancet*, **ii,** 487.

Theofilopoulos, A.N., Dixon, F.J. & Bokisch, V.A. (1974) Binding of soluble immune complexes to human lymphoblastoid cells. *J. exp. Med.* **140,** 877.

Theofilopoulos, A.N., Wilson, C.B. & Dixon, F.J. (1976) The Raji cell radioimmune assay for detecting immune complexes in human sera. *J. clin. Invest.* **57,** 169.

Woodroffe, A.J., Border, W.A., Theofilopoulos, A.N., Götze, O. Glasscock, R.J., Dixon, F.J. & Wilson, C.B. (1977) Detection of circulating immune complexes in patients with glomerulonephritis. *Kidney Int.* **12,** 268.