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IMMUNOGLOBULIN-CONTAINING CELLS IN NON-TROPICAL SPRUE

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SUMMARY

Immunofluorescent microscopy studies of jejunal biopsies from ten patients with gluten-induced enteropathy are reported. Eight biopsies were examined before and five after onset of gluten-free diet.

Fluorescent antisera specifically reacting with IgA, IgG and IgM were applied, and accordingly the cells were differentiated and quantitated.

The immunofluorescence microscopy revealed an increased amount of immunoglobulin-containing cells in both treated and untreated cases of sprue. The distribution of the immunoglobulin-containing cells within the three immunoglobulin classes was significantly altered as the proportion of IgM- and IgG-containing cells was elevated.

INTRODUCTION

Despite the fact that immunoglobulin A (IgA) is a minor component of the serum immunoglobulins, Chodirker & Tomasi (1963) found that IgA was the predominant immunoglobulin in the gastro-intestinal secretions. Correspondingly it has been shown in immunohistochemical studies using specific fluorescent antisera that the majority of the plasma cells in normal jejunal mucosa contain IgA (Rubin *et al.*, 1965; Crabbé, Carbonnara & Heremans, 1965; Hazenberg, 1968). Studies on patients with non-tropical sprue (Rubin *et al.*, 1965; Eidelman *et al.*, 1966) confirmed that the IgA-containing cells were predominant, but the distribution of immunoglobulin-containing cells of the three immunoglobulin classes was not examined.

MATERIALS AND METHODS

Subjects

(a) Eight controls aged 21–60 years.

(b) Ten patients aged 18–60 years with gluten-induced enteropathy.

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Treatment

(1) Eight patients were untreated as the biopsy used for immunofluorescence microscopy was taken. These patients were followed for 5–14 months on gluten-free diet; a typical clinical remission was recorded, but repeated intestinal biopsy was carried out in only three patients.

(2) Five patients had had a good clinical remission on gluten-free diet as the biopsy used for immunofluorescence microscopy was taken. Three of these patients had been examined prior to diet treatment but were re-examined after 9, 9 and 12 months, respectively, of gluten-free diet. In the remaining two patients immunofluorescent examination was at first carried out after 2 and 3 years of treatment.

TABLE 1. Details of patients studied

Patient	Sex	Age	Treatment	Duration of symptoms/diet (years)	Faecal fat excretion*	D-Xylose absorption†	Jejunal pathology	Immunofluorescence study performed
1	M	26	No	1	70	3·3	Severe	+
2	F	26	No	2	15	3·2	Severe	+
3	F	45	No	19	22	2·9	Severe	+
4	M	18	No	7	23	1·5	Severe	+
5	M	59	No	2	75	1·1	Severe	+
6	F	42	No	30	33	5·1	Severe	+
			+	1	4	4·4	Severe	+
7	M	49	No	8	5	8·0	Severe	No
			+	3	6	11·0	Moderate	+
8	M	60	No	4	48	2·1	Severe	No
			+	2	21	6·1	Severe	+
9	F	18	No	10	83	1·4	Severe	+
			+	3/4	18	Normal‡	Severe	+
10	F	25	No	3	8	3·1	Severe	+
			+	3/4	2	7·9	Severe	+

* Grams per day from a 3-day stool collection.

† Grams excretion in 24-hr urine collection after ingestion of 25 g. Normal greater than 5·0 g.

‡ Determined after blood values.

Regardless of treatment all biopsy specimens presented subtotal villus atrophy under the dissecting microscope as well as under the light microscope. In only one patient a slight histopathological improvement was seen after 3 years of treatment with gluten-free diet. Quantitative determinations of serum IgA, IgM and IgG revealed only minor alterations, and no agammaglobulinaemia was found.

Further details of the patients are presented in Table 1.

Biopsy specimen material

The jejunal biopsies were obtained with a hydraulic multiple biopsy tube. All specimens were taken 10–30 cm distal of Treitz' ligament. At least two biopsies were taken at the same level, one of these for conventional histopathological examination. The biopsies used for immunofluorescent studies were immediately frozen and stored at −70°C.

Preparation of fluorescein conjugates

The following antisera were obtained from Centraal Laboratorium van de Bloed Transfusiedienst van het Nederlandsche Roote Kruis, Amsterdam, Holland: (1) precipitating horse anti-human IgA (2) precipitating horse anti-human IgM, and (3) precipitating horse anti-human IgG.

A crude globulin fraction was precipitated from each antiserum by slow addition of equal volume 1·7 M $(NH_4)_2SO_4$ at 4°C. The globulin precipitate was separated by centrifugation, dissolved in phosphate buffered saline, pH 7·2, and dialysed against phosphate buffered saline in 18 hr at 0°C. The efficiency of the dialysis was controlled by Nesslers reagent. The protein content was determined by biuret and refractometry, and the solution was diluted to 1% by adding phosphate buffered saline. By adding Na_2CO_3–$NaHCO_3$ buffer pH was adjusted to 9·0. The antisera were conjugated with 0·025 mg fluoresceinisothiocyanate/mg protein giving a fluorochrome protein ratio of $3{\cdot}6 \times 10^{-3}$, $3{\cdot}6 \times 10^{-3}$ and $3{\cdot}4 \times 10^{-3}$ for IgA, IgM and IgG, respectively. The fluorochrome protein ratio was calculated from the extinction at 282 and 495 nm in a Beckmann spectrophotometer, after the formula of Wood, Thompson & Goldstein (1965). The fluoresceinisothiocyanate was added slowly at 4°C under constant stirring. The mixture was stirred for 18 hr at 4°C and then dialysed against several changes of phosphate buffered saline, pH 7·2. The dialysate was passed through a Sephadex G-25–50 column. The first yellow-green zone eluted from the Sephadex column was used. Thiomerosol 1:10,000 was added to the conjugate. Each conjugate was absorbed with guinea-pig liver powder, 100 mg powder/ml conjugate.

Labelling with fluorescent antisera

Single incubation: 4–6 μm sections were prepared in a cryostat at −20°C, placed on slides, and air-dried for 10 min. After fixation in absolute methanol for 3 min at 4°C the slides were washed in a bath of Coon's buffer for 15 min. The slides were air-dried and covered with one drop of fluorescent antiserum and left in a moist chamber for 30 min at room temperature. After incubation the slides were washed in two successive baths of Coon's buffer for 15 min and mounted in buffered glycerine (50%).

Double incubation: after cutting, methanol-fixation, and washing with Coon's buffer as described above, the slides were covered with one drop of unconjugated horse anti-human IgG, IgM or IgA sera (of the same lot as used for preparation of conjugates) and left in a moist chamber for 30 min. After incubation the sections were washed twice in Coon's buffer for 15 min and covered with one drop of fluorescent anti-IgA, anti-IgA or anti-IgM. After incubation for another 30 min the slides were washed in two successive baths of Coon's buffer for 15 min and mounted in buffered glycerine. The slides were kept at 4°C and studied within 30 hr after the staining.

Microscopy and measurements

A Reichert zetopan microscope with an Osram HBO-200 W high pressure mercury lamp was used for fluorescence microscopy. For microscopy both light- and dark-field technique was employed, but for the photomicrographs dark-field condenser was used. The microscope was equipped with a Reichert photoautomate, and coloured photomicrographs were obtained with Anscochrome 24°Din daylight film.

The photomicrographs were projected under standard magnification on a white sheet of paper. The areas to be measured were drawn by hand, cut out and weighed.

Control

The three specific antisera against human IgA, IgM and IgG were shown immunoelectrophoretically to give only one line at the expected place using whole human serum and serum samples containing homologous apparently monoclonal globulins of the different classes as antigens. Using sera with Bence-Jones proteins of type-K and type-L as antigen no reaction was found with anti-IgA and anti-IgM antisera. With Bence-Jones protein of type-L the anti-IgG antiserum produced a very fine precipitation line which indicated low anti-L activity (the immunoelectrophoretical analyses were kindly performed by B. Mansa, Biophysical Department, Statens Seruminstitut). This activity against type-L Bence-Jones protein in the anti-IgG serum seemed not to cause recognizable cross-reactions in the tissues, as serial sections where single incubation with anti-IgA, anti-IgM and anti-IgG were alternating showed that no single cell was reacting with more than one antiserum. Furthermore, to exclude cross-reaction the double incubation technique was used in all cases where quantitation was carried out. Thus, in every case the sections were preincubated alternately with the two unconjugated non-homologous antisera before staining with the conjugated serum. This preincubation diminished the background staining considerably, but left the specific staining unaffected. No differences were recorded, whether the preincubation was made with one or the other of the unconjugated non-homologous antisera.

Preincubation with the homologous unconjugated antisera abolished specific staining.

An aliquot of each fluorescent antisera was absorbed with a crude γ-globulin fraction of normal serum. After this absorption no specific fluorescence could be demonstrated.

RESULTS

Distribution of immunoglobulin

The cytoplasm of mononuclear cells in the lamina propria was the only structure giving specific fluorescence in the controls as well as in the sprue group. A faint fluorescence of the luminar end of the crypt and surface epithelium cylinder cells was considered unspecific as it could not be abolished by appropriate blocking-tests.

The fluorescence of interstitial fluid was pronounced in the sprue group and was regarded unspecific according to similar criteria. This unspecific fluorescence interfered with the interpretation, and several sections had to be made before quantitation could be carried out. In the sprue group the quantitation was made difficult by a massive infiltration of eosinophil granulocytes and mast cells, as these cells give a bright granular autofluorescence.

The specific fluorescent cells emitted a strong homogeneous applegreen fluorescence. The cells could often be recognized as plasma cells. In the control group as well as in the sprue group it was found that the cells were confluent so that the outlines of a single cell could often not be clearly distinguished. This was most pronounced in specimens from patients with sprue. In the controls the immunoglobulin-containing cells were most abundant at the basis of the villi and between the crypts, and there was a great variety of the cell density from one area to another, whereas the distribution was more uniform in the sprue patients.

Quantitation of immunoglobulin-containing cells

As the immunoglobulin-containing cells were confined to the interstitial tissue they were quantitated by relating the fluorescent area to the total area of the interstitial tissue in a

given visual field. Every single cell or group of confluent cells giving specific fluorescence was measured.

There was great variety in the density of cells giving specific fluorescence within a section, and it was therefore desirable to base the quantitation on the largest area of interstitial tissue. More than one visual field had to be photographed from a section, and the number was arbitrarily limited to six visual fields representing about one-third of the area. Furthermore, to extend the basis of the calculation, visual fields containing the greatest area of interstitial tissue were selected. As they were found more often at the basis of the villi and this region was more rich in plasma cells the population density values calculated are maximum values.

The distribution of the population densities in eighteen visual fields (three sections) was calculated for each of the antisera. The following mean values and standard deviations were found, IgA: $18{\cdot}7 \pm 8{\cdot}1$, IgM: $11{\cdot}3 \pm 3{\cdot}3$, IgG: $1{\cdot}9 \pm 1{\cdot}5$.

The area of cells giving specific fluorescence with the three antisera is shown in Fig. 1.

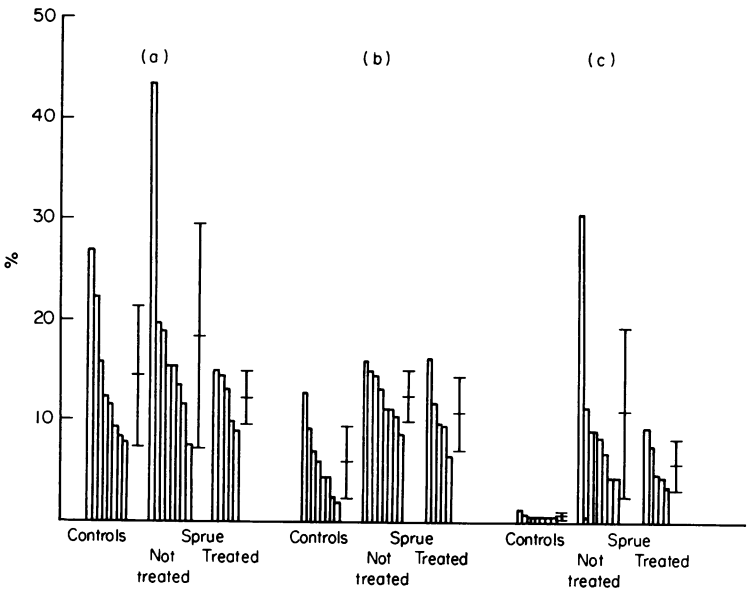

FIG. 1. Area of cells containing IgA (a), IgM (b) and IgG (c) calculated as % of interstitial tissue area in controls and sprue. Mean values ($\pm$SD) are indicated.

It appears that there is a great difference in the pattern of immunoglobulin-containing cells in the controls and in the sprue patients, whether these are treated or not.

In the control group the IgA-cells are predominant, the IgM-cells amount to about 30–60% of the former group, and the IgG-cells are very sparse. The difference between the occurrence of the three cell-types is significant (IgA–IgG: $P<0{\cdot}001$, IgM–IgG: $P<0{\cdot}001$, IgA–IgM: $0{\cdot}01>P>0{\cdot}001$). In the group of patients with untreated sprue the pattern is altered as both the number of IgM- and IgG-containing cells is markedly raised. A comparison between the controls and the untreated sprue-group shows that there is no difference in the content of IgA-cells, but there is as significantly higher number of IgM-cells ($0{\cdot}01>P>0{\cdot}001$) and IgG-cells ($0{\cdot}01>P>0{\cdot}001$) in the sprue patients.

In the group of sprue patients on gluten-free diet a similar significantly higher content of IgM-cells ($0{\cdot}05 > P > 0{\cdot}02$) and IgG-cells ($P < 0{\cdot}001$) was found compared to the controls, but there was no difference in the content of IgA-cells.

A comparison of the treated and the untreated patients with sprue revealed no differences in the content of cells containing IgA, IgM and IgG, neither was any greater difference found in the pattern of immunoglobulin-containing cells of the three patients which were examined both before and after treatment. The total amount of cells giving fluorescence with the three antisera is shown in Fig. 2.

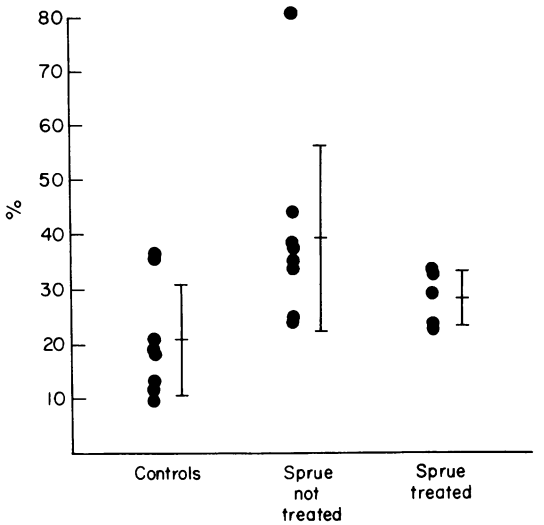

FIG. 2. Total area of immunoglobulin-containing cells calculated as % of interstitial tissue area in controls and sprue. Mean values (±SD) are indicated.

There is a higher total content of immunoglobulin-containing cells in both sprue-groups, but only in the untreated group the difference is significant ($0{\cdot}02 > P > 0{\cdot}01$).

DISCUSSION

The findings from immunofluorescence examination of jejunal biopsies from normals have been presented and discussed previously (Søltoft, 1969). The findings were essentially in accordance with reports from other laboratories (Rubin *et al.*, 1965; Crabbé *et al.*, 1965; Crabbé, 1967; Hazenberg, 1968); and a characteristic pattern with predominance of IgA-cells to IgM- and IgG-cells was found.

In the present study a significantly different pattern of immunoglobulin-containing cells was found in the sprue patients. As in normals the IgA-cells were most abundant, but the number of both IgM- and IgG-cells was markedly raised and almost equal to the IgA-cells. This was the case regardless of whether the patients were on a gluten-free diet or not. No significant difference between untreated and treated sprue patients was found, neither in the total amount of immunoglobulin-containing cells, nor in the distribution of these cells within the three immunoglobulin classes. The total number of immunoglobulin-containing cells was elevated both in treated and untreated cases of sprue. In the study of

Rubin *et al.* (1965) no quantitation of the distribution of the immunoglobulin-containing cells within the three immunoglobulin classes was done, but it is understood that the pattern was more like the controls than it was found in this series. This difference may be caused by the fact that seven of nine patients examined by Rubin *et al.* (1965) had been on a diet for 5 years, on average, and that the jejunal pathology was described as moderate, mild or even normal, whereas in this study most patients were untreated, and subtotal villus atrophy was still present in the patients on gluten-free diets. Eidelman *et al.* (1966) found an elevated number of IgA-containing cells in six patients with sprue, but the content of IgM- and IgG-cells was not mentioned and histopathological findings and dietary regime are unknown.

Crabbé (1967) quantitated the immunoglobulin-containing cells in one patient with untreated sprue and found a moderately elevated number of IgM- and IgG-cells, but the IgA-cells were still predominant.

The persistence of profound alterations in the patterns of the immunoglobulin-containing cells in jejunal biopsies more than a year after a rigorous elimination of gluten is not very surprising considering the slow and incomplete remission of the mucosal morphology.

ADDENDUM

After the study was finished it appeared that the anti-IgA preparation employed had also some specific activity against 'secretory piece' since colostrum had been used for immunization. Thus a new anti-IgA preparation was procured. This preparation was tested in serial dilution against normal human serum, light chains, myeloma and Waldenström sera, and against colostrum. It proved to possess specific reactivity only against serum IgA. Nine biopsies were re-examined with the new and the old anti-IgA preparation.

Quantitation studies showed that there was no difference in the number of interstitial cells giving specific fluorescence with the two antisera. The faint staining of the epithelial cells induced by the anti-IgA with activity against 'secretory piece' was absent with the new anti-IgA preparation, and consequently this staining cannot be considered unspecific. Studies with specific antisera against 'secretory piece' and serum-IgA are now undertaken.

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