Incorporation of L-Leucine-^{14}C into Immunoglobulins by Jejunal Biopsies of Patients with Celiac Sprue and Other Gastrointestinal Diseases

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Abstract Incorporation of L-leucine-^{14}C into proteins and immunoglobulins in vitro was determined in jejunal biopsy specimens from normal volunteers, patients with celiac sprue before and after introduction of gluten into the diet, patients with Whipple's disease in remission, and patients with immune deficiency states.

Values for incorporation of L-leucine-^{14}C into total and soluble protein by biopsies from five celiac sprue patients on a gluten-free diet were within, or slightly above, the 95% confidence limits for control data. One patient with celiac sprue and with normal intestinal histology had a normal value for incorporation into IgA; the other four patients with flat mucosas had elevated values. In Whipple's disease in remission, values for incorporation into total protein and IgA were within the control limits, whereas incorporation into soluble protein was increased. Patients with hypogammaglobulinemia or IgA deficiency had normal or elevated values for incorporation into total and soluble proteins; in these cases, however, no incorporation into IgA was detected.

Biopsies from the four celiac sprue patients studied revealed that with introduction of gluten into the diet (*a*) incorporation into total protein, soluble protein, or both, increased; (*b*) incorporation into IgA increased in all patients, and in two instances the increase was greater than the increase in incorporation into total protein; and (*c*) incorporation into IgM increased in all patients. The changes during gluten administration usually occurred before changes in gastrointestinal absorptive function or in concentration of IgA in serum could be detected.

These results indicate that gluten challenge stimulates increased local intestinal synthesis of immunoglobulins in patients with celiac sprue. The reaction occurs within days and it is possible that it plays a primary role in the pathogenesis of the disease.

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INTRODUCTION

It has become increasingly clear that the gastrointestinal tract can respond to exogenous antigens by the local production of antibodies which are secreted into the gastrointestinal tract lumen. Thus, oral immunization with live polio virus results in the appearance of antibodies in the gastrointestinal secretions, whereas parenteral immunization with killed virus produces an increase in serum antibodies, but does not produce detectable gastrointestinal antibodies (1, 2). Since IgA is the major immunoglobulin found in gastrointestinal secretions (3), and since IgA-containing cells are the predominant lymphoid cells in the gastrointestinal mucosa (4, 5), it appears that local immune responses involving the gastrointestinal tract are mediated primarily through the IgA immunoglobulin system.

It is possible that the gastrointestinal immune system participates in the production of gastrointestinal disease. This could occur through the local elaboration of antibodies which react with gastrointestinal tissue or with dietary or bacterial protein bound to such tissue, with consequent cell destruction. A mechanism such as this might play a role in the pathophysiology of celiac sprue. Thus, it could be postulated that in this disease, gluten, or some other antigen, becomes bound to the intestinal epithelium, that antibodies specific for this

antigen are then synthesized within the intestinal wall, and these antibodies react with the bound antigen to produce tissue damage. It would be important in pursuing such an hypothesis to know whether a system for local production of antibodies exists within the intestinal mucosa, and if it does, whether it responds when the patient is exposed to dietary gluten. In the present study we explored these questions by determining the incorporation in vitro of L-leucine-^{14}C into immunoglobulins by specimens of jejunal mucosa from patients with celiac sprue and other diseases as a measure of gastrointestinal immunoglobulin synthesis. We found that in some patients with celiac sprue in remission while on gluten-free diets, local intestinal IgA synthesis exceeded that of control subjects and that in all celiac sprue patients intestinal production of IgA and IgM increased soon after reintroduction of gluten into the patients' diet.

METHODS

Patients

The 17 individuals studied included 6 normal volunteers, 2 patients with Whipple's disease in remission, 2 patients with selective IgA deficiency, 2 patients with hypogammaglobulinemia, and 5 patients with celiac sprue. The 6 volunteers included 5 men and 1 woman ranging in age from 20 to 26 yr. The Whipple's disease patients included two Caucasian males, one 45 yr old and one 47 yr old; both patients had been in remission for 4 yr and were free of gastrointestinal symptoms. One of the patients with selective IgA deficiency was a 10-yr-old Negro girl with ataxia telangiectasia; this patient had a history of recurrent respiratory infection, but no laboratory or clinical evidence of gastrointestinal disease. The second patient with selective IgA deficiency was a 45-yr-old Caucasion woman with calcium malabsorption unassociated with other gastrointestinal abnormalities and with a history of recurrent respiratory infection. The two patients with hypogammaglobulinemia included a 38-yr-old Caucasian woman with late onset disease and a 24-yr-old Caucasian man with hypogammaglobulinemia since childhood but without a family history of hypogammaglobulinemia; both of these patients had recurrent respiratory infections and bronchiectasis but no history of gastrointestinal disease.

Five patients with celiac sprue were studied including four white women ranging in ages from 45 to 64 yr, and a Caucasian boy, age 12. The diagnosis of celiac sprue was based on clinical and biochemical evidence of fat malabsorption and histological demonstration of villous flattening and mononuclear infiltration of the proximal jejunal mucosa. After removal of gluten from the diet, the steatorrhea disappeared, clinical symptoms abated, and histological examination of the jejunal mucosa demonstrated marked improvement in villous architecture. Furthermore, in all five patients reintroduction of gluten into the diet led to a return of the clinical, biochemical, and histological abnormalities. At the time of this study, the patients had been on gluten-free diets for 1–5 yr, although L. H. had not adhered rigidly to her diet.

Clinical procedures

Jejunal biopsies were obtained with a four-hole multipurpose biopsy instrument, the capsule of which was positioned near the ligament of Treitz under fluoroscopic examination (6). Each patient was biopsied one to four times, and an average of three specimens were obtained with each biopsy procedure. At least one specimen from each patient was used for microscopic examination. Specimens used for studies of protein synthesis were placed immediately on iced aluminum foil, gently blotted four times, weighed, and transferred to the incubation medium within 10 min after obtaining the biopsy.

Stool fats were determined by the method of Van der Kamer, ten Bokkel Huinink, and Weijers (7). The D-xylose tolerance test was performed by the administration of 25 g of the pentose and subsequent measurement of xylose in a 5 hr urine specimen. Serum immunoglobulins were measured quantitatively by the radial immunodiffusion technique (8).

Assay of incorporation of L-leucine-^{14}C into total protein and immunoglobulins

Incubation conditions; total and soluble proteins. L-Leucine-^{14}C incorporation into protein by jejunal mucosa in vitro was measured by a modification (9) of the method of Manchester and Young (10). Each tissue specimen was incubated in 1.5 ml of Krebs-Ringer bicarbonate buffer (11) (modified to include one-half the recommended calcium concentration) to which was added: 0.05 ml of a solution containing 50 μmoles each of 20 amino acids including L-leucine, 0.25 ml of 0.85% NaCl solution, and 0.2 ml of 0.85% NaCl solution containing 10 μCi of uniformly labeled L-leucine-^{14}C (34.6 μmoles of L-leucine (New England Nuclear, Boston, Mass.). Incubations were carried out in an atmosphere of 95% O_2 and 5% CO_2 for periods of time ranging from 15 to 90 min. Incubations were terminated by transfer of the biopsy specimen, together with the incubation fluid, to an iced tissue homogenizer containing 1.0 ml of a solution containing 0.5% sodium deoxycholate (added to solubilize microsomal proteins [12]) and 10 mM L-leucine. The specimens were homogenized immediately with 20 strokes and brought to a volume of 12.5 ml with 0.15 M NaCl-phosphate buffer pH 7 containing 10 mM L-leucine. Aliquots were taken in duplicate for determination of protein content (13), and for determination of L-leucine-^{14}C incorporation into total protein. The homogenates were centrifuged at 100,000 *g* for 60 min, and duplicate aliquots were taken from the supernate for determination of L-leucine-^{14}C incorporation into immunoglobulins.

To measure L-leucine-^{14}C incorporation into total and soluble proteins, protein was precipitated from the initial tissue homogenate (total protein) and the supernatant solutions of these homogenates (soluble protein), which had been centrifuged at 100,000 *g*. Proteins were precipitated by addition of 20% TCA[1] containing 10 mM L-leucine to an equal volume of homogenate or of the 100,000 *g* supernatant solution. The precipitates were separated by centrifugation at 2600 *g*, and then resuspended in 10% TCA containing 5 mM L-leucine, heated at 90°C in a water bath for 15 min, washed three times with the TCA-leucine solu-

[1] Abbreviations used in this paper: DPO, 2,5-diphenyloxazole; NCS, *N*-chlorsuccinimide; PAS, para-aminosalicylic acid; RGG, rabbit gammaglobulin; TCA, trichloroacetic acid.

tion and once each with ethanol: ether (1:1) and ether. The dry precipitates were dissolved in 1 ml NCS solvent, and the NCS solution was dissolved in 13 ml of 0.435% DPO and toluene in glass counting vials for determination of radioactivity in a liquid scintillation spectrometer. Values for counts per minute were converted to disintegrations per minute by the channels ratio method (14).

Immunological reagents. Specific antisera were prepared in order to measure incorporation of L-leucine-^{14}C into immunoglobulins. Anti-IgA was produced by immunization of a sheep with myeloma IgA. The anti-IgA antiserum was absorbed with human IgG and serum deficient in IgA, and its specificity was determined by Ouchterlony double diffusion and radioimmunoelectrophoresis against IgG, IgM, albumin, and IgA. Anti-IgM was produced by immunization of a sheep with IgM obtained from a patient with Waldenström's macroglobulinemia. The anti-IgM antiserum was absorbed with human IgG, and its specificity was determined by Ouchterlony double diffusion against IgG, IgA, albumin, and IgM. In addition, the specificity of the anti-IgA and anti-IgM antisera was confirmed by immunoprecipitation under the experimental conditions described below against ^{125}I-labeled IgA, IgM, IgG, IgE, and human serum albumin. Anti-IgA precipitated 99.8% of the IgA-^{125}I and less than 0.5% of the other labeled proteins (which had approximately the same specific activity as the IgA-^{125}I). Anti-IgM precipitated 100% of the IgM-^{125}I and less than 2% of the other labeled proteins.

Measurement of L-leucine-^{14}C incorporation into IgA and IgM. Incorporation of L-leucine-^{14}C into IgA and IgM was determined by counting immune precipitates formed by these immunoglobulins and their specific antibodies to IgA and IgM. The precipitations were performed in 3-ml centrifuge tubes, in duplicate, on 1 ml aliquots of soluble protein fraction (100,000 *g* supernatant solution). To insure complete precipitation of the ^{14}C-labeled IgA and IgM, cold carrier immunoglobulins were added in proportions determined experimentally by precipitation curve analysis to results in antibody excess. For every determination made, complete precipitation of the immunoglobulin was confirmed by the complete precipitation of radioiodinated IgA and IgM in parallel tubes. The tubes containing the soluble protein, the specific antibody, and its carrier antigen were incubated for 1 hr at 37°C and 48 hr at 4°C. Precipitates that resulted were separated by centrifugation at 2600 *g* at 4°C, and washed three times with 0.15 M NaCl-phosphate buffer pH 7.0 containing 10 mM L-leucine. A 20% TCA solution containing 10 mM L-leucine was added to the precipitates, and the procedure described above for determination of total and soluble protein synthesis was performed.

When various antigens unrelated to gastrointestinal proteins (such as bovine serum albumin or rabbit gammaglobulin [RGG]) were added to antibodies (anti-bovine serum albumin or anti-rabbit gammaglobulin [anti-RGG]), the precipitates contained radioactivity bound nonspecifically (nonspecific counts). The amount of ^{14}C-labeled substances bound by these antigen-antibody complexes were related only to the total amount of ^{14}C-labeled proteins originally present in the solutions. These nonspecific counts constituted a blank for which the assay procedure had to be corrected. Therefore, the values for total radioactivity precipitated with anti-IgA and anti-IgM were corrected for nonspecific counts by subtraction of the values for counts brought down nonspecifically by an unrelated antibody-antigen system, in this case RGG–anti-RGG, determined in duplicate in parallel experiments. The nonspecificity of the RGG–anti-RGG binding was confirmed by immunoprecipitation under experimental conditions in the presence of ^{125}I-labeled IgA, IgM, IgG, IgE, or human serum albumin. RGG–anti-RGG bound less than 2% of each of the labeled proteins except IgG-^{125}I; 4.5% of the added IgG-^{125}I was bound.

To prove that the values for nonspecific counts bound by the specific anti-immunoglobulin precipitates (IgA–anti-IgA or IgM–anti-IgM precipitates) were the same as the values for the counts bound by the RGG–anti-RGG precipitates, the following experiment was performed. IgA–anti-IgA precipitates were formed in two separate tubes containing labeled biopsy homogenates by adding anti-IgA and carrier IgA. The precipitates bound labeled IgA as well as nonspecific counts. The precipitates were then removed and additional anti-IgA and carrier IgA were added to the supernatant solution in one tube and anti-RGG and carrier RGG were added to the second tube. The new precipitates bound an equal number of counts, indicating that they had an equivalent capacity to bind nonspecific counts when labeled IgA was not present.

To prove that RGG–anti-RGG precipitates do not bind labeled IgA, the values for IgA counts were determined by two different procedures (Table I) and compared. In procedure I, which was used routinely in the present studies, two tubes were prepared, each containing labeled homogenate. Anti-IgA and carrier IgA were added to tube 1 and anti-RGG and carrier RGG were added to tube 2. The value for counts brought down by the RGG–anti-RGG precipitate was subtracted from the value for counts brought down by the IgA–anti-IgA precipitate to obtain a value of 1013 cpm for the amount of labeled IgA in the homogenate.

TABLE I

Evaluation of Method for Determination of Incorporation of L-Leucine-^{14}C into Immunoglobulins: Studies of Nonspecific Counts

Procedure		Tube 1 Addition	Tube 1 Precipitate (a)	Tube 2 Addition	Tube 2 Precipitate (b)	IgA (a) − (b)
			cpm		*cpm*	*cpm*
I		IgA, anti-IgA	1673	RGG, anti-RGG	660	1013
II	A	RGG, anti-RGG	625	RGG, anti-RGG	695	
	B	IgA, anti-IgA	1290	RGG, anti-RGG	248	1042

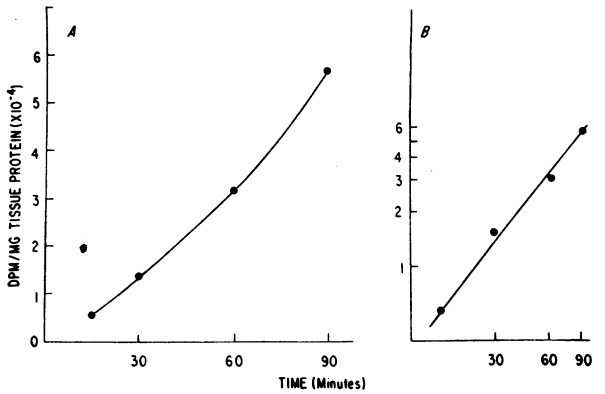

FIGURE 1 Incorporation of L-leucine-^{14}C into total protein as a function of time. (A) Linear plot. (B) Log-log plot.

In procedure II, which was carried out in two steps, A and B, anti-RGG and carrier RGG were first (step A) added to two tubes (1 and 2) each containing labeled homogenate, and the resulting precipitates were removed by centrifugation. In step B, anti-IgA and carrier IgA were added to the supernatant solution remaining in tube 1, and anti-RGG and carrier RGG were added to the supernatant solution remaining in tube 2. As in procedure I, the value for counts bound by the RGG–anti-RGG precipitate was subtracted from the value for counts bound by the IgA–anti-IgA precipitate to obtain the value for specific counts in the homogenate (1042 cpm). This value was equal, within experimental error, to that obtained without prior exposure of the labeled homogenate to RGG–anti-RGG precipitation (procedure I). This indicated that RGG–anti-RGG precipitates do not bind labeled IgA.

Relation of L-leucine-^{14}C incorporation to protein and immunoglobulin synthesis. Incorporation of L-leucine-^{14}C into a protein can be related quantitatively to the synthesis of the protein only if the specific activity of the amino acid precursor pool and the degradation rate of the protein are known (15). We attempted to diminish variation in the amino acid precursor pool in the present studies by adding a mixture of amino acids containing unlabeled leucine to the incubation mixtures. We did not, however, make direct measurements of intracellular precursor pool specific activity or protein degradation rates; therefore, conclusions we draw about rates of synthesis of proteins from the incorporation data are dependent on the reasonable assumption that these activities and rates were constant.

When an inhibitor of protein synthesis, puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio), was added to the incubation medium in a final concentration of 1 mmole/ml, L-leucine-^{14}C, incorporation into total protein was reduced by 89%, incorporation into soluble protein by 88%, incorporation into IgA by 93%, and incorporation into IgM by 100%. These observations provide evidence that L-leucine-^{14}C incorporation reflects protein synthesis.

Kinetics of L-leucine-^{14}C incorporation into proteins. When the data are plotted on a linear graph, the relation between incorporation of L-leucine-^{14}C into protein (disintegrations per minute per milligram tissue protein) and incubation time is represented by a curved line (Fig. 1 A). When plotted on a log-log graph, the data fall on a straight line (Fig. 1 B). The fact that linearity is obtained by plotting the data in logarithmic form implies that for every fractional change in time there is a constant fractional change in incorporation over the time period studied. This would be expected if the amino acid incorporation process were the product of multiple linear steps.

Statistical analysis of data. Data for incorporation of L-leucine-^{14}C were plotted logarithmically and fitted with regression lines by means of the least squares technique (Fig. 2) in order to compare results obtained in different studies. The regression lines were parallel for patients and normal individuals for any given protein studied. Thus, any time point on the regression lines could be used for comparison of studies and the 60 min value was chosen. *P* values for differences between results obtained in any two studies were calculated from the average variations of experimental values from the regression lines.

RESULTS

Studies of normal volunteers and patients with celiac sprue in remission, Whipple's disease in remission, and immune deficiency

Histological and laboratory findings. Histological examination of the jejunal biopsies taken from the normal volunteers and the patients with IgA deficiency and Whipple's disease revealed normal villous architecture and plasma cell populations. There was a persistence of PAS-positive macrophages in the mucosa of the patients with Whipple's disease although no bacillary bodies were present. In biopsies obtained from the patients with hypogammaglobulinemia, there were no plasma cells present, but the mucosal architecture was

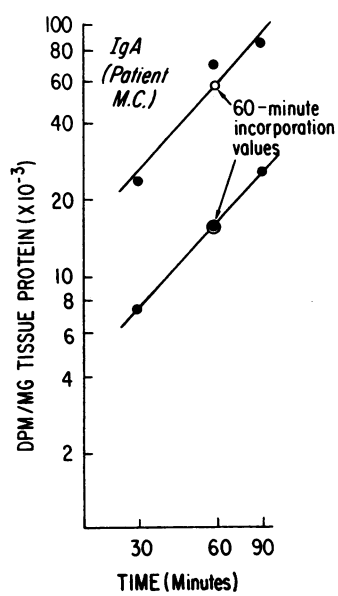

FIGURE 2 Incorporation of L-leucine-^{14}C into IgA by jejunal mucosa from patient M. C., as a function of time. Regression lines such as shown here were plotted for each study of each patient. Since the lines were parallel for each protein, it was feasible to select an arbitrary time, 60 min, for comparison of data.

otherwise normal. In the specimens of four of the five patients with celiac sprue in remission there was some persistent flattening of the villi although the lining epithelial cells were columnar and had basally oriented nuclei. The biopsy specimen of the fifth patient (G. K.) with celiac sprue had normal villi.

Results of measurements of fat and carbohydrate absorption (stool fat, serum carotene, D-xylose absorption) were normal in the normal volunteers, in the patients with Whipple's disease in remission, and in the patients with immune deficiency states. Only one of the patients (L. H.) with celiac sprue had laboratory evidence of malabsorption. This patient did not adhere rigidly to the gluten-free diet. Results of measurements of the concentration of IgA in serum (Table II) were normal in all patients studied except for one patient (A. C.) with celiac sprue in remission, and the patients with immune deficiency states. A. C. had an elevated concentration of IgA in serum, and the patients with hypogammaglobulinemia, the patient with ataxia telangiectasia, and the patient with isolated calcium malabsorption had no detectable IgA in their serum.

Incorporation of L-*leucine-*^{14}*C into total and soluble protein.* The mean control value for intestinal incorporation of L-leucine-^{14}C into total protein (Fig. 3 A) established in eight studies of six normal volunteers was 156,700 dpm/mg protein per hr with 95% confidence limits (mean ±2 SD) of 103,400–237,300. The patients with celiac sprue were studied when they were in remission on a gluten-free diet. The value for G. K., the patient with normal intestinal histology, and for two of the four patients with villous flattening were within the 95% confidence limit of the control data, whereas the values for the remaining two patients were elevated. The values for the patients with Whipple's disease in remission and patients with hypogammaglobulinemia were within the 95% confidence limits of the control data. The values for the two patients with isolated IgA deficiency were somewhat elevated.

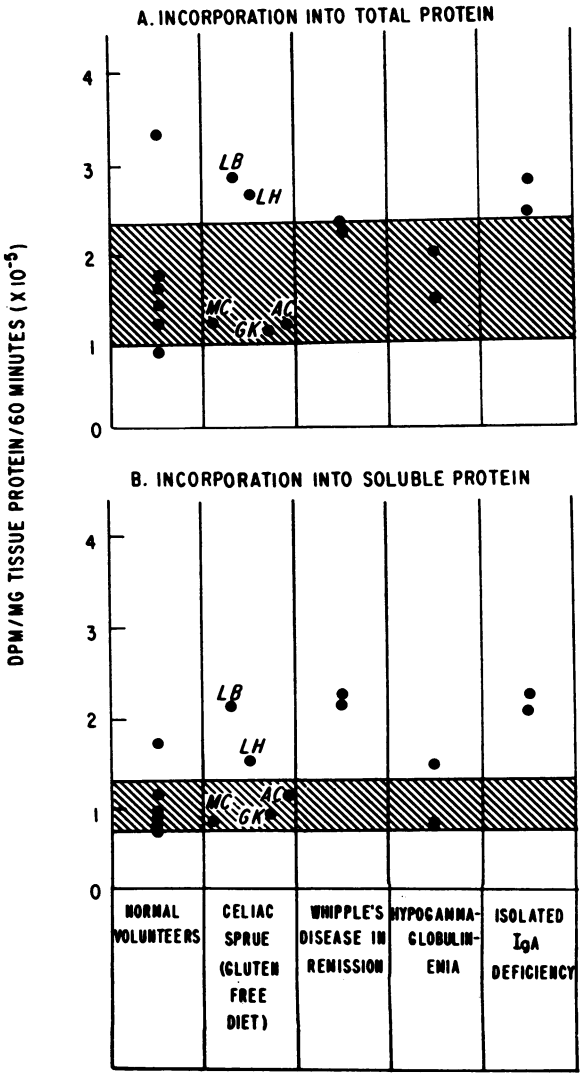

FIGURE 3 Incorporation of L-leucine-^{14}C into (A) total and (B) soluble protein by jejunal mucosa from normal volunteers and patients.

TABLE II

Serum IgA Concentration in the Patients with Celiac Sprue

Subject	Serum IgA	
	Gluten-free diet	Gluten-containing diet
	mg/ml	
Normal volunteers (50)	2.6 ±1.1 (SD)	
L. B.	2.25	2.70
M. C.	2.70	2.90
G. K.	1.10	1.05
L. H.	2.85	3.00
A. C.*	8.80	—

* Not challenged with gluten-containing diet.

The values for incorporation of L-leucine-^{14}C into soluble protein (Fig. 3 B) revealed generally similar results. Once again, the values for two of the five patients with celiac sprue were elevated, as were the values for each patient with isolated IgA deficiency. In this case, however, the values for the patients with Whipple's disease and for one of the patients with hypogammaglobulinemia were greater than the upper 95% confidence limits of the control data.

Incorporation of L-*leucine-*^{14}*C into IgA and IgM.* The mean control value for intestinal incorporation of L-leucine-^{14}C into IgA (Fig. 4 A) was 4853 dpm/mg

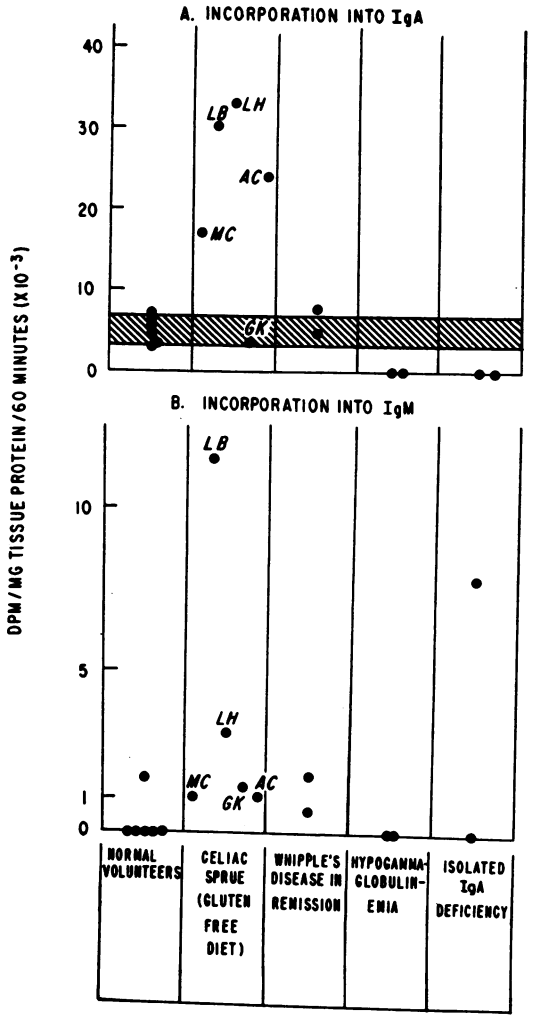

FIGURE 4 Incorporation of L-leucine-^{14}C into (A) IgA and (B) IgM by normal volunteers and patients.

protein per hr with 95% confidence limits (mean ±2 SD) of 3400–6900. Thus, in normal individuals the value for incorporation of labeled amino acid into IgA was approximately 3% of the value for incorporation into total protein. Four of the five patients with celiac sprue had abnormally high values for intestinal incorporation of L-leucine-^{14}C into IgA while they were on gluten-free diets. The value for the fifth patient, G. K., with normal jejunal histology, was within the 95% confidence limits of the control data. The value for one of the patients with Whipple's disease was within the 95% confidence limits of the control data; the value for the other patient was slightly greater than the upper limit. We could detect no significant ($P < 0.05$ for the difference between the observed value and zero) incorporation of labeled leucine into IgA for the patients with hypogammaglobulinemia or for the patients with isolated IgA deficiency.

For all but one of the normal volunteers studied, the intestinal incorporation of L-leucine-^{14}C into IgM (Fig. 4 B) could not be distinguished from zero ($P < 0.05$). Incorporation into IgM was detected for the five patients with celiac sprue on a gluten-free diet with values approximately 5–20% of the corresponding values for incorporation into IgA. This percentage range is consistent with the determined ratio of IgA-containing cells to IgM-containing cells detected in intestinal mucosa by use of specific fluorescent antiserum (4). No incorporation of labeled leucine into IgM was detected for the patients with hypogammaglobulinemia, whereas the value for one of the patients with isolated IgA deficiency (ataxia telangiectasia) was relatively high, 7800 dpm/mg protein per hr. The intestinal tissue from the other patient with isolated IgA deficiency failed to incorporate L-leucine-^{14}C into IgM.

The inability to detect incorporation of L-leucine-^{14}C into IgA by biopsy specimens from the patients with IgA deficiency and hypogammaglobulinemia is evidence that their ability to synthesize IgA in the gastrointestinal tract is impaired and is consistent with previous findings of decreased, or absent, IgA immunofluorescence of plasma cells in the intestinal mucosa of patients deficient in serum and secretory IgA (3).

Day-to-day variation. To examine variation of L-leucine-^{14}C incorporation by jejunal specimens on two different days, seven patients, two with celiac sprue, two normal controls, two patients with Whipple's disease in remission, and one with hypogammaglobulinemia, were studied. No significant ($P < 0.05$) variation was observed for any of the protein classes measured except in the case of one patient with Whipple's disease in remission who showed significant changes in incorporation into total and soluble protein but not into IgA. Because of the lack of variation, it seemed reasonable to compare studies of patients with celiac sprue before and after addition of gluten to the diet, and to attribute significant differences in observed results to effects of gluten rather than to biological variation.

Studies in patients with celiac sprue challenged with dietary gluten

Four of the five patients with celiac sprue who had been studied while in remission were fed gluten-containing diets for short periods of time (6–16 days). During the challenge, repeat determinations were made of stool fat excretion, serum carotene concentration, D-xylose absorption, and serum IgA concentration. Repeat jejunal biopsies were obtained during and at the termination of the gluten challenge, and the incorporation in vitro of

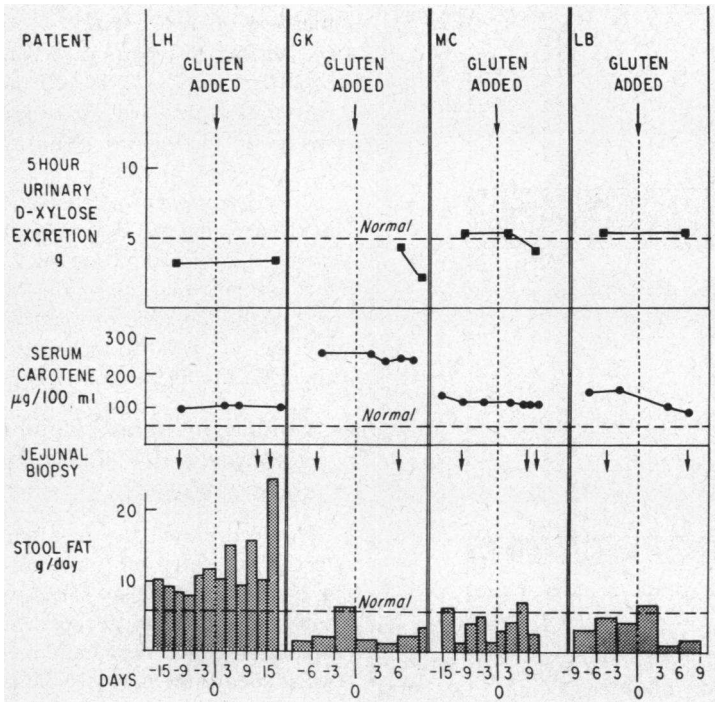

FIGURE 5 Indices of intestinal absorption of fat and carbohydrate before and during gluten challenge in patients with celiac sprue.

L-leucine-^{14}C into total protein, soluble protein, IgA, and IgM was determined.

Histological and laboratory findings. Histological examination of the jejunal biopsy specimens obtained from the four patients during gluten challenge demonstrated no change in villus-crypt ratios or mucosal thickness. However, the intestinal epithelial cells became more cuboidal and the nuclei were displaced from their normal basal location.

With one exception, there was no apparent change in the determined parameters of intestinal absorption of fat and carbohydrate during the period of gluten administration at the time jejunal biopsies were taken (Fig. 5). L. H., who had slight steatorrhea at the termination of the gluten administration, excreted 22.6 g of fat per day in her stool at the termination of the gluten challenge. The concentration of IgA in serum did not change during the period of gluten administration (Table II).

Incorporation of L-leucine-^{14}C into total protein and soluble protein. After patient G. K., with normal jejunal histology, was on a gluten-containing diet for 6 days, his intestinal incorporation of L-leucine-^{14}C into total protein was 60% higher than it was when he was on a gluten-free diet (Fig. 6 A). Although the higher value was still within the 95% confidence limit of the control data, the increase was statistically significant ($P < 0.05$). Of the three patients with flattened intestinal mucosa studied, only one (M. C.) had a statistically significant ($P < 0.05$) increase (300%) in incorporation of labeled leucine into total protein. This increase was evident on the 12th day after institution of the gluten-containing diet but not on the 9th day. The values for the other two patients, L. B. and L. H., increased, but not significantly (30 and 4%, respectively).

The effects of a gluten-containing diet on intestinal incorporation of L-leucine-^{14}C into soluble protein (Fig. 6 B) were generally similar to the effects on incorporation into total protein. A 30% increase in incorporation was detected for G. K. but this was not significant. Significant ($P < 0.05$) increases were observed for M. C. (300%) and for L. H. (50% after 16 days). The increase (30%) observed for L. B. was not significant.

In summary, it appears that for three of the four patients studied there was an increase in incorporation of L-leucine-^{14}C into total protein, soluble protein, or both, after a brief exposure to a gluten-containing diet. These findings complement earlier studies (16) which showed increased incorporation of L-leucine-^{14}C into total protein by jejunal biopsy specimens of patients with celiac sprue. The increased L-leucine-^{14}C incorporation into protein shown in those and the present studies can be related to the abnormal cell turnover observed by Creamer (17) who investigated mucosal biopsies of celiac sprue patients by use of radioautography and by counting mitoses.

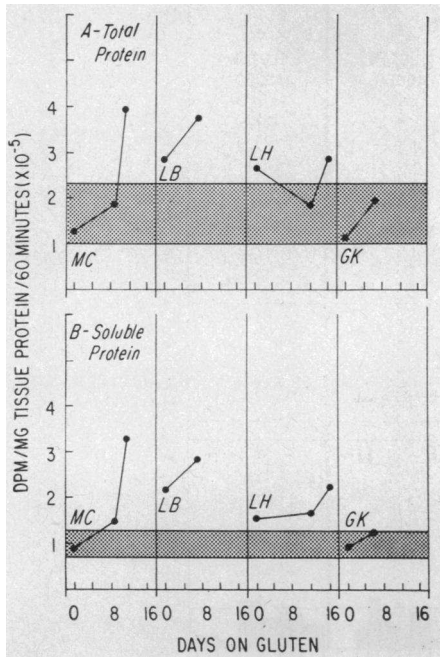

FIGURE 6 Incorporation of L-leucine-^{14}C into (A) total protein and (B) soluble protein before (day 0) and during gluten challenge by patients with celiac sprue.

Incorporation of L-leucine-^{14}C into IgA. Intestinal incorporation of L-leucine-^{14}C into IgA increased significantly for all four patients after they were challenged with gluten-containing diets (Fig. 7 A). Patient G. K., whose value for incorporation into IgA was within the 95% confidence limits of the control data before challenge, had a 3-fold increase in incorporation (from 3912 to 12,970 dpm/mg protein per hr) after challenge. The other patients, whose values for incorporation into IgA were well above the 95% confidence limits of the control data while they were on a gluten-free diet, had a 2- to 3-fold increase ($P < 0.05$) in the value for L-leucine-^{14}C incorporation into IgA after challenge with a gluten-containing diet. For three of the four patients the increase in incorporation of L-leucine-^{14}C into IgA occurred before gastrointestinal absorptive changes were detected.

Because the incorporation of L-leucine-^{14}C into total protein increased in some of the patients with celiac sprue after gluten challenge, we determined whether the fractional change in incorporation into IgA was greater than the fractional change in incorporation into total protein. In the studies of G. K., incorporation into IgA increased 3.3-fold, whereas incorporation into total protein increased 1.6-fold after gluten challenge. Thus, the fractional change in incorporation into IgA was twice the fractional change in incorporation into total protein. In the studies of the other three patients, the fractional increase in incorporation of L-leucine-^{14}C into IgA exceeded that of incorporation into total protein by factors of 1.6, 1.5, and 1.1. In all, the fractional change in incorporation into IgA was significantly greater than the change in incorporation into total protein for two of the four patients ($P < 0.1$).

Incorporation of L-leucine-^{14}C into IgM. There was insufficient data to determine day-to-day variation in the studies of incorporation of L-leucine-^{14}C into IgM. If one assumes that, as in the case of IgA, such variation was negligible, each of the patients studied showed a significant ($P < 0.05$) increase in incorporation of L-leucine-^{14}C into IgM (Fig. 7 B).

Studies in normal volunteers maintained on a gluten-free diet and then challenged with dietary gluten

We considered the possibility that the effect we observed by gluten on intestinal immunoglobulin synthesis in patients with celiac sprue was a nonspecific phenomenon attributable to abrupt reexposure to gluten after a period of gluten deprivation. As a control investigation, we studied three normal volunteers who were fed gluten-free diets for 3 wk, subjected to intestinal biopsies, placed on normal gluten-containing diets, and then subjected to intestinal biopsies again after 7 days. During these studies the fecal excretion of fat by the subjects did not change significantly.

In the study of the first volunteer the biopsy specimens were assayed for IgA synthesis as described under Methods. In the studies of the remaining two volunteers a modified and improved assay procedure was used.[2] In this procedure the biopsy specimens were incubated with L-leucine-^{14}C as before, but the newly synthesized, labeled IgA was isolated by use of a complex comprising specific anti-IgA antibodies covalently coupled to cellulose. After the incubation with L-leucine-^{14}C, the biopsy specimen was homogenized and exposed to the anti-IgA–cellulose complex so that the ^{14}C-labeled IgA was bound specifically to the complex. The complex, together with the bound IgA-^{14}C, was isolated by filtration and counted directly on a filter paper disc. The value for nonspecific binding in this procedure was obtained from the number of counts bound after incubation of labeled homogenate with anti-IgA–cellulose previously exposed to an excess of unlabeled IgA. The range of values obtained by use of this method for incorporation of L-leucine-^{14}C into IgA by intestinal biopsy specimens from normal volunteers, as well as from celiac sprue patients, agrees closely with the range of values obtained by the procedure described above under Methods. Thus, biopsy specimens from two celiac sprue patients on gluten-free diets in-

[2] Falchuk, Z. M., W. Strober, and L. Laster. Unpublished observations.

corporated 39,702 ±561 and 33,088 ±2687 counts/mg tissue protein per hr, comparing well with data obtained by the precipitation technique (see Fig. 4 A).

For the volunteers studied, the values for incorporation of L-leucine-^{14}C into IgA did not differ significantly ($P > 0.6$) in biopsies obtained before and after the exposure to dietary gluten (Table III). These observations show that when normal individuals are deprived of gluten for 3 wk and then exposed to this protein, there is no stimulation of incorporation of L-leucine-^{14}C into IgA by intestinal mucosa as occurs in patients with celiac sprue.

DISCUSSION

The results of the present studies of the incorporation of L-leucine-^{14}C into protein by intestinal mucosal biopsies lead to two conclusions. First, the human small intestine mucosa is capable of local synthesis of immunoglobulins and, second, in patients with celiac sprue, this local mechanism responds to exposure to dietary gluten by increasing the production of IgA and IgM. Furthermore, the effect of dietary gluten on intestinal incorporation of L-leucine-^{14}C into immunoglobulins exceeded the effect on incorporation into total protein in at least two of the patients studied. Thus, even if one were to assume that immunoglobulin production could increase through a mechanism other than one involving antigenic stimulation, the present results indicate that it is unlikely that the observed increased incorporation of L-leucine-^{14}C into immunoglobulins was merely a nonspecific reflection of a generalized stimulation of protein synthesis.

When the four celiac sprue patients with flattened mucosa (but not the one with histologically normal mucosa) were on a gluten-free diet, their biopsy specimens incorporated more L-leucine-^{14}C into immunoglobulins than did specimens from control subjects. This difference may be due to persistent stimulation by small amounts of dietary gluten, or to the possibility that measurements obtained with a biopsy specimen of flat mucosa are not strictly comparable to those obtained with a normal specimen. However, the latter alternative does not explain differences we observed for an individual before and after introduction of gluten into the diet. In those instances, the specimens were histologically comparable, and changes in L-leucine-^{14}C incorporation most probably reflected true differences among rates of intestinal synthesis of immunoglobulins.

The present demonstration of a local intestinal immune response bears on the question of whether or not immunologic reactions play a role in the pathogenesis of celiac sprue. The improvement in patients with celiac sprue noted after treatment with adrenal steroids may be one indication of such a role (18). More direct investigations of immunologic mechanisms in celiac sprue have included determinations of anti-gluten antibodies in serum and intestinal secretions, measurement of circulating immunoglobulins, and studies of immunofluorescent staining properties of intestinal mucosal cells.

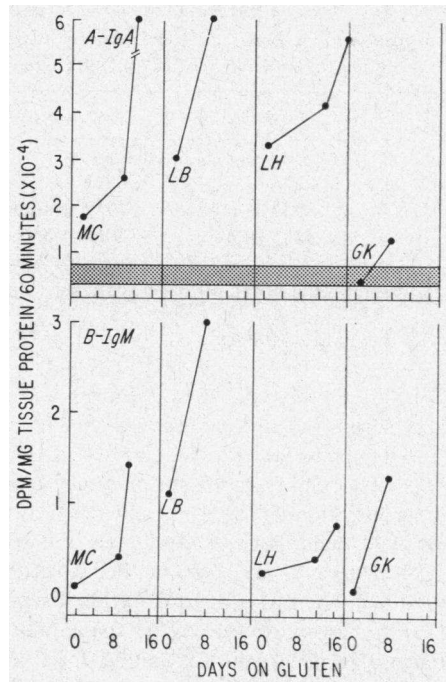

FIGURE 7 Incorporation of L-leucine-^{14}C into (A) IgA and (B) IgM before (day 0) and during gluten challenge.

Values for the concentration of immunoglobulins and specific antibodies in serum have been determined. Most investigators have found that the concentration of IgA in serum is elevated in patients with untreated celiac sprue and that after removal of gluten from the diet the concentration falls toward normal (19–21). In the serum of about a third of patients with untreated sprue the concentration of IgM was low; concentrations of IgD and IgE appeared to be normal and IgG concentrations were variable and usually near normal (19, 20, 22).

The mechanism by which the concentration of IgA in serum may be altered in sprue is not established. Eidelman, Davis, Lagunoff, and Rubin (21) found an increased absolute number of plasma cells containing IgA in jejunal mucosa of patients with celiac sprue, and Thompson, Asquith, and Cooke (23) found secretory IgA (IgA dimer associated with T-piece) in the serum of 41% of patients with celiac sprue but in only 9% of healthy individuals. These findings suggest that local gastrointestinal production of IgA is partly responsible for the increased IgA concentration in the serum of the patients. However, doubt has been cast on this hypothesis by the failure in more recent studies to detect an increase in the number of IgA-containing mucosal cells

TABLE III

Incorporation of L-Leucine-^{14}C into IgA of Normal Volunteers Pre- and Postgluten Challenge

	Prechallenge	Postchallenge
	dpm/mg protein per hr	
D. L.*	5328	6925
H. B.‡	5936 ±1212	7912 ±4836
A. Y.‡	3103 ±339	3064 ±356

* Assayed by immune precipitation as described under Methods.

‡ Assayed by anti-IgA–cellulose.

(23) and by the observation that the presence of secretory IgA in serum did not correlate with the concentration of IgA in serum or with the activity of the disease.

The other abnormality in serum immunoglobulins, the reduced concentration of IgM in celiac sprue patients, is also not well understood. Despite the low concentration of IgM in serum, and despite the detection of decreased total body synthesis of IgM in this disease, as determined by turnover studies (24), the number of intestinal mucosal plasma cells containing IgM was found to be increased (20). The concentration of IgM in serum does not appear related to the severity of the disease (19, 22) or to the presence of splenic atrophy (24, 25).

Specific circulating antibodies to gluten and gluten fractions were found by several investigators in celiac sprue (26–29). However, their relation to the gastrointestinal lesion in celiac sprue was questioned because circulating antibodies to other dietary proteins, such as milk proteins, were also demonstrated. Furthermore, anti-gluten antibodies were found in other diseases in which mucosal integrity is disrupted, such as ulcerative colitis. These findings suggest that circulating antibodies to gluten are attributable, at least in part, to abnormal permeability of the intestinal mucosa in celiac sprue to various dietary proteins, and to subsequent stimulation of internal lymphoid tissue by such proteins.

Local gastrointestinal production of anti-gluten antibodies has been regarded as unlikely because of the failure to detect gluten-binding antibodies in jejunal biopsy specimens by use of immunofluorescence techniques (5). More recently, however, Katz, Kantor, and Herskovic (30) found antibodies to a trypsin-pepsin digest of gluten in the jejunal aspirates of a high percentage of patients with untreated celiac sprue. The immunoglobulin class of these antibodies was not determined so that certain inferences regarding their origin and significance cannot be made. If the anti-gluten antibodies were in the IgA class, it would indicate that they originated in local intestinal immune systems because very little IgA is transported from the systemic circulation into the external secretions (31). However, if they were in the IgG class, it would be possible that they were transported from the serum, since about 50% of IgG immunoglobulins in external secretions are derived from the serum (31).

The present studies indicate that increased immunoglobulin synthesis does indeed occur locally in the gastrointestinal tract after gluten challenge, and moreover, that the increase in synthesis takes place soon after the introduction of gluten into the diet, and generally before functional gastrointestinal disturbances can be observed. These findings lend greater weight to the supposition that immune mechanisms are involved as one of the primary factors in the pathophysiology of celiac sprue. Thus, the proximity of an immune mechanism to the site of initial exposure to gluten makes it reasonable to postulate that an immunological reaction plays a role in the initiation of the intestinal lesion.

The additional immunoglobulins produced after gluten challenge may be specific for gluten, for other exogenous proteins, or for proteins of the epithelium which gain access to antibody-producing cells through the disruptive action of gluten. Since Rubin, Fauci, Sleisenger, and Jeffries (5) presented evidence that gluten binds to the epithelial cells of patients with celiac sprue but not those of normal individuals, it may well be that the excess immunoglobulins are composed largely of anti-gluten antibodies. Such antibodies would be free to react with gluten on epithelial cell membranes and could thereby lead to immune destruction of the epithelial cells.

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