

Boosted Systemic Immune and Local Responsiveness After Intestinal Inflammation in Orally Sensitized Guinea Pigs

MARIE-JOSÉ FARGEAS,* VASSILIA THEODOROU,* JEAN MORE,* JEAN-MICHEL WAL,† JEAN FIORAMONTI,* and LIONEL BUENO*

*Department of Pharmacology, Institut National de la Recherche Agronomique, Toulouse; and †Laboratory of Pharmacology and Immunology, Institut National de la Recherche Agronomique, Gif sur Yvette, France

Background & Aims: Intestinal inflammation resulting in disruption of the mucosal barrier function has been proposed as a cause of increased incidence of allergic diseases. This study was designed to evaluate whether intestinal inflammation is able to change the immune responsiveness to sensitization and antigen challenge responses. **Methods:** Guinea pigs orally sensitized to cow's milk proteins were either treated or not treated with trinitrobenzenesulfonic acid (TNBS) to induce intestinal inflammation and compared with control animals (not sensitized). Systemic immune and local responsiveness to antigen challenge were assessed by measuring antibody serum titers, colonic fluid secretion, mucosal histamine level, and mucus depletion. Intestinal permeability was evaluated from ^{51}Cr -ethylenediaminetetraacetic acid (EDTA) recovery and β -lactoglobulin serum level. **Results:** Immunoglobulin E titers were higher in TNBS-treated animals than in non-TNBS-treated sensitized animals. Antigen challenge in TNBS-treated animals induced a fourfold increase of colonic secretion and greater histamine and mucus depletion than in non-TNBS-treated animals. Permeability to ^{51}Cr -EDTA increased 5 days after TNBS treatment but was unchanged after antigen challenge. In contrast to controls, β -lactoglobulin was not detected in the sera of challenged sensitized and TNBS-treated animals. **Conclusions:** Intestinal inflammation increasing gut permeability enhances the sensitization process. Therefore, local anaphylactic reactions are exacerbated after antigen challenge.

Food allergies are defined as an abnormal immune response to food antigens. They are characterized by type I immediate hypersensitivity reactions whose clinical manifestations are rhinitis, urticaria, or eczema and gastrointestinal symptoms such as nausea, vomiting, abdominal pain, and diarrhea. Antigen uptake from the intestinal lumen results in the production of antibodies, mainly immunoglobulin (Ig) E but also IgG in humans^{1,2} and guinea pigs,^{3,4} which are linked to high-affinity receptors on mast cells. Subsequent exposure to the antigen

cross-links the antibodies and triggers mast cell degranulation and release of several mediators that are responsible for the typical manifestations of allergic reactions.

The cause of food allergy is poorly known but is multifactorial, including components such as heredity, age, or environment. Although the complete mechanisms of gastrointestinal allergy are not fully elucidated, it seems that the intestinal transport of allergens through the intestinal mucosa is a necessary initial step in the process. Intestinal inflammation seems to be a predisposing factor for increased sensitization of a subject. Indeed, intestinal inflammation damaging the mucosal epithelium alters the mucosal barrier permeability that normally prevents antigen uptake. As a result, a greater amount of antigens may traverse the mucosal barrier and favor allergic reactions. However, there are conflicting results concerning allergic diseases in inflammatory bowel diseases (IBDs) such as ulcerative colitis or Crohn's disease: some studies suggest an increase of atopic diseases,⁵ whereas others have noted no difference from control populations.⁶ Nevertheless, serum levels of IgG and IgM in response to cow's milk proteins are more elevated in patients with IBD than in controls,^{7,8} reflecting a specific immunization. Moreover, the use of parenteral nutrition and an exclusion diet have been shown to be beneficial in the treatment of IBD.⁹ Furthermore, enhanced uptake of bovine serum albumin has been shown in the circulation of rats in whom intestinal inflammation was induced by infestation with a nematode.¹⁰

Although there are good reasons to suppose that increased permeability to antigens provides enhanced sensitization and consequently further anaphylactic reactions after antigen challenge, direct experimental evidence is

Abbreviations used in this paper: AChE, acetylcholinesterase; EIA, enzyme immunoassay; PAS, periodic acid-Schiff; PCA, passive cutaneous anaphylaxis; S, sensitized (group); TNBS, trinitrobenzenesulfonic acid.

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missing. The present study therefore examined whether intestinal inflammation induced during the sensitization process is able to produce a larger degree of sensitization (specific IgE and IgG serum levels) and to modify the local mucosal responses after antigen challenge (anaphylaxis-induced intestinal hypersecretion). To gain insight into the mechanisms involved, intestinal permeability measures and histological and biochemical studies were designed. Guinea pigs that were sensitized to milk proteins after drinking cow's milk were used as a clinically relevant model of cow's milk allergy.^{11–13} Intestinal inflammation was triggered by intrajejunal administration of trinitrobenzenesulfonic acid (TNBS); this model of experimental inflammation has been carefully evaluated¹⁴ and is now used in guinea pigs.¹⁵

Materials and Methods

Animals

The study was performed with male Dunkin–Hartley guinea pigs (250–300 g) (Interfauna, Loches, France). Thirty were used for biochemical, histological, and intestinal secretion studies, and 24 were used for ⁵¹Cr–ethylenediaminetetraacetic acid (EDTA) permeability studies. In these two series, animals were divided in three groups: control, sensitized, and sensitized plus TNBS treatment.

Sensitization and Induction of Inflammation

In the first series, 20 animals were sensitized to cow's milk proteins by giving them only fresh pasteurized cow's milk to drink for 4 weeks. The remaining 10 animals were given water instead of milk and were used as controls (control group). Animals were fully accustomed to drinking milk 2–3 days after the beginning of the milk diet. At this time, the first half of the animals (*n* = 10) were given a 0.3-mL intrajejunal injection of TNBS in 50% ethanol at a dose of 50 mg/kg (sensitized [S] plus TNBS group) using a thin hypodermic needle under anesthesia (0.5 mg/kg acepromazine and 100 mg/kg ketamine) and aseptic conditions. This injection was given in the distal part of the jejunum, because the jejunum is known as a major site for absorption. Saline (0.9% NaCl) was administered in the other half (*n* = 10) using the same conditions as for the TNBS-treated group (S group). The control group also underwent sham operations.

This model of inflammation is an adaptation of the rat colitis TNBS-induced model described by Morris et al.¹⁶ Later on, Miller et al.¹⁴ adapted this model to induce ileitis in the guinea pig and showed that TNBS administration evokes an inflammatory process that peaks around days 7–14 and declines toward basal conditions. By day 30, the different parameters studied (granulocyte infiltration, mucosal leak of proteins, and absorptive vs. secretory state of the mucosa) are largely normalized. Previous studies in our laboratory¹⁵ showed that

TNBS administration into the guinea pig duodenum induced jejunal mucosal damage. However, using histological analysis, we have verified the induction of jejunal inflammation in guinea pigs treated with TNBS intrajejunally and killed 5 days after treatment.

The S plus TNBS group and S group were given water to drink for 3–4 days before the day of challenge. The study was then performed 25–26 days after the TNBS treatment, when the inflammatory process had almost disappeared.

On the day of the experiment (challenge day), guinea pigs of the three groups were anesthetized with urethane (2 g/kg intraperitoneally).

IgG and IgE Antibody Titers

Blood was drawn by cardiac puncture 30 minutes before the challenge to determine specific IgG and IgE titers by the passive cutaneous anaphylaxis (PCA) test. Briefly, 100 μ L of the diluted sera ranging from 1:2 to 1:256 was injected intradermally into the shaved flanks of naive guinea pigs. Two days later, 2.5 mg of β -lactoglobulin in Evans blue dye (1% in 0.9% NaCl solution) was injected intravenously in a final volume of 0.5 mL.¹⁷ One hour later, PCA reactivity was measured as the highest dilution yielding a positive response (local skin blueing with a diameter of >4 mm). Titers were expressed as the log of the highest dilution giving blue patches.

Because IgEs are thermolabile, PCA tests were also performed with sera heated at 56°C for 4 hours. The IgE antibody titers were assessed from the reduction in titers after heating.¹⁸

IgG antibodies to β -lactoglobulin were also evaluated in the serum samples by enzyme-linked immunosorbent assay.¹⁹ Multiwell microtiter plates were coated with 100 μ L β -lactoglobulin (200 ng/well). Diluted sera (1:30 to 1:65,610) were added to each well. After incubation, a rat anti-guinea pig IgG peroxidase conjugate was added. Peroxidase activity was assayed by staining with diaminio-orthophenylene in 0.05 mol/L citrate buffer (pH 4) containing 0.1% H₂O₂. The reaction was stopped with 6N SO₄H₂, and absorbance was measured at 492 nm with a microplate reader (EL 132; Bio-Tek Instruments, Winooski, VT). Positive titers were given as the log of the last dilution with an optical density that was twice that of the background.

Evaluation of Antigen-Induced Colonic Secretion

Modification of intestinal transport of water is a characteristic feature of intestinal anaphylaxis. To assess the response to antigen challenge, we measured the net water flux of an isolated colonic loop.²⁰ This method has been proven to be a good parameter to evaluate water transport abnormalities after antigen challenge in sensitized guinea pigs.²¹ A midline laparotomy was performed in anesthetized guinea pigs to expose the large intestine. A 5-cm segment of the proximal colon 3 cm from the cecocolonic junction was isolated and cannulated for intraluminal perfusion. After cleansing the contents, the loop was replaced in the abdominal cavity, which was closed,

and the colonic segment was infused with a Ringer's buffer solution containing 1 $\mu\text{Ci/L}$ [^{14}C]polyethylene glycol 4000 as a marker and cold polyethylene glycol 4000 as a carrier. The loop was infused at a constant rate (6 mL/h), and the effluent was collected for 15-minute periods during 210 minutes. After an equilibration period (90 minutes), antigen challenge was performed by adding β -lactoglobulin (100 mg) to the Ringer's solution. The antigen was infused for 30 minutes. ^{14}C activity in collected samples was measured by liquid scintillation, and water flux for every 15-minute period was calculated using the following formula: Net Water Flux ($\mu\text{L} \cdot \text{cm}^{-1} \cdot \text{h}^{-1}$) = $(1 - \text{cpm}_s/\text{cpm}_x) \times P/L$, in which cpm_s and cpm_x are ^{14}C activities in Ringer's solution and the effluent, respectively; P is the perfusion rate ($\mu\text{L/h}$); and L the length (cm) of colonic segment. Positive values represent net absorption of water, and negative values represent net secretion.

Histology

At the end of the luminal perfusion, guinea pigs were killed by cervical dislocation, and a small piece (0.5 cm) of the ileum and colon was collected and fixed in 10% buffered formalin for light microscopy. Fixed tissues were embedded in Histomed Standard (Labo Moderne, Paris, France), and 5- μm transverse sections were cut and stained with hemalum and eosin for routine histological analysis and periodic acid-Schiff (PAS) reaction for mucus evaluation.

Biochemical Determinations

Histamine release assay. Histamine level in the luminal effluent was evaluated as a marker of mast cell activation using a radioimmunoassay kit (Immunotech, Marseille, France).²² This immunoanalysis is based on the competition between acylated histamine and ^{125}I -histamine as a tracer for binding with monoclonal antibodies. Histamine level was determined in the effluent corresponding to the 15-minute period following the antigen infusion.

Myeloperoxidase activity assay. Another piece of the ileum and colon was obtained to determine myeloperoxidase activity, an enzyme specific of the azurophilic granules of polynuclear neutrophils, which is a biochemical marker of inflammatory states in the gastrointestinal tract. The method of Bradley et al.²³ was used with slight modifications. After a mincing with scissors, a small piece of either ileum or colon was homogenized in hexadecyltrimethylammonium bromide buffer (pH 6.0) using a polytron (20 seconds at maximal setting). After filtration, the filtrate was sonicated on ice and centrifuged (40,000g for 15 minutes at 4°C). The pellet was discarded, and the supernatant was assayed for myeloperoxidase activity: 0.1 mL of the supernatant was mixed with 2.9 mL of 50 mmol/L phosphate buffer (pH 6.0) containing 0.167 mg/mL *O*-dianisidine hydrochloride and 0.005% hydrogen peroxide. Absorbance at 460 nm was measured with a Uvikon 860 spectrophotometer (Kontron Instruments, Basel, Switzerland) for 5 minutes. Protein concentration was determined by the method of Lowry using a BioRad assay kit (Seine, France), and

myeloperoxidase activity was expressed as international units per micrograms of protein. One international unit of myeloperoxidase activity is defined as that required to degrade 1 μmol hydrogen peroxide per minute at 25°C.

β -Lactoglobulin determinations in serum. Blood was withdrawn by cardiac puncture 30 minutes after the end of antigen infusion, i.e., 1 hour after the beginning of antigen challenge. Determination of β -lactoglobulin level was performed in sera corresponding to 30 minutes before and 30 minutes after the challenge infusion in the three groups of animals.

Enzyme immunoassay (EIA) of β -lactoglobulin in serum was performed with a classical two-site immunometric assay using polyclonal anti- β -lactoglobulin rabbit antibodies for capture and the same antibodies, labeled with acetylcholinesterase (AChE) as tracer. Anti β -lactoglobulin antibodies were prepared from a rabbit immune serum by separation of the total Ig fraction on a protein A column. AChE labeling of purified Igs was performed according to Grassi et al.²⁴

Microplates were coated with a 10- $\mu\text{g/mL}$ solution of purified Igs in 50 mmol/L phosphate buffer, pH 7.4 (200 μL /well, 1-night incubation at room temperature). Plates were then washed and saturated with 0.1 mol/L phosphate buffer (pH 7.4) and 0.15 mol/L NaCl containing 0.1% human serum albumin and 0.01% sodium azide (EIA buffer). After 1-night incubation at 4°C, plates were extensively washed and 100 μL of serum samples at different dilutions, from 1:5 to 1:10,⁴ in EIA buffer were distributed. Normal guinea pig serum was used as control, and known concentrations of pure β -lactoglobulin in 1:10 diluted normal guinea pig serum (8 concentrations from 0 to 50 ng/mL) were distributed in the same conditions to obtain a standard curve for quantification. In the same time, 100 μL of AChE-labeled antibodies in EIA buffer were added in each well. After 1-night incubation at 4°C, plates were washed, 200 μL of the enzyme substrate (Ellman's reagent) was added, and absorbance was measured at 414 nm after 1 hour.

In these conditions, the detection limit of the assay, defined as the mean of the nonspecific binding (background measured with the negative controls) + 2SD, was approximately 0.1 ng/mL. The dynamic range of the dose-response curve extended from 0.5 to 50 ng/mL with a precision of 10%–12%.

Intestinal Permeability

The intestinal permeability study was performed in the second set of animals. Twenty-four guinea pigs were divided and treated as previously described in three groups of 8 animals: control, sensitized, and sensitized TNBS-treated animals. To assess epithelial permeability, 1 mL of the probe (^{51}Cr -EDTA, 3 μCi /guinea pig) was delivered by gastric intubation. Each animal was placed in a metabolic cage permitting separate collection of urine and feces for 24 hours. The radioactivity of urine (1 mL) was counted in a gamma counter (MR 252C; Kontron Instruments). The percentage of recovery of ^{51}Cr -EDTA for 24 hours was calculated from the administered

dose. Intestinal permeability was measured 2 days before starting milk diet and every week during the sensitization period (4 weeks). Permeability was also evaluated after challenge by adding 100 mg β -lactoglobulin to the probe.

Statistical Analysis

Values are expressed as means $\pm$ SD, and results were analyzed by analysis of variance (ANOVA) followed by a non-parametric Mann-Whitney test. Differences were considered significant for P values of ≤ 0.05 .

Results

Serum Antibody Titers

No PCA positivity was found in the control group, which had only water to drink. In contrast, the results of the PCA test, performed 3 days after the animals stopped drinking milk (S group and S plus TNBS group), showed significant differences. The mean value of the serum titers was higher ($P = 0.017$) in S plus TNBS group than in the S group (1.95 ± 0.25 vs. 1.42 ± 0.28 , respectively). Because a positive PCA test result reflects the presence of both IgE- and IgG-specific antibodies, PCA tests were performed again after heat treatment (56°C for 4 hours) to inactivate IgE. Titers from PCA tests performed with heated sera were lower and quite similar in S and S plus TNBS group (1.01 ± 0.44 vs. 0.93 ± 0.37 , respectively). Consequently, IgE-containing fractions, identified by the loss of PCA activity after heating, were significantly higher in the S plus TNBS group than in the S group (1.02 ± 0.44 vs. 0.41 ± 0.43 , respectively; $P = 0.026$).

IgG anti β -lactoglobulin titers determined by the enzyme-linked immunosorbent assay test confirmed the PCA test results. These titers were very similar in both groups: 3.81 ± 0.60 in the S group and 3.86 ± 0.40 in the S plus TNBS group.

Colonic Secretion After Challenge

Perfusion of β -lactoglobulin (unsensitized animals) induced no changes in water fluxes of a colonic loop in the control group. The loop displayed an absorptive state fluctuating from $+18.8$ (minimum) to $+51.2 \mu\text{L} \cdot \text{cm}^{-1} \cdot \text{h}^{-1}$ (maximum) per a 15-minute period during the time course study.

In sensitized animals (S group), the challenge (β -lactoglobulin added to the perfusion solution) changed the absorptive state (equilibration period) of the colonic wall in a secretory state. Basal net water flux was $+42.0 \pm 24.2 \mu\text{L} \cdot \text{cm}^{-1} \cdot \text{h}^{-1}$ in the 1-hour period before challenge and $-23.9 \pm 23.7 \mu\text{L} \cdot \text{cm}^{-1} \cdot \text{h}^{-1}$ during the 30 minutes of antigen perfusion. In the 15-minute period after the

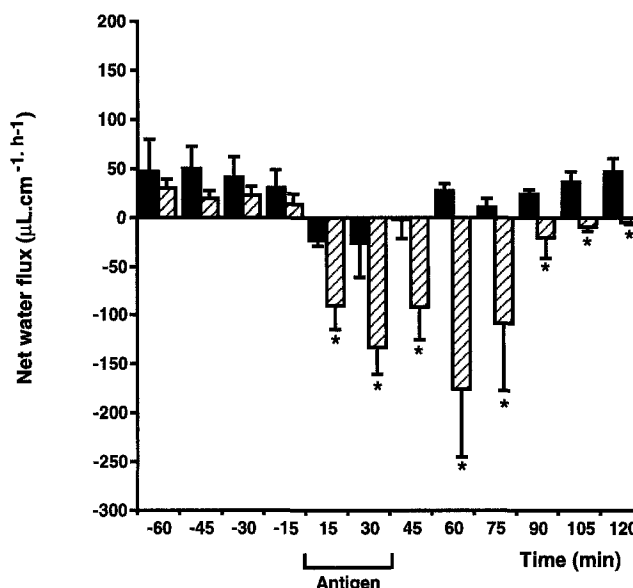


Figure 1. Net water flux in sensitized (■) and sensitized plus TNBS-treated (▨) animals measured in a colonic loop. The change of the colonic mucosa from an absorptive to a secretory state appearing after antigen challenge was considerably amplified and lasted longer in sensitized plus TNBS guinea pigs compared with sensitized ones. Each bar corresponds to the mean value ($\pm$ SD) obtained from 10 animals over a 15-minute period. Antigen infusion lasted 30 minutes. *Significantly different ($P \leq 0.05$) from values obtained in sensitized animals.

end of the β -lactoglobulin perfusion, the value of net water flux was still negative, but afterwards the absorptive state was restored (Figure 1). Basal net water fluxes were not significantly different in the S group compared with the S plus TNBS group. In contrast, the pattern of water secretion observed in the S group was highly amplified in the S plus TNBS group: during the 30-minute period of challenge, the net water flux was four times higher (-104.7 ± 31.1 vs. $-23.9 \pm 23.7 \mu\text{L} \cdot \text{cm}^{-1} \cdot \text{h}^{-1}$, respectively). Moreover, this secretion lasted more than 120 minutes. At the end of the experimental period (120 minutes after the challenge was started), the net water flux had not recovered its basal value (Figure 1).

Morphology

Transverse sections of jejunum of guinea pigs killed 5 days after TNBS treatment had evident intestinal inflammation in spite of interindividual variations in the degree of lesions (Figure 2).

Microscopic examination of ileal and colonic sections from control guinea pigs perfused by β -lactoglobulin showed no particular abnormalities. In contrast, histological study of intestinal sections from the challenged S and S plus TNBS groups showed important morphological

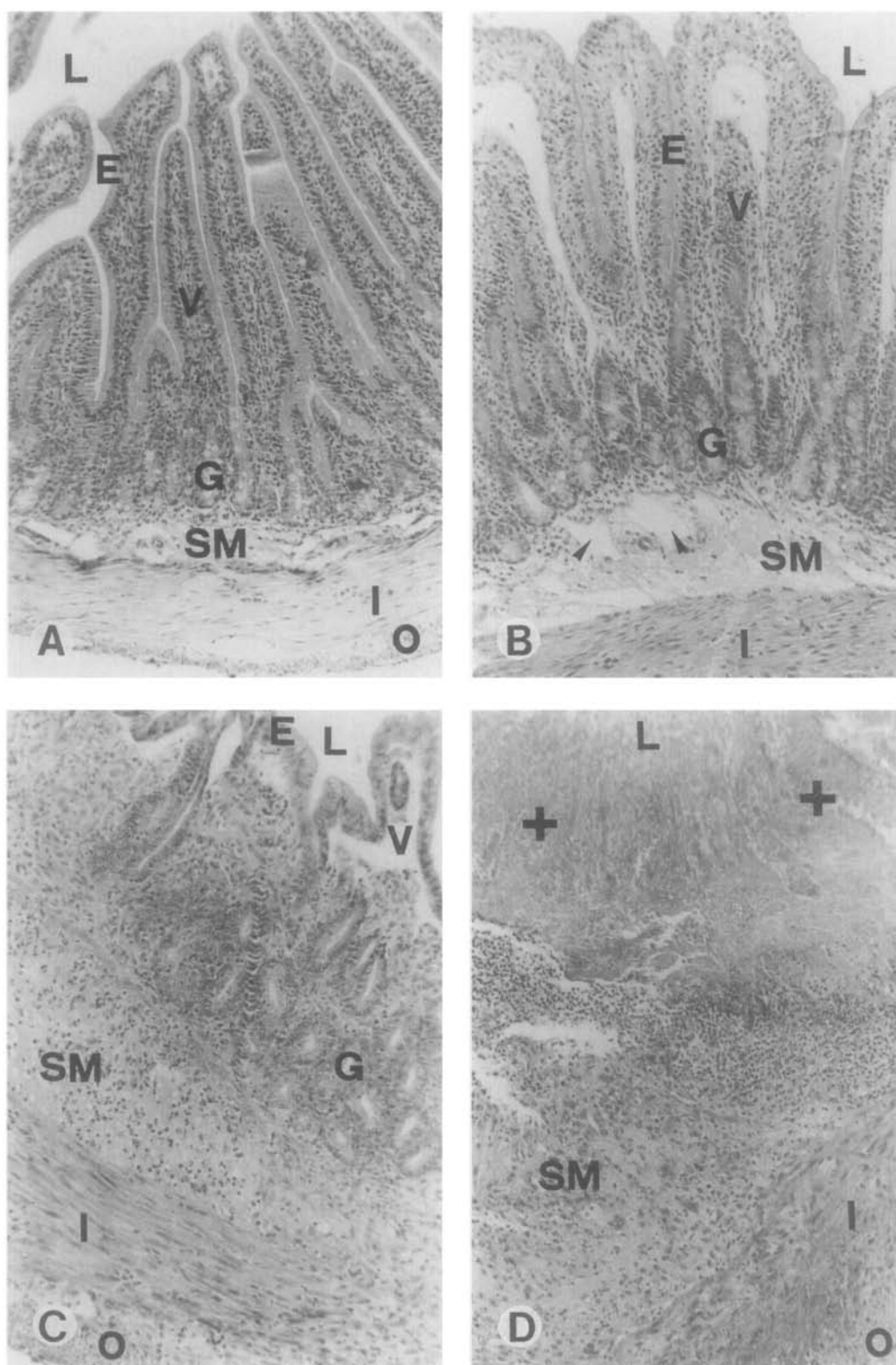


Figure 2. Transverse sections of jejunum of (A) control and (B–D) TNBS-treated guinea pigs 5 days after treatment. In TNBS-treated guinea pigs, different degrees of inflammation are observed: (B) congestive blood vessels in the submucosa (*arrowheads*) and villous edema, (C) thickening of the submucosa and mucosa invaded with mononucleated cells and neutrophils, and (D) complete necrosis of the whole mucosa (+). E, surface epithelium; G, jejunal glands; I, inner muscular layer; L, jejunal lumen; O, outer muscular layer; SM, submucosa; v, jejunal villi (hemalum-eosin stain; original magnification 100 $\times$).

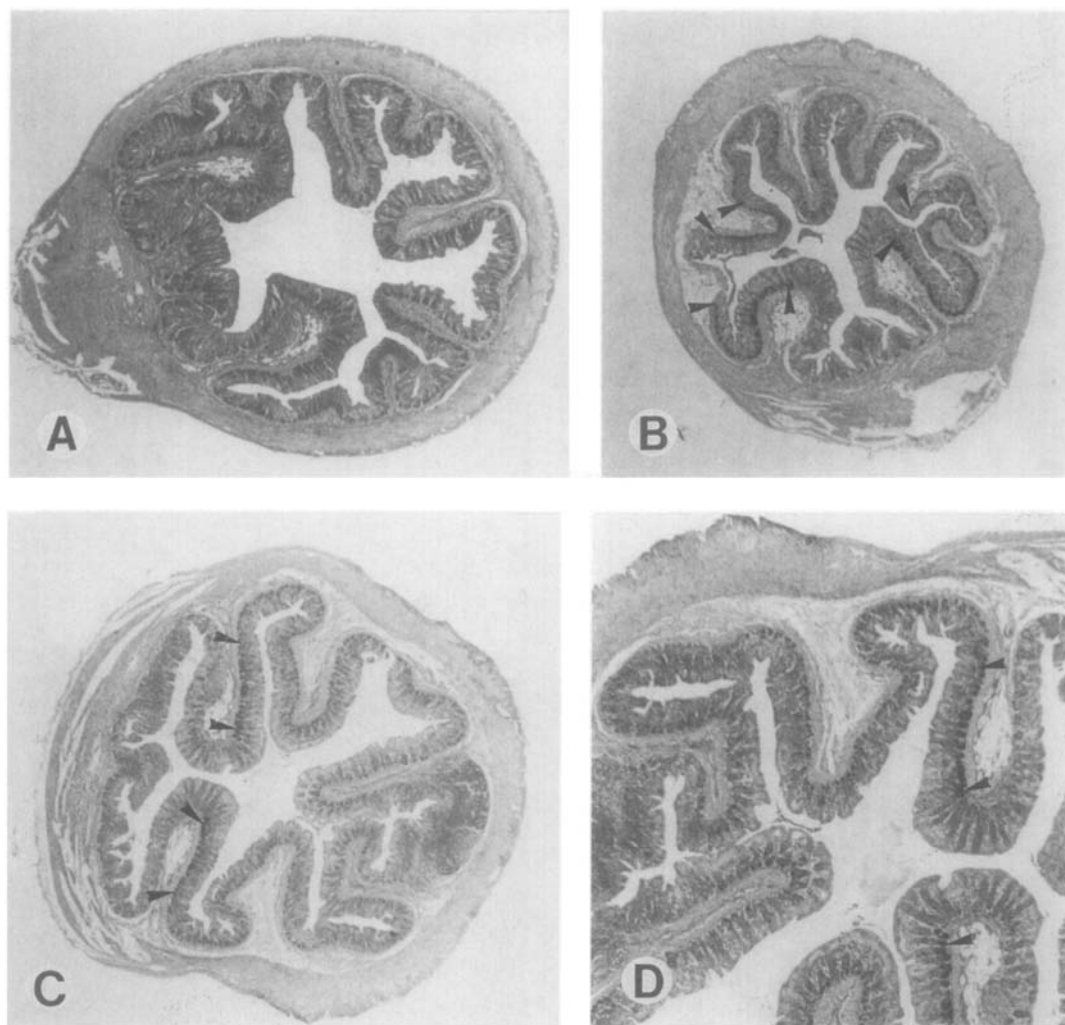


Figure 3. Transverse sections of proximal colon from antigen-challenged guinea pigs stained with the PAS reaction. The mucus is stained black. (A) Control animal (drinking water only). The whole glandular layer is stained black. (B) Sensitized animal. Note the mild depletion of the colonic glands; some areas are PAS reactive. (C) Sensitized plus TNBS guinea pig. The depletion is severe and the PAS-reactive glands are scarce. (D) High magnification of C showing PAS-reactive (black) and unreactive glands. Arrowheads delimit the portions of nondepleted glands (original magnifications: A, 15 $\times$; B, 15 $\times$; C, 15 $\times$; D, 25 $\times$).

changes that were more marked in the S plus TNBS group. In most cases, vascular dilatation, blood stasis, and edema, often localized in the submucosa, were noted. Microhemorrhages and mild erosion were occasionally observed at the villus tips. Also, goblet cells containing PAS-positive material displayed a depletion that was particularly severe in the S plus TNBS group on both the villi and the crypts (Figure 3). An important eosinophilic infiltration of the mucosa and submucosa was noticed in the S and S plus TNBS groups compared with the control group. In terms of the inflammatory process in the S plus TNBS group, no particular inflammatory cells were detected in the colon. At the jejunal level, the morphology was normalized except for the presence of rare mononucleated cells and some scattered pyknotic nuclei.

Biochemical Determinations

Histamine release. Histamine level measured in the colonic loop effluent collected for 15 minutes after the antigen challenge perfusion showed significant differences between the three groups. Histamine level was 610 ± 55 nmol/L in the control group and 1060 ± 80 and 1376 ± 75 nmol/L in S and S plus TNBS groups, respectively ($P < 0.05$).

Myeloperoxidase activity. The myeloperoxidase activity determinations made after antigen challenge corresponded to the 25th to 26th day after intrajejunal administration of TNBS or saline. These activities were quite identical at the colonic level in the control, S, and S plus TNBS groups. In contrast, at the ileal level, MPO activity was still enhanced in the S plus TNBS group (Table 1).

Table 1. Myeloperoxidase Activity in the Ileum and Colon After Antigen Challenge

	Myeloperoxidase activity (IU/mg protein)		
	Control	Sensitized	Sensitized + TNBS
Ileum	0.65 ± 0.25	0.69 ± 0.34	1.98 ± 1.05 ^a
Colon	0.12 ± 0.08	0.14 ± 0.10	0.22 ± 0.09

NOTE. Values are means ± SD (n = 10) measured 25–26 days after intrajejunal administration of TNBS.

^aP < 0.05 vs. control.

Antigen recovery. Sera from the control group (not sensitized) gave negative results, i.e., nondetectable β -lactoglobulin concentrations before challenge. After challenge, β -lactoglobulin was found in the serum of all the nonsensitized animals at concentrations ranging from 50 to 800 ng/mL.

Conversely, in the other experimental groups (S and S plus TNBS groups), no detectable concentrations of β -lactoglobulin (>0.5–1 ng/mL) were observed neither before nor after challenge in any of the animals.

Epithelial Permeability Tests

In the basal period, the percentage of the probe recovery was nearly 6%. The control group had no alterations of intestinal permeability throughout the experiment. In the S group, there was a decrease in permeability at the end of the first week of milk diet ($4.9\% \pm 0.80\%$ vs. $6.2\% \pm 2.0\%$); however, this difference was not statistically significant ($P > 0.05$). In contrast, in the S plus TNBS group, intestinal permeability was significantly increased at the end of the first week of milk diet, i.e., on day 5 after TNBS ($9.4\% \pm 1.9\%$ vs. $6.1\% \pm 1.7\%$ in the basal period). Afterwards, the values normalized. After challenge, no changes were noticed in the three groups.

Discussion

Our results show that, in guinea pigs, the induction of an experimental inflammatory process during the sensitization period enhances not only the systemic immune response by increasing the serum level of specific antibodies but also the local reaction after antigen challenge whereby the intestinal hypersecretion and severe mucus discharges are observed.

The model of food allergy used in our study seems clinically more relevant than other models in which sensitization is achieved by intraperitoneal or intravenous administration of food proteins together with adjuvants to stimulate production of antibodies. In our model, guinea pigs are sensitized orally simply by giving milk

to drink for 4 weeks. Isolated colonic epithelia of animals sensitized by this technique have been shown to respond to β -lactoglobulin challenge by a characteristic chloride ionic secretion.¹² The experimental inflammation induced by TNBS has also been evaluated in detail by Miller et al.¹⁴ who used this compound to produce ileitis in guinea pigs. In agreement with their findings, we found that myeloperoxidase activity (a marker of inflammation) determined 25–26 days after TNBS is unchanged at the colonic level but is still roughly doubled at the ileal level compared with controls, whereas 1 week after TNBS it peaks at fourfold higher values,¹⁴ in accordance with the inflammation observed in our study. In contrast, microscopic examinations of the small intestine nearly 4 weeks after TNBS administration do not show accumulation of inflammatory cells, whereas the rare presence of pyknotic nuclei indicates that intestinal organization has already taken place after inflammation. The presence of the eosinophilic infiltration can be explained by the fact that eosinophils in local tissues are typical markers of type I allergic reactions.

PCA tests performed with nonheated and heated sera show that specific antibody titers are higher in TNBS-treated sensitized animals than in only sensitized ones. This is particularly true for IgE, whereas IgGs are quite similar in both groups. This result confirms that PCA test positivity corresponds to the presence of both IgG and IgE antibodies, as has already been observed,¹⁹ and that hypersensitivity reactions are accompanied by production of both IgG and IgE antibodies.¹⁸ This higher sensitization in the TNBS-treated group can be explained by the increase of epithelial permeability measured 5 days after TNBS injection, a time when the inflammatory state peaks. Indeed, damaged mucosal barrier can account for the increased permeability and consequently increased passage of antigen leading to a stronger stimulation of immunocompetent cells in the lamina propria of intestine. In agreement with this explanation, patients with IBD have a higher incidence of positive results in skin tests to food antigens than control subjects.²⁵ Moreover, a specific immunization to milk protein antigen evidenced by the increased titers of antibodies to milk proteins has been shown in patients with ulcerative colitis and Crohn's disease compared with controls.^{7,8} This fact may reflect a higher permeability of the diseased mucosa to permit the passage of dietary antigens resulting in enhanced sensitization to food components.

The most striking result of our study is the clear-cut hypersecretion observed after challenge in TNBS-treated guinea pigs vs. only sensitized ones. This fluid accumulation is a characteristic feature of intestinal anaphylaxis

triggered by antigen challenge in sensitized animals and results from a chloride secretion correlated with fluid accumulation.^{12,26,27} Chloride secretion and water accumulation are hallmarks of diarrheas observed in patients with gastrointestinal food allergies. In our study, this fluid accumulation is four times higher and lasts longer in TNBS-treated animals. Taken together, it seems to suggest that inflammation in allergic patients aggravates gastrointestinal symptoms.

It is well documented that intestinal fluid balance alterations are induced by mediators released after mast cell activation.^{21,28} Indeed, these changes are associated with mucosal histamine decrease,²⁹ villous edema,²⁶ and goblet cell mucus secretion.³⁰ In our model, colonic effluent histamine level is significantly increased in TNBS-treated guinea pigs after antigen challenge, suggesting that a greater number of mast cells are activated. On the other hand, increased mast cell number³¹ and mediator release³² have been shown in IBD as well as in animal models of IBD.³³ Moreover, histological evaluation of mucus by the PAS reaction indicates that goblet mucous cells are severely depleted in sensitized TNBS-treated guinea pigs, whereas goblet cells display a mild depletion in sensitized animals. It is known that the thickness and composition of the mucus coat covering the microvilli protect the mucosa against antigen penetration and that increasing mucus discharge may provide an additional defense mechanism for limiting antigen passage.³⁴ This result is in accordance with a previous report showing that release of goblet cell mucus is enhanced by antigenic stimulation in orally immunized animals, whereas there is no mucus release in parenterally immunized animals.³⁰ Another study, performed with rats sensitized by intraperitoneal injection of ovalbumin, concludes that release of mucus is not a feature of intestinal anaphylaxis.²⁹ These conflicting results can be explained by the fact that the mode of immunization induces different immunologic responses: IgA production seems to be increased when immunization is induced by oral route, whereas IgG appears preferentially after parenteral injection.³⁵ These observations suggest that local immune response is not necessarily dependent on systemic response and that IgA plays a role in the defense mechanisms of intestinal epithelium mucosa.

The fact that β -lactoglobulin is detected in the serum of challenged control rats but not in orally sensitized and then challenged rats seems to suggest that oral sensitization is responsible for the failure of detection in the later group. A reasonable explanation to this finding would be the formation of β -lactoglobulin–mucosal IgA immune complex and, hence, neutralization of the β -lactoglobulin.

Alternatively, it is possible that the part of β -lactoglobulin that escapes the mucosal IgA trap is absorbed and binds ultimately with the specific IgG and IgE in the serum to form humoral immune complexes. Actually, attempts to detect such circulating immune complexes by immunoelectrophoresis and blotting techniques have failed (results not shown), presumably because blood collected by cardiac puncture would not allow such a detection due to the degradation of immune complexes after a hepatic first-pass metabolism. Our finding is in accordance with other studies showing that the mucosal uptake of antigen is consistently reduced in previously immunized animals compared with nonimmunized animals^{36,37} but in disagreement with a recent study in which antigen recovery was significantly increased after antigen challenge in rats sensitized by subcutaneous ovalbumin coupled with intraperitoneal *Bordetella pertussis* as an adjuvant.³⁸

Our results of permeability studies after challenge seem to contradict other results indicating an increase of intestinal permeability measured with ⁵¹Cr-EDTA. However, these studies are based on parenteral immunization to food proteins^{38,39} or worm parasites.⁴⁰ In contrast, in the same model as ours, it has been shown that the ionic conductance, an index of paracellular permeability, does not change after challenge.¹⁹ Because ⁵¹Cr-EDTA is transported by a paracellular route across the tight junctions,⁴¹ we can assume that the paracellular route of permeation is not altered after challenge.

There are few experimental studies about relationships between intestinal inflammation and immune responsiveness. Apical challenge with β -lactoglobulin has been shown to change the short-circuit current reaction only if a controlled lesioning with ultraviolet irradiation has been applied on the apical surface of sensitized guinea pig colons.⁴² A more recent study using colonic sheets of sensitized guinea pigs suggests that inflammation produces hyperresponsiveness of the colonic mucosa.⁴³ Our study clearly indicates that intestinal inflammation increases the local responsiveness to antigen most likely through the activation of a greater number of immunocompetent cells, particularly mast cells. Furthermore, the choice of immunization route in food allergy models is a crucial component of the response.

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Address requests for reprints to: Marie-José Fargeas, Ph.D., Department of Pharmacology, Institut National de la Recherche Agronomique, 180 Chemin de Tournefeuille, BP3, 31931 Toulouse, France. Fax: (33) 61-28-53-10.

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