

Preliminary report

# Parenteral immunization to $\beta$ -lactoglobulin modifies the intestinal structure and mucosal electrical parameters in rabbit

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## Abstract

Systemic and local immune responses and the intestinal structure were examined in parenterally  $\beta$ -Lg-sensitized rabbits. Immunization led to high IgG titers against  $\beta$ -Lg. In a Ussing chamber, a sensitized ileum had a higher short-circuit current (Isc) and potential difference (PD) than a control following in vitro  $\beta$ -Lg challenge. Histological study indicated that presence of the sensitizing antigen affected and considerably modified the structure of the intestinal mucosa in sensitized rabbits when compared to controls. These alterations were revealed by active atrophy and marked infiltration of the lymphocytes. These findings indicate that antigen exposure results in morphological changes and abnormalities affecting the transport of water and electrolytes. This study provides a clearer understanding of the physiopathological mechanisms of allergy to cow's milk protein. © 2004 Published by Elsevier B.V.

**Keywords:** Cow's milk protein allergy; Anaphylaxis; Rabbit; Intra-Epithelium Lymphocytes; Histology; Intestine

## 1. Introduction

Cow's milk protein allergy is a major cause of food hypersensitivity in children. Of the milk proteins,  $\beta$ -lactoglobulin, the predominant protein in bovine

whey, has been shown to be a major antigen capable of interacting with the intestinal mucosa and stimulating cells in the gut-related immune system [1–3]. The sensitization of animal models (rat, mouse, rabbit) by the parenteral administration of cow's milk protein has been demonstrated to generate immunoglobulins that are of a similar specificity to those produced by humans [4–6] and to alter the intestinal barrier, as characterized by reduced brush-border enzymes, villus atrophy and crypt hyperplasia [7]. Moreover, exposure of the intestinal mucosa to milk antigens in

**Abbreviations:** IELs, Intraepithelial lymphocytes;  $\beta$ -Lg,  $\beta$ -Lactoglobulin; Isc, Short-circuit current; PD, Potential difference; G, Conductance.

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the previously sensitized guinea pig resulted in an imbalance of water and electrolyte transport, associated with the active secretion of water and electrolytes and increased permeability to the antigen [8]. The aim of the present study was to verify the effects in rabbits of the parenteral administration of  $\beta$ -lactoglobulin on the architecture of intestinal villi and intra-epithelium lymphocytes, and to determine whether a direct interaction between the sensitizing antigen and the mucosa could elicit a local response.

## 2. Materials and methods

### 2.1. Animals

Forty-two male and female New Zealand rabbits weighing 2–3 kg were studied. The rabbits were non-immunized ( $n=12$ ) or immunized with either  $\beta$ -lactoglobulin ( $\beta$ -Lg; Sigma, France) ( $n=18$ ) or  $\alpha$ -lactalbumin ( $\alpha$ -La; Sigma) ( $n=12$ ) by subcutaneous injections at numerous different points along the spinal cord [9]. On the first day, the animals received 2 mg  $\beta$ -Lg or  $\alpha$ -La dissolved in 0.5 ml physiological saline, and then on days 7, 14, 21, 28, 35, 49 and 56 they received a dose of 1 mg  $\beta$ -Lg or  $\alpha$ -La dissolved in 0.5 ml physiological saline and 0.5 ml incomplete Freund's adjuvant (Sigma).

### 2.2. Blood sampling and anti- $\beta$ -Lg or $\alpha$ -La IgG determinations

Blood was drawn from the marginal vein of each animal's ear. Blood samples were collected at day 0 before immunization and at day 56 after immunization. The blood was centrifuged at 2500 rpm for 10 min at 4 °C. The serum was removed and stored in aliquots at –25 °C. Specific anti- $\beta$ -Lg or anti- $\alpha$ -La IgG were assayed in serum samples using an enzyme-linked immunosorbent assay (Elisa). Multi-well micro-titre plates (Nunc, Polylabo, France) were coated for 1 h at 37 °C with 100  $\mu$ l of  $\beta$ -Lg or  $\alpha$ -La (2  $\mu$ g ml<sup>–1</sup>), before being washed with phosphate buffered saline containing 0.05% Tween 20 (pH 7.4). A total of 100  $\mu$ l of serially diluted serum (1/30–1/65610) was added to each well, and the plates were then incubated for 1 h 30 min at 37 °C and washed. A total of 100  $\mu$ l of goat anti-IgG peroxidase conjugate

(1:3000, Sigma) was then added to each well and the plates were left for 1 h at 37 °C. After washing of the plates, peroxidase activity was assayed by adding 0.2 mg ml<sup>–1</sup> diamino-orthophenylene in 0.05 M citrate buffer, pH 4, containing 0.05% H<sub>2</sub>O<sub>2</sub>. After external stirring at room temperature in the dark, the reaction was stopped by 6N H<sub>2</sub>SO<sub>4</sub> and absorbance was measured at 492 nm using an Uniskan plate reader (Labsystem).

### 2.3. Ussing chamber experiments

After removal of the ileum, a fragment was opened flat and mounted in a Ussing chamber (exposed area: 0.1 cm<sup>2</sup>). Both surfaces of the tissue were bathed with 2 ml Ringer solution (pH 7.4), maintained at 37 °C and oxygenated (O<sub>2</sub>: 95%, CO<sub>2</sub>: 5%). Bathing solutions for the mucous and serous membranes were connected to calomel electrodes via 3M KCl agar bridges in order to measure the trans-epithelial potential difference (PD), and to Ag–AgCl electrodes for the passage of current through the system. Open circuit conditions were used in experiments designed to measure electrophysiological parameters. Conductance (G) was evaluated regularly by passing a 10- $\mu$ A current through the preparation, and the short-circuit current (I<sub>sc</sub>) was then calculated taking account of fluid resistance. After an initial period of 30 min, 1 g l<sup>–1</sup> or 0.1 g l<sup>–1</sup> of the antigen was added to the mucosal and the serous compartments, respectively.

### 2.4. Histology

Histological studies were performed on isolated intestinal fragments from control and  $\beta$ -Lg-sensitized rabbits. In each group, 18 ileal fragments were removed, and were either not incubated ( $n=6$ ) or incubated for 15 ( $n=6$ ) or 30 ( $n=6$ ) min at 37 °C in carbogen oxygenated Ringer solution in the presence of 0.1 mg ml<sup>–1</sup>  $\beta$ -Lg. The fragments were fixed in AFA (a 2% formalin, 5% acetic acid, 75% ethyl alcohol solution), embedded in paraffin and stained with hematoxylin-eosin for subsequent histological analyses. These two dyes were used to reveal the nucleus and the cytoplasm, respectively. Briefly, paraffin was removed in toluene (56 °C, 2 min) and the fragments were rehydrated in four successive

ethanol-water solutions (100°, 95°, 90°, 70°) for 2 min and then washed in water. The fragments were then incubated for 3 min in hematoxylin solution (5 g/l hematoxylin, 100 g/l potassium alun, 2.5 g/l mercuric oxide in water), washed with water, incubated for 5 min in eosin solution (20 g/l eosin in ethanol), rinsed twice in 70% ethanol, incubated for 1 min in toluene, and mounted in strips for microscopy. Measurements of the height of villi at the jejunum were performed using an optical microscope equipped with a micrometer. For each tissue, three independent counts were completed, enumerating 100 enterocytes, so that IEL values could be obtained for 100 enterocytes.

### 2.5. Statistics

The results were analyzed using Student's *t*-test and were presented as mean values  $\pm$  SEM. A *p* value  $<0.05$  was considered as significant.

## 3. Results

After parenteral immunization of the animals, a significant anti- $\beta$ -Lg or anti- $\alpha$ -La IgG response was measured in  $\beta$ -Lg and  $\alpha$ -La immunized animals, respectively. In contrast, no anti  $\beta$ -Lg and anti- $\alpha$ -La antibody responses were detected in control animals.

### 3.1. Electrical parameters of the intestinal mucosa

Intestinal specimens were mounted in a Ussing chamber. In control tissues, addition to the mucosa of 1 g l<sup>-1</sup> BSA,  $\beta$ -Lg or  $\alpha$ -La did not produce any modification to Isc, PD or G values. In tissues from  $\beta$ -Lg immunized animals, addition to the mucosa of 1 g l<sup>-1</sup> BSA did not produce any changes to Isc, PD or G, but the addition of either 1 g l<sup>-1</sup>  $\beta$ -Lg to the mucosal reservoir or 0.1 g l<sup>-1</sup>  $\beta$ -Lg to the serosal reservoir resulted in a significant stimulation of Isc and PD ( $p < 0.05$ ). In tissues from  $\alpha$ -La immunized animals, the addition of  $\alpha$ -La to the mucosal reservoir also resulted in a significant stimulation of Isc and PD ( $p < 0.05$ ). Under all conditions, tissue conductance (G) remained stable throughout the experiments, thus suggesting preservation of the epithelial integrity of tissues mounted in Ussing chambers (Table 1).

Table 1

Effect on electrical parameters (Isc, PD, G) of mucosal (1 g l<sup>-1</sup>) or serosal (0.1 g l<sup>-1</sup>) application of milk protein antigens (BSA,  $\beta$ -Lg or  $\alpha$ -La) on ileal fragments mounted in Ussing chambers from control and  $\beta$ -Lg or  $\alpha$ -La immunized rabbits

|                                                        | $\Delta$ Isc<br>( $\mu$ A/cm <sup>2</sup> ) | $\Delta$ PD<br>(mV) | $\Delta$ G<br>(mmho/cm <sup>2</sup> ) |
|--------------------------------------------------------|---------------------------------------------|---------------------|---------------------------------------|
| <i>Control animals (n=12)</i>                          |                                             |                     |                                       |
| BSA 1 g l <sup>-1</sup> Mucosal                        | 0.85 $\pm$ 3.11                             | 0.36 $\pm$ 0.32     | 2.07 $\pm$ 2.21                       |
| $\beta$ -Lg 1 g l <sup>-1</sup> Mucosal                | 2.16 $\pm$ 3.62                             | 0.11 $\pm$ 0.44     | 3.39 $\pm$ 3.33                       |
| $\alpha$ -La 1 g l <sup>-1</sup> Mucosal               | 2.33 $\pm$ 5.12                             | 0.27 $\pm$ 0.21     | 3.90 $\pm$ 2.52                       |
| <i><math>\beta</math>-Lg Immunized animals (n=18)</i>  |                                             |                     |                                       |
| BSA 1 g l <sup>-1</sup> Mucosal                        | -2.90 $\pm$ 2.63                            | 0.38 $\pm$ 0.25     | 2.68 $\pm$ 3.23                       |
| $\beta$ -Lg 1 g.L <sup>-1</sup> Mucosal                | 6.25 $\pm$ 1.85*                            | 0.11 $\pm$ 0.01*    | 0.49 $\pm$ 0.52                       |
| $\beta$ -Lg 0.1 g l <sup>-1</sup> Serosal              | 11.35 $\pm$ 3.11*                           | 0.35 $\pm$ 0.11*    | 3.03 $\pm$ 3.11                       |
| <i><math>\alpha</math>-La Immunized animals (n=12)</i> |                                             |                     |                                       |
| $\alpha$ -La 1 g l <sup>-1</sup> Mucosal               | 9.60 $\pm$ 1.00*                            | 0.41 $\pm$ 0.15 *   | 1.80 $\pm$ 2.12                       |

Results are expressed as means  $\pm$  SEM of the difference in Isc, PD and G values measured during the 120 minute period before and after introduction of the protein, respectively.

\* significantly different from zero ( $p < 0.05$ ).

### 3.2. Optical microscopy observations

When observed under the optical microscope, the intestinal mucosa in the control group exhibited long, thin villi surrounded by a simple, cylindrical, and single-layer epithelium, mainly composed of tall cells with a flat striatus containing regular nuclei at the base and corresponding to the enterocytes. The chorion appeared to be fibrous and polymorphous and presented mononucleated elements, which were in small abundance and corresponded to cells of the immune system, i.e. lymphocytes. In contrast, the intestinal mucosa of  $\beta$ -Lg-immunized rabbits presented major atrophy characterized by villi surrounded by a pseudo-stratified epithelium containing cubic cells with dystrophic nuclei. At the level of the chorion, inflammatory infiltration was extremely marked (Fig. 1).

### 3.3. Length of villi and lymphocyte infiltration

When compared with controls, the average length of villi was significantly reduced ( $p < 0.0001$ ) in the intestine of  $\beta$ -Lg-sensitized animals. This length was reduced by half, indicating marked atrophy originating from lymphocyte infiltration and the response of the gut-associated immune system. In control rabbits,

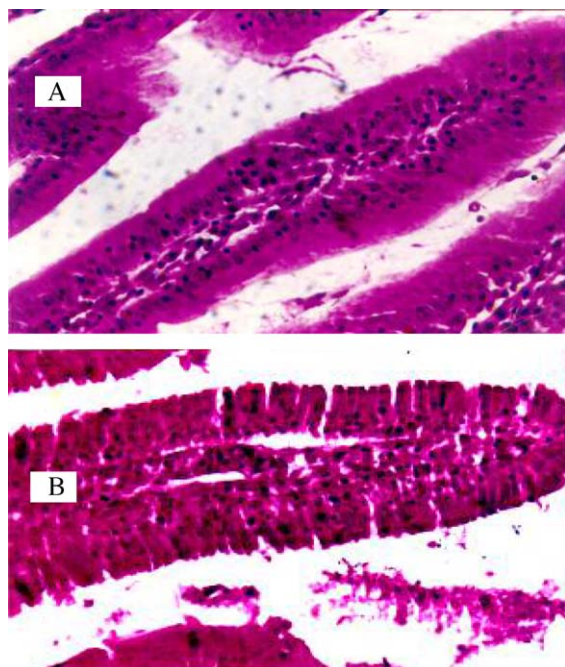


Fig. 1. Optical microscopy (Gx40) of intestinal ileal fragments from (A) a non-immunized rabbit (control), and (B) a  $\beta$ -Lg immunized rabbit, challenged in vitro with  $0.1 \text{ g l}^{-1}$   $\beta$ -Lg for 15 min and stained with hemalun-eosin.

the IEL count revealed a lymphocyte-to-enterocyte ratio of approximately 3:5. The immunization rabbits with  $\beta$ -Lg did not just induce atrophy of the villi, it also caused a considerable increase in the IEL value compared with controls ( $p < 0.005$ ). The observed average in the group of immunized rabbits was  $110.41 \pm 4.79$  lymphocytes per 100 enterocytes. When compared with the controls, the lymphocyte count had doubled (Table 2).

#### 4. Discussion

The findings of the present study agree with the idea that a direct interaction between the sensitizing antigen and immune cells in the intestinal mucosa is associated with local immune reactivity to the antigen in sensitized animals.

The results demonstrated that exposure to the antigen of intestinal ileal fragments from  $\beta$ -Lg or  $\alpha$ -La-sensitized animals produced an increase in spontaneous electrogenic activity related to disturbances of water and electrolyte transport. This study also indicated that sensitization by the  $\beta$ -Lg antigen considerably altered the structure of the intestinal mucosa compared to that found in controls, as shown by pronounced atrophy of the mucosa, marked inflammatory infiltration of lymphocytes in the chorion and the presence of a large number of intra-epithelial lymphocytes. Exposure of the intestine of sensitized animals to the antigen was likely to produce an activation of immune cells of the lamina propria and to elicit a local immune response associated with the electrogenic activities observed.

One important point was that the increase in lymphocyte infiltration and villus atrophy in  $\beta$ -Lg-sensitized rabbits might constitute an element of intolerance to  $\beta$ -Lg. These results are in agreement with the finding that during acute diarrhoea in children, an increase in the levels of anti- $\beta$ -Lg IgG antibodies was observed, and that IgG antibodies had an effect on the development of anti-cow's milk IgE antibodies [17,18]. When the sensitizing antigen was eliminated from the diet of children who could not tolerate cow's milk protein, a reduction in the levels of intra-epithelial lymphocytes was observed [11]. In the

Table 2

Villus length and IEL count in ileal fragments from control and  $\beta$ -Lg-sensitized rabbits

|                                 | Rabbit group | In vitro $\beta$ -Lg challenge |                   |                     |
|---------------------------------|--------------|--------------------------------|-------------------|---------------------|
|                                 |              | No challenge                   | 15 min            | 30 min              |
| Villus length ( $\mu\text{m}$ ) | Control      | $5.28 \pm 0.12^a$              | $5.13 \pm 0.17^a$ | —                   |
|                                 | Immunized    | $2.80 \pm 0.16^b$              | $2.51 \pm 0.23^b$ | $2.76 \pm 0.30^b$   |
| IEL count (/100enterocytes)     | Control      | $48.88 \pm 5.03^a$             | $50 \pm 6^a$      | —                   |
|                                 | Immunized    | $110.41 \pm 4.79^b$            | $115 \pm 5.68^b$  | $122.22 \pm 6.13^b$ |

Values are expressed as means  $\pm$  SEM. Ileal fragments were either not incubated (no challenge;  $n=6$ ) or incubated for 15 ( $n=6$ ) or 30 ( $n=6$ ) min at  $37^\circ\text{C}$  in carbogen oxygenated Ringer in the presence of  $0.1 \text{ g l}^{-1}$   $\beta$ -Lg. Villus length and IEL values with the same letter are not significantly different.

context of cow's milk protein intolerance, intra-epithelial lymphocytes have been suspected of exhibiting stronger cytotoxicity, which in turn is implicated in pathogenesis [12]. Alterations and damage in the area of the villi after an injection of saponine have been observed in rats immunized to  $\beta$ -Lg [13].

Our results thus confirm that the local reaction observed after exposure to the sensitizing antigen was the consequence of a reaction between this antigen and the immune cells of the lamina propria [8,14–16]. They also indicate that the intestinal barrier both transfers antigenic information to the immune system and represents a target for mediators of the immune response. It is accepted that intolerance to cow's milk protein is characterized in children by increased intestinal permeability to milk protein antigens [8–10]. Reactivity to the antigen by the intestine has been observed with both  $\beta$ -Lg and  $\alpha$ -La sensitized animals, thus suggesting that the local immune response is not specific to  $\beta$ -Lg. Antigen permeability may therefore represent an important step in the potential of milk protein antigens to induce oral sensitization and reactivity.

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