

## Decrease in Systemic Tolerance to Fed Ovalbumin in Indomethacin-Treated Mice

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### Abstract

The oral administration of non-steroidal anti-inflammatory drugs (NSAID) to animals induces a quick increase in intestinal permeability and secondary inflammatory lesions of the intestine. The mechanisms leading to the inflammatory lesions are hypothetical. The increased intestinal permeability could allow a greater mucosal and systemic penetration of fed antigens and bacterial products leading to an abnormal mucosal and systemic immune and inflammatory response toward these materials. We examined the effect of oral dosing with indomethacin on ovalbumin serum levels and the systemic immune response to ovalbumin in mice fed with ovalbumin. The ovalbumin serum level was higher in indomethacin-treated mice and the increase was proportional to the dose of indomethacin. It was associated with epithelial and subepithelial lesions. Moreover, the systemic humoral and, to a lesser extent, the cellular tolerance were partially abrogated in the treated mice. These findings suggest that the oral administration of indomethacin in mice induces an increased passage of fed antigen through the intestinal epithelium associated with a decrease in systemic tolerance to this antigen. The reason for this decrease remains unclear. Besides a disequilibrium between systemic and mucosal immune responses, a loss of integrity of the intestinal epithelial cells and a direct immunomodulating effect of indomethacin may also be involved. This decrease in systemic tolerance to luminal antigen could be involved in the development of NSAID enteropathy.

**Keywords** : NSAID enteropathy ; Immune tolerance ; Immunization ; Intestinal permeability

### Introduction

Non-steroidal anti-inflammatory drug (NSAID) enteropathy is a well-documented entity in humans [1]. It is characterized by an increased intestinal permeability, modifications of intestinal absorption and motility and jejuno-ileal inflammation. Sometimes, ulcerative lesions, bleeding, perforations or the more specific diaphragm disease can occur. Studies in animals [2] have shown two steps in this NSAID enteropathy: first, microvascular damages, imbalance in eicosanoids, mitochondrial damage and increased intestinal permeability within hours following the administration of one dose of NSAID; secondly, oedema, ulcers and inflammatory reaction developing 12-24 h after NSAID. This inflammatory reaction may become chronic if the NSAID administration is prolonged. This second step is relatively non-specific and depends on luminal agents. It could be diminished or even suppressed in germ-free animals [3], after antibiotic therapy [4] or in case of fasting [5]. The mechanisms leading from an increased intestinal permeability and early mucosal modifications to lesions and more chronic inflammation are hypothetical. An increased mucosal and systemic penetration of antigens, superantigens, bacterial cell wall fragments and infectious agents is suspected to lead to a breakdown in systemic immune tolerance, to a perturbation of the mucosal immune response and to a nonspecific inflammatory response in the mucosa. The aim of our study was to clarify whether oral administration of indomethacin is associated with an increased penetration of fed soluble antigen and with a decrease in systemic tolerance toward this fed antigen.

### Material and Methods

#### *Animals*

Female Balb/c mice, ranging from 4 to 8 weeks of age, were obtained from the Iffa Credo Laboratories in

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Brussels, Belgium. Their mean weight was  $20 \pm 2.7$  g.

### *Antigen*

Crystallized and lyophilized ovalbumin grade V was obtained from Sigma Chemical Co., St. Louis, Mo., USA.

### *Study Design*

At day 0, 90 mice were administered an oral dose of indomethacin. Eighteen hours' later, they were administered a single oral dose of ovalbumin. Three hours after ovalbumin administration, 30 of them were killed by cervical dislocation. They were used for serum ovalbumin measurement and for histological examination of the intestine. Other mice were immunized subcutaneously with ovalbumin at day 7, and 30 of them were killed by cervical dislocation at day 17 for measurements of anti-ovalbumin antibody levels and an *in vitro* lymphocyte proliferation test. The last 30 mice were used for a specific delayed hypersensitivity test *in vivo*. Mice were divided into six groups representing different experimental conditions (table 1). Five mice were used for each experimental condition.

**Table 1.** Study design

	Indomethacin mg/kg	Oral ovalbumin mg	Subcutaneous ovalbumin, $\mu$ g
Control +	0	0	100
Control -	0	0	0
Indo 0	0	10	100
Indo 0.5	0.5	10	100
Indo 2.5	2.5	10	100
Indo 5	5	10	100

### *Indomethacin Administration*

Natrii indomethacinum (Merck, Sharp and Dohme) was diluted in saline to different concentrations: 100  $\mu$ g/ml, 500  $\mu$ g/ml and 1 mg/ml. It was administered by an intragastric tube under light ether anesthesia. One group of mice (Indo 0) was injected with saline only.

### *Oral Pre-Immunization*

Mice were fed a single dose of 10 mg ovalbumin in 100  $\mu$ l of saline by an intragastric tube.

### *Subcutaneous Immunization*

Ovalbumin was precipitated in  $Al(OH)_3$  at 2 mg/ml. Fifty microlitres of this suspension were injected subcutaneously at the base of the tail with a 30-gauge needle.

### *Measurement of Serum Ovalbumin*

Mice were bled by cardiac puncture. Fifty to 200  $\mu$ l of serum were obtained from each mouse and stored at  $-20^\circ C$ . Serum ovalbuminaemia was determined by RIA. We used an inhibition dosage with a double-antibody method. Briefly, sera were incubated for 16 h at  $20^\circ C$  in tubes with  $^{125}I$ -ovalbumin (grade V, Sigma) and a rabbit anti-ovalbumin. IgG (Organon Teknika). Then a goat antirabbit antibody was added and mixed slightly during 4 h at  $20^\circ C$ . Tubes were then centrifuged and the supernatants were adsorbed. The radioactivity of the pellets were counted (cpm). A reference curve was made simultaneously with different concentrations of ovalbumin and the ovalbuminaemia of the different sera was calculated from this reference curve.

### *Histology*

Jejunal and ileal segments were fixed in Bouin's solution and embedded in paraffin. Five-micrometre sections were routinely stained with haematoxylin-eosin. Periodic acid-Schiff staining was performed when necessary.

### *Immune Evaluation*

Systemic humoral response to ovalbumin was determined by an antibody assay. Anti-ovalbumin serum levels were measured by ELISA. Mice were bled by cardiac puncture. Fifty to 200  $\mu\text{l}$  of serum were obtained from each mouse and stored at  $-20^{\circ}\text{C}$ . Microtitre plates were coated with 100  $\mu\text{l}$  of an ovalbumin solution (100  $\mu\text{g}/\text{ml}$ ) per well at  $4^{\circ}\text{C}$  for 24 h. Plates were rinsed 3 times with a washing solution (PBS + Tween 0.5%). Plates were then incubated for 1 h at room temperature with a bovine powdered milk solution (PBS + Tween 0.5% + powdered milk 1%) to block non-specific sites. Plates were rinsed 3 times with the washing solution. Sera diluted at 1/10, 1/40, 1/160 and 1/640 were added (50  $\mu\text{l}/\text{well}$ ) for 1 h at  $37^{\circ}\text{C}$ . Reference serum was obtained by serum pooling from mice immunized several times subcutaneously with ovalbumin; it was diluted at 1/500, 1/2,000, 1/8,000 and 1/32,000. Plates were rinsed 3 times with the washing solution. The immunoglobulin total was detected with peroxidase-conjugated goat antimouse immunoglobulin (Dako) used at 1/500. Plates were incubated at  $37^{\circ}\text{C}$  for 1 h and then rinsed 3 times with the washing solution. The reaction was developed with O-phenylenediamine dihydrochloride for 5 min (50  $\mu\text{l}/\text{well}$ ). The reaction was stopped with  $\text{H}_2\text{SO}_4$  (25  $\mu\text{l}/\text{well}$ ). Plates were read with a spectrophotometer type LP 200 at 405 nm. The level of anti-ovalbumin antibody in the serum for each mouse was expressed as units of reference serum antibody level.

Systemic cellular response to ovalbumin was determined by a lymphocyte proliferation test *in vitro* and by a specific delayed-type hypersensitivity (DTH) *in vivo*. Inguinal lymph nodes (draining lymph nodes of the tail) were used as a source of cells for an antigen-induced proliferation: 400,000 cells were cultured in flat-bottom microwell plates in 0.2 ml of culture medium (RPMI 1640 Gibco + de complemented fetal calf serum 10% + non-essential amino acids 1% + sodium pyruvate 1% + penicillin and streptomycin + mercapto-ethanol 0.1%). Cells were cultured in culture medium alone or with ovalbumin 100  $\mu\text{g}/\text{well}$ . Moreover, a control of the non-specific mitogenic activity was made for each condition using pokeweed mitogen 1%. Cultures were triplicated for each condition. The plates were incubated in a humidified 5%  $\text{CO}_2$ , 95% air atmosphere for 72 h and were pulsed with 1  $\mu\text{Ci}$  (in 10  $\mu\text{l}$ ) of  $^3\text{H}$ -thymidine for the final 24 h of culture. Cultures were harvested with an automated sample harvester and radioactive measurement was determined in a Packard liquid scintillation analyser. The result was expressed, for each mouse, as the ratio between the mean proliferation, in counts per minute, of the triplicated culture with and without ovalbumin (stimulation indices). DTH to ovalbumin was evaluated by a footpad testing. Fifty microlitres of physiological saline or heat-aggregated ovalbumin diluted in saline (100  $\mu\text{g}/50 \mu\text{l}$ ) were injected intradermally, respectively, into the right and left footpads. Volumes of the footpads were measured with a Mitutoyo micrometer before and 48 h after the injection. The volume variations between the left and the right footpads were compared. The results were recorded as the difference between the volume variations of the right and the left footpads ( $\mu\text{m}$ ).

### *Statistical Analysis*

Results are expressed as means  $\pm$  SEM of the results obtained with the 5 mice for each experimental condition. Comparisons of data were made using a two-tailed t test.

## **Results**

### *Serum Ovalbuminaemia*

Results are shown in table 2. There was a significant increase between non-fed mice (negative and positive controls) and ovalbumin-fed mice. There was also a significant increase between non-treated mice and indomethacin-treated mice. This increase was more pronounced for higher doses of indomethacin.

**Table 2.** Serum ovalbuminaemia (ng/ml)

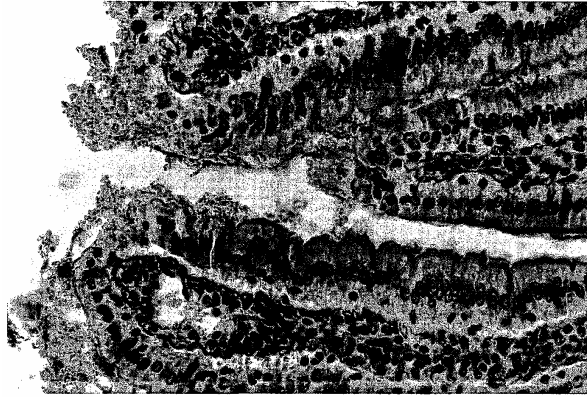
Control (+and-)	Indo 0	Indo 0.5	Indo 2.5	Indo 5
6.06 $\pm$ 0.42	22.2 $\pm$ 2.67	58.6 $\pm$ 17.32	134 $\pm$ 25.8	192 $\pm$ 58.5

### *Histology*

Jejunal and ileal specimens from mice treated with indomethacin 0.5 mg/kg did not show any significant difference with those from untreated mice. After treatment with indomethacin 2.5 mg/kg, mucosal lesions

appeared, characterized by a basal interstitial oedema detaching epithelial cells from the underlying basement membrane. Specimens from mice treated with indomethacin 5 mg/kg showed basal interstitial oedema and necrosis and shedding of epithelial cells located at the top of the villi (fig. 1).

**Fig. 1.** Jejunum 21 h after oral dosing with 5 mg/kg of indomethacin. Subepithelial oedema and focal epithelial necrosis and desquamation Periodic acid-Schiff.  $\times 150$ .



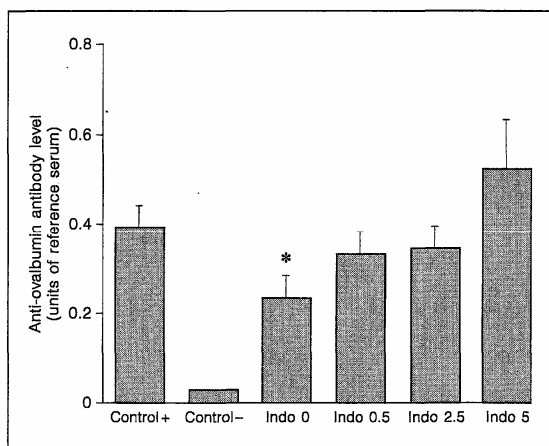
#### *Humoral Systemic Immune Response*

Results are shown in figure 2. There was a significant decrease in the serum anti-ovalbumin antibody level induced by subcutaneous immunization with ovalbumin in ovalbumin-fed mice. However, this decrease was not significant in indomethacin-treated, ovalbumin-fed mice any more, and there was even an increase in the serum anti-ovalbumin antibody level in mice treated with indomethacin 5 mg/kg.

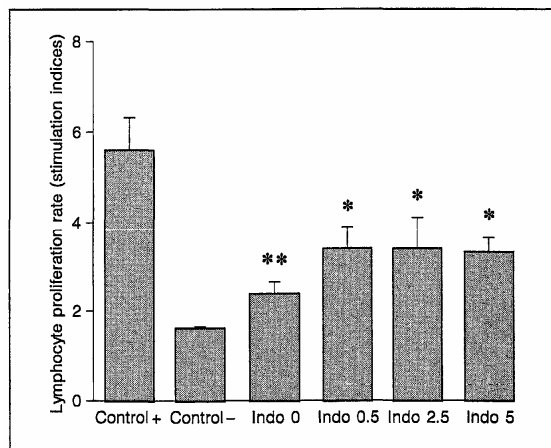
#### *Cellular Systemic Immune Response Evaluated by a Lymphocyte Proliferation Test*

Results are shown in figure 3. There was a significant decrease in the specific lymph node cell proliferation rate induced by subcutaneous immunization with ovalbumin in ovalbumin-fed mice. This decrease was specific because the mitogenic activity elicited by pokeweed mitogen 1% was unchanged. This significant decrease was still present in indomethacin-treated mice, even in those treated with 5 mg/kg indomethacin. Although the decrease was weaker in indomethacin-treated mice, it was not significantly different from the non-treated mice.

**Fig. 2.** Serum anti-ovalbumin antibody levels, 10 days after subcutaneous immunization, in the different experimental conditions. There was a significant decrease in previously ovalbumin-fed mice (indo 0,  $p < 0.05$ ). However, the decrease in indomethacin-treated mice was no longer significant and there was even an increase in the mice treated with indomethacin 5 mg/kg.



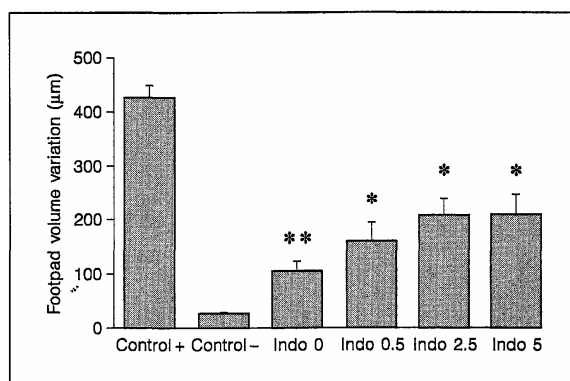
**Fig. 3.** Inguinal lymph node cell proliferation rates in the presence, of ovalbumin, 10 days after subcutaneous immunization at the base of the tail, in the different experimental conditions. There was a significant decrease in the lymphocyte proliferation rate in previously ovalbumin-fed mice both in non-treated ( $p < 0.004$ ) and indomethacin-treated mice ( $p < 0.05$ ).



#### Cellular Systemic Immune Response Evaluated by DTH

Results are shown in figure 4. There was a significant decrease in footpad swelling after intradermal injection of ovalbumin in ovalbumin-fed mice. This decrease was still significant in indomethacin-treated mice. However, it was less pronounced, and the difference between non-treated mice and mice treated with indomethacin 2.5 or 5 mg/kg was significant.

**Fig. 4.** DTH to ovalbumin, 10 days after subcutaneous immunization, in the different experimental conditions. There was decrease in the DTH intensity in ovalbumin-fed mice. This decrease was significant both in non-treated ( $p < 0.0001$ ) and indomethacin-treated mice ( $p < 0.002$ ).



#### Discussion

This study provides some novel observations of the modifications in intestinal permeability and systemic immune response to fed antigen following oral dosing with indomethacin.

An increased intestinal permeability following oral administration of indomethacin has been shown both in humans and animals using different tests and probes [6-9]. However, permeability to molecules normally present in the digestive tract, which could play a role in the pathophysiology of NSAID enteropathy (e.g. fed antigens, bacterial products), has rarely been studied. Our results show an increased ovalbumin serum level in ovalbumin-

fed mice following oral dosing with indomethacin. This increase was proportional to the dose of indomethacin. It probably reflects an increased passage of undegraded ovalbumin through the digestive epithelium. This increased permeability was associated with histological changes characterized by subepithelial oedema and, for the highest dose of indomethacin, epithelial desquamation.

Systemic immune tolerance to a fed antigen is a well-established phenomenon in mice [10]. Some conditions, such as cyclophosphamide pretreatment [11] or graft-versus-host reaction [12] have been associated with an abrogation of this tolerance. Moreover, abnormalities of the systemic and mucosal immune responses to luminal antigens have been implicated in different human diseases such as coeliac disease and inflammatory bowel diseases [13, 14]. Our results show a breakdown in humoral but only a slight decrease in cellular systemic tolerance to fed ovalbumin in indomethacin-pretreated mice. Such a dissociation between humoral and cellular tolerance induced by fed antigen has already been described in different experimental models [10, 15], suggesting different mechanisms of induction. Suppressible cells [16], clonal anergy [17] and soluble factors, that is fragments of ovalbumin [18], have been implicated in tolerance induction for cell-mediated immune response. On the other hand, the mechanism of humoral tolerance remains poorly understood [10]. In our study the increased passage of ovalbumin into the blood could allow an increased stimulation of the general immune system not sufficiently suppressed by the mucosal tolerance induction and responsible for the humoral tolerance breakdown. As the epithelial cells may play a role in the induction of tolerance at the mucosal level [19], their dysfunction or destruction induced by indomethacin may also contribute to the defect observed in tolerance induction. Alternatively, indomethacin by itself may have interfered with the induction of systemic tolerance. Indomethacin inhibits the production of several eicosanoids, that have, at physiological concentrations, some immunomodulatory effects [20, 21]. Particularly, in another system of systemic tolerance induction in mice, involving injection of de-aggregated human  $\gamma$ -globulin followed by injection of aggregated human  $\gamma$ -globulin, indomethacin has been shown to inhibit the induction of a systemic tolerance for humoral response [22]. It has also been shown to block the suppression of lymph node cells by gut epithelial cells *in vitro* [23]. Whatever its mechanism, the development of a systemic humoral response to an antigen present in the digestive tract has been shown to induce a mucosal inflammation in an experimental model [24]. Therefore, the humoral tolerance breakdown to a fed antigen in indomethacin-pretreated mice may participate in the development of mucosal inflammation in NSAID enteropathy. Although there was not a breakdown in cellular tolerance, the tolerance level achieved in indomethacin-treated mice was lower than in non-treated mice. This decrease in cellular tolerance was found both in the lymphocyte proliferation test and in the DTH response. An increase in cellular immune responsiveness toward a luminal antigen may also be involved in the development of mucosal lesions [11] and may thus play a role in NSAID enteropathy.

In conclusion, oral administration of indomethacin is followed by an increased passage of fed ovalbumin from the lumen of the digestive tract into the blood, which is probably due to epithelial and subepithelial alteration. This is followed by a decrease in systemic (mainly humoral) tolerance to ovalbumin. Although the precise mechanism of this decrease in tolerance remains to be elucidated, an increased stimulation of the general immune system, a bypass of intestinal epithelial cells or a direct immunomodulating mechanism of indomethacin may be involved. This decrease may participate in the development of inflammatory lesions characteristic of NSAID enteropathy.

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