

Active Colitis Exacerbates Immune Response to Internalized Food Antigens in Mice

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Key Words

Intestinal inflammation · Colitis · Food allergy · Sensitization · Mucosal immunology

Abstract

Background: Previous studies have indicated that colitis increases intestinal permeability to food antigens. This condition also generates an immunoreactive milieu in the gut, which may exacerbate or counteract allergy reactions. This, along with the fact that both colitis and allergy are being co-diagnosed more frequently, means the scientific interest on the immune relation between these pathologies is increasing. We evaluated the immune response to an internalized food antigen that was initiated during a concomitant active intestinal inflammatory response. **Methods:** An ovalbumin (OVA)-induced immune response was analyzed in healthy mice and in mice suffering from colitis induced by the administration of dinitrofluorobenzene/dinitrosulfonic acid (DNFB/DNS) at the moment of OVA challenge. The OVA-induced clinical score and allergy response both in plasma and in splenocyte cultures from these animals were compared. **Results:** Although no differences were observed in the allergy clinical score, the concomitant active colitis led to an

increase in the immune response to OVA antigen, as shown by increased spleen size and OVA-induced splenocyte proliferation, exacerbated expression of total and OVA-specific IgG1 levels, increased colonic IL-4 expression and OVA-induced IL-4 and IL-5 cytokine expression in spleen cells. **Conclusions:** Our results indicate that animals with active colitis undergo an exacerbated immune response to an internalized antigen. This finding could be relevant for the allergy management of patients presenting simultaneously with chronic colitis.

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Introduction

Abnormal immunologic reactions against endogenous or exogenous antigens, infectious agents, intestinal microbiota or environmental factors contribute to the pathogenesis of several intestinal conditions, including inflammatory bowel disease (IBD) or irritable bowel syndrome (IBS) [1, 2]. Since 1961, when Truelove [3] proposed that ulcerative colitis (UC) was due to an abnormal response towards food allergens such as milk, the hypothesis that allergic reactions to diet antigens are

involved in the etiology and pathogenesis of IBD has remained controversial [4–9]. In fact, practices based on the elimination of a particular food or the use of elemental diets are still common among many IBD and IBS patients, especially those with Crohn's disease (CD) [8–11].

Concerning these initial proposals, a strong association between allergy and UC has been consistently shown in several studies, despite the fact that a causative relation between food allergy and IBD has not been fully demonstrated to date. In this regard, the incidence of cow's milk protein allergy in IBD patients is higher than that observed in a healthy population (20.9% in UC and 8.5% in CD patients compared to 2.8% in the normal population) [12] or even higher and reaching 50% of prevalence in UC patients when referring to general allergic diseases not limited to cow's milk protein allergy [13].

The involvement of allergy as a cause or consequence of UC was further analyzed by D'Arienzo et al. [13]. They found a low rate of sensitization in UC patients before the appearance of clinical symptoms was demonstrated. This observation, together with the finding that the first appearance of intestinal symptoms in most UC patients occurs simultaneously with or precedes allergic symptoms [13], suggests that UC facilitates the onset of allergic symptoms in subjects sensitized to environmental antigens. Moreover, many patients with celiac disease are sensitized to many other food proteins. This observation may reflect either a hypersensitive immune status or an increase in sensitization because of enhanced gut permeability caused by the celiac disease [14]. Finally, another potential explanation for the priming capacity of colitis to cause food allergy could be related to bias in the immune response involved in these two pathologies. In this regard, UC and atopy share a Th2 profile [15, 16]. However, a Th1 component has been extensively described in intestinal inflammation, even in UC patients [17, 18]. This component would exert an opposite action, inhibiting the initiation of the humoral Th2 response induced by allergens.

Of concern is the fact that not only is the incidence of food allergies and colitis rising in the Western world [19, 20], but the age of onset is getting younger [21]. Thus the potential interaction and exacerbation of these two pathologies is of increasing interest. Food allergy is one of the major causes of hypersensitivity in children, affecting approximately 2% of the general population [22]. Allergic food deprivation is currently the only approach available to combat such responses. This medical practice is especially relevant in patients codiagnosed with IBD,

where a nutritional deficit of particular food components could be deleterious.

Experimental animal models are commonly used to simplify and study complex human pathologies such as allergy or colitis [23–33], and they allow the discovery of interactions and common elements of particular signaling events and molecular pathways. With this aim, we examined the exacerbating or inhibitory potential of active intestinal colitis on the allergic response to an internalized food antigen, using the dinitrofluorobenzene/dinitrosulfonic acid (DNFB/DNS) model of colitis [27] and a well-established model of allergy based on ovalbumin (OVA) intraperitoneal (IP) sensitization and challenge. The DNFB/DNS model in mice induces a transient colitis that extends for 3 days and is associated with a mild intestinal inflammation. The altered immune response induced in this model could be considered an unconventional Th2-biased response with an increase on IL-5, IL-10 and humoral response but also Th1 cytokines such as IFN γ , TNF α or IL-17 after subsequent DNS exposures, similarly to what have been described by other UC models such as dextran sodium sulfate-induced colitis in mice [27].

Our findings indicate that the altered immune response induced by DNFB/DNS during colitis is sufficient to exacerbate the allergic response generated against an internalized food antigen. This finding thus supports the notion that patients with colitis may have a more severe response to antigens to which they have previously been sensitized.

Material and Methods

Reagents

All chemicals were purchased from Sigma Chemical Co. (Madrid, Spain) unless otherwise stated. Antibodies for ELISA were purchased from Bethyl Laboratories (Montgomery, Tex., USA: immunoglobulins) or from Biosource (Camarillo, Calif., USA: cytokines). Bovine casein ELISA kit was obtained from Crystal Chem Inc. (Downers Grove, Ill., USA).

Animals

Female 8-week-old BALB/c mice were purchased from Harlan (Barcelona, Spain) and housed at 22°C with a 12-hour-light controlled cycle. They were kept on a casein-based chow diet (Harlan Laboratories, Indianapolis, Ind., USA) under specific-pathogen-free conditions. This study was carried out following the European Union Directive (86/609/EEC) for the protection of vertebrates used for experimental and other scientific purposes, in compliance with the Helsinki Declaration. Animal use was approved by the Animal Research Committee of the University of Granada (No. 2011-355-CEA).

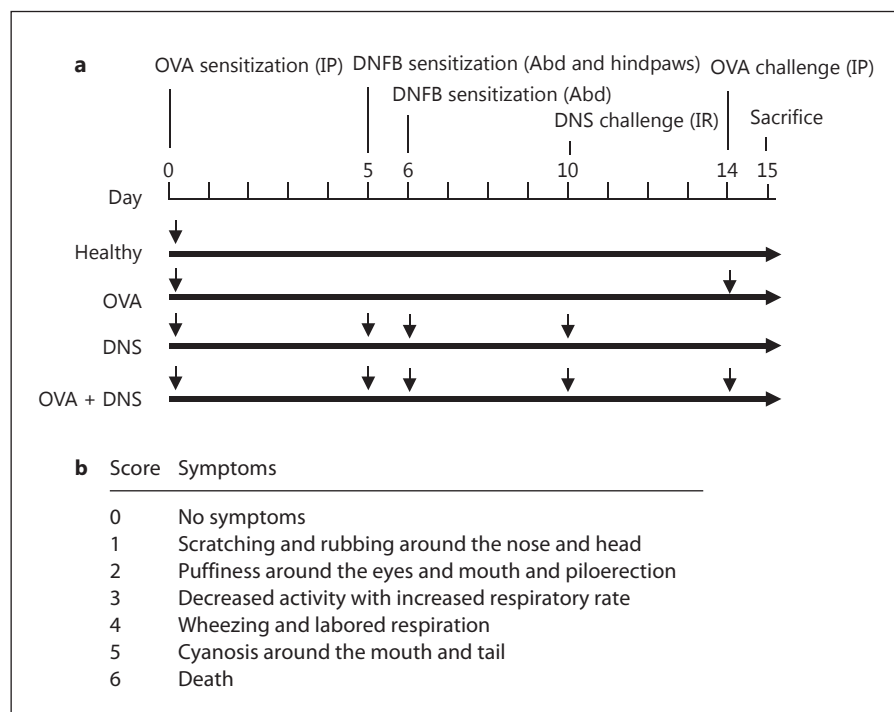


Fig. 1. a Protocol scheme. **b** Score of hyper-sensitivity symptoms. Thirty minutes after OVA challenge, the symptoms of hyper-sensitivity were scored on a scale from 0 (no symptoms) to 6 (death) as shown and previously described [33]. Abd = Abdomen; IR = intrarectal administration.

Animal Models

After a 7-day acclimation period, the mice were weighed and randomly distributed into 4 experimental groups (healthy, OVA, DNS and OVA + DNS; $n = 10$ per group; fig. 1a).

OVA Sensitization

OVA sensitization was induced at day 0 in all groups by IP administration of 200 μ l of a solution containing 1 mg of OVA II per mouse plus Imject Alum adjuvant [(40 mg/ml $\text{Al}(\text{OH})_3$ /40 mg/ml $\text{Mg}(\text{OH})_2$); Pierce, Rockford, Ill., USA]. Control animals (i.e. the healthy group) did not receive any other treatment.

Colitis Induction (DNFB/DNS Model)

In 2 other groups (DNS and OVA + DNS), colitis was induced as previously described [27]. On day 5 (after OVA sensitization), mice were sensitized by epicutaneous administration of DNFB (0.6% in acetone-olive oil 4:1) on the shaved abdomen (50 μ l) and on the paws (50 μ l divided between the hindlegs). On day 6, the mice received a boost of DNFB only on the abdomen (50 μ l). On day 10, animals from both groups were challenged intrarectally with 0.6% DNS dissolved in 10% ethanol. The challenge was conducted under light inhalation anesthesia (3% halothane).

OVA Challenge and Allergy Score

Finally, on day 14, the OVA and OVA + DNS groups were subjected to an IP challenge with OVA (1 mg/mouse plus Imject Alum adjuvant, as previously described).

Using a scoring system modified slightly from previous reports [28, 33], we evaluated hypersensitivity responses 30 min after OVA challenge (fig. 1b). An extra half point for each score was obtained when the symptom became visible at ≤ 10 min after the challenge.

Symptom evaluation was performed by two independent observers who were blind to the treatments.

Blood and Tissue Sample Collection

Twenty-four hours after OVA challenge, all mice were sacrificed by means of a sodium pentobarbital overdose. Before death, the blood of each animal was collected in an EDTA tube by cardiac puncture and the plasma fraction was obtained by centrifugation and stored at -80°C until analysis. Once the mice were dead, the spleen and colon were removed. The spleen was weighed and reserved for splenocyte cell cultures, as previously described [30], while colon samples were placed on an ice-cold plate, cleaned of fat and mesentery and then blotted on filter paper [31]. Each colon specimen was weighed, and the length was measured under a constant load (2 g). Afterwards, the colon was opened longitudinally and evaluated for visible damage and the number of colonic patches, which appear as bulges in the tissue, was counted with the naked eye.

Myeloperoxidase Determination

Myeloperoxidase (MPO) activity in colon homogenates was measured following the technique described by Krawisz et al. [34]. The results were expressed as MPO units per gram of wet tissue. One unit of MPO activity was defined as that degrading 1 μ mol of hydrogen peroxide per minute at 25°C .

Splenocyte Cultures

To obtain primary splenocytes, the removed spleens were homogenized in DMEM plus 1% penicillin/streptomycin. After centrifugation (1,500 rpm for 5 min) erythrocytes were lysed with buffer (1.7 M NH_4Cl , 0.12 M KHCO_3 and 9 mM EDTA) for 30 min at 4°C . Resting cells were counted by using a hemocytometer and

they were cultured in medium containing DMEM plus 10% FBS in order to perform proliferation and stimulation assays. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. Splenocytes were cultured in 24-well plates and stimulated with Concanavalin A (ConA; 5 µg/ml), LPS (50 µg/ml) or OVA (100 µg/ml) for 48 or 96 h, and the supernatants were collected and frozen until ELISA analysis.

Proliferation Assay

Spleen-derived cells were cultured in 24-well plates (3×10^6 cells/ml) in 0.5 ml of media and stimulated with ConA (5 µg/ml), LPS (50 µg/ml) or OVA II (100 µg/ml) for 24 h. Cell proliferation was measured by ³H-thymidine (ICN Pharmaceuticals Inc., Costa Mesa, Calif., USA) incorporation as previously described [30, 32].

Determination of Casein, Immunoglobulin and Cytokine Levels

Plasmatic and cell culture supernatant protein level measurements were performed by ELISA, following the manufacturer's recommendations. Blood was centrifuged (3,500 g for 10 min at 4°C), and plasma aliquots were collected and frozen at -80°C. No dilutions were required for the cytokine determinations of plasma and splenocyte culture supernatants. No plasma dilutions were performed for the analysis of casein levels. For immunoglobulin measurements, dilutions of 1:10,000 were performed for the determination of total IgG1, IgG2a and IgA in plasma, and 1:2 for IgE. Secondary antibodies (Bethyl Laboratories) were diluted 1:10,000 for total IgG1, IgG2a and IgA and 1:5,000 for IgE.

Levels of specific IgG1 to OVA were measured in nondiluted plasma. Plates (96-well) were coated with 20 µg/ml of OVA grade II in coating buffer (0.5 M Na₂CO₃). After overnight incubation at 4°C, plates were washed 3 times with wash solution (50 mM Tris, 0.14 M NaCl and 0.05% Tween 20) and blocked with 50 mM Tris, 0.14 M NaCl and 1% BSA. Plasma samples were then added to the plates and incubated for 1 h at room temperature (25°C). Plates were washed 3 times, and 100 µl of 1:1,000 diluted goat antimouse IgG1 antibody conjugated with peroxidase (Bethyl Laboratories) was added for 1 h at 25°C. Staining was performed with 3,3',5,5'-tetramethyl-benzidine (TMB) for 30 min at 25°C in the dark and stopped with 0.1 M H₂SO₄. The plates were then read at 450 nm. All analyses were performed in triplicate.

Statistical Analysis

Statistical significance was calculated with the Student t test in the case of parametric parameters such as the ELISA results. All tests were performed with one tail following the 2-sample equal variance model (homoscedastic). For the hypersensitivity score, the Mann-Whitney U test was used to determine statistical significance. In all cases, data are represented as the mean ± SEM, and figures correspond to representative data from one of at least three independent experiments.

Results

The selection of the animal models to be used and the treatment protocol were the main concerns of the study (see Discussion). After several approaches, we selected

the DNFB/DNS colitis model and the OVA-induced allergy model by IP administration. We considered these models highly suitable for the proposed goal of the study because both protocols have differentiated routes of allergen administration and an adequate duration (fig. 1a). Furthermore, both models induce only slight alterations on the physiology and behavior of the animals, while producing a clear effect on the immune system. Accordingly, no significant alterations in body weight were seen during the 10 days before colitis induction (challenge with DNS, day 10). DNS-induced colitis led to a trend to transient loss of or no gaining of body weight, this loss being more evident in concomitant allergic animals (OVA + DNS), reaching statistical significance when compared with healthy mice, despite the fact that the allergic process per se did not affect body weight in noncolitic animals (OVA) (fig. 2a). No difference in food intake or water consumption was observed among any of the groups. No differences were detected in the transient incidence of diarrhea/soft stools in either colitic group (data not shown) that could have been responsible for the body weight variations observed.

The IP challenge with OVA induced a fast and robust allergy response characterized by an initial intense scratching by the animals followed by a generally reduced activity, piloerection, wheezing and labored breathing. Moreover, almost one third of the animals showed cyanosis around the mouth and nose. Similar symptoms were observed in both allergy groups (fig. 2b), although a fast onset of the symptoms was observable in the animals suffering concomitant colitis.

The animals were sacrificed 24 h after OVA challenge (day 15), and intestinal and immunologic parameters were analyzed to evaluate the effects on colitis and allergy induced by the two hypersensitivity pathologies. As expected, major macroscopic alterations were not seen in gut parameters after single or combined DNS or OVA challenges after 5 days of the intrarectal DNS challenge (fig. 3a) nor in the number of visible Peyer's patches (0.8 ± 0.4 , 1.4 ± 0.6 , 2.2 ± 0.8 and 2.6 ± 1.1 in the healthy, OVA, DNS and OVA + DNS groups, respectively; not significant), except for a small intestine distension observed in groups subjected to a single challenge (fig. 3b). Moreover, no differences were observed in the colonic inflammatory infiltrate measured as MPO activity (fig. 3c) or in the gut permeability measured as detection of casein protein fragments from the diet in the plasma of animals from the different groups (7.58 ± 1.26 , 9.06 ± 1.89 , 8.72 ± 1.19 and 10.11 ± 2.25 pg/ml in the healthy, OVA, DNS and OVA + DNS groups, respectively; not significant).

Fig. 2. No clinical exacerbation of allergy symptoms was observed in colitic or non-colitic animals. **a** Body weight modifications were recorded daily throughout the experiment and are represented as body weight increase (%) vs. animal weight at day zero. Data correspond to mean $\pm$ SEM. The Student t test was performed to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group. **b** Allergy symptoms were scored 30 min after OVA challenge following the scoring system shown in figure 1b. The Mann-Whitney U test was performed to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; †† $p < 0.01$, compared to the DNS group.

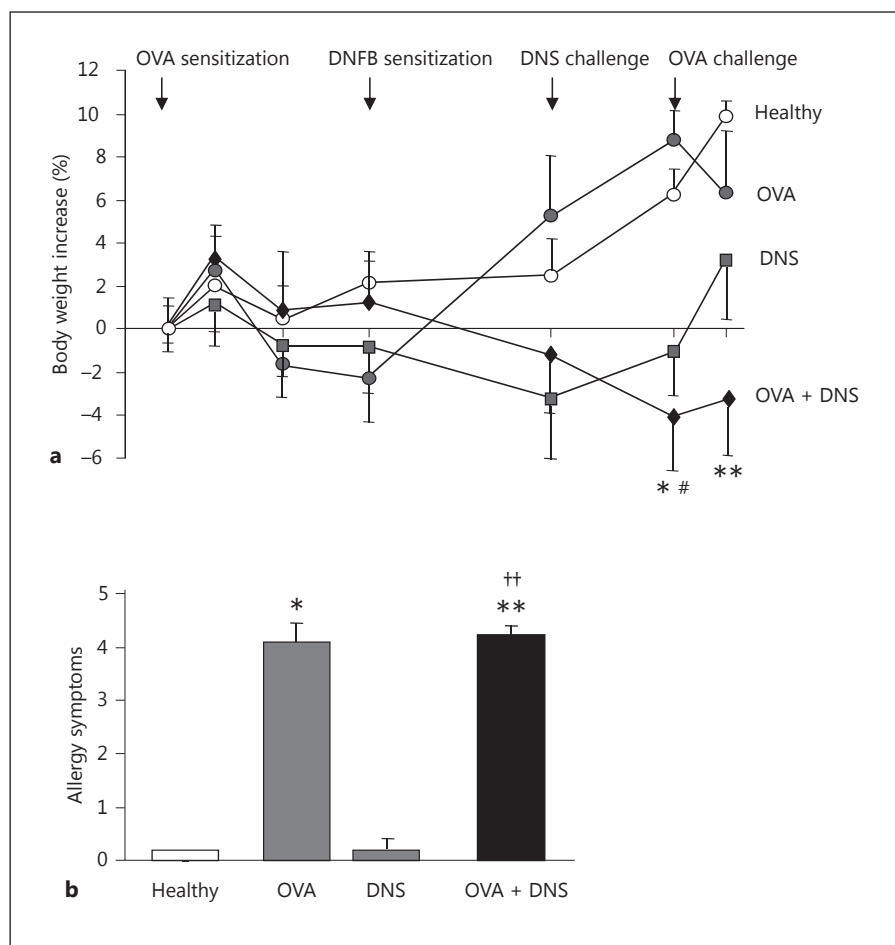
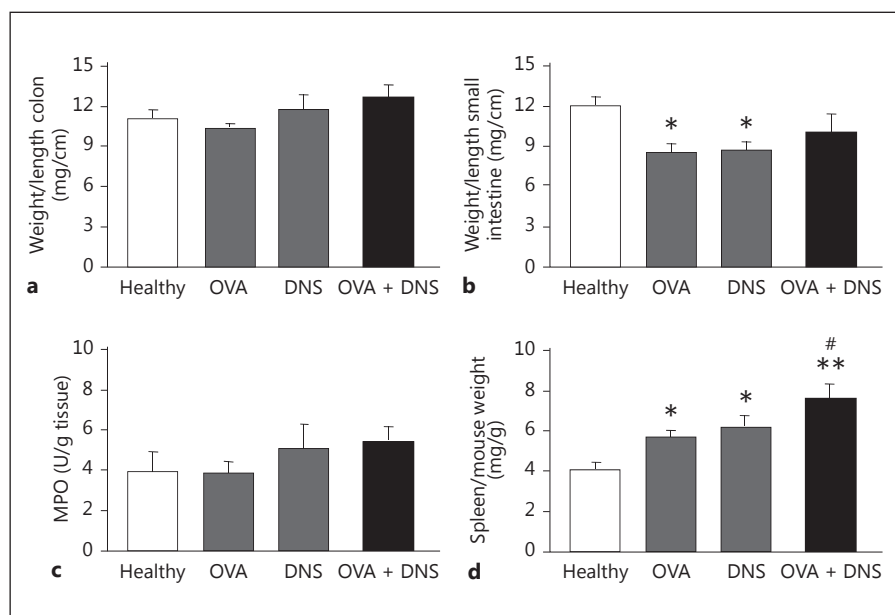


Fig. 3. Intestinal and macroscopic effects. Weight/length ratio of the colon (**a**) and small intestine (**b**), MPO activity in colon homogenates (**c**) and spleen weight (**d**) were measured 24 h after OVA challenge in all animals (as described in Material and Methods). All results are expressed as the mean $\pm$ SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group.



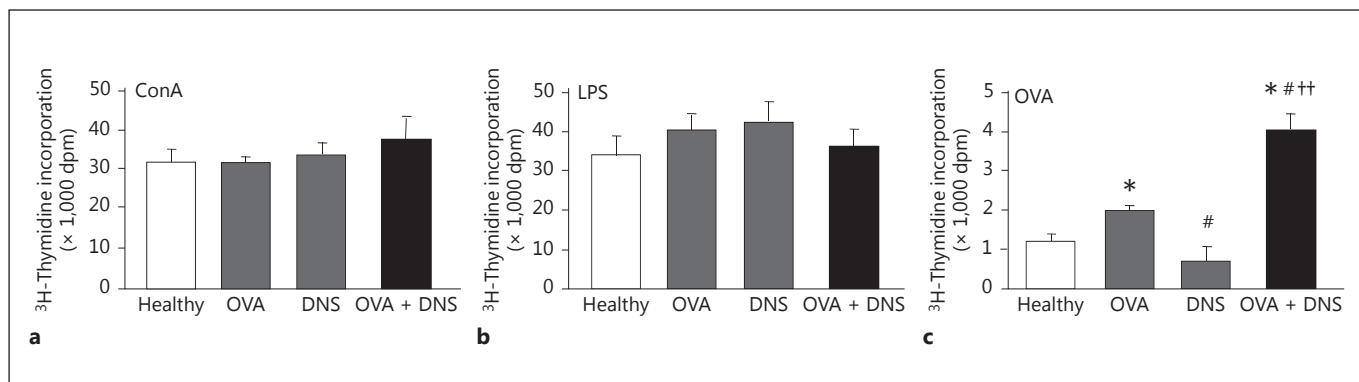
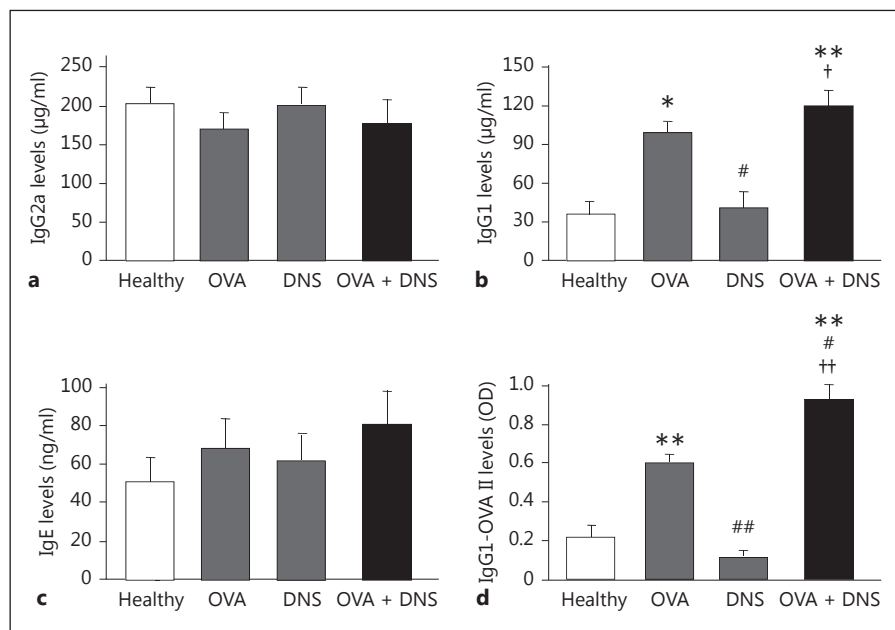


Fig. 4. OVA-induced lymphocyte proliferation was increased in allergic animals with concomitant colitis. Lymphocyte proliferation was measured by ³H-thymidine incorporation in splenocytes obtained from all animal groups cultured in the presence of 5 µg/ml ConA (**a**) or 10 µg/ml LPS (**b**) for 48 h or in the presence of 100

µg/ml purified OVA grade II (Sigma) (**c**) for 96 h. Results are expressed as 1/1,000 of the mean dpm ± SEM. The Student t test was used to determine statistical significance. * *p* < 0.05, compared to healthy animals; # *p* < 0.05, compared to the OVA group; †† *p* < 0.01, compared to the DNS group.

Fig. 5. Active colitis increased the plasma levels of total and OVA-specific IgG1 induced by OVA challenge. Plasma from all mice was collected after sacrifice and used to measure total IgG2a (**a**), total IgG1 (**b**), total IgE (**c**) or OVA-specific IgG1 (**d**) levels by ELISA (as described in Material and Methods). Results are expressed as the mean plasma concentration (**a–c**) or as total absorbance units (**d**) ± SEM. The Student t test was used to determine statistical significance. * *p* < 0.05, ** *p* < 0.01, compared to healthy animals; # *p* < 0.05, ## *p* < 0.01, compared to the OVA group; † *p* < 0.05, †† *p* < 0.01, compared to the DNS group.



Despite the slight intestinal effects at the analyzed time point, the incidence of the two pathologies was observable thanks to alterations in the immune system. Accordingly, a significant enlargement of the spleen was observed after OVA or DNS challenge and was exacerbated by the concomitant pathologies (fig. 3d). This spleen enlargement was associated with an increase in the number of splenocytes (7.98 ± 1.1 , 11.5 ± 0.9 , 12.2 ± 0.9 and 11.1 ± 0.5 million cells/ml in the healthy, OVA, DNS and OVA

+ DNS groups, respectively). To analyze the immunoreactivity of the splenocytes, cell proliferation was evaluated by ³H-thymidine incorporation after stimulation of cells with ConA (T cell stimulus), LPS (B cell stimulus) or OVA (antigen-specific lymphocytes) (fig. 4). The data obtained pointed to the high antigen selectivity of the OVA-induced allergy model, since no increased splenocyte proliferation was observed after general lymphocyte activators such as ConA or LPS (fig. 4a, b), while a sig-

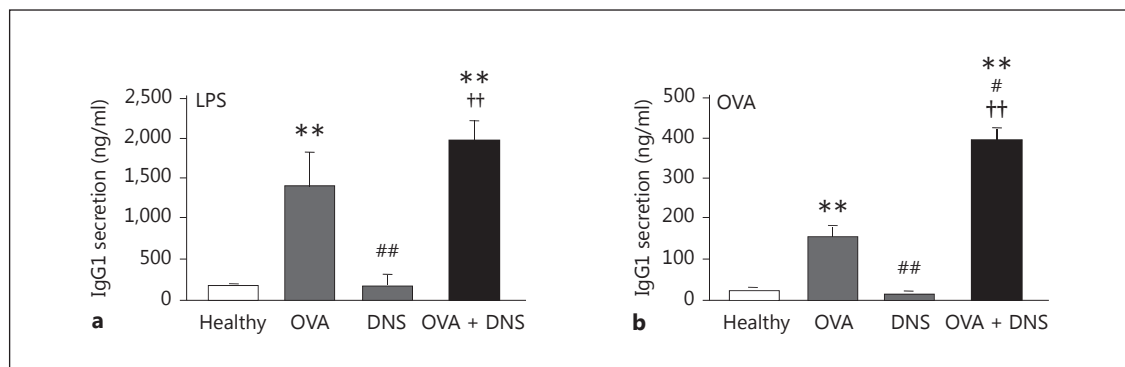


Fig. 6. Active colitis increased the secretion of total and OVA-specific IgG1 in splenocytes from concomitantly allergic animals. Lymphocyte immunoglobulin secretion was measured in splenocytes obtained from all animal groups cultured in presence of 10 µg/ml LPS (**a**) or 100 µg/ml purified OVA grade II (Sigma) for

96 h (**b**). Results are expressed as the mean concentration ± SEM. The Student t test was used to determine statistical significance. ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, ## $p < 0.01$, compared to the OVA group; †† $p < 0.01$, compared to the DNS group.

nificant increase in response was observed in the OVA group after the ex vivo stimulation with the OVA allergen (fig. 4c). Moreover, although no increased proliferation was seen in the DNS group subjected to any of the stimuli used, the colitic process induced by DNS duplicated the in vitro splenocyte response induced by OVA stimulation in colitic animals (DNS + OVA) (fig. 4c).

The exacerbation of the specific allergy response to OVA in animals suffering concomitant colitis was also observed when analyzing the humoral response. Accordingly, total IgG2a plasma levels of all treated groups resembled those of healthy animals (fig. 5a). Only differences in total IgG1 levels (fig. 5b), the main immunoglobulin that drives allergy in mouse models [28, 33], but not IgE (fig. 5c), were observed between treated groups, where increased levels were detected in both OVA-induced allergy groups compared to the 2 nonallergic groups (healthy and DNS-colitic). Again, the specificity of the response was demonstrated through the analysis of the OVA-specific IgG1 plasma levels, which increased 3-fold in the OVA group and even more in OVA + DNS animals (fig. 5d). Consistent with the immunoglobulin plasma levels, IgG1 secretion induced by LPS- or OVA-activated splenocytes was also increased in the allergy group and was exacerbated in allergic animals that also had colitis (fig. 6).

Finally, to further analyze the immune response induced, the cytokine response exerted in the two pathologies was also measured in the plasma and splenocytes. Although significant differences in plasma levels were not observed in any of the treatment groups (not shown) or

in the supernatants of cultured splenocytes activated with ConA (table 1), a clear Th2-biased increase in cytokine response was detected in spleen T cells activated with OVA in the allergy groups. This response increased in magnitude in allergy animals with concomitant colitis (fig. 7). In this regard, the allergic process induced by OVA challenge in colitic animals significantly increased the secretion of IL-2, IL-4 and IL-5 in splenocytes. This observation suggests an increase in OVA-reactive lymphocyte clones in the spleen of these animals, a phenomenon that was not significant (although a trend was noted) in noncolitic allergy animals (fig. 7), which achieved significance only for IL-5 secretion.

Discussion

Intestinal hypersensitivity conditions such as IBD, IBS, celiac disease or food allergy are the result of interactions between immunologic, genetic and environmental factors. The coexistence of IBD and allergy has become more frequent in recent decades, and many studies indicate a connection between these two pathologies [4–13, 20–22]. At present, we do not know whether food sensitization is a risk for or a result of intestinal inflammation. However, what is clear is that almost one third of unselected patients with gastrointestinal inflammatory diseases report adverse reactions to food as a cause of their symptoms [35]. Food commonly identified by colitic patients as causing symptoms includes milk, peanuts, citrus fruits, wheat, eggs and fish, all of which are correlated

Fig. 7. Active colitis modified the cytokine profile induced in response to an internalized food allergen. Cytokine secretion was measured in splenocytes from all animal groups cultured in the presence of 100 µg/ml purified OVA grade II (Sigma) for 96 h. Cytokines were measured in the nondiluted culture supernatants by ELISA, following the manufacturer's instructions. Results are expressed as the mean concentration ± SEM. The Student t test was used to determine statistical significance. * $p < 0.05$, ** $p < 0.01$, compared to healthy animals; # $p < 0.05$, compared to the OVA group; † $p < 0.05$, †† $p < 0.01$, compared to the DNS group.

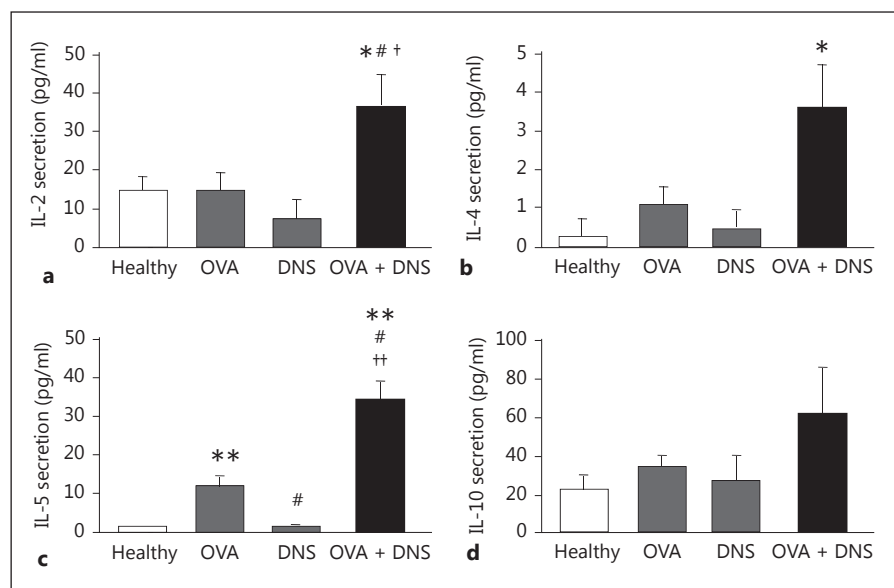


Table 1. ConA-induced cytokine expression in spleen cells

	Healthy	OVA	DNS	OVA + DNS
IL-2	1,581.1 ± 143.2	1,193.2 ± 50.6	1,351.1 ± 55.9	1,114.5 ± 171.4
IL-4	594.8 ± 137.2	570.0 ± 90.4	493.4 ± 101.4	299.6 ± 38.5
IL-5	59.1 ± 23.3	44.6 ± 6.7	20.09 ± 2.4	36.0 ± 10.2
IL-10	539.9 ± 45.9	523.9 ± 64.7	664.9 ± 43.4	507.1 ± 10.8

Data correspond to cytokine levels in pg/ml from splenocyte cultures obtained from each animal (n = 10 per group) and are represented as the mean ± SEM. No statistical difference was observed in any of the comparisons.

with sensitization [9, 12, 36–38]. As a result, elimination diets or elementary diets have become commonplace for some colitic patients in recent decades [10, 39]. In this regard, more than 20% of patients in the active phase of UC [40] or CD [41] benefit from milk-free diets. Moreover, it should also be considered that food allergy may be present in patients who do not report adverse reactions to food, as they may not recognize that a certain food triggers their symptoms because the allergens are multiple or ubiquitous and therefore difficult to identify by experience [35]. Taking all these data together, there is a clear correlation between IBD and food allergy.

Since human pathological research has been widely supported by laboratory data thanks to the use of diverse experimental animal models, our objective was to analyze the relation between allergic response and colitis using simplified experimental models. For this purpose, our

first and more complex task was to select the most suitable experimental models resembling the pathophysiological mechanism for the correlation between the two pathologies observed in humans. Three main interrelated mechanisms have been hypothesized that could be responsible for the association between colitis and allergy, namely increased intestinal permeability, antigen cross-reactivity and the existence of a biased immune response [4, 14, 34].

It is currently well accepted that alterations in the mucosal barrier and permeability may occur during intestinal inflammation, which could facilitate the entrance of antigens and, therefore, the onset of the hypersensitivity symptoms in subjects sensitized to environmental antigens involved in allergy and IBD, despite the fact that this hypothesis has not been confirmed [13, 14] and is even neglected [42] in some studies. However, mucosal barrier

disruption in experimental models is highly variable and leads to the entrance of several food and gut antigens that will finally cause an unspecific immune response, thus leading to a misleading evaluation of the results obtained. Therefore, to achieve the main desired characteristics for any experimental model, i.e. the robustness and reproducibility of the data obtained, we made the compromise of eliminating the altered intestinal permeability from the equation, despite being aware that by doing so we are limiting the similarities to the complex human situation. Based on this, we selected the DNFB/DNS colitis model because it induces mild inflammation that does not alter mucosal integrity [27], confirmed by the fact that no significant differences were observed in gut permeability to intact proteins. Moreover, the selection of a modified IP OVA-induced allergy model [43] allows the evaluation of an allergic response caused by a specific internalized antigen at the desired concentration, thus reducing the variability and lack of specificity of the induced response.

On the other hand, the cross-reactivity of antibodies against allergens and antibodies related to intestinal inflammation, caused by an exacerbated response to similar mucosal antigens, has also been postulated to be responsible for the association between the two pathologies. This cross-reactivity has been found between some inhalant and food antigens involved in colitis, such as Betv1, Mald1 [14, 44], and between food antigens and autoantigens involved in other autoimmune diseases such as type I diabetes [45]. In this regard, the selection of DNFB/DNS and OVA antigens in our mouse models, the controlled and casein-based diet used and the absence of alteration of intestinal integrity greatly reduce the likelihood of cross-reactivity between DNS-modified antigens and OVA. Accordingly, our results demonstrate that the observed exacerbation of the allergic response was specific for OVA and did not result from a general upregulation or hyperreactivity of the systemic immune system in the colitic animals. Due to this, no increase was observed in total immunoglobulins (except IgG1) and the increased immune response in splenocytes (increased production of IgG1, IL-4 and IL-5) was observable only after splenocyte stimulation with purified OVA but not with general stimuli.

Therefore, our data suggest that the main driver of the exacerbated allergic response observed in the colitic animals was due to the immune modulation resulting during the intestinal inflammation induced by the DNFB/DNS model. In this regard, an increased allergy response to OVA due to a biased Th2 environment induced by haptens such as TNBS has been previously described [46]. However, it should be noted that the immune exacerbat-

ed response observed was not due to an additive effect of the immune mediator levels induced during colitis to those at the time of OVA challenge, as the immune response induced by the DNFB/DNS model was mild and returned to basal levels after the first 3 days [27] – shown by the poor altered immune response observed in the DNS group. This was also a reason to select the DNFB/DNS model in Balb/c mice rather than use other haptens or mouse strains with an increased immune response that could interfere with the evaluation and interpretation of the results observed.

In this regard, the colitic process and the biased immune response originating during the DNFB/DNS sensitization/challenge period may have produced transitory immune factors or stimuli that reinforce the maintenance or perpetuation of the OVA-specific clones generated during OVA sensitization. Several factors have been described as participating in the maintenance and perpetuation of memory B cells such as IL-4, B-cell activating factor, nerve growth factor [47, 48] or even IL-18 [49], an inflammatory cytokine that also plays a critical role in mucosal repair and homeostasis. Moreover, the Th2 cytokines and mediators produced locally in high amounts during colitis, namely IL-3, IL-5 and GM-CSF (by eosinophils) and SCF or IL-4 (by mast cells), are of major importance not only for the development and recruitment of allergy effector cells but also for the enhancement of their functional activity and for increasing mediator release in response to classical triggering agents [14]. Accordingly, IgE-mediated food allergy is more frequently described in UC [13, 37, 50] than in CD [51, 52] in relation to the more Th2-biased pathomechanism of UC. In support of this notion, an increased *in vitro* response to OVA stimulation, but not to other stimuli, was observed in splenocytes obtained from the OVA + DNS group in comparison to all the other groups. Moreover, the involvement of the acquired immune system in the exacerbated response was also evidenced by the enlargement of the spleen and an increase in IL-2 expression.

Taken together, our results clearly demonstrate that an exacerbated immune response to internalized OVA antigen occurs in animals with active colitis; this was the main objective of the study. However, experimental models are not always fully satisfactory, and this one is not an exemption. The IP OVA model induced a robust allergy symptomatology that impeded the detection of clinical differences between the colitic and noncolitic groups. Moreover, we observed a distension of the small intestine in both the DNS and OVA groups. Although this feature did not modify the gut permeability of the animals and has

also been observed in other intestinal hypersensitivity models [27, 29], the etiology of this phenomenon remains unknown. Finally, a poor systemic immune response, i.e. no changes in levels of plasma cytokines or total IgE antibodies, was observed in our models, despite the fact that these molecules are common markers of human allergy. Nevertheless, the lack of systemic cytokine disturbances and the non-IgE IgG1-biased response are common features of several allergy models [24, 25, 27–29].

The observations presented in this work might be relevant for subjects codiagnosed with colitis and food allergy who present with a highly reactive immune response and increased gut permeability. More studies are required

to evaluate the potential effect of colitis on the sensitization risk to new antigens. In this regard, an increased control of allergy reactivity and the implantation of strategies to avoid or reduce the increased risk of anaphylaxis in these subjects should be explored.

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