Emerging Therapy in Arthritis: Modulation of Markers of the Inflammatory Process

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ABSTRACT The induction of tolerance has been proposed as a therapeutic strategy for arthritis aiming to decrease progression of the pathology, probably by promoting suppressor mechanisms of the autoimmune response. This work aimed to confirm whether the treatment with vitamin D3 could synergize oral tolerance induced by hydrolyzed collagen peptides, in our experimental model of antigen induced arthritis in New Zealand rabbits. Clinical observation of the phenomenon indicates that simultaneous treatment with hydrolyzed collagen peptides and vitamin D3 was beneficial when compared with no treatment, for arthritic animals, and for arthritic animals that received treatment with only hydrolyzed collagen peptides or vitamin D3. Treatment with hydrolyzed collagen peptides caused diminished proinflammatory cytokine levels, an effect synergized significantly by the simultaneous treatment with vitamin D3. The anatomical-pathological studies of the animals that received both treatments simultaneously showed synovial tissues without lymphocytic and plasma cell infiltrates, and without vascular proliferation. Some of the synovial tissue of the animals of these groups showed a slight decrease in Galectin-3 expression. We propose that simultaneous oral treatment with vitamin D3 and hydrolyzed collagen peptides could increase the immunoregulatory effect on the process of previously triggered arthritis. We used articular cartilage hydrolysate and not collagen II because peptides best expose antigenic determinants that could induce oral tolerance. Oral tolerance may be considered in the design of novel alternative therapies for autoimmune disease and we have herein presented novel evidence that the simultaneous treatment with vitamin D3 may synergize this beneficial effect. *Microsc. Res. Tech. 00:000–000, 2016*.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune pathology. Because of its high prevalence rate (1% of the adult population) and because it affects quality of life and increases the development of arthritis-related diseases, as well as mortality, with the consequent economic/health impact, the study of this pathology should be considered a global and national priority (Cardiel, 2006). The occurrence of RA is characterized by joint inflammation, with proliferation of the synovial membrane and cartilage and juxta-articular bone erosion, which becomes progressive. Its etiology is multifactorial, although it is known that the balance of pro- and anti-inflammatory cytokines is altered (Blandizzi et al., 2014; Firestein and Zvaifler, 1990; Sarrio et al., 2006; Inoue et al., 2006). The subsequent activation of metalloproteinases at the synovial membrane level and the production of nitric oxide and reactive oxygen species due to the hypoxia caused by proliferation could lead to tissue damage (Firestein and Zvaifler, 1990). Synoviocytes undergo hyperplasia and angiogenesis possibly occurs to support the growth of the synovial lining. In RA, the main sources of proinflammatory cytokines are T cells and macrophages (Inoue et al., 2006). Proinflammatory cytokines play a decisive role in the generation of the inflammatory and destructive response (Smolen et al., 2005).

Citrullination, a reaction that converts arginine residue into citrulline residue, is essential for autoimmunity of RA (Chang et al., 2013). Citrulline is formed from arginine residues in peptides or proteins by the enzyme peptidylarginine deiminase (PAD). It has been hypothesized that during rheumatoid synovitis, PAD is released into the extracellular space, locally inducing citrullination of arginine residues in several proteins such as type I and II collagens. The citrulline antibody appears early in the course of RA and is present in the blood of

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most patients. When the citrulline antibody is detected in a patient's blood, there is a 90–95% likelihood that the patient has RA and that his/her pathophysiology is probably implicated (De Rycke et al., 2005). Pathologically, antibodies to citrullinated proteins are expected to be produced in the synovial compartment: PAD generates citrulline residues by deamination of arginine residues of proteins. Isoforms 2 and 4 of PAD are expressed in mononuclear cells isolated from synovial fluid (Foulquier et al., 2007). These data suggest that the presence of citrullinated proteins in the RA synovium causes antigen-driven maturation of B cells at the site of inflammation. Although absent in healthy synovium, PAD4 mRNA is readily transcribed and translated by polymorphonuclear neutrophils infiltrating the synovial tissue during inflammation. As a consequence, several synovial proteins are subjected to citrullination. One of these proteins has been identified as fibrin, which has been reported to be citrullinated also in the synovium of patients with RA. Antibodies directed to citrulline-containing proteins are highly specific for RA and can be detected in up to 80% of patients with RA (Foulquier et al., 2007).

It is known that during the progression of RA there is also an imbalance of galectins, a highly conserved family of lectins, which play an essential role in processes related to the regulation of the immune response (Rabinovich and Toscano, 2009). In particular, Galectin-3 would have a proinflammatory role, promoting, among other actions, monocyte chemotaxis and activation of macrophages. It has been shown that this galectin is present in the inflamed synovium of RA patients, mainly in the areas of destruction of articulation (Ohshima et al., 2003), and that it has a pathogenic role in the development and progression of the disease (Forsman et al., 2011). Galectin-3 stimulates synovial fibroblasts to secrete proinflammatory chemokines and cytokines, favoring the recruitment of mononuclear cells and the persistence of inflammation and joint destruction. Synovial fibroblasts are also an important source of Galectin-3 (Filer et al., 2009).

In addition, several pieces of evidence support the hypothesis that a vitamin D (1,25D) imbalance may be a factor in the etiology of autoimmune diseases mediated by T cell responses, such as RA. It is known that 1.25 D exerts an antiproliferative action in inflammatory responses and acts as a powerful immunosuppressant (Cordero et al., 2002; Dusso, 2014; Dusso et al., 1997). Interactions between vitamin D metabolism and inflammation indexes through secretion of tumor necrosis factor-alpha (TNF-α), which are also especially involved in osteoclastic resorption and therefore in bone loss processes observed in RA, have been suggested (Frediani et al., 2006).

Several studies have addressed the association between low vitamin D levels and RA activity, and clinical disability manifestations (Cutolo et al., 2006). Some preliminary data indicate a possible association between seasonal changes of vitamin D serum levels, latitude, and disease activity in RA patients. This must not be surprising, as the immunomodulatory effects of vitamin D are clear and have been attributed to protective effects in autoimmune disorders such as some chronic inflammatory bowel diseases, multiple sclerosis, and type I diabetes.

Because RA is an inflammatory incapacitating disease it is necessary to consider emerging therapeutics, without unwanted side effects. The induction of tolerance has been proposed as a therapeutic strategy that aims to decrease the progression of the pathology, probably by promoting suppressor mechanisms of the autoimmune response. Oral tolerance is initiated in the gut-associated lymphoid tissue that comprises the mucosal epithelium, lamina propria, Peyer's patches, and mesenteric lymph nodes. Several lines of evidence have suggested that Peyer's patches, which are lymphoid nodules interspersed among the intestinal *villi*, are essential for mucosal immune responses and oral tolerance to soluble antigens (Backstrom and Dahlgren, 2004; Feldmann et al., 2004; Sigal, 2005). Oral tolerance has long been recognized as a physiological mechanism of immune unresponsiveness to dietary antigens and indigenous bacteria that maintains tissue integrity by preventing harmful delayed-type hypersensitivity responses in the intestine (Backstrom and Dahlgren, 2004). Although deletion might contribute to oral tolerance in certain situations, other mechanisms, such as clonal energy or active suppression, are necessary to maintain efficient tolerance, since the thymus continuously releases naive lymphocytes (Dubois et al., 2009).

We have presented preliminary evidence in our model of antigen-induced arthritis, in which oral tolerance treatment with hydrolyzed collagen would decrease the progression of the inflammatory pathology (Abramson et al., 2014; Mortarino et al., 2008; Toledo et al., 2009). On the other hand, vitamin D, as a pleiotropic hormone, may synergize the immunotolerance process (because of the evidence that low levels of vitamin D are related to a decrease in the proportion of TCD4+ cells that would differentiate towards the formation of regulatory or suppressor T cells, directly related to tolerance phenomena) (Christakos et al., 2006). Given this background, we aimed to confirm these findings at a clinical and imaging level, as well as to obtain indicators accounting for the effects of the therapeutic strategies provided. Interferon-gamma (IFN-γ) contributes to the decrease in vitamin D and the conversion of vitamin D to its active hormonal form in the circulation during human inflammation (suggesting that 25(OH)D decreases and conversion to 1,25(OH)D increases with increasing IFN-γ in the circulation) (Barker et al., 2012). To this end, we decided to supply our experimental model with cholecalciferol (vitamin D3) rather than directly with the active metabolite of vitamin D, to prevent any undesirable effects related to hypercalcemia, which high doses of the latter could generate.

MATERIALS AND METHODS

Experimental Model

Three-month-old female New Zealand rabbits ($\sim$3 kg) were kept in individual cages, with a standard diet (Asociación de Cooperativas Argentinas, Buenos Aires, Argentina) and water ad libitum (five groups, $n = 5$ of each).

Protocols for animal studies were approved by the Institutional Ethics Committee of the School of Medical Sciences of the Universidad de Rosario, Santa Fe, Argentina, and Resolution No. 713/2012. The control

group (C, $n = 5$) received placebo arthritis induction. Animals subjected to arthritis induction ($n = 20$) were subdivided in four groups according to the oral treatment received (A; AL; AD; and ADL; $n = 5$ each group). Oral treatments were randomly assigned to the animals according to their number, and using a table of random numbers (Evans and Olson, 1998). Before the onset of the experiment it was established that none of the oral treatments affected the variables under study in the control group.

Induction of Arthritis in the Knee Joint of Rabbits. Arthritis was induced as previously described (Sanchez-Pernaute et al., 2003). Briefly, the animals received intradermal immunizations in their backs of an emulsion of ovalbumin (OVA) (Sigma Chemicals, USA) (5 mg mL^{-1} in NaCl 0.9%) and complete Freünd's adjuvant (Sigma, USA) in a 1:1 proportion. After 15 days they were reimmunized. Five days later, 1 mL of OVA (5 mg mL^{-1} in NaCl 0.9%) was injected into their knees. Group C received an injection of 1 mL vehicle (NaCl 0.9%).

Twenty days after the second induction, we verified whether there was inflammatory state in rabbits subjected to arthritis induction, by clinical studies and magnetic resonance (MR) studies. This procedure allowed us to avoid unnecessary slaughter of animals and to analyze the synovial tissue anatomopathologically at this stage, as previously performed (Abramson et al., 2014). The MR studies were performed to confirm that all knees of the animals presented hydroarthrosis in the articular space and alterations in the subchondral bone, detected as hyperintensity. The MR studies were carried out with a magnetic imaging scanner RMN General Electric "Vectra" 0.5 Te, coronal coil, with the following sequence: Inversion Recovery TR 2000, TE 60, NEX 3, time of acquisition 6 min, characterizing three different anatomical spaces: joint space, subchondral bone, and periarticular soft tissues.

Oral Treatments

Hydrolyzed collagen peptides, kindly provided by *Sucesores Alfredo Villar S.A. Laboratories*, Rosario, Argentina, was obtained by enzymatic hydrolysis of undenatured articular cartilage extracted from the lower limbs of 2-year-old cattle, cholecalciferol (vitamin D3) was kindly provided by Laboratorios Gador, Argentina.

Oral treatments were developed for 3 months, 20 days after the second immunization: (1) hydrolyzed collagen peptides, 0.500 mL/rabbit/day (AL group); (2) cholecalciferol (vitamin D3), 2,000 U/rabbit/day (AD group); (3) hydrolyzed collagen peptides, 0.500 mL/rabbit/day and cholecalciferol (vitamin D3), 2,000 U/rabbit/day, simultaneously (ADL group); and (4) an oral placebo dose rabbit/day (A group).

Clinical Studies. Performed twice: (1) to confirm that there was an inflammatory process, which took place 20 days after the second immunization; and (2) to evaluate differences between groups 3 months after the treatments. Three observers independently evaluated each of the two knees of each rabbit clinically, according to the following score: 0 if it was unaltered; 1 if it was swollen and showed no demonstrable pain by palpation; 2 if it was swollen and demonstrated pain by retracting the knee in palpation. This means that each rabbit would have a score that could range from 0 (if neither knee was affected) to 4 (in the case that both knees were swollen and tender). Values provided by each observer were averaged for each rabbit. Subsequently, data between groups were statistically compared using the Kruskal Wallis, and Mann Whitney tests.

Extraction of Serum

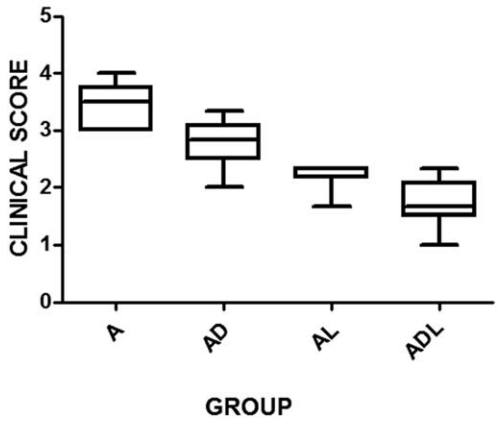
Conventional norms were respected for the extraction of sera, which were fractionated in Aliquots of 50 μL and preserved for no more than 30 days at −4°C, for the determinations of cytokine levels and IgG anti-citrullinated protein.

Study of IgG Anti-citrullinated Protein. We applied the indirect immunofluorescence (IFI) technique. This method was applied because it has been shown that in RA patient sera anti-citrullinated antibodies are present that react with filaggrin proteins expressed in the rat esophageal epithlelium (Dubois-Galopin, 2007; Simon et al., 1993), and also because we previously demonstrated that it was a simple determination of IgG anti-citrullinated protein in our experimental model (Mortarino et al., 2008). Prior to the determination, cryostatic sections were obtained from the esophagus of a 4-month-old Sprague Dawley mouse post-mortem according to conventional techniques. The sections were immediately immersed in small cryopreservation containers and preserved in liquid nitrogen. Samples were preserved in a freezer at −70°C until cryostatic sections of 6 μm (slides) were obtained, in order to be used in conventional IFI technique. Serum samples were diluted in phosphate buffered saline (PBS) containing 0.1% Tween-20, and were incubated on the slides for 30 min. The slides were rinsed twice with buffer for 5 min and incubated for 45 min with a polyvalent anti-rabbit immunoglobulin fluoresceinated conjugate (SIGMA)—the sera from animals of group C were diluted 1/40 to avoid unspecific reactions. The sera of each animal group (A, Al, AD, ADL) were titrated and the last serum dilution exhibiting evidence of fluorescence was considered the titration end-point. Data between groups were statistically compared using the Kruskal Wallis, and Mann Whitney tests.

Assessment of Pro-inflammatory Cytokines. The levels of TNF-α and IFN-γ were assessed by the enzyme immunoassay technique (Quantikine laboratories from R&D Systems). Determinations were performed in triplicate. In these studies the assumptions of ANOVA were fulfilled. The assumptions that the dependent variable is approximately normally distributed for each category of the independent variable were analyzed using the Shapiro-silk test of normality, and homogeneity of variances was analyzed applying the Levene's test. The level of statistical significance was determined by ANOVA and the Tukey Test.

Slaughter of Animals

After 3 months of treatment all animals were sacrificed with five times the dose used for general anesthesia for New Zealand rabbits (acepromazine 1 mg kg^{-1}, ketamine 35 mg kg^{-1}, xylazine 18 mg kg^{-1}), according



Groups	p
A – AD – AL - ADL	<0.0001*
A – AD – AL	0.003*
AD – AL - ADL	0.006*
A – AD – ADL	0.001*
A – AD	0.027#
A – AL	0.003#
A – ADL	<0.0001#
AD - AL	0.041#
AD - ADL	0.004#
AL - ADL	0.065#

*Kruskal Wallis Test #Mann Whitney U test

Fig. 1. Severity of arthritis; clinical score estimated. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously.

to the Bioethical Committee of the Facultad de Ciencias Médicas, Universidad Nacional de Rosario.

Anatomopathological Studies of Synovial Tissues. Sections of the synovial tissues of the left knees obtained from sacrificed animals were fixed in formaldehyde at 10% in PBS. After immersing in deionized water and dehydration, they were soaked in paraffin and 5-μm sections were cut, set on a glass support pretreated with polylysine, and subjected to conventional hematoxylin-eosin staining. The severity of the pathological status was evaluated by optical microscopy, considering whether there was inflammatory infiltrate, synovial hyperplasia, angiogenesis, and edema. Three independent observers studied 50 fields per sample. Criteria for characterization of the synovial membrane fields used in previous work were applied, in high power field; (Abramson et al., 2014): normal synovial tissues (without inflammatory cells), low inflammation tissue (<5 inflammatory cells/field), moderate inflammation tissue (5–10 inflammatory cells/field, and edema), severely inflamed tissue (more than 15 inflammatory cells/field, and angiogenesis and edema).

Analysis of Galectin-3 Synovial Expression by Western Blot. The synovial tissue of the right knees obtained from sacrificed animals was homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 1 mM PMSF and a protease inhibitor cocktail 1Xl (Pierce)) in a homogenizer and left on ice for 30 min. The solution was centrifuged at 4°C for 10 min at 15,000 rpm and supernatants were stored at −20°C. After quantifying protein extraction, samples corresponding to 150 μg were mixed 1:1 with 2XSDS-PAGE loading buffer, boiled for 5 min, cooled on ice, and resolved on a 15% PAGE under denaturing conditions. After electrophoresis, the separated proteins were electroblotted onto nitrocellulose membranes and reversible Ponceau staining was applied to check equal loading of gels. The membranes were probed with a 1:2,000 dilution of an anti-Galectin-3 antibody, produced and kindly provided by Dr. Gabriel Rabinovich from the Lab. de Inmunopatología - IBYME—CONICET, Argentina, (Harjacek et al., 2001). The membranes were then washed and incubated with a 1:3,000 dilution of a horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology, USA). The secondary antibody was detected by means of chemiluminescence using an ECL kit (Pierce). Ponceau staining of proteins in the nitrocellulose membrane was used as loading control (Romero-Calvo et al., 2010).

RESULTS

Clinical Studies

Clinical observation showed significant differences between rabbits with induced arthritis (A) versus controls, as reported previously (Abramson et al., 2014; Mortarino et al., 2008; Toledo et al., 2009). The simultaneous treatment of collagen hydrolysates cholecalciferol significantly reduced clinical scores of rabbits, not only in comparison with group A, but also with those obtained with those animals of the groups that received a single treatment (Fig. 1).

Anatomical–Pathological Studies of Synovial Tissues

The control group showed unaffected synovial tissue (Fig. 2a). The arthritic group revealed severely inflamed tissue (Fig. 2b). The AL group was characterized as moderate inflammation synovial tissue, because the numbers of inflammatory cells and synoviocyte proliferation were reduced, but the tissue still presents some edemas (Fig. 2c). Histological examination of the AD group revealed a state of low inflammation tissue, so the thickness of the cell lining layer and the density of the mononuclear cell infiltrate were significantly lower (Fig. 2d). The synovial tissue of rabbits with arthritis that received both treatments simultaneously (ADL) showed fewer areas with hyperplasia in only two samples (3 areas/50 fields). This group did not show inflammatory cell infiltrates, and was without vascular proliferation (Fig. 2e). So these groups were considered as normal synovial tissues.

Serum Levels of Anti-citrulline IgG Antibodies

These were considerably higher for group A than for the control group ($P < 0.01$). While the ADL group

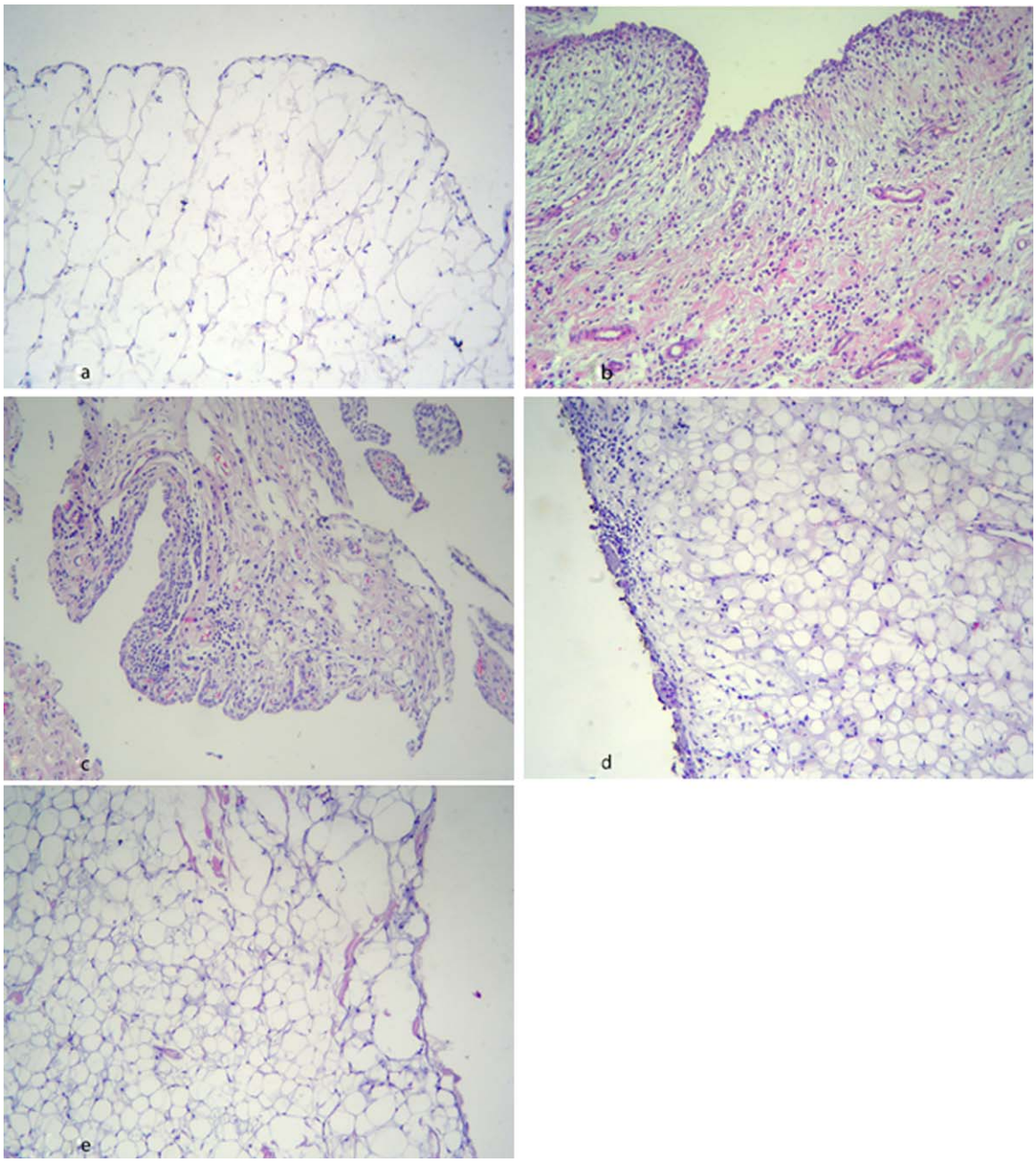


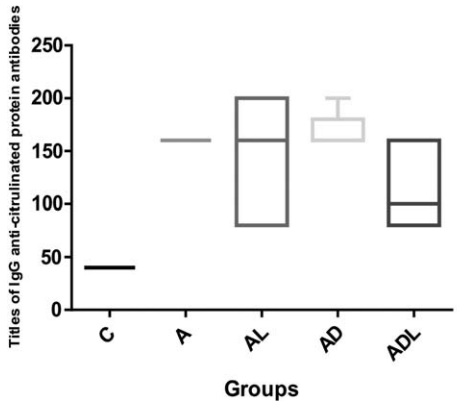
Fig. 2. Optic microscope images of synovial tissues histological sections (×10). **a**:C. **b**: A. **c**: AL. **d**: AD. **e**: ADL. C group; control group. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

showed 3/5 rabbits with lower levels of anti-citrulline antibodies in relation to group A, the differences were not so significant (Figs. 3 and 4).

Serum Levels of Proinflammatory Cytokines

IFN-γ levels. IFN-γ levels in A were significantly higher than in C ($P < 0.01$). All treatments promoted a decrease in the levels of this cytokine ($P < 0.01$); although they were higher than in C, there was a tendency to be normalized. Notably, groups AD and ADL showed no significant differences from the control group (Fig. 5a).

TNF-α levels. The levels of this cytokine in A were significantly higher than those in C ($P < 0.05$). All treatments promoted a decrease in the levels of this cytokine ($P < 0.01$), revealing no significant differences with respect to the control group. The levels of **TNF-α** in ADL were significantly lower than in AD or AL ($P < 0.05$) (Fig. 5b).



Groups	p
C - A – AD – AL - ADL	0.004*
C - A	0.004#
C - AD	0.004#
C - AL	0.004#
C - ADL	0.004#

*Kruskal Wallis Test; #Mann Whitney U test

Fig. 3. lgG anti-cytrulinated protein with indirect immunofluorescence (IFI) technique. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously.

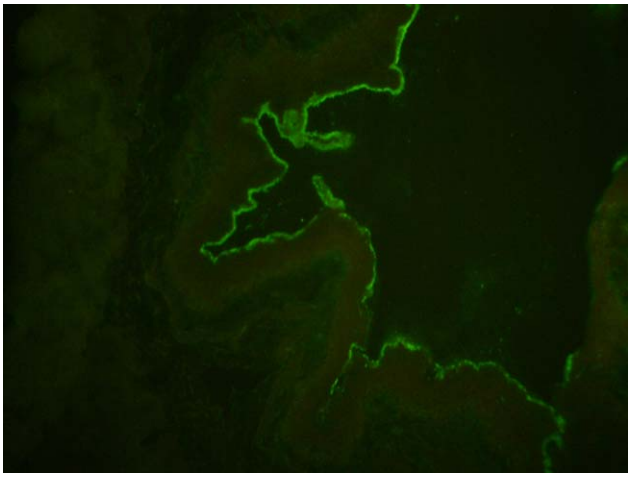


Fig. 4. Representative image obtained by indirect immunofluorescence (IFI) technique (×40). Indirect measurement of anti-citrullinated proteins IgG, present in serum of arthritic rabbits (diluted serum 1/40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Galectin-3 Expression in Synovial Tissue

To visualize the samples in a single gel, samples from each group were randomly selected in order to test the samples of the same group in the same gel. Immunodetection showed bands at 30 kDa, corresponding specifically to Galectin-3 (Fig. 6a). The analysis of these bands showed that the expression of Galectin-3 in the control group was low whereas in group A was significantly increased. Group AL showed variability in the results: in one of the extracts, the expression was lower, whereas in the other extract the response was similar to that of group A. This is consistent with previous clinical results (Mortarino et al., 2008), where animals from group AL showed high or quite low response to the treatment. In group AD, no significant effects were generally observed in the expression of Galectin-3, but in some of them there was a slight decrease. Finally, protein expression in group ADL was significantly different from that of group A, which was consistent with clinical and histological results. To check for equal loading, we used Ponceau staining of nitrocellulose membranes after transfer and prior to immunoblotting (Fig. 6b).

DISCUSSION

The present study evidences the benefits of our therapeutic approach in this experimental model of arthritis. It is important to remark that the inflammatory phenomenon was already established when we started the treatment, which is necessary for the future extrapolation of these results to human studies. Clinical observation indicates that the simultaneous treatment with hydrolyzed collagen and cholecalciferol were beneficial and that this difference was significant when compared with both the untreated rabbits and those receiving lysates or vitamin D3 only.

Our model of arthritis showed increased levels of anti-citrulline antibodies, in line with that found in humans with RA by De Rycke (2005). While the ADL group showed animals with lower levels of anti-citrulline antibodies in relation to group A, (3/5 animals), the differences were not so significant, probably because there were still remnants of citrullinated epitopes that may awaken the autoimmune response or because the level of anti-citrulline has not yet decayed enough to avoid detection. Studies conducted in the longer term would be needed to validate this.

Treatment with hydrolysate collagens in our arthritic experimental model caused diminished pro-inflammatory cytokine levels, an effect synergized with the simultaneous treatment with vitamin D3. The anatomical-pathological studies of synovial tissues revealed a beneficial effect of concomitant treatment of hydrolyzed collagen and vitamin D3.

It has been recognized that vitamin D plays an important role in reducing the risk of many chronic diseases, including RA, systemic lupus erythematosus, insulin-dependent diabetes mellitus, multiple sclerosis, several cancers, heart and infectious diseases (Hiraki et al., 2012; Sen and Ranganathan, 2012; Weng et al., 2007).

Epidemiological, genetic and basic studies indicate a potential role of vitamin D in the pathogenesis of

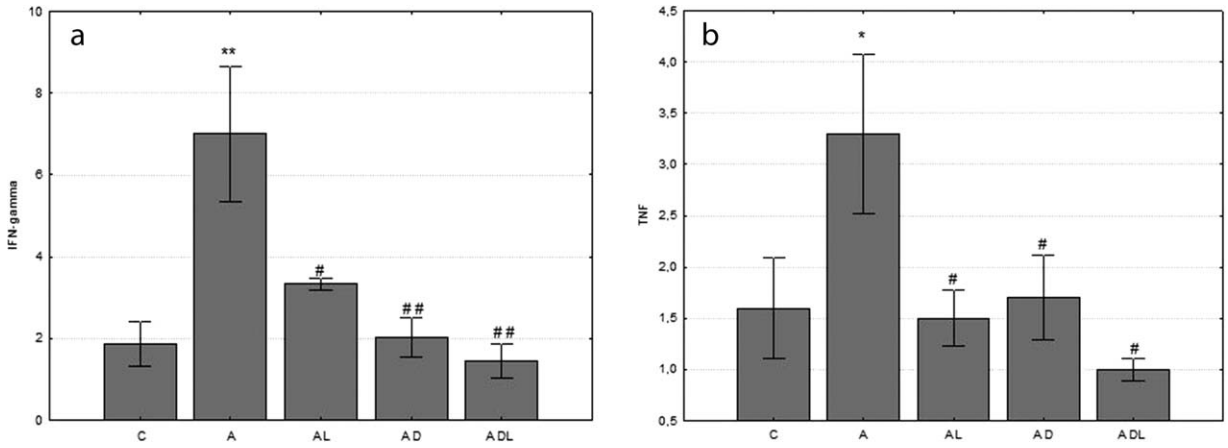


Fig. 5. a and **b**: Proinflammatory markers. Mean values (± SD;) (*, $P < 0.05$; **, $P < 0.01$ vs. C) (#; $P < 0.05$; ## $P < 0.01$ vs. A) of proinflammatory cytokines, IFN-gamma and TNF-alfa, for each study group). C group; control group. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously.

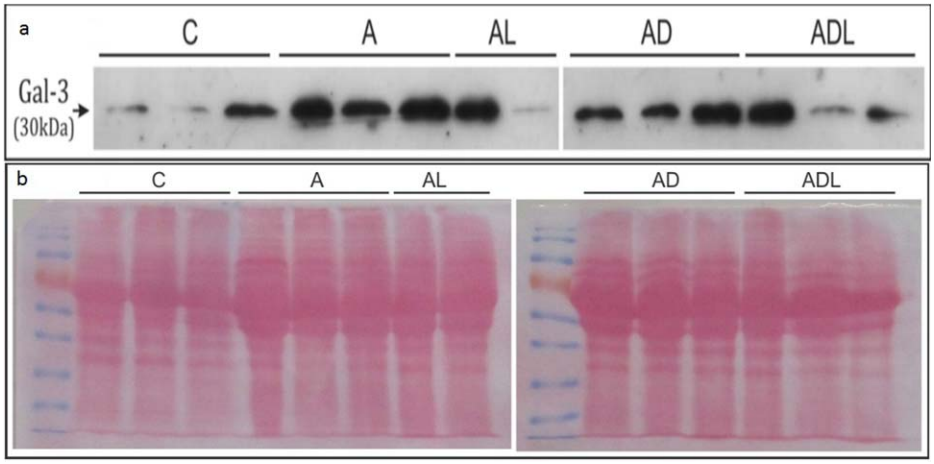


Fig. 6. a: Bands obtained after Galectin-3 immunodetection in Western blotting. In immunodetection, bands were obtained at the height of 30 kDa, corresponding specifically Galectin-3. C group; control group. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously. **b.** As loading pattern, Ponceau staining of nitrocellulose membranes after transfer and prior to immunoblotting was used. C group; control group. A group: The animals received placebo dose day/rabbit. AL group: The animals received hydrolyzed collagen peptides, 0.5 mL/day/rabbit. AD group: cholecalciferol (vitamin D3); 2,000 U pro-vit D/day/rabbit. ADL group: The animals received hydrolyzed collagen peptides, 0.500 mL/day/rabbit, and 2,000 U cholecalciferol (Vitamin D3)/day/rabbit simultaneously. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

certain autoimmune diseases, most of which demonstrate a correlation between low levels of vitamin D and disease appearance or manifestations (Agmon-Levin et al., 2013). A meta-analysis showed an association between total vitamin D intake and RA incidence: individuals in the group with the highest total vitamin D intake were found to have a 24.2% lower risk of developing RA than those in the group with lowest vitamin D intake (Song et al., 2012).

Our results are strengthened in the light of new results obtained by Barker et al., who observed an increase in proinflammatory cytokines in subjects with vitamin D deficiency (Barker et al., 2012). It has been shown that vitamin D could induce innate tolerance by promoting tolerogenic dendritic cells (Gregori et al., 2001; Griffin et al., 2001; Holick, 2007; Peelen et al., 2011; Shapira et al., 2010; Toubi and Shoenfeld, 2010). Additionally, both humoral and cellular adaptive responses are affected by vitamin D. Decreased proliferation and antibody production by B cells have been documented following exposure to vitamin D (Chen et al., 2007).

Pathophysiological studies from other laboratories have confirmed that hypovitaminosis D in genetically predisposed subjects can impair self-tolerance by compromising the regulation of dendritic cells, of regulatory T-lymphocytes, and of Th1 cells (Antico et al., 2012). Cross-sectional studies in the Massachusetts

area have shown that levels of vitamin D <30 ng mL^{-1} are present in a significant percentage, not only in patients with autoimmune disease, but also in healthy subjects (Mitchell et al., 2012).

Thus, we propose that the simultaneous treatment with cholecalciferol and oral L could increase the immunoregulatory effect on the process of arthritis triggered previously, reaffirming results published by others who realized that T cells have receptors for vitamin D (Cantorna, 2011, 2012).

Recent studies suggest that the reduced serum levels of vitamin D commonly seen in RA patients may increase fibroblast-like synoviocyte-mediated cartilage and bone invasion and erosion, thus supporting a plausible role for vitamin D supplementation to prevent or reduce bone and joint destruction in accordance with the results of our MR studies (Laragione et al., 2012). The 1,25-dihydroxyvitamin D3 inhibits cell proliferation, immunoglobulin production, and the release of cytokines through binding to Vitamin D binding protein (VDBP) (Yan et al., 2012). The VDBP also mediates bone resorption by activating osteoclasts.

Vitamin D would interfere with the invasive properties of synovial fibroblasts, which have a central role in the development of synovial hyperplasia and joint destruction in RA (Laragione et al., 2012). The decrease in the invasive and inflammatory capacities of synovial fibroblasts would explain the decrease in the levels of Galectin-3, as these cells are an important source of this lectin (Forsman et al., 2011).

Our results are in accordance with the conclusion obtained by Rabinovich et al., (2002), who indicated that Galectin-3 plays a selective role in modulating cytokine and chemokine production, thus illuminating Galectin-3 as a novel target for intervention in autoimmune inflammatory pathologies. Interestingly, Forsman et al. (2011) showed that Galectin-3 plays a pathogenic role in the development and progression of antigen-induced arthritis and that the disease severity is accompanied by alterations of antigen-specific IgG levels, systemic levels of TNF-α and IL-6, and frequency of IL-17-producing T cells. It should be remarked that in our experiments, we used L and not collagen II, because peptides best expose antigenic determinants that induce oral tolerance. In this way, administration of hydrolyzed collagen peptides could become independent of the patient's digestive capacity and could help to reduce the risk of treatment failure in RA patients. Oral tolerance may be used to design novel alternative therapies for autoimmune disease and we have herein presented novel evidence that the simultaneous treatment with vitamin D3 may synergize this beneficial effect.

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