

CYTOKINE AND NITRIC OXIDE LEVELS IN A RAT MODEL OF IMMUNOLOGIC PROTECTION FROM ADJUVANT-INDUCED ARTHRITIS

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In the present study we investigated the correlation between the progression of adjuvant arthritis induced by *Mycobacterium butyricum* and the production of nitric oxide and some pro- and anti-inflammatory cytokines in arthritic rats and in rats treated with low intra-peritoneal doses of *Mycobacterium* 3 and 10 days after arthritis induction. The intra-peritoneal administration of *Mycobacterium* antigen significantly inhibited disease development. Compared to healthy rats, a rise in serum and peritoneal pro-inflammatory cytokines was observed in all arthritic rats already from the 14th day. The treatment with intra-peritoneal *Mycobacterium* was associated with a significant reduction in IL-6 serum concentrations and a slight decrease of IFN- γ production by peritoneal macrophages. Nitrite/nitrate plasma and peritoneal levels were significantly higher in all arthritic rats. Intra-peritoneal administration of *Mycobacterium* caused a further increase in nitrite/nitrate plasma concentrations, while no differences were evident in nitric oxide production by peritoneal macrophages. From our data it is evident that among the variables here investigated, IL-6 seems to be the more representative marker of the disease and of the treatment effect. A possible role of nitric oxide as a modulator rather than a direct mediator in this model of inflammation is discussed.

Rheumatoid arthritis is a chronic autoimmune disorder characterized by accumulation of activated T cells, macrophages and plasma cells in the joints. Although the cause of this disease is still unknown, recent studies have shown that cytokines secreted by the activated cells are likely to play a major role in the maintenance of the inflammatory process (1). In particular, the importance of cytokines such as IFN- γ , IL-1, IL-2 and IL-6 in the pathogenesis of arthritis has been demonstrated *in vivo* in different animal models (2, 3). In addition, it has been shown that IL-10, an anti-inflammatory cytokine secreted by activated T cells, monocytes and B cells (4, 5), is spontaneously produced by synovial membrane cells derived from patients with rheumatoid arthritis (6).

Aberrations in nitric oxide (NO) metabolism

have been reported in a number of different models of pathophysiological conditions, including acute (7) and chronic (8, 9) inflammation, associated with an overproduction of NO that either directly or indirectly may promote tissue injury (10, 11). So, it is not surprising that changes in NO production have been found to be associated with arthritic immunopathies (12) though the exact relationship between NO and the disease pathogenesis is not well defined.

Adjuvant arthritis (AA) is an extensively studied model in the rat which shares many clinical and histopathological features with human rheumatoid arthritis: it is generally considered as a T-cell dependent chronic inflammatory disease of autoimmune origin (13). In previous studies we showed that the intra-peritoneal (i.p.) treatment of

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rats with *Mycobacterium butyricum* (Mb) on days 3 and 10 after arthritis induction, at concentrations 10 times lower than the inducing one, led to a significant antigen-specific and long-lasting suppression of arthritis development (14).

The aim of this study was to investigate the correlation between the development and progression of AA and the production of pro- and anti-inflammatory cytokines in arthritic rats (non protected animals) and in rats with the disease treated with low i.p. doses of Mb (protected animals). Moreover, we investigated the nitrite/nitrate serum levels, that provide an accurate measurement of NO production *in vivo* (15) in protected versus non protected animals.

MATERIALS AND METHODS

Induction and evaluation of AA

AA was induced in 36 inbred male Lewis rats (Charles River, Italy), weighing 125-150 g, by injection into the right hindpaw of Freund's complete adjuvant, 0.6 mg of heat-killed Mb (Difco, Detroit, MI, USA) suspended in 0.1 ml of paraffin oil. Animals were weighed every three days and at the same time the severity of arthritis was assessed by measuring the controlateral paw swelling with an electronic water plethysmometer (Mod. 7150, Ugo Basile, Milan, Italy). After 14, 21 and 28 days arthritis development was evaluated by the same blind observer: primary and secondary arthritic lesions were scored on an arbitrary scale (arthritic index) as follows: left and right hind feet each 0-7, left and right fore feet each 0-4, tail 0-5, ears 0-2, nose and eyes each 0-1.

Treatment and blood samples

Arthritic animals were randomly assigned to these following different treatments (18 animals per group): i.p. injection of 0.1 ml of paraffin oil on day 3 and 10 after arthritis induction (group AA) or i.p. injection of 60 µg of Mb suspended in 0.1 ml of paraffin oil at the same times (group AA+Mb). A group of 18 healthy rats was also included in the study.

Blood samples were collected by cardiac puncture from ether-anaesthetized rats at weekly intervals. Blood was centrifuged and the sera or plasma were stored at -20°C until testing for laboratory parameters.

Peritoneal lavages and macrophages collection

At days 14, 21 and 28 peritoneal cells were harvested

from six rats of each group. Cells obtained by peritoneal lavages were washed three times in sterile PBS, counted and suspended at a concentration of 1×10^6 /ml in Iscove's modified Dulbecco's medium supplemented with FCS 10%, 100 U/µl penicillin, 100 mg/ml streptomycin, 1.5 mM L-glutamine, 2 mM β-mercaptoethanol, plated in a 24-well microplate and incubated at 37°C, 5% CO₂. After 2 h, plates were washed to remove non-adherent cells and macrophages were cultured for 24 h. Then culture media were collected and stored at -20°C until analysis.

IL-6, IL-10 and IFN-γ bioassays in serum and macrophage culture media

To measure IL-6 activity, the murine IL-6 dependent hybridoma cell line 7TD1 was used, according to a previously described method (14): the standard was a human recombinant IL-6 (Boehringer Mannheim, Germany).

IL-10 and IFN-γ were measured by using an ELISA method (Cytoscreen™ Immunoassay kit, Biosource Int., Camarillo, California, USA): antibodies specific for rat IL-10 or rat IFN-γ have been coated into the wells of microtiter plates, followed by the addition of a biotinylated second antibody. During the first incubation, the antigens bind simultaneously to the immobilized antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, streptavidin-peroxidase enzyme is added: this binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of IL-10 or IFN-γ present in the original specimen.

Nitrite/nitrate measurement

The total plasma nitrite amount (nitrite/nitrate) was assayed by using a colorimetric method (Cayman, Ann Arbor, MI, USA). Briefly, in a 96-well microplate 40 µl of plasma, 40 µl of assay buffer, 10 µl of enzyme co-factor and 10 µl of nitrate reductase were placed. Samples were incubated for 3 h at room temperature to allow a complete conversion of nitrate to nitrite. To evaluate the total nitrite amount, 100 µl of Griess reagent (1:1 of 0.1% naphthylethylene-diamine dihydrochloride/1% sulfanilamide in 5% H₃PO₄) were added, allowing the color to develop for 10 min and

reading the absorbance at 540 nm using a microplate reader. Concentration of total plasma nitrite/nitrate was determined using a 0-35 μM range standard curve.

Macrophage culture media were assayed for nitrite production using Griess reagent. For this purpose, 100 μl of Griess reagent were added to 100 μl of sample allowing the color to develop for 10 min and reading the absorbance at 540 nm using a microplate reader. Nitrite concentration was determined using a 0-35 μM range nitrite standard curve.

Statistics

The evaluation of the data was carried out by applying Student's "t" test. A p value < 0.05 was considered as significant.

RESULTS

The intra-paw administration of Freund's complete adjuvant induced the arthritis in all treated rats with a lag-time of 10-12 days after injection. The arthritic index of the three groups of rats is shown in Fig. 1: the i.p. administration of a low dose Mb antigen (60 μg) 3 and 10 days after arthritis induction significantly inhibited disease development from the 14th day until the 28th day. This effect was also confirmed by the reduction of controlateral hindpaw volume (2.28 ± 0.39 ml in AA rats vs 1.63 ± 0.38 ml in AA+Mb rats at 21st day, data not shown).

The serum concentrations of IL-10, IL-6 and IFN- γ in the three experimental groups are reported in Figure 2. Compared to healthy rats, a rise in cytokines was observed in all arthritic rats already from the 14th day, even if this effect was significant only for the pro-inflammatory cytokines IL-6 and IFN- γ . The temporal trend was different for the three cytokines: IL-10 slightly increased from the 14th to the 28th day; IL-6 peaked on the 21st day after arthritis induction, when the disease showed the maximum expression; IFN- γ peaked on the 14th day just after the beginning of arthritis development. The treatment with i.p. low doses of Mb caused a slight decrease of IL-10 at 14th day followed by an increase, even if not significant, at 21st and 28th days; IL-6 serum concentrations were markedly decreased by i.p. Mb at 21st day; IFN- γ showed a slight but not significant increase in Mb-treated rats at 14th and 21st days.

Compared to healthy rats, peritoneal macrophages collected from arthritic rats produced significantly more IL-6 and IFN- γ with a peak at 21st day, while IL-10 increased only at 14th day. The treatment with i.p. Mb caused a slight but not significant decrease of IFN- γ production by peritoneal macrophages, while it did not affect the release of the other tested cytokines (Fig. 3).

$\text{NO}_2^-/\text{NO}_3^-$ levels measured in plasma were significantly higher in arthritic rats, compared to healthy rats, at 14th and 21st days after AA induction.

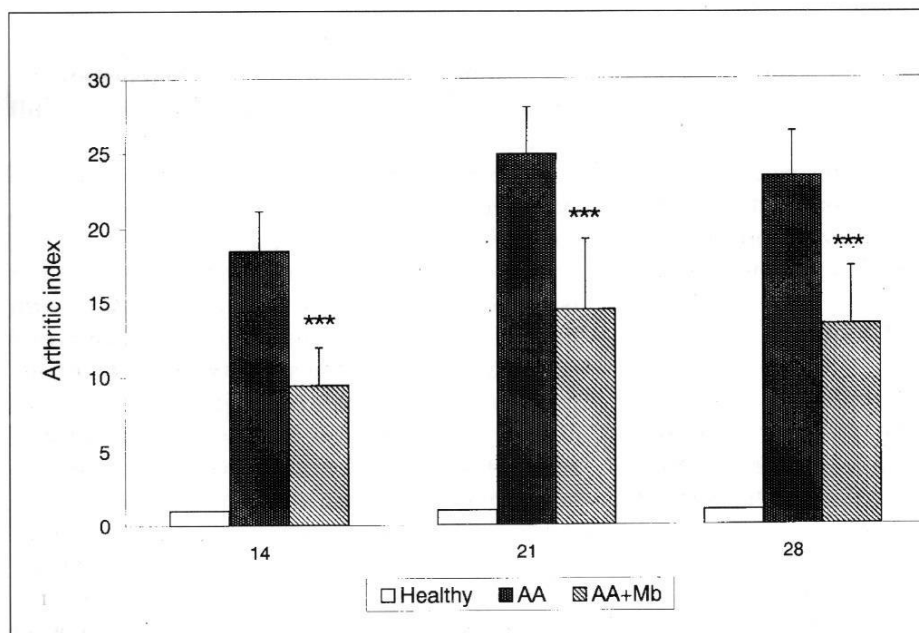


Fig. 1. Arthritic index of adjuvant arthritis at 14th, 21st and 28th day in rats receiving i.p. 0.1 ml paraffin oil (AA) or 60 μg Mycobacterium butyricum (AA+Mb). Data are expressed as means \pm S.D. *** $p < 0.001$ (AA+Mb vs AA).

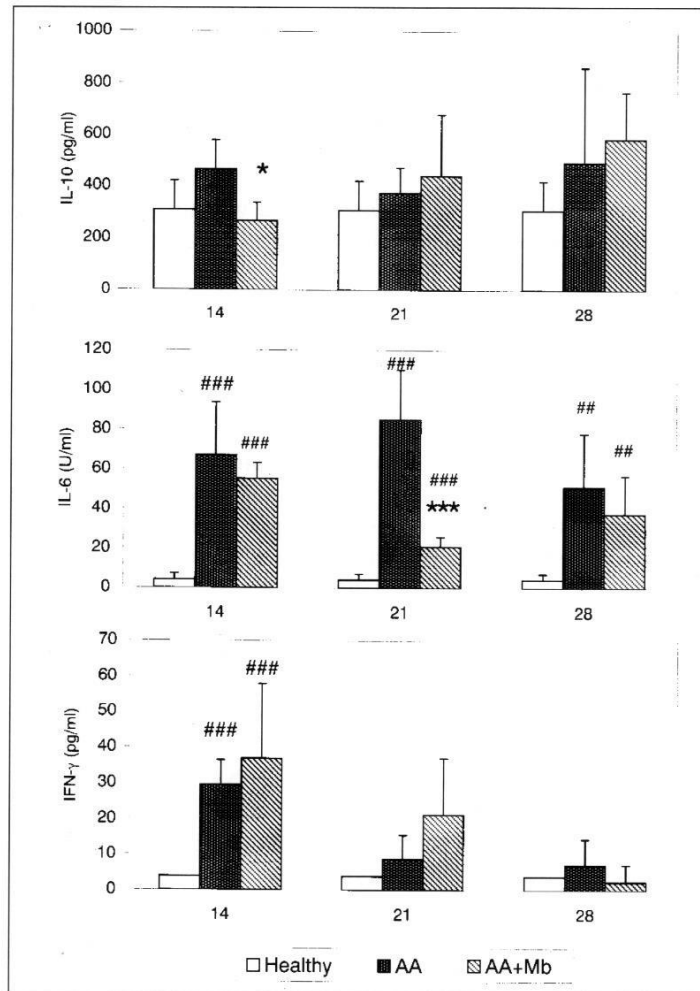


Fig. 2. IL-10, IL-6 and IFN- γ levels in the serum of healthy and arthritic rats with (AA+Mb) and without (AA) i.p. injection of *Mycobacterium butyricum* 14, 21 and 28 days after arthritis induction. Data are expressed as means \pm S.D. ## $p < 0.005$ and ### $p < 0.001$ (AA vs healthy); * $p < 0.05$ and *** $p < 0.001$ (AA+Mb vs AA).

Treatment with i.p. Mb caused a further increase in $\text{NO}_2^-/\text{NO}_3^-$ levels that was statistically significant, compared to AA rats, at 21st day (Fig. 4).

In vitro NO production by peritoneal macrophages markedly and significantly increased in all AA rats compared to healthy, but no differences were evident in relation to Mb treatment (Fig. 5).

DISCUSSION

AA develops in susceptible rat strains after the injection of Mb (16). The first step is an immune response that produces specific lymphocyte clones, the second phase is the development of a local and systemic inflammatory response associated with an increase in multiple cytokines, NO production and circulating acute-phase reactants (17-19). These abnormalities are a hallmark of the

intense inflammatory response, but the contribution of each component to disease progression is still uncertain.

From our data it is evident that AA can be prevented or treated via administration of the causative antigen given at a 10-fold lower dose, confirming previous observations that this disease is antigen-specific, since the injection of rats with the uncorrelated antigens casein (14) and *E.coli* (20) did not reduce the severity of arthritis. The protocol presented here (i.p. injection of Mb 3 and 10 days after adjuvant arthritis) derives from a series of previous experiments (21) and appears to be the best one, giving significant suppression of the disease without causing peritoneal damage.

Preliminary studies suggested (20) that the resolution of this systemic pathology could be due either to the high production of antibodies, induced

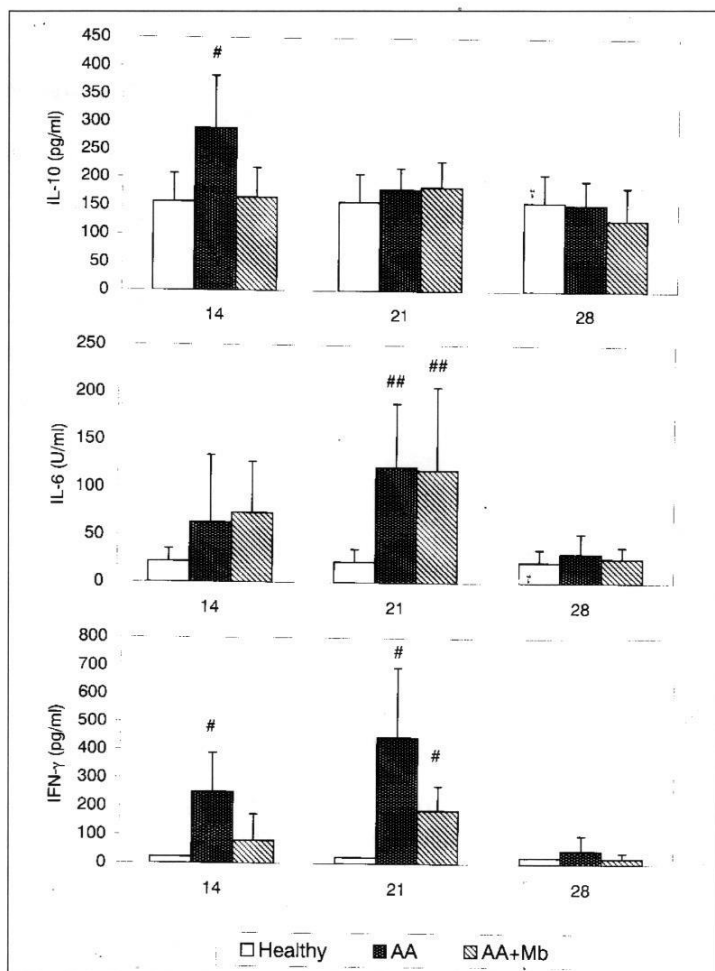


Fig. 3. Production of IL-10, IL-6 and IFN- γ by macrophages of healthy and arthritic rats with (AA+Mb) and without (AA) i.p. injection of Mycobacterium butyricum 14, 21 and 28 days after arthritis induction. Data are expressed as means \pm S.D. # $p < 0.05$ and ## $p < 0.005$ (AA vs healthy).

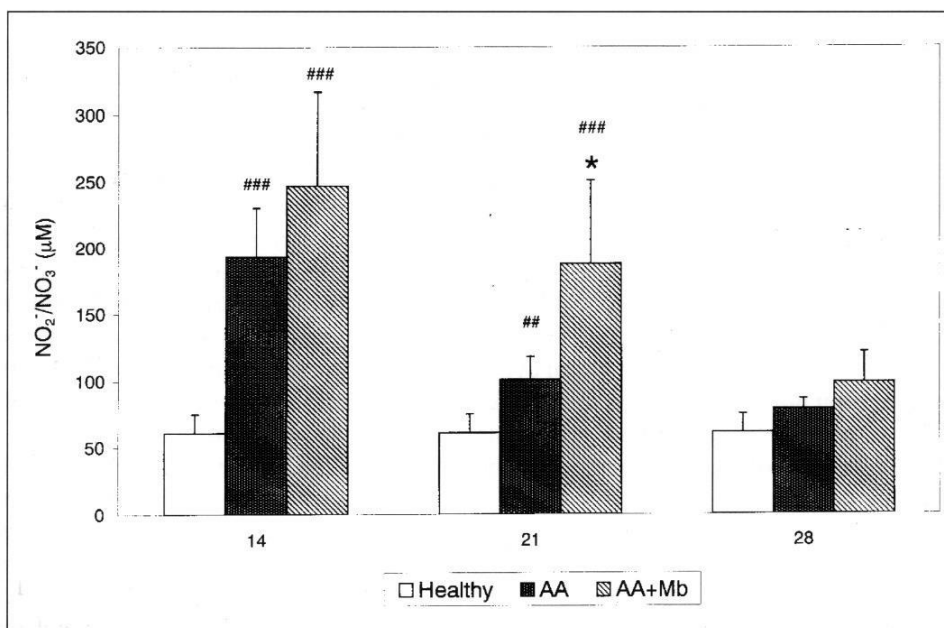


Fig. 4. Nitrite/nitrate (NO₂⁻/NO₃⁻) plasma levels in healthy and arthritic rats with (AA+Mb) and without (AA) i.p. injection of Mycobacterium butyricum 14, 21 and 28 days after arthritis induction. Data are expressed as means \pm S.D. ## $p < 0.005$ and ### $p < 0.001$ (AA vs healthy); * $p < 0.01$ (AA+Mb vs AA).

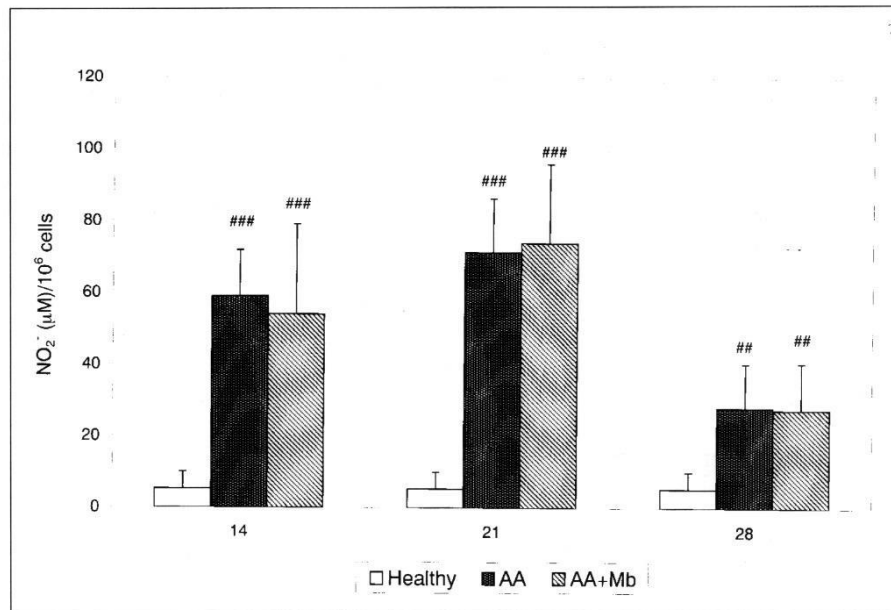


Fig. 5. Production of nitrite (NO_2^-) by peritoneal macrophages of healthy and arthritic rats with (AA+Mb) and without (AA) i.p. injection of *Mycobacterium butyricum* 14, 21 and 28 days after arthritis induction. Data are expressed as mean \pm S.D. ## $p < 0.005$ and ### $p < 0.001$ (AA vs healthy).

by the i.p. Mb challenge, and/or to a regulation by cytokines and NO, since they seem to have defined roles at various times during the course of autoimmune arthritis in the rat.

In this paper we studied the dynamics of some key-cytokines and of NO production in relation to arthritis and Mb treatment either in the circulation, since AA is a systemic disease, or in peritoneal macrophages, since the protection was obtained using the i.p. route of administration.

In our model of adjuvant arthritis, the rats developed an autoimmune disease with systemic effects and a significant increase, compared to controls, of the proinflammatory cytokines IL-6 and IFN- γ either in the serum or in peritoneum. However, the time-course of the two cytokines was different because only IL-6 appeared to be associated with the severity of systemic inflammation.

The i.p. injection (3 and 10 days after arthritis induction) of low doses of the same antigen involved in the pathology led to a decrease of the proinflammatory cytokines but not of IFN- γ , thus confirming the different role of the two cytokines in the disease. A possible explanation of the treatment effect could be an immune deviation in protected animals with a functional shift from a Th1 to a Th2 response, where type 2 cytokines (IL-4 and IL-10) can inhibit the production of the proinflammatory cytokines (IL-1, IL-6 and TNF- α) and numerous

macrophage functions (22). However, this hypothesis was not confirmed by our data, showing only a modest (but not statistically significant) increase of IL-10 during time in i.p. treated rats. In every case, from our results serum IL-6 seems to be the more representative marker, since it is well correlated to both the disease kinetics and the effects of i.p. Mb.

The different pattern of cytokines observed in the circulation and in the supernatant of peritoneal macrophages in the two groups may indicate that the two compartments, the peritoneum and the vascular bed, have been independently involved in cytokine production, as reported in other models of focal infections (23, 24). In fact, from our data the modulation of the IL-6 macrophage production by Mb does not occur in the peritoneal site but in the bloodstream. Moreover, it has been observed by other authors (25) that local peritoneal cytokines do not diffuse readily into the circulation. Further studies are necessary to clarify whether other sites of cytokine production (e.g. lymphonodes, spleen) may be more relevant to study these processes of immune activation and regulation.

The induction of iNOS in response to increased cytokine production is a non specific event which occurs in a wide variety of cell types; the finding of increased NO production cannot be viewed as specific for a given clinical syndrome, but rather as a reflection of an immune-activated state in

which inflammatory cytokines and other mediators have up-regulated iNOS in different tissues. In particular, excessive NO is produced during the course of a variety of rheumatic diseases including RA and increased levels of nitrite in serum and synovial fluids of RA patients have been detected (12). Ueki et al. (26) demonstrated a significant relationship between serum NO_x levels and disease activity in RA. The authors also found a significant correlation between NO and levels of TNF- α and IL-6. However, a key concept is that there is species and cell variability regard to the regulation of iNOS expression: for example, while iNOS is *in vitro* readily induced by IL-1 β and TNF- α in murine macrophages, these cytokines do not effectively induce iNOS in human leukocytes (27).

Enhancement of NO production may correlate with antibody protection "in vivo" (28). Other authors (15) suggest that a stimulation of iNOS in the early phase of adjuvant-induced arthritis, when the immune system is sensitized and activated by the antigenic stimuli, may be essential for the resolution of the disease. This well agrees with our data showing nitrite/nitrate plasma levels higher in protected animals, where the diseases ameliorates, indicating a further iNOS upregulation. Moreover, in these animals the pro-inflammatory IL-6 serum levels are lower compared to unprotected animals.

Instead, considering peritoneal macrophages, *in vitro* NO production is not modified by low doses of Mycobacterium. A possible explanation could be that the evaluation was made in isolated and manipulated cells. Otherwise, IFN- γ resulted significantly higher in arthritic rats and this could explain why IL-10 and nitrite/nitrate were similar in both groups. In fact, production of IL-10 is under the control of IFN- γ (29) and NO in macrophages is generated by iNOS which is itself inducible by IFN- γ (30). In every case, since AA is a systemic disease, it seems more appropriate to take into account, rather than peritoneal nitrite/nitrate data, the values in the blood, where also other cell types can contribute to iNOS upregulation.

In conclusion, in this model of chronic inflammation NO should probably be regarded as a mediator, together with the antibodies (20), in the resolution of the disease, possibly for its vasodilating properties favouring the drainage of mediators and antigens and then accelerating the process of recovery.

Further studies are required to evaluate constitutive and inducible NO synthase activity in the microenvironment of target tissues and to elucidate the role of NO in this mechanism of protection.

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