Lack of causal relationship between inducibility/severity of adjuvant arthritis in the rat and disease associated changes in production of nitric oxide by macrophages

Zdeněk Zidek, Daniela Franková, Berta Otová

Abstract

Objective—To investigate the formation of nitric oxide (NO) by peritoneal macrophages in three inbred strains of rats differing both in their susceptibility to the induction of adjuvant arthritis (AA) and in the severity of the disease.

Methods—AA was induced by intraplantar injection of *Mycobacterium tuberculosis* (*M.tb*) in paraffin oil. Isolated peritoneal macrophages were cultured for 24 hours and supernatants were assayed for nitrite using Griess reagent.

Results—All rats of the LEW and BN strains became diseased, but the F344 strain included both responders and non-responders. No significant interstrain differences were observed in the generation of NO by macrophages from control animals. Nitrite concentrations were remarkably enhanced in all *M.tb* treated animals, regardless of the absence or presence of AA, and did not parallel its severity. Altered production of NO by macrophages from adjuvant treated rats was normalised *in vitro* in the presence of lipopolysaccharide.

Conclusions—Our findings suggest that the activity of constitutive or inducible NO synthase in peritoneal macrophages cannot be regarded as a determinant of genetically controlled disease inducibility and severity. Secretion of latent forms of certain NO downregulatory factors during development of AA may be implicated.

(*Ann Rheum Dis* 1995; 54: 325–327)

Although exact understanding of the aetiology and pathogenetic aspects of adjuvant induced arthritis (AA) in the rat remains to be established, it is generally regarded as a T cell dependent chronic inflammatory disease of autoimmune origin.^{1,2} Activated T lymphocytes are a well recognised major source of a number of cytokines,³ some of which, either per se or in co-operation with other macrophage derived factors (monokines), stimulate biosynthesis of nitric oxide (NO).⁴ Cytokines such as interferon gamma (IFN γ), tumour necrosis factor α (TNF α), interleukin (IL)-1, IL-2, or IL-6, have been shown to be implicated in the development of immune arthritis.^{5–8} It is not surprising, therefore, that

changes in NO production have been found to be associated with arthritic immunopathies,⁹ though the actual relationship of NO to disease pathogenesis is not well defined.

The aim of this study was to investigate changes in the capability of macrophages to generate NO during development of AA in rats, and to examine if these changes are related to the inducibility or severity of the disease.

Materials and methods

ANIMALS

Female rats of the inbred strains Lewis (LEW), Brown Norway (BN) and Fischer (F344) (weights 150–160 g) were obtained from Charles River Wiga, Germany.

INDUCTION OF ARTHRITIS AND ITS EVALUATION
Adjuvant arthritis was induced by intraplantar injection of 0.5 mg *Mycobacterium tuberculosis* (strain H37RA, Difco) (*M.tb*) in 0.1 ml of liquid paraffin. Control animals were injected with saline. Swelling of the uninjected paw was evaluated using a plethysmometer (Ugo Basile 7150).

CELL CULTURES

Peritoneal cells were collected 5, 11, and 21 days after the *M.tb* injection, resuspended in RPMI-1640 medium, and placed in 96 well microplates (2.5×10^5 cells/well). After a standard adherence procedure, the medium was replaced with complete RPMI-1640 medium containing 10% heat inactivated fetal bovine serum (Flow ICN), 50 μ g/ml gentamicin, 2 mmol/l L-glutamine, and 5×10^{-5} mol/l 2-mercaptoethanol (Sigma). The cultures were maintained for 24 hours at 37°C, in 5% carbon dioxide, in a humidified Heraeus incubator. Samples from individual animals were run in triplicate. In a parallel series, cells were incubated in the presence of lipopolysaccharide (LPS) (*Salmonella typhimurium*, Sigma), 5 μ g/ml. The final volume per well was always 100 μ l.

NITRITE ASSAY

The amount of nitrite (expressed as nmol/10⁶ cells) in the macrophage supernatants, taken as

Institute of Pharmacology,
Academy of Sciences
of Czech Republic,
Prague, Czech
Republic
Z Zidek
D Franková

Department of
Biology, 1st Faculty of
Medicine, Charles
University, Prague,
Czech Republic
B Otová

Correspondence to:
Z Zidek,
Institute of Pharmacology,
Laboratory of Immunology,
Academy of Sciences of
Czech Republic, Vítězská
1083, 142 20 Prague 4,
Czech Republic.

Accepted for publication
11 November 1994

Swelling of the uninjected paw in adjuvant treated rats during the observation period of 21 days

Strain	Group	n	Volume of swelling (ml)		
			Day 11	Day 15	Day 21
LEW	Control	6	0.01(0.02)	0.02(0.01)	0.01(0.03)
	<i>M.tb</i> treated	6	0.40(0.11)*	1.02(0.13)***	1.23(0.08)***
BN	Control	6	0.01(0.01)	0.02(0.03)	0.03(0.05)
	<i>M.tb</i> treated	6	0.04(0.03)	0.08(0.04)	0.63(0.12)**
F344	Control	6	-0.02(0.02)	0.03(0.03)	0.03(0.02)
	<i>M.tb</i> treated (responders)	6	0.08(0.03)*	0.15(0.04)*	0.46(0.12)*
	<i>M.tb</i> treated (non-responders)	4	-0.03(0.04)	0.03(0.02)	0.05(0.03)

Values are mean(SEM). *M.tb* = *Mycobacterium tuberculosis*.

Significantly different from zero increase: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

a measure of NO production, was determined using a Griess reagent and read at 540 nm.

STATISTICAL ANALYSIS

Analysis of variance and Student's *t* test were used to establish the significance of differences between the group means.

Results

DEVELOPMENT OF ADJUVANT ARTHRITIS

As shown in the table, AA (swelling) developed in all animals of the LEW ($n = 6$) and BN ($n = 6$) strains, whereas only four of 10 treated F344 rats became arthritic. The onset of AA was delayed in BN and F344 strains compared with LEW rats. The most severe response was observed in LEW animals.

NITRITE FORMATION AND IN VITRO EFFECT OF LIPOPOLYSACCHARIDE

Figure 1 shows NO formation on day 21 in control animals, AA responders and non-responders, in the presence or absence of LPS.

No significant strain differences were found in NO production by macrophages from

Figure 1 Concentration of nitrite (nmol/10⁶ cells) in supernatants of peritoneal macrophages collected from LEW (□), BN (▨) and F344 (■) rats on day 21 after intraplantar injection of *M. tuberculosis*. Macrophages were cultured either alone or in the presence of lipopolysaccharide (LPS) 5 µg/ml. Values are means and SEM for six animals (four animals for F344 non-responders). No non-responders occurred in the LEW and BN strain. AA = adjuvant arthritis.

control animals not treated with *M.tb*, but production was significantly enhanced ($p < 0.001$) after in vitro incubation of cells with LPS.

Macrophages from adjuvant treated rats produced nitrites in markedly increased amounts ($p < 0.001$) which differed between the genotypes. However, when the cells were cultured in the presence of LPS, their augmented ability to produce NO was reduced to that of LPS untreated macrophages from control animals not injected with *M.tb*, except for those of strain BN, in which the return to normal values was incomplete.

The disease free F344 animals (non-responders) showed an increased production of NO which was indistinguishable from that detected in their F344 diseased (responders) counterparts. In this group, however, the increase was not affected by the in vitro presence of LPS.

A trypan blue exclusion test showed that 87–93% of cells from both control and arthritic animals remained viable at the end of the 24 hour period of culture, regardless of the presence or absence of LPS.

DYNAMICS OF CHANGES IN PRODUCTION OF NO
Significantly increased production of NO was observed throughout the study period (days 5, 11, and 21) (fig 2). NO production remained increased in the in vitro presence of LPS on day 5 ($p > 0.20$), but had decreased significantly on days 11 and 21 ($p < 0.001$).

Discussion

These results demonstrate that peritoneal macrophages from rats injected with mycobacterial adjuvant produced enhanced amounts of NO and that this effect preceded the onset of secondary arthritic lesions. These findings are compatible with those of Ialenti *et al*¹⁰ who showed that the severity of arthritis in Lewis rats was paralleled by pharmacologically manipulated production of NO by peritoneal macrophages. Others have reported a reduction of arthritic swelling in rats by inhibition of L-arginine dependent formation of NO.^{11 12} Considered alone, these data might suggest that increased production of NO is one of the pathogenetic mechanisms of AA; however, our comparative genetic study does not support such an unambiguous conclusion. We found also that macrophages from *M.tb* treated (but disease-free) rats of the F344 strain produced similarly enhanced concentrations of NO. Furthermore, we found no association between severity of the AA and the magnitude of the increase in NO production; rather, the increase in production of NO was greater in BN rats which showed a very mild arthritic response, and least in LEW rats with the most severe AA. Moreover, changes in NO production in F344 mild responders were similar to those found in LEW rats. In addition, no quantitative differences in formation of NO existed between AA susceptible and AA resistant F344 rats.

Figure 2 Production of nitrite (nmol/10⁶ cells) by peritoneal macrophages from LEW rats injected with mycobacterial adjuvant. Macrophages were cultured either alone (□) or in the presence of lipopolysaccharide 5 µg/ml (■). Values are means and SEM for six animals; those at time 0 represent untreated controls.

Overall, these findings show that increased production of NO by peritoneal macrophages from adjuvant treated rats is not a correlate of genetically controlled disease inducibility, nor is it a biochemical marker of disease severity. It remains to be clarified, however, whether systemic changes in NO production, assessed here in peritoneal macrophages, reflect the situation at the site of inflammation. It cannot be excluded that local formation of NO is essential for the manifestation of arthritis. In any case, our findings support the view that NO should be regarded as a modifier rather than a direct mediator of the disease. Further pharmacogenetically designed studies to monitor constitutive and inducible NO synthase activity directly in the microenvironment of target joints and adjacent tissues will be undertaken to elucidate this problem.

Our results also show that macrophages from arthritic rats lose their enhanced capacity to generate NO when they are cultured in vitro with LPS. This effect was only expressed in AA

responders, and was fully manifested at the time of onset of the swelling. It is difficult to explain the mechanism and biological significance of this phenomenon at present. Further studies are required to ascertain if 'arthritic macrophages' can secrete latent forms of downregulatory factors of NO synthesis, which may be rendered biologically active when exposed to LPS. Such biological properties are known to be inherent to transforming growth factor β -1^{13 14} which, interestingly, has been shown to be implicated in arthritic disease.⁶

This work was supported by Grant No 307/94/0964 awarded by the Czech Republic.

- Kayashima K, Koga T, Onoue K. Role of T lymphocytes in adjuvant arthritis. II. Different subpopulations of T lymphocytes functioning in the development of the disease. *J Immunol* 1978; 120: 1127-31.
- Cohen I R. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. *Ann Rev Pharmacol* 1991; 9: 567-89.
- Mosmann T R, Schumacher J H, Street N F, et al. Diversity of cytokine synthesis and function of mouse CD4⁺ T cells. *Immunol Rev* 1991; 123: 209-29.
- Deng W, Thiel B, Tannenbaum C S, Hamilton T A, Stuehr D J. Synergistic cooperation between T cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. *J Immunol* 1993; 151: 322-9.
- Cooper S M, Sriram S, Ranges G E. Suppression of murine collagen-induced arthritis with monoclonal anti-Ia antibodies and augmentation with IFN- γ . *J Immunol* 1988; 141: 1958-62.
- Thorbecke G J, Shah R, Leu C H, Kuruvilla A P, Hardison A M, Palladino M A. Involvement of endogenous TNF- α and TGF- β during induction of collagen type II arthritis in mice. *Proc Natl Acad Sci USA* 1992; 89: 7375-9.
- Maury C P J. Interleukin 1 and the pathogenesis of inflammatory disease. *Acta Med Scand* 1986; 220: 291-4.
- Theisen-Popp P, Pape H, Muller-Peddinghaus R. Interleukin-6 (IL-6) in adjuvant arthritis of rats and its pharmacological modulation. *J Immunopharmacol* 1992; 14: 565-71.
- Farrell A J, Blake D R, Palmer R M J, Moncada S. Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. *Ann Rheum Dis* 1992; 51: 1219-22.
- Ialenti A, Moncada S, Di Rosa M. Modulation of adjuvant arthritis by endogenous nitric oxide. *Br J Pharmacol* 1993; 110: 701-6.
- McCartney-Francis N, Allen J B, Mizel D E, et al. Suppression of arthritis by an inhibitor of nitric oxide synthase. *J Exp Med* 1993; 178: 749-54.
- Oyanagui Y. Nitric oxide and superoxide radical are involved in both initiation and development of adjuvant arthritis in rats. *Life Sci* 1994; 54: 285-9.
- Gilbert R S, Herschman H R. Transforming growth factor beta differentially modulates the inducible nitric oxide synthase gene in distinct cell types. *Biochem Biophys Res Commun* 1993; 195: 380-4.
- Corradin S B, Buchmuller-Rouiller Y, Smith J, Suardet L, Mauel J. Transforming growth factor beta 1 regulation of macrophage activation depends on the triggering stimulus. *J Leukoc Biol* 1993; 54: 423-9.