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

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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.

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 × 10^3 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 µg/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 µg/ml. The final volume per well was always 100 µl.

NITRITE ASSAY
The amount of nitrite (expressed as nmol/10^6 cells) in the macrophage supernatants, taken as
Values are mean(SEM). *p < 0.05; **p < 0.01; ***p < 0.001.

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 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.

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 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 in the arthritic swelling in rats by inhibition of L-arginine-dependent formation of NO. 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.
Lack of causal relationship between inducibility/severity of adjuvant arthritis in the rat

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 mediator rather than a direct inhibitor 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

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