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Specific and long-lasting suppression of rat adjuvant arthritis by low-dose *Mycobacterium butyricum*

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Abstract

We have tested the therapeutic effect of intraperitoneal injections of *Mycobacterium butyricum* on the development of adjuvant arthritis in rats and we have explored the specificity and the duration of effectivity of this treatment. Rats with induced arthritis were injected intraperitoneally with the causative antigen, *Mycobacterium butyricum*, at concentrations 10 times lower than the inducing one, on the 3rd and 10th day after arthritis induction. The severity of the disease was assessed on the basis of physical (arthritis index, paw swelling) and biochemical (serum interleukin-6) parameters. The treatment with *Mycobacterium butyricum* led to a significant suppression of adjuvant-induced arthritis. This therapeutic effect was both antigen-specific, because intraperitoneal aspecific inflammation did not prevent the disease, and long-lasting. The results obtained in this model confirm the possibility of modulating the autoimmune process even when the immunological response is already triggered, suggesting new therapeutic strategies, more suitable than preventive vaccination, in human autoimmune diseases.

Keywords: Adjuvant arthritis; Cytokine; Immunoregulation

1. Introduction

Adjuvant arthritis is an extensively studied model of rheumatoid arthritis, which is induced in rats by injection of heat-killed mycobacteria in mineral oil (Freund's complete adjuvant).

The pathogenic mechanism of the disease appears to be mediated by T cells. In fact, adjuvant arthritis can be transferred to naive rats by T lymphocyte clone, whose antigen specificity has been identified as the peptide 180–188 of the mycobacterial 65-kDa heat shock protein (hsp65). This protein belongs to the hsp60 family and shares about 50% amino-acid identity with the mammalian homologue and has been shown to be elevated in inflamed synovia of arthritic patients (Karlsson-Parra et al., 1990; Boog et al., 1992) and in rats with adjuvant arthritis (Kleinau et al., 1991). Numerous experiments have shown that immunization with mycobacteria, hsp65, or peptide 180–188 confers protection against several forms of experimental arthritis induced by Freund's complete adjuvant (Billingham et al., 1990; Yang et al., 1990), streptococcal cell walls (Van den Broek et al., 1989), collagen type II (Billingham et al., 1990; Ito et al., 1991) or synthetic adjuvants as CP20961 (Billingham et al., 1990) and pristane (Thompson et al., 1990). However, hsp65 and its peptide 180–188 are not arthritogenic on their own (Billingham et al., 1990). The protective effects appear to be mediated by T cells specific for the 256–270 epitope of hsp65, indicating that different epitopes on the same protein could activate arthritogenic or protective T cell populations (Anderton et al., 1995).

In a previous study we showed that multiple low doses of *Mycobacterium butyricum* in oil intraperitoneally (i.p.) administered in a preventive or curative way, suppressed adjuvant arthritis development. Unfortunately, this treatment caused peritoneal inflammation in experimental ani-

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mals. On the contrary, a single i.p. injection of *Mycobacterium butyricum* in oil was able to delay the development of adjuvant arthritis, without signs of local inflammation (Conforti et al., 1995).

In the present work we both confirmed the therapeutic effects of the same antigen that induces adjuvant arthritis and developed the optimal schedules to obtain this protection. Furthermore, in order to rule out possible interference of aspecific peritoneal inflammation, we have studied the effect of casein-induced peritonitis on our experimental model.

We also investigated adjuvant arthritis development after a second arthritogenic injection of Freund's complete adjuvant in protected and non-protected arthritic animals, pointing to the long-lasting nature of the tolerance to antigen.

Finally, we measured the serum levels of interleukin-6 in protected and unprotected arthritic animals, as interleukin-6 is known to be involved both in human rheumatoid arthritis (Houssiau et al., 1988) and in animal arthritic models (Theisen-Popp et al., 1992).

2. Materials and methods

2.1. Animals

Inbred male Lewis rats (Charles River, Italy) weighing 160–180 g at the start of the experiments were used. The animals were kept under standardized conditions on a standard diet and water ad libitum.

2.2. Induction and evaluation of adjuvant arthritis

Adjuvant arthritis was induced by Freund's complete adjuvant injection, 0.6 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI, USA) suspended in 0.1 ml of paraffin oil, into the hindpaw. The day of adjuvant injection was designated as day 0.

The animals were weighed every 3 days and the severity of arthritis was assessed at the same time by measurement of contralateral paw swelling with an electronic water plethysmometer (Model 7150, Ugo Basile, Milan, Italy).

After 14, 21 and 28 days, the severity of arthritis development was evaluated. Primary and secondary arthritic lesions were scored on an arbitrary scale (arthritic index) as follows: left and right hindpaws each 0–7, left and right forepaws each 0–4, tail 0–5, ears 0–2, nose and eyes each 0–1. The maximum possible score per animal was 32, as described by Conforti et al. (1995). The plethysmometer operator and the observer recording the arthritic index scores were unaware of the treatment regimen and of the group to which each rat was assigned.

Average paw swelling and arthritic index in groups of treated animals were compared with those of the control group, and percent inhibition was calculated.

2.3. Treatment with i.p. Mycobacterium butyricum in oil

Arthritic animals were randomly assigned to two different treatment groups as indicated below: the first group (arthritic controls, 6 animals) received i.p. 0.1 ml of paraffin oil on the 3rd and the 10th day after arthritis induction; the second group (10 animals) was treated at the same times with i.p. 60 μg of *Mycobacterium butyricum* suspended in 0.1 ml of paraffin oil, i.e., at a 10-fold lower dose than the arthritogenic one.

A group of non-arthritic rats which received intraperitoneally 60 μg of *Mycobacterium butyricum* on the 3rd and 10th day after the start of the experiment, and a group of healthy rats ($n = 6$ in each) were also included.

2.4. Re-induction of adjuvant arthritis

Three months after the first induction, the same arthritic animals were subsequently injected with an arthritogenic dose of Freund's complete adjuvant into the base of the tail. This second route of administration was chosen so as to not interfere with the first paw injection.

Simultaneously, adjuvant arthritis was induced in the base of the tail in the group of healthy controls purchased at the time of the first induction and kept under standard conditions (age-control group) in our animal quarters.

2.5. Serum interleukin-6 bioassay

Blood samples were collected by heart puncture from three rats of each group at weekly intervals. Blood was centrifuged and the sera were stored at −20°C until the time of testing for interleukin-6 activity.

To measure interleukin-6 activity, the murine interleukin-6-dependent hybridoma cell line 7TD1 was used (Theisen-Popp et al., 1992). Cells were cultured in culture flasks in Iscove's modified Dulbecco's medium (IMDM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, 1.5 mM L-glutamine, 50 μM 2-mercaptoethanol, 0.1 mM hypoxanthine and 16 μM thymidine (Sigma, St. Louis, MO, USA) in a humidified atmosphere (8% CO_2). Cells were cultured for 3 days in 96-well, flat-bottom microtitre plates (2×10^4 cells/well) with serial dilutions of the sera. Cell proliferation was determined by means of the hexosaminidase reaction (Landegren, 1984). Briefly, 60 μl of 7.5 mM hexosaminidase in 0.1 M citrate buffer (pH 5.0) containing 0.5% Triton X-100 was added to the cells and the plates were then incubated at 37°C, 100% humidity, for 4 h. The colour reaction was developed and enzyme activity was blocked by the addition of 0.1 M glycine buffer pH 10.4, 90 μl per well. Extinction was measured in a microplate

reader (Reader 400, SLT Labs Instruments) at 405 nm using 620 nm as reference. The standard used in this assay was a human recombinant interleukin-6 (Boehringer-Mannheim, Mannheim, Germany).

2.6. Aspecific inflammation

A leukocyte-rich peritoneal inflammatory exudate was induced in a group of 5 animals by i.p. injection with 1 ml of sterile 1% casein in saline, as described by Bellavite et al. (1983), on the 3rd and 10th day after arthritis induction in order to study the effect of an aspecific inflammation on adjuvant arthritis development. The control group ($n = 5$) received i.p. 1 ml of saline on identical occasions. After the first casein administration, peritoneal lavages were performed and the number of cells obtained was determined by flow cytometry.

2.7. Statistical analysis

The significance of differences in hindpaw volume was evaluated with the analysis of variance (ANOVA) for mixed design, considering treatment (adjuvant arthritis vs. adjuvant arthritis + i.p. *Mycobacterium butyricum*) as the between-subject variable and time (0–28 days) as the within-subject variable (Myers, 1979). Effects of two treatments at a particular experimental period were compared using simple contrasts. The variance of hindpaw volume was very unstable between different groups: thus, performance of the analysis of variance demanded a preliminary logarithmic transformation (Fleiss, 1986). A significance level of 0.05 was chosen.

To evaluate the effects of reinduction on the arthritic index in each group, Friedman two-way analysis of variance by ranks was used. To assess the significance of differences between treated and untreated groups at each time interval, the Wilcoxon-Mann-Whitney test was performed and P values were multiplied by four (the Bonferroni correction) to allow for the multiple comparison.

The data for interleukin-6 are expressed as arithmetic means ± S.D. of quadruplicate cultures in the proliferative assay. Statistical differences were evaluated with Student's *t*-test.

3. Results

3.1. Effects of i.p. administration of Mycobacterium butyricum on arthritis development

Fig. 1 shows the time course of volume changes of the contralateral hindpaw of healthy and arthritic rats during the development of adjuvant arthritis. The hindpaw of arthritic control rats started swelling on days 10–11, the

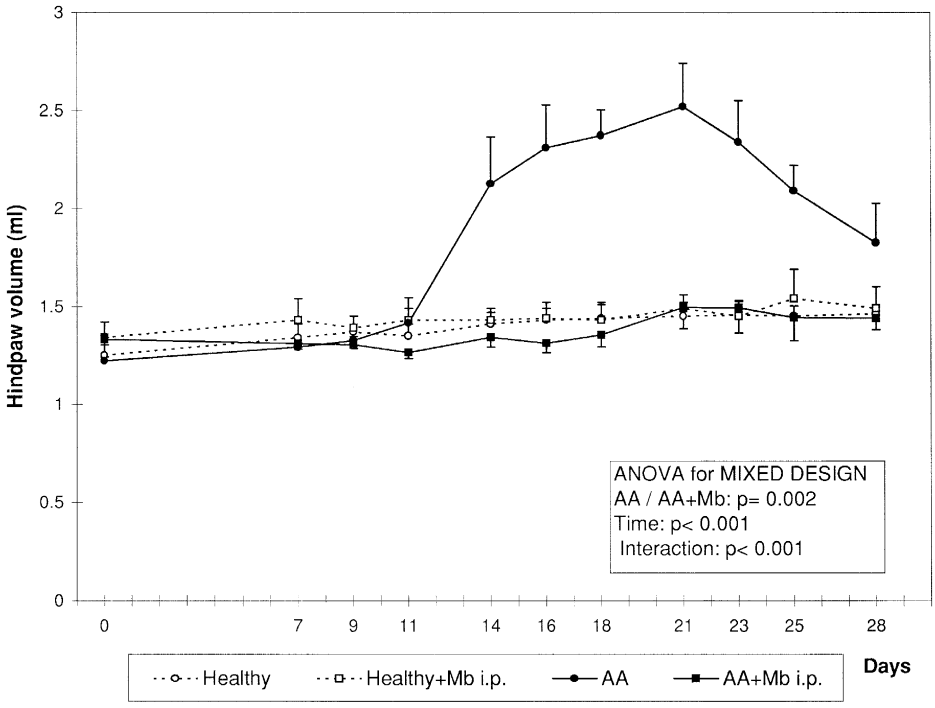

Fig. 1. Time-course of volume changes of the contralateral hindpaw in healthy rats (○), healthy rats receiving i.p. 60 μg *Mycobacterium butyricum* (Mb) in 0.1 ml paraffin oil, on the 3rd and 10 th day after the start of the experiment (□), arthritic rats receiving i.p. paraffin oil 0.1 ml (●) or 60 μg Mb in 0.1 ml paraffin oil (■) on the 3rd and 10th day after the arthritis induction. Arthritis was induced by injection of 0.1 ml Freund's complete adjuvant (containing 0.6 mg Mb) into the right hindpaw. Each value represents the mean ± S.D. Statistical analysis with an ANOVA shows significant differences between arthritic rats (AA) and arthritic rats receiving Mb i.p. (AA + Mb) as indicated in the figure's frame.

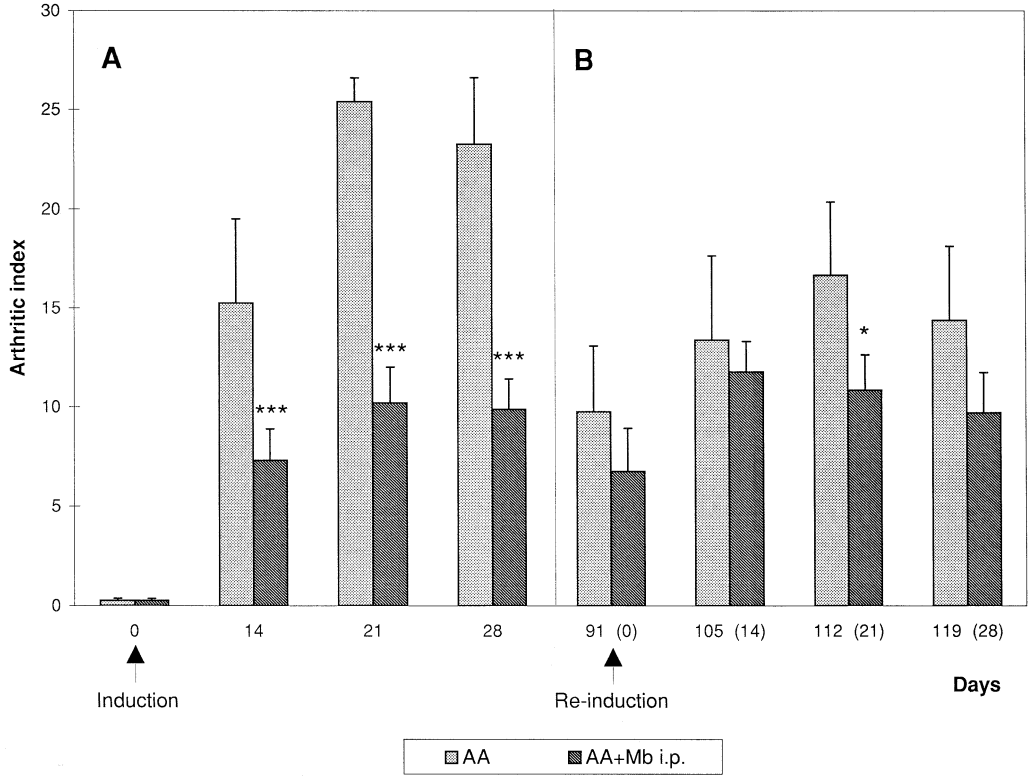

Fig. 2. Arthritic index (range 0–32) of adjuvant arthritis in rats receiving i.p paraffin oil 0.1 ml (AA) or 60 μg Mb in 0.1 ml paraffin oil (AA + Mb) on the 3rd and 10th day after arthritis induction, during first induction (Fig. 2A) and subsequent reinduction 3 months later (Fig. 2B). Data represent means ± S.D. *** $P < 0.001$, * $P < 0.05$, significantly different from AA group (Mann-Whitney's *U*-test).

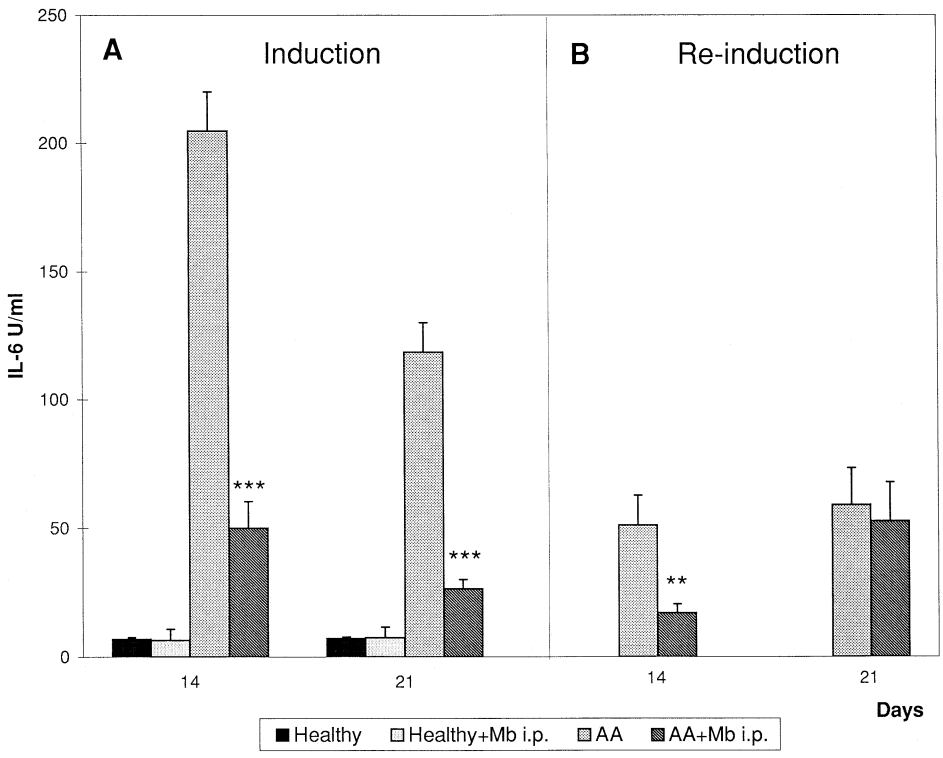

Fig. 3. Interleukin-6 (IL-6) levels in the serum of healthy and arthritic rats with and without i.p. injection of *Mycobacterium butyricum* (Mb) in oil, 14 and 21 days after the first (Fig. 3A) and the second arthritis induction (Fig. 3B). Interleukin-6 was determined using the interleukin-6-dependent cell line 7TD1 as described in Section 2. Columns represent the means ± S.D. from three rats per group, in quadruplicate. ** $P < 0.001$, * $P < 0.01$, significantly different from AA group (Student's *t*-test).

Table 1
Effect of peritoneal aspecific inflammation on the severity of adjuvant arthritis in rats

Treatment	Day 14 [a]		Day 21 [a]	
	Arthritic index [b]	Paw volume	Arthritic index [b]	Paw volume
AA [c]	16.12 ± 5.23	1.93 ± 0.38	23.25 ± 6.44	2.0 ± 0.37
AA + casein [d]	20.37 ± 4.27	2.38 ± 0.46	28.0 ± 1.0	2.49 ± 0.36

[a] Data represent means ± S.D. for 5 rats.
[b] Primary and secondary arthritic lesions were scored on an arbitrary scale (range 0–32).
[c] Adjuvant arthritis (AA) was induced by injection of 0.6 mg of *Mycobacterium butyricum* (Mb) suspended in 0.1 ml paraffin oil into the hindpaw.
[d] Rats were treated intraperitoneally with 1% casein on the 3rd and 10th day after arthritis induction.

oedema reached its maximum expression on day 21 and then slowly decreased. The i.p. administration of 60 μg of *Mycobacterium butyricum* on the 3rd and the 10th day suppressed the contralateral paw swelling during the disease.

The extra-articular manifestations of adjuvant arthritis were also significantly reduced ($P < 0.001$), > 52% inhibition, at each time considered (14, 21, 28 days after arthritis induction) as shown in Fig. 2A.

In addition we have shown that the i.p injection of *Mycobacterium butyricum* on days 3 and 10 in healthy rats was not itself arthritogenic as indicated by hindpaw volume in Fig. 1. No extra-articular manifestations were observed either.

3.2. Effects of i.p. administration of Mycobacterium butyricum on subsequent adjuvant arthritis induction

Fig. 2B shows the effect of adjuvant arthritis re-induction by injection of Freund's complete adjuvant into the base of the tail, in protected and unprotected arthritic animals. On day 0 (Fig. 2B) the unprotected animals presented with residual arthritic lesions and responded to a lesser extent to a second adjuvant arthritis induction, as is shown by the comparison of the increase (Δ) from days 0 to 21 of arthritic indexes for adjuvant arthritis induction ($\Delta = 25.4$) and for adjuvant arthritis re-induction ($\Delta = 6.8$). This cannot be entirely explained by the aging process, as the Δ of the arthritic index in healthy and age-matched animals was 18.6 (data not shown).

The rats treated with an i.p. injection of *Mycobacterium butyricum* in oil developed a secondary arthritis milder than that of the unprotected rats. The reduction of the arthritic index was statistically significant on day 21 in comparison to that in the arthritic unprotected rats (Fig. 2B).

3.3. Interleukin-6 serum level during the induction and re-induction of arthritis

Fig. 3 summarizes the serum levels of interleukin-6 on the 14th and 21st days after the first and the second induction of adjuvant arthritis. Interleukin-6 activity increased markedly in the serum of arthritic control rats in comparison to that of healthy controls after the first arthritis induction, reaching its maximum level on day 14 (Fig. 3A). It then decreased to 25.63 ± 3.46 U/ml at the time of reinduction (day 91, data not shown).

The i.p. administration of *Mycobacterium butyricum* in oil to arthritic rats significantly reduced the interleukin-6 serum levels ($P < 0.001$, Fig. 3A) in parallel with its effect on the arthritic symptoms. This reduction persisted even in the case of the re-induction of adjuvant arthritis (Fig. 3B). The i.p. injection of *Mycobacterium butyricum* in oil to healthy rats did not cause any increase in interleukin-6 serum levels, as indicated in Fig. 3A.

3.4. Effects of an aspecific peritoneal inflammation on adjuvant arthritis development

This possibility was explored by i.p injecting casein in arthritic rats. The peritoneal inflammatory process was assessed from counts of lymphocytes, granulocytes and monocytes in peritoneal wash. All three cell types were 3 times higher in the casein-treated rats than in untreated ones.

The effects of the aspecific peritoneal inflammation on adjuvant arthritis are shown in Table 1: no significant differences were found between the arthritic indexes of casein-treated rats and non-treated arthritic animals.

4. Discussion

A lot has been done, using numerous experimental rodent models, to look for treatment protocols to prevent or to cure autoimmune diseases by administration of the causative antigen (specific immunosuppression or tolerization) according to two general strategies:

(a) Preventive vaccination, either via parenteral injection of whole antigen, hsp65 and peptide 180–188 (Billingham et al., 1990) or via oral (oral tolerance) administration of autoantigens (Nagler-Anderson et al., 1986; Zhang et al., 1990; Khoury et al., 1992). Oral tolerance appears to be possible only before an immune response to the antigen has been induced (Yoshino, 1995).

(b) Post-induction treatment was reported to be effective by some authors (Yang et al., 1990; Currey, 1970)

and, in our case, was effective with the whole antigen (*Mycobacterium butyricum*) given at a 10-fold lower dose and via a route other than the arthritogenic one (i.p. versus subplantar), 3 and 10 days after arthritis induction.

In our model, the choice of this treatment protocol derived from a series of experiments designed to explore the feasibility of tolerance induction in adjuvant arthritis rats (Conforti et al., 1995). On the one hand, several injections of *Mycobacterium butyricum* were able to completely suppress adjuvant arthritis development but caused heavy peritoneal damage, on the other hand a single administration of *Mycobacterium butyricum* 10 days after the induction of adjuvant arthritis was capable of delaying the onset of the disease but the therapeutic effect was low. Thus, the protocol presented here (i.p. injection of *Mycobacterium butyricum* 3 and 10 days after adjuvant arthritis induction) appears to be the best protocol, giving almost complete suppression of the disease. This is emphasised by the reduction of interleukin-6 serum levels in treated arthritic rats in comparison to the control group.

It has been shown that interleukin-6 serum levels correlate significantly with disease activity in adjuvant arthritic rats and can be influenced by drug treatment (Theisen-Popp et al., 1992; Leisten et al., 1990). Non-steroidal antiinflammatory drugs, such as indomethacin, only partially inhibited paw swelling concomitant with a slight decrease of serum interleukin-6, whereas treatment with immunosuppressants such as dexamethasone or cyclosporin A was associated with complete normalization of serum interleukin-6 levels (Brauer et al., 1994). Our results confirm the increase of interleukin-6 in serum during adjuvant arthritis and show that i.p. injection of low doses of *Mycobacterium butyricum* to non-arthritic rats does not cause either systemic inflammatory reaction or increase in interleukin-6.

The strong decrease of interleukin-6 levels observed in arthritic animals treated with intraperitoneal *Mycobacterium butyricum* strongly supports the hypothesis that this treatment suppresses the disease by interfering with the production of pro-inflammatory, multifunctional cytokines.

The role and importance of the regulatory T cell emerged in the models of adjuvant arthritis prevention, both via vaccination and oral tolerance (Zhang et al., 1990). Far less is known about the cellular processes supporting the curative effect of i.p. injection of the antigen. The present work tried to clarify some aspects of this post-induction approach.

The first aspect is the specificity of the i.p. treatment to the extent that the control treatments (oil alone or unrelated antigen such as casein) were ineffective. The second aspect is the long-lasting effect of an i.p. injection which can protect the animals from the second arthritogenic inoculum. These two aspects induced us to discard the hypothesis of 'distracting inflammation' which was evoked to explain the protective effect mediated by zymosan, interleukin-1 or other unspecific inflammatory substances (Killar and Dunn, 1989; Caccese et al., 1992; Joosten et al., 1994).

Furthermore, the i.p. injection of *Mycobacterium butyricum* is not pathogenic on its own and is effective if given 3 and 10 days after the arthritogenic inoculum. This time interval is sufficient to develop fully the antigen-specific Th cells, and even the effector lymphocytes. The i.p. injection of the antigen is able to trigger a regulatory mechanism which in turn shuts off a fully developed autoimmune attack.

A single injection given at day 10 is less effective; this could be due to the inability of regulatory cells to stop fully developed effector arthritogenic lymphocytes, or to limit the damage to the joints, which is irreversible when established.

It is important to emphasise that i.p. injection of the antigen generates long-lasting protection, seemingly supported by long-lived T cells.

At present the two main hypotheses about the nature of these T regulatory cells are: (a) suppressor cells are either antigen- or idiotypic-specific (Zoller and Andrighetto, 1985); (b) 'immune deviation' with activation of Th_2 cells (Röcken et al., 1996; Finkelman, 1995).

The observation (unpublished) that the treatment given 2 days before, or simultaneously with arthritis induction is less effective than the same treatment given 3 and 10 days later suggests that the presence of Th and/or effector cells is necessary for the development of the protective effect, which argues in favour of the suppressor hypothesis.

However, our results show that adjuvant arthritis induced by paw injection of *Mycobacterium butyricum* is autoregulating. The arthritic index measured after the second adjuvant arthritis induction was much less than the same index measured after the first inoculum. This indicates that an arthritogenic injection is able to trigger regulatory cells. Further studies will clarify the role of the peritoneal cavity in the process of down-regulation of arthritogenic lymphocytes via activation of antigen and/or idiotype-specific suppressor cells.

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