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INTRAPERITONEAL ADMINISTRATION OF ADJUVANT INHIBITS THE DEVELOPMENT OF ADJUVANT ARTHRITIS IN RATS

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In recent years, considerable efforts have been made to develop effective therapy for autoimmune diseases by specific suppression of the autoreactive immune process without affecting the remainder of the immune system. In our study we evaluated the protective effects and therapeutic potential of *Mycobacterium butyricum* (Mb), the causative antigen inducing adjuvant arthritis (AA), an experimental model of autoimmune disease in the rat. The antigen was administered to rats by a different route and at concentrations 10 and 100 times lower than the inducing one. Arthritis was induced by injection of 0.6 mg of Mb in paraffin oil into the hindpaw, and the severity of disease was assessed by measurement of contralateral paw swelling every three days and primary and secondary lesions were scored on an arbitrary scale after 14, 21, and 28 days. Animals were assigned to different groups and treated intraperitoneally with different doses and schedules of Mb. The administration of 60 μg of Mb every two days, starting 6 days before arthritogenic injection until the second day after, led to a significant inhibition of the arthritic process ($p< 0.001$ of the arthritic index). Treatment of animals with 60 μg of Mb every two days, from day 2 to day 21 after arthritis induction caused almost total suppression of lesions. However, in both treatment schedules, animals showed important signs of peritoneal inflammation. The administration of single injection of 60 or 6 μg of Mb 10 days after arthritis induction led to an inhibition of

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arthritic index reaching the maximum percentage on day 14 (26% and 24% with 60 and 6 μg respectively) and was able to delay the development of oedema foot volume, without signs of local inflammation. These results confirm the ability to modulate the autoimmune process even when the immunological response is far advanced, suggesting new strategies in the therapy of human autoimmune diseases.

Adjuvant arthritis (AA) is an experimental model of autoimmune disease used for the study of rheumatoid arthritis in man, which is induced in rats by subplantar or intradermal injection of a suspension of killed mycobacteria in mineral oil.

The aetiology of this pathology has not been established, though it appears to be mediated by immunological mechanisms: the disease can be transferred or modulated by T lymphocytes (A2 cell line), which are specific for mycobacteria and responsive to cartilage (1,2,3). Support for an autoimmune aetiology has been provided in recent years by several studies, suggesting a highly conserved 65-kD heat shock protein (hsp) of mycobacteria as the primary causative antigen (4,5). A nonapeptide representing the amino acid sequence 180-188 of this 65-kD mycobacterial hsp is probably the critical epitope for AA, and a possible explanation for adjuvant disease is molecular mimicry of a cartilage proteoglycan by this epitope (6,7).

Identification of the causative antigen of the autoimmune reaction in AA makes it possible to prevent or modulate the disease in an antigen-specific way. A number of experiments have already been conducted to study the potential of mycobacteria, hsp65, or the peptide 180-188 as vaccines to induce specific immunosuppression by modulation of the immunoresponse.

Pretreatment intraperitoneally (ip) with mycobacteria, hsp65 or peptide 180-188 prior to the arthritogenic challenge resulted in complete prevention (8,9,10), while the therapeutic treatment with peptide 180-188 only moderately suppressed the development of arthritis (11). Suppression of adjuvant arthritis has also been obtained by oral administration of Type II collagen (12). The immunological mechanism leading to tolerance to AA by antigen administration is still a matter of debate.

The aim of this study was to develop the optimal treatment regimen for studying the protective effects and the possible therapeutic potential of the same aetiological antigen inducing AA, administered to rats in subarthritogenic doses and via a route other than induction.

MATERIALS AND METHODS

Induction and evaluation of arthritis

Seventy inbred male Lewis rats (Charles River) weighing 185.5 ± 15.5 at the start of the experiment were used. Adjuvant arthritis was induced by injection of 0.6 mg of heat-killed *Mycobacterium butyricum* (Mb, Difco) suspended in 0.1 ml of paraffin oil into the hind paw. The day of adjuvant injection was designated as day 0.

Animals were weighed every three days and at the same time the severity of arthritis was assessed by measurement of contralateral paw swelling with an electronic water plethysmometer (Mod. 7150, Ugo Basile, MI, Italy).

After 14, 21 and 28 days, the severity of arthritis development was also 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 (13). The plethysmometer operator and the observer recording the arthritic index scores were unaware of the treatment regimen and the group to which each rat was assigned.

Average paw swelling and arthritic index in groups of treated animals were compared with those of a group of controls, and percentage inhibition was calculated.

Treatment regimens

Animals were randomly assigned to 7 different groups (10 animals per group) and treated with multiple or single administrations of Mb as indicated below:

Around-induction treatment starts 6 days before arthritis induction and lasts until the 2nd day after the arthritogenic injection (5 administrations in all).

Group 1 (controls) received 0.1 ml of paraffin oil ip every two days,

Group 2 received 60 μg of Mb suspended in 0.1 ml paraffin oil ip every two days.

Post-induction treatment was administered from day 2 to day 21 after arthritis induction (10 administrations in all).

Group 3 (controls) received 0.1 ml of paraffin oil ip every two days,

Group 4 received 60 μg of Mb suspended in 0.1 ml paraffin oil ip every two days.

Treatment day 10: the substances were administered once on the 10th day after induction.

Group 5 (controls) received 0.1 ml of paraffin oil ip

Group 6 received 60 μg of Mb suspended in 0.1 ml of paraffin oil ip

Group 7 received 6 μg of Mb suspended in 0.1 ml of paraffin oil ip

Statistical analysis

Differences in hindpaw volume were evaluated with repeated ANOVA for a mixed design, considering treatment (control, 60 μg, 6 μg) as the between-subject variable and time 0-28 days as the within-subject variable (14). Comparison between two treatments at a particular experimental period was performed with simple contrasts. Variance of hindpaw volume was very unstable between different groups: thus, to perform analyses of variance a preliminary logarithmic transformation proved necessary (15). A significance level of 0.05 was chosen.

For the comparison of arthritic index scores between treated and untreated groups, the non-parametric Mann-Whitney test was used.

RESULTS

Figure 1 shows the arthritic indices espressed as means in rats treated with multiple doses around and after the induction of AA. The animals receiving 60 μg of Mb showed significant inhibition of arthritic lesions in comparison to controls with both treatment regimens ($p < 0.001$). The inhibition lasted until day 28th and was higher when the rats were treated after induction (61% and 38% at day 21 respectively).

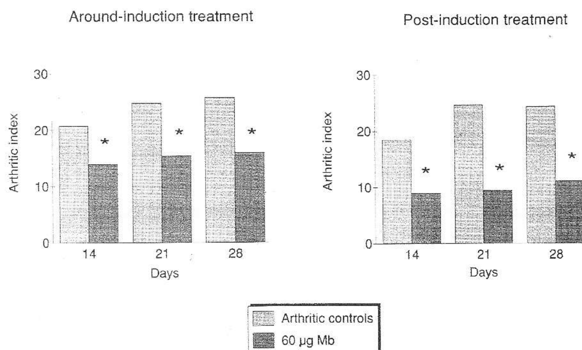

Figure 1. *Arthritic index (range 0-32) of adjuvant arthritis in rats receaving multiple administrations ip of paraffin oil (controls) or 60 μg of Mb; Around-induction treatment: 5 injections on days -6, -4, -2, 0, 2. Post-induction treatment: 10 injections from day 2 to 21. * $p<0.001$, significantly different from arthritic control group (Mann-Whitney's U test).*

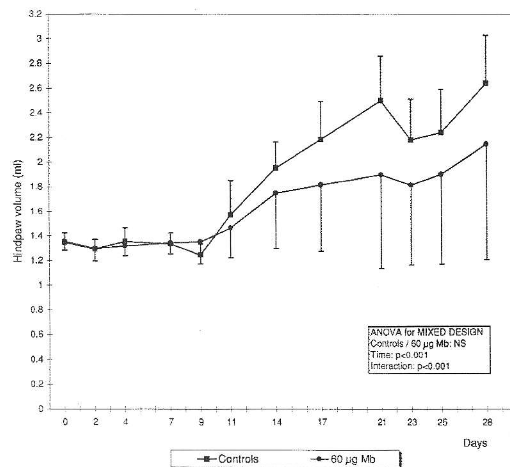

Figure 2. *Contralateral paw volume of control rats and rats receiving around-induction treatment: 5 injections ip of paraffin oil or 60 μg of Mb on days -6, -4, -2, 0, 2. Data represent mean ± SD of 10 rats.*

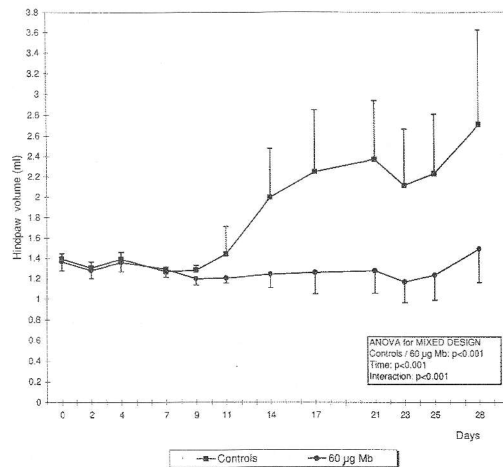

Figure 3. *Contralateral paw volume of control rats and rats receiving post-induction treatment: 10 injections ip of paraffin oil or 60 μg of Mb from day 2 to 21. Data represent mean ± SD of 10 rats.*

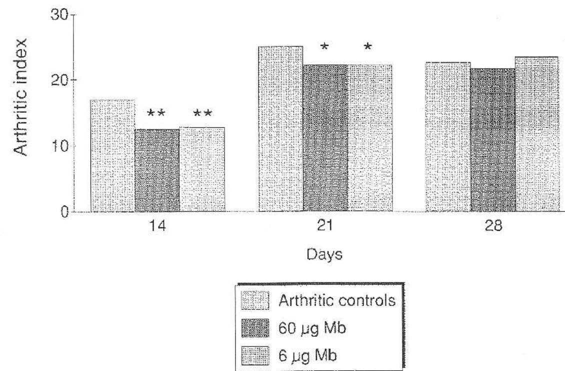

Figure 4. *Arthritic index (range 0-32) of adjuvant arthritis in rats receiving a single ip injection of paraffin oil (controls), 60 or 6 μg of Mb 10 days after induction of disease (day 10 treatment). ** p<0.001, * p<0.05, significantly different from arthritic control group (Mann-Whitney's U test).*

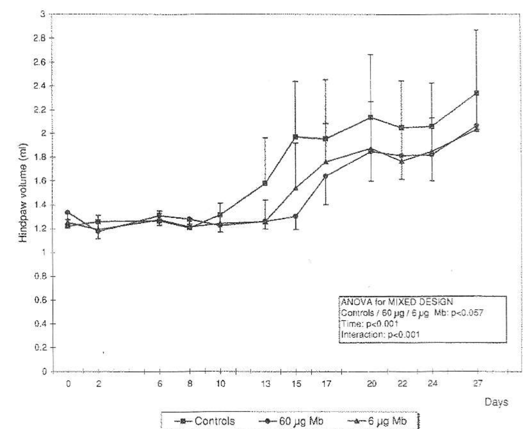

Figure 5. *Contralateral paw volume in rats treated with a single ip injection of paraffin oil (controls), 60 or 6 μg of Mb 10 days after induction of adjuvant arthritis (day 10 treatment). Data represent mean ± SD of 10 rats.*

In the same groups of rats, contralateral paw volume was also measured as an additional index of arthritic disorder. The results are shown in Figures 2 and 3. In both control groups, hindpaw volume remained stable until day 9 and progressively increased thereafter, doubling its initial value in 28 days. Hindpaw swelling was partially prevented by around-induction treatment with Mb (Fig.2) and was completely abolished after post-induction treatment (Fig.3). As a consequence, hindpaw volume was similar in treated and untreated groups up to days 9-11, while a significant difference was observed from day 21 to day 28. The different hindpaw volume time course in the two groups is confirmed by statistical analysis: ANOVA for a mixed design revealed a significant interaction between time and treatment. Moreover, with post-induction treatment the difference between treated and untreated groups was so large that the whole effect of treatment was also significant.

The two treatment regimens caused a marked reduction in arthritic manifestations, but were accompanied by signs of peritoneal inflammation. Most of the animals treated with repeated doses of Mb showed abdominal distention and intraperitoneal exudates 10 to 15 days after the start of treatment. At the end of the experiment (day

28), autopsy revealed gut swelling, redness of the serosa, multiple granulomata, and occasional haemorrhages. These observations prompted us to look for a "therapeutic" regimen using a reduced number of ip administrations and a lower dose.

The effects of a single administration of different concentrations of Mb on arthritis development are shown in Figures 4 and 5. A significant inhibition of arthritis as evaluated by the arthritic index score was observed in rats treated once 10 days after induction (Fig. 4). The maximum percentage inhibition occurred at day 14 (26% and 24% with 60 μg and 6 μg, respectively) and the effect lasted only until day 21 (11% with both doses).

Hindpaw volumes are shown in Figure 5. The time course of paw swelling in the arthritic control rats was similar to the previous groups, showing an increase in volume from day 11 to day 28. The injection of 60 or 6 μg of Mb on post-induction day 10 reduced paw swelling in the contralateral paw in a dose-depending manner.

The 60 and 6 μg doses of Mb delayed the development of oedema by four and two days, respectively. In the following days the effects of the two doses are similar, showing 30% inhibition of the arthritic process. Statistical analysis showed significant interaction between the groups as outlined in the legend to Figure 5.

DISCUSSION

In this work we report on a series of experiments designed to explore the feasibility of a new model of tolerance induction in AA rats. Marked to slight reductions in inflammatory symptoms, according to different treatment protocols, were obtained by intraperitoneal injection of Mb in rats in which arthritis was induced by the same agent.

The effect termed "low dose tolerance" has been known now far about 30 years, and several authors have demonstrated that injecting animals with small doses of an antigen may induce specific tolerance to it (16, 17, 18). Unresponsiveness to mycobacterial adjuvant has been induced in rats by previous treatment with subarthritogenic doses of the same inoculum (19), and transfer of this unresponsive state has been obtained by viable lymph node cells from unresponsive animals (20). Such suppressor cells have been identified as A2-line T lymphocytes, and two subclones of line A2 were found either to induce arthritis (clone A2b) or to prevent or therapeutically reduce AA (clone A2c) (1).

The mycobacterial driving antigen for T cell clones A2b and A2c has been identified as in the heat shock protein 65 kD, and the epitope responsible for AA may be the nonapeptide sequence 180-188. Vaccination against AA has also been obtained by hsp 65 kD administered in immunogenic form in oil (4), and with pretreatment with peptide 180-188.

Autoantigen tolerance has been reported using peptides derived from the target antigen in other experimental models of autoimmune diseases such as diabetes and experimental autoimmune encephalomyelitis (EAE).

Good results have also been obtained in the suppression of AA and EAE by oral administration of type II collagen and myelin basic protein, respectively (12, 21).

Oral tolerance is clearly distinct from tolerance induced by systemic administration of soluble antigens, and its mechanisms of action are quite different. It has been shown that oral tolerance caused by low antigen doses may be mediated by regulatory T-cells that induce active suppression, whereas high doses of antigen can induce clonal anergy (22). Active suppression would be generated by antigen-specific suppressor T cells in the Peyer's patches of mice, which later populate systemic lymphoid tissues (23). In rats with EAE oral administration of myelin basic protein has been reported to stimulate CD8+ T cells to produce trasforming grow factor (TGFB)-(24), and the administration of exogenous TGF-(suppress EAE and other experimental autoimmune disease (25), indicating a possible inhibitory role for this cytokine.

In the present study the administration of multiple low doses of Mb intraperitoneally before and after induction of disease is able to suppress arthritis, but causes heavy peritoneal damage. In this case we cannot rule out the possibility that aspecific inflammation may have diverted the target of immunocompetent primed cells.

However, a single administration of Mb on day 10 does not damage the abdominal cavity and is capable to delaying the onset of disease, which generally occurs between days 12 and 14, and the severity of the lesions is also reduced by this treatment. Other dosage and schedules of treatment and possibly the effect of oral administration of antigen will be further investigated.

At the present, we have confirmed the protective effect of low doses of Mb in AA and modulation of the disease by a single dose given just before the symptoms of arthritis become evident. The possibility of inverting the immune process, once a destructive autoimmune reaction is underway, is crucial for new strategies in the therapy of human autoimmune diseases.

A recent report in NOD mice which spontaneously develop autoimmune diabetes describes successful therapy with a peptide of the heat shock protein administered subcutaneously, once the disease is far advanced (26). The authors explain the mechanism of this tolerance in terms of the activation of a network of anti-idiotypic T cells which down-regulate autoimmune T effector cells.

Further studies are needed to clarify the mechanism of immunomodulation in our experimental conditions, especially through evaluation of inflammatory and suppressive cytokines such as IL-1, IL-6, TNF-α, IFN-γ, TGF-β and IL-4 and through the characterization of lymphocytes obtained from cultures of rat paw articulations.

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