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EVALUATION OF THE ANTI-INFLAMMATORY EFFECT OF DIPYRIDAMOLE, ATP *PER SE* AND IN COMBINATION WITH METHOTREXATE IN FREUND'S ADJUVANT INDUCED ARTHRITIS IN RATS

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory disease which mainly affects synovial joints and leads to damage of articular cartilage and ankylosis of joints. The effect of various drug interventions like the methotrexate, dipyridamole and ATP alone and in combination on RA were studied. RA was introduced with the help of Freund 's adjuvant (0.5ml/200gms wistar rat) in 6 Separate Wistar rat groups. Freund 's complete adjuvant (FCA) is the combination of mineral oil, an emulsifier mannide monooleate with 1mg/ml heat killed dry mycobacterium tuberculosis H37Ra. The mycobacterium stimulate the immune system and enhance the immune response. On 11th day after FCA the various animals groups were treated with drug interventions and after 10 days of treatment the paw edema of the respective rats were assessed by a mercury displacement plethysmograph (INCO Corp., India). During these studies the changes in paw edema by drug treatment of animals were analyzed. The results of this study are discussed in this article.

Key Words: Freund's adjuvant, dipyridamole, methotrexate, arthritis, ATP

Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disorder of unknown aetiology that is characterized by symmetric synovitis and the propensity to cause joint destruction, disability and premature death. Disease-modifying anti-rheumatic drugs (DMARDs) slow the natural course of the disease, reduce joint damage and pain, and retard loss of function and disability. Treatment plays a key role in controlling the inflammation of rheumatoid arthritis and minimizing joint damage. Treatment usually entails a combination of drug therapy and other non-drug therapies. The levels of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1-β (IL1-β) and interleukin-6 (IL-6) are increased in arthritic joints (1), and these cytokines have been implicated in the joint destruction and tissue dysfunction seen in rheumatoid arthritis (2).

The advocation of low dose methotrexate (MTX) is the most widely used anti-rheumatic drug and it is the 'gold standard' against which other systemic medications are compared (3). . It has been suggested that at least part of the anti-inflammatory effects of the disease modifying anti-rheumatoid drugs methotrexate and sulphasalazine may be attributable to the release of the endogenous nucleoside adenosine by these drugs (4). It has as its target the enzyme dihydrofolate reductase, which is required for reduction of dihydrofolate to tetrahydrofolate (5). Adenosine, an additional active metabolite of MTX has been found to be potent anti-inflammatory effects, and earlier studies strongly support the notion that the potent anti-inflammatory effect via suppression of inflammatory cytokines such as tumour necrosis factor (TNF)-alpha, interleukin-6, or macrophage inhibitory protein-1 alpha. It was further found that the anti-inflammatory effect of adenosine is mediated via A2A and the A3 adenosine receptors.

The highly selective A3 adenosine receptor (A3AR) agonist 1B-MECA had an anti-inflammatory effect in collagen-induced arthritis in DBA1 mice and adjuvant-induced arthritis (AIA) in rats. Adenosine is increasingly regarded as an important endogenous regulator of inflammatory processes. All the adenosine receptors currently recognised—A1, A2A, A2B and A3 can modify the release of inflammatory mediators or reactive oxygen species from immune-competent cells (6, 7). Most attention has been devoted to the A2 receptors, with the A2A subtype being shown to decrease the secretion of TNF-alpha (8, 9) and IL-12 (10,11) as well as suppressing the mitochondrial respiratory burst (12, 13). There is also evidence that similar activity may be shown by A2B receptors (14, 15) and A3 receptors (16, 17), while A1 receptors can promote the release of some cytokines (18) but suppress IL-6 secretion. The concept of a key role for adenosine receptors in inflammatory processes is supported by the up-regulation, especially of A2A and A2B receptors, that occurs in activated monocytes and

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macrophages. Dipyridamole is a widely established drug for the treatment of angina pectoris and cerebrovascular disease. Its main mechanism of action is to inhibit the cellular uptake and metabolism of adenosine, with resulting vasodilatory and anti-aggregatory effects. On the other hand, ATP is also found to be converted to adenosine and may have prominent roles endogenously (19). We have now investigated the hypothesis that an increase in adenosine levels in patients, induced by chronic treatment with dipyridamole, may in turn lead to a reduction in pro-inflammatory cytokine release, and could alleviate the symptoms of rheumatoid arthritis. This study was hitherto conducted to ascertain the anti-arthritic effect of dipyridamole and ATP and synergistic effect with methotrexate in adjuvant induced arthritis in albino Wistar rats.

Materials and Methods

Drugs

Dipyridamole and ATP were obtained from Sigma chemical company (USA). Dipyridamole was dissolved in 0.1 N HCl and ATP was dissolved in sterile water before using it for injection. Methotrexate and water for injection was obtained from the CMC pharmacy, Vellore.

Test animals

Wistar Lewis rats of either sex weighing around 175 to 225 gms were used in the studies. Animals were housed in cages under standard lab conditions (12:12 hr light dark cycle at 25 ± 5 degree C). The rats were fed with commercial rat diet and water *ad libitum* and were divided in to 6 groups, containing 6 animals in each group. The ethical guidelines for the involvement of the animals used in experiments were followed in all the tests.

Induction of arthritis

Arthritis was induced by a modified method described earlier (20). Arthritis was induced by injecting 0.5 ml Freund's complete adjuvant (FCA) containing 1mg/ml of dry heat killed Mycobacterium butyricum of sterile paraffin oil in to plantar surface of right hind foot of all the animals.

Experimental design

Animals were divided in to 6 groups of 6 rats in each group. On the 11nth day after injecting FCA, the animals in the various groups received the respective drugs as follows:- Group 1 received saline 0.5 ml of 0.9% NaCl and served as the control. Group 2 received MTX at a dose of 0.2 mg/kg on alternate days for a total period of 10 days. Group 3 received Dipyridamole at a dose of 10mg/kg for 10 days. Group 4 received ATP at a dose of 0.75 mg/kg for a period of 10 days. Group 5 received MTX 0.2mg/kg on alternate days along with Dipyridamole 10 mg/kg daily for 10 days. Group 6 received MTX 0.2 mg/kg on alternate days ,along with ATP 0.75 mg/kg daily for a total period of 10 days. After 10 days of treatment the paw edema of the respective rats were assessed by a mercury displacement plethysmograph (INCO Corp., India). The percentage inhibition of paw edema was calculated using the formula 1-Vt/Vc X100. Where by paw volume before arthritis(Vt) –paw volume after intervention(Vc)/ paw volume before arthritis(Vt) X 100

Statistical analysis

The statistical analysis was conducted for control and test group of animals and comparisons were done between the control and test groups containing n=6 number of animals. The statistical tests applied were the Wilcoxon signed ranks test and Man Whitney test and it was used for the analyses of the data. $P<0.05$ were considered to be statistically significant.

Results

The results of this study are shown in Figure 1 and Table 1. Treatment of different animal groups (n=6) with dipyridamole (10mg/kg) and methotrexate (0.2mg/kg) caused substantial and significant improvement in paw edema caused by FCA injection. Similarly ATP (0.75mg/kg) was injected to the rats and it also caused a 36.11% reduction in paw edema. The improvement in paw edema was evaluated by a mercury displacement plethysmograph. The combination of methotrexate and dipyridamole produced a potentiated 46.39 % reduction in paw edema after treatment with these drugs for a duration for 10 days. However the combination of methotrexate and ATP did not produce appreciable improvement as per our results and caused only a 9.72 % reduction in adjuvant arthritis.

Discussion

In our study, MTX produced a beneficial effect on FCA induced arthritis and this can be attributed to various factors. Amongst the first line of drugs available for rheumatoid arthritis, the foremost important is the drug MTX which is given at a low dose. It is now widely accepted that the mechanism of action of MTX in RA is due to the inhibition of the enzyme AICAR transformylase by the MTX polyglutamates, which are formed after MTX is taken up by the inflammatory cells (21,22). Since AICARiboside directly inhibits ADA (adenosine deaminase which deaminates adenosine in to inosine) and AICAR inhibits AMP deaminase, the intracellular accumulation of AICAR could lead to release of adenosine and AMP (converted by 5' nucleotidase to adenosine in the extracellular space) in to the extracellular space. It is this increase in adenosine concentration in the extracellular space which is responsible for the anti inflammatory effect seen in patients treated with MTX is proved in

experiments conducted by Cronstein et al (23,24). To further prove that this adenosine released was responsible for the anti-inflammatory effect, was evident in other experiments where agents like adenosine kinase inhibitors (25-27), sulfasalazine were used and were shown to have anti inflammatory effect as well by an adenosine dependent mechanism,in both *in vitro* and *in vivo* experiments. More support that MTX enhances adenosine release in humans, was provided by recent studies which showed that, children treated with MTX for childhood leukaemia, had increased adenosine concentration in the cerebrospinal fluid.

Similarly a reduction in paw edema was seen after treatment with dipyridamole and ATP to further the notion that adenosine is responsible for alleviating the symptoms of rheumatoid arthritis. ATP has been shown to be converted to adenosine endogenously and relieve the symptoms of arthritis (19), however it was less potent then dipyridamole and MTX. The anti-inflammatory action caused by the drugs dipyridamole could be because of it's reuptake inhibition of adenosine at the inflammation site. The study by German et al (28) showing that dipyridamole enhances plasma adenosine levels in human beings supports our claim that dipyridamole can cause increase in adenosine levels. And the concentrations of adenosine required to inhibit inflammatory cell function are similar to those observed in vivo (29) and hence an increase in adenosine caused by dipyridamole can very well have an anti-inflammatory action. The adenosine thus released extracellularly, interacts with adenosine receptors on the cell surface and produces reduction in inflammation by suppression of inflammatory cytokines such as tumour necrosis factor (TNF)-alpha, interleukin-6 (30). Of particular interest is the interaction of adenosine on A2a receptor on stimulated neutrophils, that leads to inhibition of release of toxic oxygen metabolites, and adhesion to a variety of different cell types, and suppresses neutrophil mediated injury both *in vitro* and *in vivo* (31,32). With the above evidence, we also observed whether the adenosine reuptake-inhibitory action of dipyridamole would be sufficient to cause an anti inflammatory action in the above experiment, and similarly whether the adenosine released from the metabolism of ATP would have any anti inflammatory action as well. Our results portray that dipyridamole and methotrexate treatment *per se* significantly attenuated the paw edema. These results are novel and interesting. We also tried to evaluate, whether combining the drugs dipyridamole with methotrexate had any synergistic effect, on the basis that the adenosine released from MTX and adenosine reuptake inhibition by dipyridamole, increased the extracellular concentration of adenosine and this could lead to an enhanced anti inflammatory effect in what can be described a possible drug synergism. Our results thus confirm a salient role for dipyridamole and methotrexate combination in alleviating the paw edema induced by FCA.

Figure 1: Figure 1 illustrates the bar graphs depicting the effects before and after injections of dipyridamole (10mg/kg), methotrexate (0.2mg/kg) and ATP (0.75mg/kg) on Freund's complete adjuvant induced paw edema in albino rats. The values depicted are from 6 experiments each.

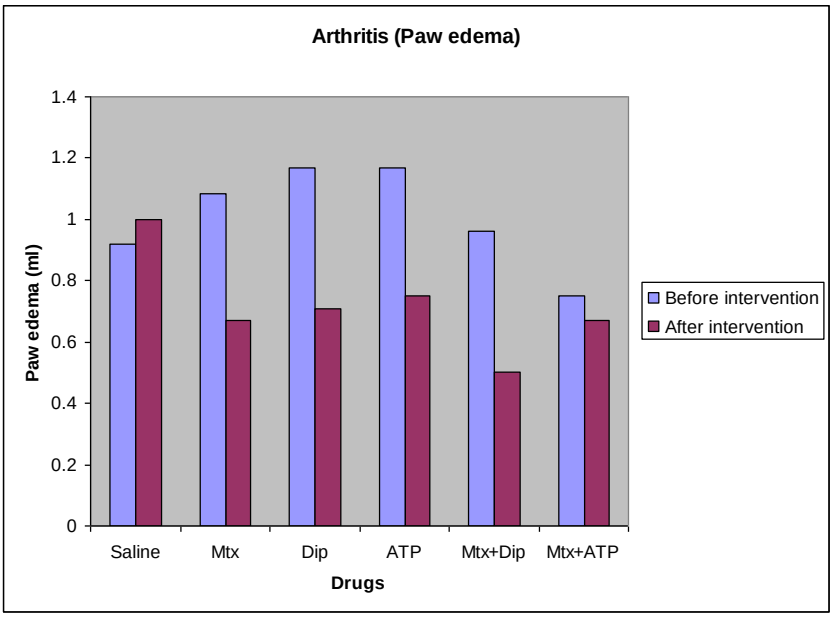

Table 1: Table 1 shows the effect of dipyridamole (10mg/kg), methotrexate (0.2mg/kg) and ATP (0.75mg/kg) on paw edema induced by Freund's complete adjuvant injection. The percentage change from control group (saline treated) is compared with treated group and statistical significance is given. The values depicted are from 6 experiments each.

Serial No.	Treatment	% change from control	'P' Value
1)	Saline	-9.72	P<0.05
2)	Dipyridamole (10mg)	40.83	P<0.05
3)	Methotrexate (0.2 mg)	37.5	P<0.05
4)	ATP (0.75mg)	36.11	P<0.05
5)	Methotrexate + Dipyridamole	46.39	P<0.05
6)	Methotrexate + ATP	9.72	P>0.05

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