Original Article

The Effect of Malaria-Induced Serum Tumor Necrosis Factor-Alpha on Epiphyseal Bone Formation of Rats

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Abstract

Aim: Malaria infection has long been associated with stunted growth in children and by extension bone formation. There is, however, a paucity of information on the specific factors or substances that are responsible for this observation. The objective of this study was, therefore, to find out whether malaria-induced tumor necrosis factor-alpha (TNF-α) influenced in any way the formation of the epiphyseal bone layer of Sprague Dawley rats. **Materials and Methods:** Thirty-two Sprague Dawley rats aged 4 weeks were used in this study, and the animals were randomly allocated to four groups (Group A–D), with each group having 8 animals. Animals in Group A were given oral artemether/lumefantrine combination drug only, animals in Group B were inoculated with *Plasmodium berghei* (NK65) only, those in Group C were inoculated with *P. berghei* (NK65) and treated with oral artemether/lumefantrine, while animals in Group D were neither inoculated with the malaria parasite nor given artemether/lumefantrine therapy. Blood was drawn from the animals at predetermined intervals to measure serum TNF-α levels. **Results:** Recurrent bouts of murine malaria caused serum TNF-α levels to be significantly elevated even after the infected animals were successfully treated. The elevated serum TNF-α levels were found to correlate with the amount of bone tissue deposited at the distal femoral epiphysis. **Conclusion:** Malaria-induced TNF-α most likely delays bone tissue formation and may be a major contributing factor in the development of stunted growth in children.

Keywords: Bone, malaria, stunting, tumor necrosis factor-alpha

Introduction

Tumor necrosis factor-alpha (TNF-α) is a major cytokine produced during malaria infections. The release of TNF-α is responsible for the pyrexia that is observed during infections,[1-5] and it also plays an important role in inhibiting parasite multiplication.[2,3,6] Elevated levels of TNF-α have, however, been linked to the severity of disease and the development of complications, such as cerebral malaria and anemia.[2,5,7,8] TNF-α influences the activity, growth, and maturation of osteoblasts and osteoclasts,[9-13] and elevated serum levels have been found to play an important role in the pathogenesis of inflammatory bone disorders such as rheumatoid arthritis.[11,12,14] The fact that TNF-α influences the activity of bone cells raises the question as to whether its release during malaria infections may also have an effect on bone tissue formation. There appear to be no studies in literature that has attempted to answer this question. This study, therefore, sought to investigate the effect, if any, that malaria-induced TNF-α may have on epiphyseal bone formation.

Materials and Methods

Thirty-two Sprague Dawley rats aged 4 weeks were used for this study. The rats were given standard laboratory chow and water in the morning and evening throughout the experimental period. Animals were treated humanely in conformity with the local institutional guidelines for animal experimentation.

Animals were randomly assigned to four groups (Group A–D) of 8 animals each.

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- Group A: Rats were given oral artemether/lumefantrine combination drug only (dosage: 3.2 mg/kg/body weight) two times a day for 3 days
- Group B: Rats were given an inoculum of 0.2 ml of *Plasmodium berghei* (NK65)-infected blood
- Group C: Rats were given an inoculum of 0.2 ml of *P. berghei* (NK65)-infected blood and treated with oral artemether/lumefantrine (dosage 3.2 mg/kg/body weight) two times a day for 3 days
- Group D: Rats were neither inoculated with parasitized blood nor given artemether/lumefantrine therapy.

Thick film slides were subsequently prepared from tail vein blood to calculate the parasite density, after which treatment was started. Three weeks after treatment was completed, thick films were again prepared to establish that there were no parasites in the blood after which blood samples were drawn from the tail veins using 25G hypodermic needles. Animals were then made to rest for one more week after which the experimental protocol was repeated.

All animals were anesthetized with chloroform and subsequently sacrificed after the second cycle of the experiment. Both femurs were removed from the thighs of the animals. The bones were decalcified in 8% formic acid solution over a 24-h period and subsequently transected. The distal portion was then bisected in the coronal plane. Both halves were places in single cassette and embedded with paraffin wax. The blocks were sectioned serially at 4-μm thickness using a Leica RM 2125RT microtome (Leica Biosystems Nussloch GmbH, Germany). Every 25th section was selected for stained using hematoxylin and eosin dye.

Using AmScope MD35 640 × 480 Pixel Still and Video USB Digital Eyepiece Camera (Amscope, USA), microscopic images of the histological slides were captured. Slides that were deemed suitable for the purpose of this study were those that showed the full thickness of both the epiphyseal growth plate and the bone epiphyseal layer. Using the digital measuring functions of the AmScope MD35 camera (Amscope, USA), the thickness of the epiphyseal bone layer was measured. As much as possible, the lines of measurements were oriented to that of the longitudinal axes of the chondrocyte columns found within the epiphyseal growth plate.

All blood samples collected were made to sit for 2 h at room temperature to enable the sample to clot adequately, after which they were centrifuged for 15 min at 1000 ×*g* at 8°C. Serum was separated from the cells and stored in Eppendorf tubes at −80°C until the time of use.

Samples were thawed before assay. Serum concentrations of TNF-α were measured using Biomatik rat TNF-alpha ELISA kit (Biomatik, USA). The assays were conducted strictly following the manufacturer's instructions without any modification. The optical density of each well was determined using the Accuris microplate reader MR-9600 (Accuris Instruments, USA). Serum TNF-α concentrations were automatically calculated using the microplate reader in picograms per milliliter.

Analysis

IBM Statistical Package for the Social Sciences (SPSS) version 21 (IBM Corp., Armonk, N.Y., USA). was used for data analysis. The mean thickness and standard deviation of the epiphyseal bone layers for each group was calculated. The mean serum TNF-α level for each group was also calculated after each cycle. The differences between the group means were analyzed using a one-way analysis of variance (ANOVA) test. An least significant difference *post hoc* (multiple comparisons) test was then run to identify the group (s) that had statistically significant difference (s) in their mean serum TNF-α levels at $P = 0.05$. Pearson correlation was also used to analyze the linear relationship between mean bone thickness and mean serum TNF-α level of the different experimental groups at a significance of 0.05.

Results

All animals in Group B died by postinfection day 14; hence, no results were obtained for Group B. In the three remaining Groups (A, C, and D), it was noted that the mean serum TNF-α concentrations at the end of the second cycle were higher than those recorded at the end of the first. These differences were, however, not statistically significant [Table 1].

After the first cycle of the experiment, the mean serum concentration of TNF-α for the various groups was compared using one-way ANOVA. It was observed that there was a significant difference in the means of the various groups ($P < 0.05$). *Post hoc* analysis showed that the mean serum concentration of TNF-α differed significantly between Group C and Group D animals and also between Group A and Group C animals [Table 2].

At the end of the second cycle of the experiment, the mean serum concentration of TNF-α for the various groups was again compared using one-way ANOVA. There was a significant difference in the mean serum concentration of the various groups ($P = 0.002$).

Post hoc analysis showed that the mean serum concentration of TNF-α differed significantly between Group A and Group C animals and also between Group C and Group D animals [Table 3]. Similar results were observed at the end of the first cycle.

Table 1: Serum tumor necrosis factor-alpha level of the different experimental groups after the first and second cycles

Group	Mean±SD (pg/ml)		Significant at 0.05
	First cycle	Second cycle	
A	18.52±1.60	18.03±0.86	0.273
C	29.51±1.22	34.08±4.15	0.121
D	19.01±1.06	19.61±0.82	0.297

SD: Standard deviation

In all the three groups, there was a negative correlation between the mean bone thickness and the serum TNF-α concentration; the correlation was, however, only significant with regard to animals in Group C [Table 4].

Discussion

When the mean serum TNF-α concentration of the various groups in this study was compared at the end of the first cycle of the experiment, it was found that there was a statistically significant difference between the mean TNF-α concentration of Group C and D animals ($P < 0.05$). There was also a significant difference between the serum TNF-α level of Group C and A animals ($P < 0.05$).

Similar observations were also made when the serum TNF-α concentration of the various groups was compared at the end of the second cycle of the experiment. The serum TNF-α concentration of the animals that were infected with the malaria parasite was thus significantly higher than the animals in the other groups. It is well documented that in the acute phase of both human and murine malaria infections, the level of TNF-α in the bloodstream increases as part of the body's response in combating the infection.[4,7,15,16] The elevated levels of TNF-α have been linked to severity of the disease as well as to the development of complications, such as cerebral malaria and anemia.[2,5,7,8]

The findings of this study, however, suggest that even after the acute phase of the infection, TNF-α levels continue to be elevated, and this was clearly evident by the fact that 21 days after the completion of the antimalarial therapy, and with the plasmodium parasite eradicated from the blood, serum concentrations of TNF-α were still found to be significantly elevated.

Thus, serum TNF-α concentrations, although generally accepted to fall from their extremely high levels after the acute phase of infection,[4,7,15] may probably never return to their original baseline value even after adequate treatment. This observation is supported by the findings of some studies carried out in malaria endemic zones, in which it was noted that persons who experienced frequent bouts of malaria continued to exhibit elevated levels of plasma TNF-α, even during noninfective periods.[7,17]

Within Group C animals, it was observed that the mean TNF-α concentration at the end of the second cycle was greater than

Table 2: Least significant difference *post hoc* multiple comparisons of the mean serum tumor necrosis factor-α level of the different experimental groups after the first cycle

Group	Group	Mean difference	Significant
A	C	−11.48	0.001*
	D	−0.99	0.395
C	D	10.49	0.002*

*Significant at 0.05

Table 3: Least significant difference *post hoc* multiple comparisons of the mean serum tumor necrosis factor-α level of the different experimental groups after the second cycle

Group	Group	Mean difference	Significant
A	C	−15.57	0.001*
	D	−1.09	0.676
C	D	14.48	0.002*

*Significant at the 0.05

Table 4: Correlation between mean bone thickness and mean serum tumor necrosis factor-α level of the different experimental Group (A-D) after the second cycle

Group	
Group A	
Pearson correlation	−0.305
Significant (two-tail)	0.617
Group C	
Pearson correlation	−0.922*
Significant (two-tail)	0.026
Group D	
Pearson correlation	−0.420
Significant (two-tail)	0.580

*Correlation is significant at the 0.05 level (two-tailed)

that recorded at the end of the first cycle. This difference was, however, not statistically significant. From this observation, a question arises as to whether with each new episode of malaria there would be a further increase in the serum TNF-α levels and whether the increment of TNF-α levels would have increased in perpetuity or it would have eventually plateaued. The data generated from this study was not adequate to answer these questions and, therefore, create an area of interest for future research.

Also of interest was the observation that the levels of serum TNF-α recorded in Group A animals did not differ significantly from those observed for animals in Group D after both cycles, suggesting that the presence of the antimalarial drug artemether/lumefantrine did not significantly elevate the level of serum TNF-α. It is, therefore, possible to infer that the elevated levels of TNF-α observed in Group C animals (inoculum and treatment) were mainly due to the presence of the malaria infection and that the administration of the antimalarial drug contributed minimally to the production of TNF-α.

At the end of the second cycle of the experiment, it was observed that there was a negative correlation between the serum TNF-α concentration and the mean epiphyseal bone thickness of animals in all the three groups. This correlation was, however, only significant in the rats that had been infected with the malaria parasite. There appears, therefore, to be some association between the amount of bone tissue deposited at the epiphysis of Sprague Dawley rats and the elevated serum TNF-α levels. This negative correlation suggests that bone tissue formation is adversely affected by elevated serum TNF-α produced during recurrent bout of murine malaria.

Indeed, the elevated levels of TNF-α have been implicated in the pathogenesis of bone diseases such as rheumatoid arthritis and related forms of inflammatory arthritis.[11,12,14] This study, however, appears to be the first to provide evidence that TNF-α produced as a result of recurrent bouts of malaria infection possibly has an adverse effect on bone formation and deposition.

The sustained elevated TNF-α levels observed may thus play an important role in the etiology of stunted growth which has been associated with malaria infections.[18-22] The mechanism by which elevated serum TNF-α adversely affected bone formation was not investigated in this study; however, there is a large body of literature which shows that TNF-α promotes resorption of bone[11,12,23,24] while at the same time inhibiting its formation.[14,25,26] It does this by regulating the differentiation of the cell lineage of osteoclasts and osteoblasts.[9-14,27]

Conclusion

This study shows that recurrent bouts of murine malaria cause the concentration of serum TNF-α to remain raised even after appropriate treatment. This elevated level of TNF-α most likely plays a crucial role in retarding bone tissue formation and may thus contribute to the development of stunting in children, particularly in areas where malaria is endemic. The findings from this study may also offer an explanation as to why there may be a delay in the fusion of the epiphyseal growth plate in the sub-Saharan African population, as has been reported by some researchers.[28-30]

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Conflicts of interest

There are no conflicts of interest.

References

1. Miller LH, Good MF, Milon G. Malaria pathogenesis. Science 1994;264:1878-83.
2. Perlmann P, Troye-Blomberg M. Malaria and the immune system in humans. Malaria Immunol 2002;80:229-42.
3. Malaguarnera L, Musumeci S. The immune response to *Plasmodium falciparum* malaria. Lancet Infect Dis 2002;2:472-8.
4. Boström S, Giusti P, Arama C, Persson JO, Dara V, Traore B, *et al*. Changes in the levels of cytokines, chemokines and malaria-specific antibodies in response to *Plasmodium falciparum* infection in children living in sympatry in Mali. Malar J 2012;11:109-20.
5. Sarangi A, Mohapatra PC, Dalai RK, Sarangi AK. Serum IL-4, IL-12 and TNF-alpha in malaria: A comparative study associating cytokine responses with severity of disease from the coastal districts of Odisha. J Parasit Dis 2014;38:143-7.
6. Clark IA, Cowden WB. The pathophysiology of falciparum malaria. Pharmacol Ther 2003;99:221-60.
7. Dodoo D, Omer FM, Todd J, Akanmori BD, Koram KA, Riley EM. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production *in vitro* predict clinical immunity to *Plasmodium falciparum* malaria. J Infect Dis 2002;185:971-9.
8. Lyke KE, Burges R, Cissoko Y, Sangare L, Dao M, Diarra I, *et al*. Serum levels of the pro-inflammatory cytokines interleukin-1 beta (IL-1_), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and il-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. Infect Immun 2004;72:5630-7.
9. Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, *et al*. Tumor necrosis factor α stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL–RANK interaction. J Exp Med 2000;191:275-86.
10. Kim N, Kadono Y, Takami M. Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis. J Exp Med 2005;202:589-95.
11. Teitelbaum SL. Osteoclasts; culprits in inflammatory osteolysis. Arthritis Res Ther 2005;8:201.
12. Schett G, Gravallese E. Bone erosion in rheumatoid arthritis: Mechanisms, diagnosis and treatment. Nat Rev Rheumatol 2012;8:656.
13. Mbalaviele G, Novack DV, Schett G, Teitelbaum, SL. Inflammatory osteolysis: A conspiracy against bone. J Clin Invest 2017;127:2030-9.
14. Zhao B. TNF and bone remodeling. Curr Osteoporos Rep 2017;15:126-34.
15. Mugweru J, Waihenya R, Kimani F, Ng'ang'a Z, Matiru V, Muregi FW. Cytokine levels associated with experimental malaria pathology during *Plasmodium berghei* ANKA infection in a mouse model. J Clin Immunol Immunopathol Res 2013;5:1-8.
16. Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, *et al*. Malaria immunity in man and mosquito: Insights into unsolved mysteries of a deadly infectious disease. Ann Rev Immunol 2014;32:157-87.
17. Peyron F, Vuillez JP, Barbe G, Boudin C, Picot S, Ambroise-Thomas P. Plasma levels of tumor necrosis factor during a longitudinal survey in an endemic area of malaria. Acta Tropica 1990;47:47-51.
18. Hautvast JL, Tolboom JJ, Willems JL, Mwela CM, Monnens LA. Consequences of infections for three□month length increment in young

children in rural Zambia. *Acta Paediatr* 2000;89:296-301.
19. Lee G, Yori P, Olortegui MP, Pan W, Caulfield L, Gilman RH, *et al*. Comparative effects of vivax malaria, fever and diarrhoea on child growth. Int J Epidemiol 2012;41:531-9.
20. Kang H, Kreuels B, Adjei O, Krumkamp R, May J, Small DS. The causal effect of malaria on stunting: A mendelian randomization and matching approach. Int J Epidemiol 2013;42:1390-8.
21. Ferreira ED, Alexandre MA, Salinas JL, de Siqueira AM, Benzecry SG, de Lacerda MV, *et al*. Association between anthropometry-based nutritional status and malaria: A systematic review of observational studies. Malar J 2015;14:346-9.
22. Gari T, Loha E, Deressa W, Solomon T, Lindtjørn B. Malaria increased the risk of stunting and wasting among young children in Ethiopia: Results of a cohort study. PLoS One 2018;13:e0190983.
23. Martensson K, Chrysis D, Savendahl LL. Interleukin-1 and TNF act in synergy to inhibit longitudinal growth in fetal rat metatarsal. J Bone Miner Res 2004;19:1805-12.
24. Goldring SR, Purdue PE, Crotti TN, Shen Z, Flannery MR, Binder NB, *et al*. Bone remodelling in inflammatory arthritis. Ann Rheum Dis 2013;72 Suppl 2:52-5.
25. Osta B, Benedetti G, Miossec P. Classical and paradoxical effects of TNF-α on bone homeostasis. Front Immunol 2014;5:48.
26. Algate K, Haynes DR, Bartold PM, Crotti TN, Cantley MD. The effects of tumour necrosis factor□α on bone cells involved in periodontal alveolar bone loss; osteoclasts, osteoblasts and osteocytes. J Periodont Res 2016;51:549-66.
27. Yao Z, Lei W, Duan R, Li Y, Luo L, Boyce BF. RANKL cytokine enhances TNF-induced osteoclastogenesis independently of TNF receptor associated factor (TRAF) 6 by degrading TRAF3 in osteoclast precursors. J Biol Chem 2017;292:10169-79.
28. Lewis CP, Lavy SB, Harrison WJ. Delay in skeletal maturity in Malawian children. J Bone Joint Surg Br 2002;84:732-4.
29. Dembetembe KA, Morris AG. Is greulich-pyle age estimation applicable for determining maturation in male Africans? S Afr J Sci 2012;108:1-6.
30. Brown AA, Derkyi-Kwarteng L, Amonoo-Kuofi HS. Study on the time frame for ossification of the medial clavicular epiphyseal cartilage by X-ray in Ghanaian students. Int J Morphol 2013;31:491-6.