International Journal of Tropical Medicine 1 (3): 97-99, 2006

Characterization of Intracellular Toxin from Periodontopathic *Prevotella intermedia* Isolates in Malaysia

[1]W.H. Himratul-Aznita and [2] A. Ansary
[1]Department of Oral Biology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia
[2]Open University, Jalan Sultan Ismail, Kuala Lumpur, Malaysia

Abstract: Periodontal disease is a global public health problem, including in Malaysia. The destruction of the periodontium has been associated with subgingival plaque microflora. *Prevotella intermedia* has been categorized as a periodontopathic bacteria as it contributes to the development and progression of periodontal disease. Therefore, a study on *P.intermedia*'s virulent properties is important for the understanding of periodontal disease. Thus, the aim of the study is to characterize the virulence effect of the intracellular toxin. In the study, intracellular toxin of *P.intermedia* from ninety isolates were obtained and injected subcutaneously in male balb/c mice, aged between 8-12 weeks old. The results showed that the intracellular toxin from all *P.intermedia* isolates were able to cause the development of localized lesions on the skin of balb/c mice when 0.1, 0.2 and 0.3 mL filtrates were used. In addition, it was found that the degree of skin lesions on the balb/c mice was dose dependent as the infective dose was found to correspond to the development of larger lesion accompanied by skin coagulative necrosis. In the second part of the study, the intracellular toxin was heated for 1, 5, 15, 30, 45 and 60 min at different temperatures of 37 and 60°C prior to the infection in mice. It was discovered that the intracellular toxin was not stable and sensitive towards high temperature and is thermolabile when the activity was terminated upon heating to 60°C as none of the injected mice developed skin lesion.

Key words: *P. intermedia*, skin lesion, intracellular toxin, stability

INTRODUCTION

Periodontal disease is an inflammation of the gingival tissues due to the penetration of products of the subgingival plaque bacteria around the teeth[1] and has been implicated as a major cause of periodontal destruction that leads to tooth loss and is a global public health problem including in Malaysia. One of the most important microorganisms involved in periodontitis is *Prevotella intermedia*. The black-pigmented pleomorphic rod Gram-negative anaerobe has been reported to contribute to the development and progression of periodontal disease[2-5]. The bacteria has been suggested to be involved in the etiology and pathogenesis of periodontal disease due to their high prevalence in periodontal pockets[6]. Thus, the virulent properties of the species are very important for the pathogenesis of periodontal disease. Therefore, the study was undertaken with the objective of characterizing the virulent effect of *Prevotella intermedia* intracellular toxin from clinical isolates in Malaysia.

MATERIALS AND METHODS

Bacterial strains and culture conditions: All ninety *P.intermedia* clinical isolates were from our research collection. The isolates were grown in enriched tryptic soy agar supplemented with 5 μl mL^{-1} hemin, 0.5 μl mL^{-1} menadione and 5% defibrinated blood. All microbiological manipulations were performed using continuous anaerobic techniques, as *Prevotella intermedia* is an anaerobic organism. In addition to the clinical isolates, *Porphyromonas gingivalis* ATCC 33277 was used as the positive control and Phosphate Buffered Saline (PBS) was used as negative control in the studies.

Preparation of intracellular toxin: For preparation of the intracellular toxin, the bacterial cells were washed and resuspended in 1.0 mL 0.85% normal saline and sonicated for 30 sec. The sonication was repeated 3 times after which the suspension was centrifuged and the sonicated pellet was discarded. The supernatant fluid was filtered through a 0.22 μm membrane and retained for further use. 0.1, 0.2 and 0.3 mL of the intracellular toxin were used to

Corrresponding Author: Himratul-Aznita, Department of Oral Biology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia

inject the mice subcutaneously at two dorsal sites of the male balb/c mice aged between 8-12 weeks, provided by The Central Animal House, Faculty of Medicine, University of Malaya under the ethical code of (BM/09/04/03/WHA(R)). The presence, size, consistency and location of each ulceration lesion was evaluated. Mice were also examined daily to assess their general health status. Six mice were used for every strain tested, in order to confirm the accuracy and the reproducibility of the experiment.

Temperature lability of *Prevotella intermedia* toxin: The toxin from all isolates was heated for 1, 5, 15, 30, 45 and 60 min at 37 and 60°C, respectively. Toxic activity were tested using 0.2 mL heat-treated toxin and injected subcutaneously at two dorsal site of the balb/c mice as described above.

Histological analysis: Skin lesions were biopsied and fixed in 10% formalin at room temperature overnight. After fixation, the excised skin lesions were embedded in paraffin before being sectioned into 5μm thick and stained using haematoxylin and eosin stains.

RESULTS AND DISCUSSION

The intracellular toxin of ninety *Prevotella intermedia* clinical isolates were injected subcutaneously in mice to determine their toxicity which were observed as lesions on the skin. The study has shown that all mice developed lesion when 0.1, 0.2 and 0.3 mL were used Table 1. However, infection with 0.1 intracellular toxin produced mild skin lesions, while increasing the infective dose to 0.2 and 0.3 mL was found to correspond to the development of larger lesions with higher level of severity for all mice. Lesion size varied from small areas to coagulative necrotizing lesion of the epidermis layer in mice receiving high doses of the toxin. It is therefore, the response to develop skin lesion on balb/c mice and the degree of lesions severity was dose dependent, which is in agreement with those of other research results[7-9] on periodontopathic anaerobes. In addition, all infected mice showed signs of mild to moderate cachexia relative to the doses used, with ruffled hair and occasionally with hair loss for some of the severely infected mice. This may be caused by inflamed follicles, which were involved in the inflammatory process. As there was no mortality in any of the mice after the injection, all infected mice were euthanized prior to skin biopsy for histology slide preparation.

Table 1: The diameter of lesion areas developed with 0.1, 0.2 and 0.3 mL of intracellular toxin of *P. intermedia* clinical isolates

	Number of mice infected		
Diameter of lesion area (mm)	0.1 mL	0.2 mL	0.3 mL
Less than 1 mm (mild skin lesion)	73	1	-
1 mm – 4 mm (moderate skin lesion)	17	86	59
More than 4 mm (severe skin lesion)	-	3	31
Total of isolates	90	90	90

Table 2: Toxic activity of heated intracellular toxins of *P. intermedia* clinical isolates

	Number of *P. intermedia* isolates causing the mice to develop lesions											
	37°C						60°C					
Mice(lesion)												
Mice (injected)	1'	5'	15'	30'	45'	60'	1'	5'	15'	30'	45'	60'
6/6	1	1	2	1	2	2	0	0	0	0	0	0
5/6	20	22	19	21	21	22	0	0	0	0	0	0
4/6	39	36	38	37	36	36	0	0	0	0	0	0
3/6	26	26	25	25	27	24	0	0	0	0	0	0
2/6	4	4	6	5	4	6	0	0	0	0	0	0
1/6	0	1	0	1	0	0	0	0	0	0	0	0
0/6 (none)	0	0	0	0	0	0	0	0	0	0	0	0
Total isolates infected	90	90	90	90	90	90	0	0	0	0	0	0

In order to study the intracellular toxin stability to heat, the toxins from all isolates were heated for 1, 5, 15, 30, 45 and 60 min at 37 and 60°C, respectively. It was found that the toxin activity of all *P.intermedia* isolates was stable at 37°C eventhough being heated for as long as 60 min. Injection with toxins heated at 60°C failed to induce any infection, as there was no lesion detected on the skin of the infected balb/c mice Table 2.

Upon histological examination, localized lesions were found to be non-invasive and localized at the infection site, a finding similar to other reported results[7,9]. The size of the lesions varied from small areas to coagulative necrotizing lesion of the epidermis layer in mice receiving high doses. Skin necrosis was also observed which led to sloughing and scab formation. In extreme cases there was complete destruction of portions of the epidermis layer.

CONCLUSION

Finally, it can be concluded that the intracellular toxins produced by *P.intermedia* clinical isolates in Malaysia are virulent and thermolabile.

ACKNOWLEDGEMENT

This study was supported by Vote F grants F0391/2001A, F0350/2002A and F0350/2002D from University of Malaya, Kuala Lumpur, Malaysia. The authors gratefully acknowledge Mohd-Ariff, Z. for the technical assistance throughout the preparation of this study.

REFERENCES

1. Kilian M., 1981. Degradation of immunoglobulins A1, A2 and G by suspected principal periodontal pathogens. Infection and Immunity, 34: 757-765.
2. Zambon J.J., 1996. Periodontal diseases: Microbial factors. Annals of Periodontology, 1: 879-925.
3. Van der Weijden, M., F. Timmerman, E. Reijerse, G.N. Wolffe A.J. van Winkelhoff, U. van de Velden, 1994. The prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in selected subjects with periodontitis. J. Clin.Periodontology (Copenhagen). 21: 583-588.
4. Wennstrom J.L., G. Dahlén, J. Svensson and S. Nyman, 1987. *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius*: Predictors of attachment loss. Oral Microbiology and Immunology, 2: 158-163.
5. Slots J., L. Bragd, M. Wikström and G. Dahlén, 1986. The occurrence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* and *Bacteroides intermedius* in adults. J.Clin. Periodontology, 13: 570-577.
6. Slots J., 1981. Importance of Black-Pigmented *Bacteroides* in Human Periodontal Disease. In: Host-Parasite Interactions in Periodontal Diseases. (Eds. R.J. Genco and S.E. Mergenhagen) Symposium Proceedings of American Society for Microbiology, 4-6 May 1981, Buffalo, New York, Washington D.C. pp: 27-45.
7. van Steenbergen T.J.M., P. Kastelein, J.J.A. Touw and J. de Graff, 1982. Virulence of black-pigmented *Bacteroides* strains from periodontal pockets and other sites in experimentally induced skin lesions in mice. J. Periodontal Res. 17: 41-49.
8. Kastelein P., T.J.M. van Steenbergen, J.M. Bras and J. de Graff, 1981. An experimentally induced phlegmonous abscess by a strain of *Bacteroides gingivalis* in guinea pigs and mice. Antonie van Leeuwenhoek, 47: 1-9.
9. Sundqvist G., M.I. Eckerbom, a.P. Larsson and U.T. Sjören 1979. Capacity of anaerobic bacteria from necrotic pulps to induce purulent infections. Infection and Immunity, 25: 685-693.