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Cellular immune response following Russell's viper (*Daboia russelii siamensis*) bites in Myanmar

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Cellular immune response of 14 proven Russell's viper (*Daboia russelii siamensis*) bite cases from Thayawady Hospital was monitored from 12 to 117 weeks following the bites in 1992 by performing mitogen induced lymphocyte proliferation to phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and Russell's viper (*D.r.siamensis*) venom and tolerance of PHA and PWM induced lymphocytes to venom challenge. Immune status of a Russell's viper bite reptile keeper at 36 and 40 weeks after the bite and a venom handler of Myanmar Pharmaceutical Factory were also available for study. In general, response to PHA, PWM and venom induced lymphocyte proliferation of the victims and the venom handler was within normal range, however these parameters waned with time in the former. PHA induced lymphocytes could tolerate venom challenge more than PWM induced lymphocytes throughout the study (75-88% vs 63-74%).

INTRODUCTION

Russell's viper bite is a common problem of our farmers. It carries a high morbidity and mortality rate of 7710 (6529-8994) and 7.13% (4.9-8.5%) (mean and range) respectively based on poisonous snake bites collected by the Department of Health Planning from the whole country (1998-2003) [1]. Prophylactic immunization with venoid [2] may be another alternative to reduce morbidity and mortality of snake bite. Understanding of immune response of Russell's viper bite may help in planning for active immunization programme. Study on humoral response following snakebite including Russell's viper has been reported [3-7]. However, information on study of cellular immune status following snakebite was scanty [8] and such study will further improve our understanding on cellular immune response following snakebite. This study concerns with monitoring of cellular immune response of Russell's viper bite cases from 12 to 117 weeks after the bite.

MATERIALS AND METHODS

Monitoring of immune status of 14 proven Russell's viper (*Daboia russelii siamensis*) bite cases of Thayawady Hospital was carried out from 12 to 117 weeks in 1992. Follow- ups were carried out at 12, 24, 48, 78 and 96 week after the bite and samples collected were transported to the Venom Research Laboratory, Department of Medical Research, Yangon in cold chain and were processed on the same day. The following tests were performed in order to monitor the cellular immune response of the subjects; mitogen induced lymphocyte proliferation to phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and venom, tolerance to venom challenge by PHA and PWM induced lymphocytes. Relatives of the snakebite victims who have the same social economic status as the patients served as controls. While carrying out the study, we came across a case of Russell's viper bite in a reptile keeper (AM) 36 weeks ago from the snake farm of

Myanmar Pharmaceutical factory and a venom handler (BN) (over 30 years service) from the same factory and it was of our interest to study immune status of them in order to see responses elicited in different situations.

Preparation of lymphocytes

Twenty milliliters of heparinised blood were layered onto 10 ml of Ficoll-paque reagent (Pharmacia) and centrifuged at 1700 rpm for 20 min. at 20-25ºC. Cells from buffy coat layer were collected and washed with RPMI 1640 medium twice at 1500 rpm for 15 min. and 1000 rpm for 5 min. Diluted cell stocks (1-2 x 10^6/ml in RPMI medium with 10% foetal calf serum) were kept cold on ice.

Response to mitogens

1. *Phytohaemagglutinin (PHA) induced lymphocyte proliferation*

One-hundred microliter of $1x10^6$ cell/ml, 90 µl of RPMI and 10 µl of 10 µg/ml PHA solution were added to 96-well flat bottom culture plate, mixed and incubated at 37 ºC in 5% CO_2 for 72 hours. Then 10 microlitre of 1 uci/ml ^{3}H-thymidine were added to the culture 18 hours before harvesting onto a glass fibre and were counted in gamma counter. Stimulation indexes were calculated from the results. Triplicate samples were put up for the test and control. Another set of PHA induced lymphocytes were harvested, pooled and washed at the end of 72- hour culture and were used for venom challenge experiment.

2. *Pokeweed mitogen (PWM) induced lymphocyte proliferation*

A similar experiment was set up as in PHA stimulation test but 10 µl of 1:20 dilution of PWM was used in place of PHA and were cultured at 37ºC in 5% CO_2 for 7 days. Ten microliters of 1uci /ml ^{3}H-thymidine were added to the culture 18 hours before harvesting and then counted in gamma counter as in PHA response. Stimulation indexes were calculated from the results as before. Another set of PWM induced lymphocytes were harvested on day 7, pooled, washed and used for venom challenge experiment.

Venom induced lymphocyte proliferation

To 100 ul of $1x10^6$/ml of cell suspension in 96-well flat bottom culture plate, 90 ul of RPMI medium and 10 ul of varying concentrations of Russell's viper venom (300 ng/ml, 30 ng/ml, 3 ng/ml and 300 pg/ml) were added and incubated at 37°C with 5% CO_2 for 72 hours. Ten microliters of 1 uci/ml ^{3}H-thymidine were added to the culture 18 hours before harvesting and then counted in gamma counter as in the other mitogen response tests. Stimulation indexes were calculated from the results. For control, 100 ul of cell suspension and 100 ul of RPMI medium were used.

Venom challenge experiments

Venom challenge experiment of PHA and PWM induced lymphocytes was carried out as below.

Fifty microliters of either cell suspension (1 x 10^6 cell/ml) was incubated with 20 µl of varying concentrations of Russell's viper venom (doubling dilution of 500 µg/ml of venom to 7.8 µg/ml) at 37ºC for 1 hour and centrifuged at 1500 rpm at 4ºC for 10 minutes. Cell viability was assessed using trypan blue indicator. At least 200 cells were counted in a haemocytometer under light microscopy for percentage of viable cells. For control, cell suspension was incubated with the medium instead of venom solution.

RESULTS

PHA induced lymphocyte proliferation

PHA induced lymphocyte proliferation of the snakebite victims is shown in Figure 1. Maximum stimulation index was observed at 24-30 weeks following the bites and the response of the PHA induced lymphocyte proliferation was not suppressed throughout the study period. The stimulation index of PHA induced lymphocyte proliferation of

the venom handler (BN) and that of Russell's viper bite reptile keeper (AM) at 36 and 40 week after the bite were also not suppressed (data not shown).

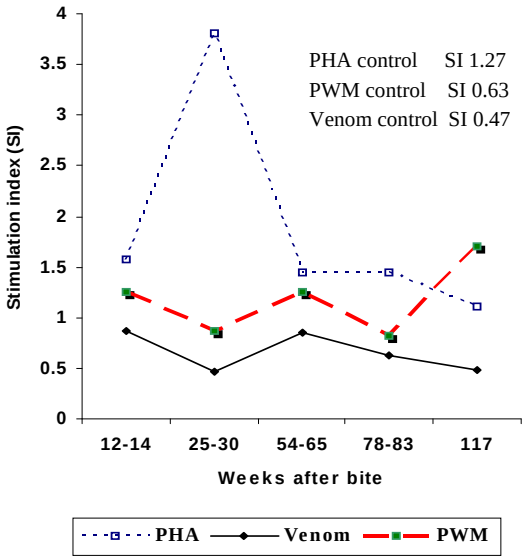

Fig.1. Mitogens (PHA and PWM) and Russell's viper venom induced lymphocyte proliferation of the snakebite victims

PWM induced lymphocyte proliferation

PWM induced lymphocyte proliferation of the victims is shown in Figure 1. A fluctuating response of PWM induced lymphocyte proliferation of the victims was observed throughout the study (12-117 weeks).

Venom induced lymphocyte proliferation

Maximum stimulation index of the venom induced lymphocyte proliferation was observed in the controls and the victims at 12 week after the bite (n=4) with 32 ng/ml of the venom and required 800 ng/ml (n= 4) at 28 week and 20 ug/ml (n= 4) at 60 week (data not shown).

Stimulation indexes of the venom induced lymphocyte proliferation of the victims are shown in Figure 1. A fluctuating response of venom induced lymphocyte proliferation was observed and it declined from 48-60 weeks onwards. The stimulation indexes of the venom induced lymphocyte proliferation of the venom handler were 8.72 and that of the bitten reptile keeper at 36 week (6.93) and 40 week (0.63) (controls 0.64 ± 0.32 venom).

Venom challenge experiment (Tolerance of PHA and PWM induced lymphocytes to venom induced killing)

Tolerance of PHA induced lymphocyte to venom challenge

Tolerance of PHA induced lymphocyte to venom challenge. When PHA induced lymphocytes of the victims were challenged with 10 ug (500 ug/ml) of the venom, 37% of the lymphocytes from the controls and 75-89% of the lymphocytes from the patients could withstand the venom challenge (10 ug) throughout the period of study (Figure 2). PHA induced lymphocytes of the venom handler could withstand (100%) challenge of 5 ug of the venom, in snakebite victims (n=5) 1.25 ug at 54 week, 0.62 ug at 78 week and the bitten reptile keeper 2.5 ug at 36 week and 0.62 ug at 40 week (controls 0.62 ug venom).

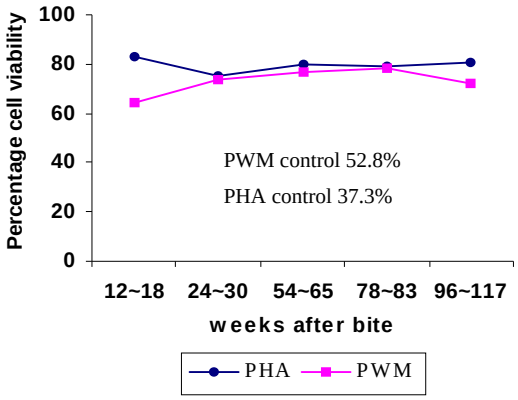

Fig. 2. Tolerance of PHA and PWM induced lymphocytes to the Russell's viper venom induced killing (10ug)

Tolerance of PWM induced lymphocytes to venom challenge

When PWM induced lymphocytes were challenged with the same dose of the venom

(10 ug) 53% of the lymphocyte of the controls and 64-74% of the lymphocytes from the victims could withstand venom challenge (10 ug) throughout the study (Figure 2). The PWM induced lymphocytes of the venom handler could withstand (100%) 2.5 ug of the venom, snakebite victims (n=5) 1.25 ug at 54 week, 0.62 ug at 78 week, the bitten reptile keeper 1.25 ug at 36 week and 0.31ug at 40 week (controls 0.31ug venom).

DISCUSSION

The pattern of PHA induced lymphocyte proliferation of snakebite victims (peaked at 24-30 weeks after the bite and declined with time) is similar to that of the humoral response of the victims reported earlier [7]. The cellular immune response of the victims from 12 to 117 weeks after the bites showed no suppression of the (PHA) response. Study on cellular immune response to *Crotalus durissus terrificus* in BALB/c mice also showed no inhibitory effect on cellular immune response [8]. Variable response of PWM and venom induced lymphocyte proliferation could be due to cytotoxic effect of the venom. Crude venom and venom components have been shown to be cytotoxic to animal tissue, lymphocyte, BALB/c splenic lymphocytes and BALB/cAn splenic B and T cells [9-12].

Venom induced lymphocyte proliferation declined with time (40-48 weeks onwards) in the snakebite victims and more venom was needed to induce response however it remained high in the venom handler (BN) who has been engaged in powdering desiccated crystal venoms for over last 30 years. It is speculated that immune response of the venom handler has been boasted with the venom either through inhalation or absorption of it through skin. It has been documented that year- long immunization with repeated increasing doses of venom n man produced protective neutralizing antibody against immunizing venom [13-15]. Protective immunity developed in a traditionally immunized subject who has received many immunizations over a period of 26 years could withstand a king cobra bite [16].

In the present study, development of tolerance to the venom in the snakebite victims and the venom handler is highlighted. PHA induced lymphocytes are more sensitive to venom killing compared to PWM induced lymphocytes (37% vs 53%). Mitogen stimulated BALB/cAn splenic T and B cells were found to be sensitive to cytotoxic effects of snake venoms [9]. BALB/c splenic T lymphocyte was found to be approximately 10,000 times more senitive to cardiotoxin D from *Naja naja siamensis* than B lymphocytes [12]. Following the bite, PHA induced lymphocytes developed more tolerance to the venom than PWM induced lymphocytes (75-88% vs 63-74%). A greater tolerance to venom challenge by PHA/PWM induced lymphocytes was observed in the venom handler compared to the victims, probably the result of frequent boastings with the venom in the former. Tolerance of the lymphocytes of the snakebite victims and the venom handler remained high throughout the study but decline in tolerance was noted in the bitten reptile keeper after 36 weeks. More venom was needed to induce lymphocyte proliferation as time waned in the victims.

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