

INDUCTION OF MODEL TIME-RELATED AIRWAY INFLAMMATION IN WISTAR RATS WITH CRUDE EXTRACT OF *PERIPLANETA AMERICANA*

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ABSTRACT

Pulmonary inflammation in bronchial airways is an obvious hallmark in bronchial asthma. Asthma-like responses were induced in wistar rats with crude extract of *Periplaneta americana*. Wistar rats were sensitized with the crude extract on day 0, and challenged twice with the extract on days 14 and 21. Bronchoalveolar lavage fluid (BAL) extracted from crude extract-induced rats and Phosphate buffered saline (PBS)-induced control rats were examined at 12 hourly intervals up to 72 hours post induction. The crude extract-induced rats displayed a high level of airway inflammation characteristic of cockroach-induced asthma compared with the PBS-induced rats. This involved increased total leukocyte counts, percentage neutrophils, lymphocytes and eosinophils at all time points examined. There were peak inflammation times of 24 hours for eosinophils and lymphocytes and 36 hours for neutrophils. The results showed evidence of late airway inflammation in BAL of *P. americana* crude extract-induced wistar rats comparable to airway inflammation of antigen-sensitized human asthmatics. These results are discussed together with the significance of wistar rat asthma model in the current herbal asthma therapy validation trials.

KEYWORDS: Inflammation, Asthma, Induction, *P. americana*, Rats.

INTRODUCTION

A major feature of bronchial asthma is the pulmonary inflammation of leukocyte cells, particularly eosinophils in the bronchial airways (Wills-Karp, 1999; Seminario and Gleich, 1994). Other leukocyte cells similarly found to infiltrate into lung tissue and obtained in the bronchoalveolar lavage (BAL) fluid in asthma, include neutrophils, lymphocytes and Monocytes (Fahy *et al*, 1995; Rankin *et al*, 2000; Kim *et al*, 2001; Zhou *et al*, 1998). The Chronic inflammation in the lungs of asthmatics causes them to respond with reversible airway narrowing when stimulated in ways that have minimal effects on non-asthmatics (Hogg, 1997).

The role of indoor allergens in the Pathogenesis of asthma is well recognized and often linked with the recent trend of increasing morbidity and mortality rates of asthma Worldwide (Sporik *et al*, 1990; Woolcock and Peat, 1997). Cockroach allergen has long been recognized as a major allergen in asthma (Pollart *et al*, 1989; Platts-mills *et al*, 1991; Cali *et al*, 1992; Gelber *et al*, 1993). The presence of cockroach allergen at home and other confined environment sensitizes people and causes asthma (Steinberg *et al*, 1987; Kang *et al*, 1989; Sarpong *et al*, 1997). The cockroach specific asthmatic responses appear at various time points after airway challenge with the allergen, exhibiting immediate, late, dual or persistent pattern along with variable degrees of Peripheral eosinophilia (Kang *et al*, 1979; 1992). It has been concluded that Cockroach allergen possesses a characteristic that is prone to specifically induce asthmatic airway inflammation in the host (Arruda *et al*, 2001).

Previous reports have shown a guinea pig asthma model sensitized with aerosolized cockroach allergen, which developed asthma-like inflammation with the characteristics largely resembling human cockroach-induced asthma (Kang *et al*, 1995; Zhou *et al*, 1998). It was also reported that such sensitized guinea pigs showed cockroach-specific airway obstructions at various time points with an abundance of inflammatory cells in BAL fluid (Chen *et al*, 1998; Zhou *et al*, 1998).

There has been a search for appropriate asthmatic model to enable trial of most and newly discovered herbal asthma therapies also to assess the side effects or toxicity of

these therapies. Mice and guinea pigs have been used (Kang *et al*, 1995; Zhou *et al*, 1998; Chen *et al*, 1998; Campbell *et al*, 1998; Kim *et al*, 2001), but there have been relatively few trials with rats using cockroach allergens. A report indicated that the response of inflammatory cells in a rat model of asthma is similar to that found in humans (Bice and Seagrave, 2000). The rat model of asthma is the closest to human asthma in terms of duration and pattern, showing principally a late phase asthmatic response, and it will be beneficial to use rats in these trials. It is also relevant to assess the extent to which *Periplaneta americana* (Cockroach) allergen can induce asthmatic responses in the Nigerian environment.

We therefore initiated the studies to examine the responses of Wistar rats in a time-related manner, to sensitization with crude extract of *Periplaneta americana*, the most prevalent domiciliary cockroach in Nigerian environment; also to assess the effect of crude aqueous extracts of some herbal asthma therapies in asthmatic responses in Wistar rats model. We report here the results of a time-related airway inflammatory response in Wistar rats induced with crude extract of *Periplaneta americana*.

MATERIALS AND METHODS

- i. **Experimental animals:** Female Wistar rats weighing 180-200g and aged 12 weeks were obtained from the animal resource center of the Department of Zoology, University of Calabar, Calabar, Nigeria. They were housed in plastic cages with wire screen tops, in the animal research facility of the Department of Biochemistry, College of Medical Sciences, University of Calabar, Calabar, Nigeria, and allowed to acclimatize for two weeks. The animal room was maintained under standard Laboratory conditions at room temperature of $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$, relative humidity of 46% with adequate ventilation and a 12 hour light and dark cycle. Food and water was provided the rats ad libitum. Permission for animal studies was obtained from the animal ethics Committee of the College of Medical Sciences, University of Calabar, Calabar, Nigeria.
- ii. **Preparation of *Periplaneta americana* Crude extract:**

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Life whole bodies of *P. americana* (American cockroach) were obtained from Several house holds in Calabar, Cross River State, Nigeria. They were caught at night using prepared garri paste dipped in soup as bait. This was kept on the floor of the Kitchen to attract the Cockroaches, which were killed and stored in a corked glass container with air holes. The wings, legs and antennae of the cockroaches were removed, and the pooled denuded samples were weighed to give 28g. this was washed in distilled water, chopped into small pieces with razor blade and crushed in a mortar with pestle to give a homogeneous paste. Sterile phosphate buffered saline, pH 7.2, was added as homogenizing buffer (560ml) to the paste in the mortar and left to stand at 4°C for 24 hours. The mixture was homogenized at high speed in an electric blender (Gelman Hawskey, England) for 60 seconds. The homogenate, cleared of debris was centrifuged (Galenkamp) at 1,000 revolutions per minute for 15 minutes at room temperature. The supernatant was decanted and filtered through a cheese cloth. The filtrate was collected into sterile sample tubes and stored in a refrigerator at -4°C until used.

- iii. Establishment of sub lethal Sensitization and intratracheal Challenge dose (LD₅₀ test): A LD₅₀ test was performed to determine the sub lethal sensitization and challenge dose of the Crude extract of *Periplaneta americana* to be used in the main experiment. The test was carried out using 54 healthy Wistar rats which were randomly assigned to three groups each containing eighteen rats, and each group sensitized with a different concentration of the Crude extract obtained by dilution of the stock *P. americana* crude extract as follows:
 - (a) Undiluted Crude extract
 - (b) Crude extract diluted with Phosphate buffered saline (PBS) at 1:5 V/V
 - (c) Crude extract diluted with Phosphate buffered saline (PBS) at 1:10 V/V

Further, each group (Concentration level) was divided into 3 sub groups (dosage amount) of 6 rats each. Sub groups were each administered with dosage amount of Crude extracts viz. 0.5ml, 1ml and 1.5ml. Thus, groups represented concentration levels while sub groups represented dosage amounts. Each rat was sensitized with interperitoneal (ip) injection of the appropriate dosage amount of the concentration level of crude extract to which it belonged together with equal amount of PBS vehicle. It was observed that all eighteen rats given different dosage amounts (0.5ml, 1ml, 1.5ml) of undiluted crude extract (group a) developed sickness and died within 3 days of sensitization. Similarly, all the eighteen rats given different dosage amounts (0.5ml, 1ml, 1.5ml) of crude extract dilution 1:5 v/v PBS (group b) died within 4 to 10days of sensitization. However, the rats given different dosage amounts of crude extract dilution 1:10 v/v PBS (group c) survived for 14 days and showed no visible ill effects.

- iv. Validation of induction of asthma-like inflammatory response: Induction of asthma-like airway inflammation with *P. americana* crude extract was validated using the surviving sensitized rats of group C. The rats in the three dosage amounts were challenged twice with the respective dosage amounts (0.5ml, 1ml, 1.5ml) of crude extract diluted 1:10 V/V PBS. The intratracheal challenge on the test rats were done twice, on days 14 and 21. Thereafter Bronchoalveolar lavage fluid was harvested from each rat and examined for leukocyte inflammation. It was observed that BAL fluid of rats challenged with 1ml and 1.5ml had many burst cells and cell debris, while BAL of rats challenged with 0.5ml showed good total leukocyte and differential count. Therefore the

crude extract dilution of 1:10 V/V PBS (group C) and dosage of 0.5ml were used for subsequent induction of model asthma-like responses in the main experiment.

- v. Induction of asthma-like airway inflammation in wistar rats: This was achieved by sensitization of wistar rats with *P. americana* crude extract diluted 1:10 V/V PBS and intratracheal challenge of the rats with the crude extracts.

Seventy-two healthy female Wistar rats were randomly assigned into two groups of 36 rats each. One group was sensitized on day 0 by giving each rat an intraperitoneal (ip) injection of 0.5ml *P. americana* crude extract with 0.5ml PBS vehicle. The other group of rats was given an ip injection of 1ml PBS placebo. On days 14 and 21, the sensitized rats in the treatment group were given intratracheal challenge with 0.5ml of the crude extract while in an unconscious state with Chloroform. The following procedure was used: the rat was placed for 10 seconds in a desiccator containing a pad of cotton wool soaked in 95% chloroform. The unconscious rat was removed and laid on its back on an inclined wooden board (1mx1m). The body weight was supported by taping the base of the tail to the wooden board. The jaw was opened and the tongue gently extended with forceps and thus position the epiglottis with the trachea open. An aliquot of the crude extract (0.5ml) was placed at the base of the oro-pharynx, which was subsequently inhaled. This technique produces a reliable delivery of fluid droplets to the lungs. The challenged rat was returned to the cage to regain consciousness.

The control group rats were similarly challenged, but each with 0.5ml of PBS. The technique was repeated for each rat on day 21 to achieve two separate challenges with *P. americana* crude extract and placebo respectively. The sensitization and challenge protocol for this wistar rat asthma model was modified from the methods of Campbell *et al*, (1998) and Kim *et al*, (2001).

- vi. Collection and analysis of Bronchoalveolar lavage (BAL) fluid: At 12 hourly intervals up to 72 hours post induction, BAL fluid was collected from six rats and analysed. The rats were euthanized by cervical dislocation and dissected on a wooden board. The trachea was exposed and intubated by a sterile polyethylene catheter (size 11, Bard, Georgia, USA).

The Catheter was passed into the lungs and BAL fluid was collected by lavaging with two separate aliquot of 1ml PBS each through the catheter and emptied into sterile specimen bottle containing 0.77m EDTA. The wash was centrifuged at 1,000 revolutions per minute for 15 seconds and the cell pellet stored. Total leukocyte count was done after diluting the BAL with Turk's solution and the cells counted in the improved Neubauer counting chamber (Fortuna, Germany). Differential slides were prepared and stained with Leishman's stain and differential cell counts were obtained in the microscope (CHB Olympus, Japan 35974) using laboratory counter with keys (Clay Adams, England). The cell counts of neutrophils, lymphocytes and eosinophils were expressed as a percentage based on a total of 200 cells counted.

STATISTICAL ANALYSIS

All values were expressed as mean counts $\pm$ SEM. Differences between treatment means were compared by analysis of variance (ANOVA). When the overall F value was significant, pairwise comparison was performed between groups using student's 't' test.

RESULTS

Assessment of induction of asthma-like airway inflammation in wistar rat asthma model was done by estimating total leukocyte cell counts and percentage differential cell infiltration in the BAL fluid.

Total leukocyte counts and percentages of neutrophils, lymphocytes and eosinophils in BAL fluid of *Periplaneta americana* crude extract-induced Wistar rats were compared with those of PBS-induced control wistar rats at 12 hourly intervals up to 72 hours post induction.

1. Total leukocyte cell counts in BAL fluid at 12 hourly intervals post induction.

The results on table 1 revealed that the total leukocyte counts in *P. americana* crude extract-induced Wistar rats were significantly higher ($P < 0.01$) than in PBS-induced control Wistar rats at all the time points post induction examined. The increase in total leukocyte counts in BAL fluid of *P. americana* crude extract-induced rats was noticed at 12 hours post induction and reached a peak at 24 hours post induction. Thereafter, there was a slight fall at 36 hours until 72 hours post induction, though the values remained significantly ($P < 0.01$) higher than those in PBS-induced control rats. There was no time-related increase in total leukocyte counts in BAL fluid of PBS-induced control rats.

2. Percentage neutrophils in BAL fluid at 12 hourly intervals post induction.

On table 2 is shown the percentage neutrophils in BAL fluid of *P. americana* crude extract-induced rats and PBS-induced control rats. The results showed that *P. americana* crude extract-induced rats had significantly ($P < 0.01$) higher values than PBS-induced control rats at all the time points post induction examined. The increase in percentage neutrophils in BAL fluid of crude extract-induced rats was noticed at 12 hours post induction and reached a peak at 36 hours post induction. Thereafter, there was a slight fall in the values at 48 hours until 72 hours post induction, though the values remained significantly ($P < 0.01$) higher than those in PBS-induced control rats. There was no time-related increase in percentage neutrophils in BAL fluid of PBS-induced control rats.

3. Percentage lymphocytes in BAL fluid at 12 hourly intervals post induction.

Table 3 shows the percentage of lymphocytes in BAL fluid of *P. americana* crude extract-induced rats and PBS-induced control rats. The result showed that crude extract-induced rats had significantly ($P < 0.01$) higher values than PBS-induced control rats at all the time points post induction examined. The increase in percentage lymphocytes in BAL fluid of crude extract-induced rats was noticed at 12 hours post induction and reached a peak at 24 hours post induction. Thereafter, there was a slight fall in the values at 36 hours until 72 hours post induction, though the values remained significantly ($P < 0.01$) higher than those in PBS-induced control rats. There was no time-related increase in percentage lymphocytes in BAL fluid of PBS-induced control rats. The values remained fairly stable at all time points post induction examined.

4. Percentage eosinophils in BAL fluid at 12 hourly intervals post induction.

Table 4 shows the percentage eosinophils in BAL fluid of *P. americana* crude extract-induced rats and PBS-induced control rats. The result showed that crude extract-induced rats had significantly ($P < 0.001$) higher values than PBS-induced control rats at all the time points post induction examined. The increase in percentage eosinophils in BAL fluid of crude extract-induced rats was noticed at 12 hours post induction and reached a peak at 24 hours post induction. Thereafter, there was a slight fall in the values at 36 hours until 72 hours post induction, though the values remained significantly ($P < 0.001$) higher than those in PBS-induced control rats. There was no time-related increase in percentage eosinophils in BAL fluid of PBS-induced control rats. The values remained fairly stable at all time points post induction examined.

INSERT HERE TABLES 1, 2, 3 AND 4.

TABLE 1: Total leukocyte cell counts ($\times 10^6$) in BAL fluid at different time points post induction in *P. americana* crude extract-induced Wistar rats.

Treatment	12hrs	24hrs	Duration 36hrs	48hrs	60hrs	72hrs
PBS-induced Control	3.4 $\pm$ 0.10	3.5 $\pm$ 0.37	3.8 $\pm$ 0.14	3.4 $\pm$ 0.33	3.7 $\pm$ 0.33	3.4 $\pm$ 0.37
Crude extract- Induced	12.8 $\pm$ 0.22 ^a	19.4 $\pm$ 0.97 ^a	16.4 $\pm$ 0.31 ^a	15.8 $\pm$ 0.37 ^a	15.1 $\pm$ 0.87 ^a	14.4 $\pm$ 0.31 ^a

Values are means of 6 determinations $\pm$ SEM.

The superscript 'a' indicates significant difference in the results of crude extract-induced rats compared with PBS-induced control rats at $P < 0.01$.

TABLE 2: Percentage neutrophils in BAL fluid at different time points post induction in *P. americana* crude extract-induced Wistar rats.

Treatment	12hrs	24hrs	Duration 36hrs	48hrs	60hrs	72hrs
PBS-induced Control	6.5 $\pm$ 0.36	4.9 $\pm$ 0.18	6.1 $\pm$ 0.19	5.9 $\pm$ 0.31	5.6 $\pm$ 0.37	5.9 $\pm$ 0.31
Crude extract- induced	18.1 $\pm$ 0.58 ^a	20.8 $\pm$ 0.38 ^a	21.9 $\pm$ 0.41 ^a	19.1 $\pm$ 0.25 ^a	12.7 $\pm$ 0.47 ^a	18.4 $\pm$ 0.54 ^a

Values are means of 6 determinations $\pm$ SEM.

The superscript 'a' indicates significant difference in the results of crude extract-induced rats compared with PBS-induced control rats at $P < 0.01$.

TABLE 3: Percentage lymphocytes in BAL fluid at different time points post induction in *P. americana* crude extract-induced Wistar rats.

Treatment	Duration					
	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
PBS-induced Control	4.9±0.35	3.9±0.19	4.4±0.22	4.7±0.33	4.3±0.47	4.6±0.39
Crude extract-Induced	15.5±0.25 ^a	23.1±0.31 ^a	19.2±0.62 ^a	20.1±0.47 ^a	19.8±0.41 ^a	19.4±0.58 ^a

Values are means of 6 determinations ± SEM.

The superscript 'a' indicates significant difference in the results of crude extract-induced rats compared with PBS-induced control rats at P<0.01.

TABLE 4: Percentage eosinophils in BAL fluid at different time points post induction in *P. americana* crude extract-induced Wistar rats.

Treatment	Duration					
	12hrs	24hrs	36hrs	48hrs	60hrs	72hrs
PBS-induced Control	2.8±0.32	2.1±0.12	2.2±0.15	2.1±0.15	2.7±0.42	2.4±0.37
Crude extract-Induced	14.2±0.43 ^a	36.1±1.17 ^a	26.4±0.97 ^a	24.4±0.80 ^a	22.4±0.64 ^a	21.1±0.24 ^a

Values are means of 6 determinations ± SEM.

The superscript 'a' indicates significant difference in the results of crude extract-induced rats compared with PBS-induced control rats at P<0.001.

DISCUSSION

The study demonstrated that airway inflammation occurred in bronchoalveolar lavage fluid of *Periplaneta americana* crude extract-induced Wistar rats. This was observed within 12 hours post induction and continued up to 72 hours post induction. The significant (P<0.01) increase in infiltration of leukocytes notably, neutrophils, lymphocytes and eosinophils occurred at all time points post induction examined. There was demonstrated evidence of time-related increases in inflammatory cells in BAL fluid of *P. americana* crude extract-induced Wistar rats. One significant finding in this study was that increase in total leukocytes counts in BAL fluid of *P. americana* crude extract-induced Wistar rats reached a peak at 24 hours post induction. Similarly, increases in percentage lymphocytes and eosinophils reached a peak at 24 hours post induction, whereas increase in percentage neutrophils reached a peak at 36 hours post induction. Reports of time-related infiltration process involving leukocyte counts, neutrophils, lymphocytes and eosinophils in asthmatic Wistar rat model have not been found in the literature. Reports however, showed that pulmonary eosinophilia peaked at 48 hours after the last cockroach allergen challenge in a sensitized and challenged mouse model (Campbell *et al.*, 1998; Kim *et al.*, 2001). It was also reported that peak eosinophilia occurred at 3 hours after the last allergen challenge in an ovalbumin sensitized and challenged mouse model (Gonzalo *et al.*, 1996), where as eosinophilia peaked at 24 hours post challenge in a cockroach extract-challenged guinea pig model (Zhou *et al.*, 1998; Chen *et al.*, 2001).

Generally, reports showing increases in total leukocyte counts, eosinophils and neutrophils in BAL fluid following ovalbumin sensitization and challenge of Brown Norway rats abound in literature (Kips *et al.*, 1992; Gilmour and Selgrade, 1996; Palmans *et al.*, 2000; Vanacker *et al.*, 2001). The results in this study are consistent with these earlier reports. Increased eosinophilia in antigen-challenged human

asthmatics had been correlated with concurrent increase in mRNAs expressing IL-4, IL-5 and IL-13, which were associated with T-lymphocytes (Robinson *et al.*, 1993; 1993^a). In these reports CD4⁺ (Th2) T-lymphocytes were implicated in production of cytokines IL-4, IL-5 and IL-13, that were critical for eosinophil inflammation in asthma (Seminario and Gleich, 1994; Rankin *et al.*, 2000). The observation of peak infiltration time of 24 hours for both eosinophils and lymphocytes in this study is consistent with the reports of correlation in activities of these cells in the asthmatic inflammation process. Reports in antigen-induced monkeys have implicated neutrophilia in the late phase airways obstruction (Wegner *et al.*, 1990; Gundel *et al.*, 1991). These reports also show that eosinophils and neutrophils are activated by different effector molecules, VCAM-1 and E-selectin respectively. The observation of different peak infiltration times of eosinophils and neutrophils (24 hours and 36 hours respectively) in this Wistar rat asthma model is consistent with earlier reports of the activation of these cells.

The late phase asthmatic response is characteristic of human asthma (Bice and Seagrave, 2000) and is well known to accompany influx of inflammatory cells notably neutrophils, eosinophils and lymphocytes (Bice and Seagrave, 2000; Chen *et al.*, 2001). The induction in this Wistar rat model of airway inflammatory responses in BAL fluid at various time points has highlighted a prominent leukocytosis with marked eosinophilia, lymphocytosis and neutrophilia at 24 hours and above, which correlates well with late phase asthmatic response previously reported in cockroach allergen specific airway inflammation in human asthma (Kang *et al.*, 1979; 1992). This is consistent with previous finding that the response of inflammatory cells in the rat model of asthma is similar to that of humans (Bice and Seagrave, 2000).

Conclusion: We conclude that time-related asthmatic responses have been induced following the administration of crude extract of *P. americana* thus adding to the list of novel Wistar rat model of asthma which closely resembles human

asthma. The present finding is significant as it will enable the trial of many newly discovered herbal asthma therapies in the asthmatic responses in Wistar rats and help to validate the efficacy of these herbal therapies in the alleviation of asthma.

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