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ANTI-INFLAMMATORY ACTIVITY OF 6MFA, A COMPOUND OBTAINED FROM FUNGUS *ASPERGILLUS OCHRACEUS*

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SUMMARY: 6MFA, an interferon-inducing substance obtained from fungus, *Aspergillus ochraceus*, has shown anti-inflammatory activity both in acute and chronic animal models of inflammation. It was found that 6MFA was equally effective in inhibiting both exudative as well as granulative phase of inflammation. The compound suppressed also cellular migration during inflammatory process and potentiated significantly the anti-inflammatory activity of indomethacin. The compound was devoid of analgesic or antipyretic activity.

The probable mechanism of action of this compound is not fully understood. However, the possibility of triggering the induction of endogenous anti-inflammatory substance(s) along with interferon(s), or interaction of induced interferon(s) directly or indirectly with the prostaglandin system has been attributed.

INTRODUCTION

6MFA (see a footnote on page 146), a new microbial compound containing polysaccharide, protein and nucleic acid (double-strand RNA) obtained from fungus, *Aspergillus ochraceus*, ATCC 28706 (1,2), has been shown to possess anti-Japanese-encephalitis activity in a murine model (3,4).

Interferon induction has been shown to be the basis of anti-viral resistance activity of 6MFA (3). Tests have also indicated that immune response of the vertebrate host plays a significant role in concert with interferon response to achieve the mouse protection (5,6).

Interferon-inducing agents like ploy I/C, tilorone and statolon have shown to elicit significant anti-inflammatory activity (7-11). In addition, rheumatoid

arthritic patients receiving interferon therapy showed significant improvement in grip-strength and motility of joints (12).

With these background information, we decided to investigate the effect of 6MFA on different animal models of inflammation and also attempted to explain possible mode of action of this compound.

MATERIALS AND METHODS

Male albino rats of Hindustan Antibiotic (HA) strain weighing 120-150 g were used throughout the study. The rats were kept on a light-dark cycle (10 hr light and 14 hr dark) and maintained on HA diet and water ad libitum in our animal quarters.

Drug solutions and treatment schedule: 6MFA Lot 7-84 (which has shown 100% and 70% anti-viral activity in Japanese encephalitis (JE) and Semliki Forest virus (SFV) mouse models respectively) was used for all the experimental studies and solutions were prepared in distilled water just before the experiment. Indomethacin was obtained from Merck Sharp and Dhome, Bombay, and prepared as a suspension in a 0.5% (w/v) carboxymethyl cellulose solution. The time interval between 6MFA administration and the injection of various inflammatory agents, viz., carrageenin, albumin, Freund's adjuvant and UV erythema, was kept 17 hr, because at this interval 6MFA elicited maximum effect. However, in cotton pellet granuloma and granuloma pouch experiments, 6MFA was administered twice at an interval of 4 days to minimize hyporesponsiveness of the second dose with respect to interferon induction.

Carrageenin - induced edema: Carrageenin edema was induced by injecting subcutaneously carrageenin into the plantar region of the hind-paw of rats according to the method of Winter et al (13).

A group of rats were treated with 6MFA (10 mg/kg intraperitoneally) 17 hr prior to indomethacin and carrageenin was injected 1 hr after indomethacin treatment. ED₅₀ of indomethacin with and without 6MFA treatment for carrageenin edema inhibition was calculated.

Albumin - induced edema: Albumin edema was produced by injecting subcutaneously 0.1 ml of 2% (w/v) bovine albumin (Sigma Chemicals Co., St. Louis, MO), prepared in normal saline, into the plantar region of the hind-paw of rats.

Freund's adjuvant edema [18 hr arthritis test - Sofia et al (14)]: Rats were injected subcutaneously 0.1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) into the plantar region of the right hind-paw.

Ultraviolet - induced erythema: Albino guinea pigs weighing 250-300 g were prepared for UV exposure as described by Winder (15). Three circular areas (6 mm

diameter) of the right flank were exposed to UV radiation for 30 sec from a quartz lamp. Following UV exposure the intensity of erythema at different time intervals was visually graded by a trained observer unaware of the treatment schedule using an arbitrary scale 0-3 as follows:

- 0 — No evident erythema.
- 1 — A mild reaction, pale pink color.
- 2 — An intermediate response between 1 and 3.
- 3 — An intense reaction, deep reddish pink in color.

The maximum score for a three point exposure in an animal was 9.

Drugs, 6MFA (100 mg/kg intraperitoneally) and indomethacin (10 mg/kg orally) were administered 17 and 1 hr, respectively, prior to UV exposure. Six guinea pigs were used for each dose level.

Cotton pellet granuloma: Cotton pellet granuloma was induced in rats according to the method of Winter and Portar (16). Four sterilized cotton pellets each weighing 10 mg were implanted two on either side of the ventral region. On the 9th day, pellets were removed together with granuloma and dried at 70 C for 6 hr and weighed. The increase in dry weight was taken as a measure of granuloma formation.

6MFA (100 mg/kg intraperitoneally) was administered twice at an interval of 4 days, commencing a day prior to cotton pellet implantation. Indomethacin (3 mg/kg orally) was administered daily for 8 days from the day of cotton pellet implantation. The granuloma inhibition with the drug treatment was calculated by comparing with granuloma formation in the untreated control group.

Granuloma pouch: A granuloma pouch was produced on the shaven back (dorsal side) of the rat by injecting subcutaneously 25 ml of air. Croton oil 0.5 ml of 0.5% (v/v) prepared in corn oil was injected into this pouch (17). On the 10th day, rats were sacrificed by ether anesthesia; exudates from the pouches were harvested and volumes were recorded with the help of a syringe. Thereafter, the pouches were carefully dissected and made free from extraneous tissue. The dry weights of the pouches were recorded after drying in an oven at 70 C for 6 hr or till a constant weights was obtained.

To see the effect of 6MFA on cellular response (the basic inflammatory response) of the croton oil-induced granuloma pouch, the differential cell counts in the exudate of intrapouch-treated rats were made on stained smears using counting chambers. The cell counts were made with respect to total volume of the exudate.

Statistical analysis: Results were expressed as mean $\pm$ SEM. The effect of various pretreatment on base-line parameters was assessed by student 't' test (two tailed) and P-values greater than 0.05 were considered as not significant.

ED₅₀ values of indomethacin and 6MFA, with 95% confidence limits for the inhibition of different types of edemas were calculated by the method of Litchfield and Wilcoxon (18).

RESULTS

6MFA treatment inhibited significantly carrageenin, albumin and Freund's adjuvant-induced edemas. The ED₅₀ values of 6MFA and indomethacin in different edemas are shown in Table I. The ED₅₀ values of 6MFA and indomethacin were different in different edemas and their potencies in these inflammatory models were of the order, Carrageenin > albumin > Freund's adjuvant.

Table I. ED₅₀ of 6MFA in different experimentally induced hind-paw edema in rats

Type of inflammation	ED ₅₀ (19/20 Confidence limits) mg/kg	
	6MFA (ip)	Indomethacin (per os)
Carrageenin edema	22.0(16.0-31.0)	2.6(1.7-3.9)
Albumin edema	48.0(36.0-63.0)	7.2(5.6-9.4)
Freund's adjuvant edema	62.0(24.8-130.6)	11.2(8.6-14.6)

6MFA was administered intraperitoneally at different doses 17 hr prior to carrageenin and albumin injection. Paw volumes were measured on a plethysmographic apparatus before and 3 hr after carrageenin or albumin injection. Mean edema volume was determined and inhibition was calculated with respect to its vehicle-treated group. In case of Freund's adjuvant edema, 6MFA was administered intraperitoneally 1 hr prior to Freund's adjuvant injection and paw volumes were measured before and 17 hr after Freund's adjuvant injection. Indomethacin was administered orally 1 hr prior to carrageenin, albumin or Freund's adjuvant injection.

It was also found that pretreatment with 6MFA (10 mg/kg intraperitoneally) at a noneffective dose (less than 15% edema inhibition) potentiated the anti-inflammatory (AI) activity of indomethacin significantly. ED_{50} for 6MFA + indomethacin was 1.2 (0.9-1.6) mg/kg and for indomethacin alone was 2.6 (1.7-3.9) mg/kg.

The time course development of UV erythema in guinea pigs indicate that within 15 min after UV exposure, erythema appeared in some animals and by 30 min all the animals developed distinct lesions, pale pink in color. The intensity of the reaction rapidly increased reaching maximum by 5 hr. At the maximum, the lesions appeared deep reddish-pink in color (Fig. 1).

The erythema persisted with more or less of the same intensity for about 6 hr when a slow subsidence began. The reaction was noticeable even at 24 hr though the lesions were then more dusty in color.

Treatment with 6MFA delayed the onset and development of UV erythema (Fig. 1). The intensity of erythema response was comparatively less in 6MFA and

Fig. 1. Each point represents the mean observations of six guinea pigs; the vertical bars indicate the standard deviation of mean.

Table II. Effect of 6MFA on Croton oil-induced granuloma in rats

Treatment and dose	Route	Exudate volume (ml) Mean $\pm$ SEM	Granulation tissue(g) Mean $\pm$ SEM
Control	—	7.3 $\pm$ 0.7	1.3 $\pm$ 0.24
6MFA (100 mg/kg)	ip	5.3 $\pm$ 0.3 P 0.05	0.8 $\pm$ 0.1 P 0.03
Indomethacin (3 mg/kg)	po	6.1 $\pm$ 0.8 N.S.	1.0 $\pm$ 0.3 N.S.
6MFA (2 mg)	Intra- pouch	3.5 $\pm$ 0.25 P < 0.001	0.4 $\pm$ 0.1 P < 0.001
Indomethacin (0.5 mg)	Intra- pouch	5.0 $\pm$ 0.8 P < 0.03	0.7 $\pm$ 0.4 P < 0.01

A group of rats with granuloma pouch received 6MFA (100 mg/kg) by the ip route twice in the 8 days' period at an interval of 4 days. Another group of granuloma pouch rats were received 6MFA (2 mg/rat) via the intrapouch route once only.

Similarly, indomethacin (3 mg/kg) was administered orally daily for 8 days to a group of rats with the granuloma pouch, and another group received (0.5 mg/rat) via intrapouch once only. The rats from all groups were sacrificed on the 9th day after the induction of the pouch and effects of drugs were compared with the untreated control group. Each consisted of 8-10 rats.

indomethacin-treated guinea pigs than the untreated control for the observation period. However, delay in erythema development was significantly greater in 6MFA-treated animals than in indomethacin-treated animals.

In cotton pellet granuloma experiments, treatment with 6MFA (100 mg/kg intraperitoneally, twice in 8 days) and indomethacin (daily 3 mg/kg orally for 7 days) elicited 25% and 50% inhibitions, respectively, in granular tissue formation.

Fig. 2. Each point represents 6-8 observations, the vertical bars indicate the standard error of mean, *P < 0.05, **P < 0.01.

In granuloma pouch experiments, treatment with 6MFA both by the intraperitoneal and intrapouch routes showed marked inhibition in exudate and granular tissue formation (Table II). Administration by the intrapouch route showed greater effect than by the ip route. Indomethacin treatment showed significant effect on exudate and granular tissue formation only by the intrapouch route (Table II). Increased cell counts, viz., leukocytes, polymorphs and monocytes, in the exudate of the granuloma pouch were decreased significantly with the treatment with both 6MFA and indomethacin (Fig. 2). The decrement in cell counts in the 6MFA-treated group was more marked than that in the indomethacin-treated group.

Therapeutic index of 6MFA and indomethacin in different edemas indicate that 6MFA has a large therapeutic index than does indomethacin (Table III).

6MFA did not elicit any analgesic activity in mice (Swiss strain) when tested by the tail-clip, shock and thermal methods. 6MFA also failed to show any anti-pyretic activity in yeast-induced pyrexia rats.

Table III. Therapeutic index of 6MFA in carrageenin, albumin and adjuvant arthritis (18 hr arthritis test)

Drug	Therapeutic index = $\frac{LD_{50}}{ED_{50}}$		
	Carrageenin edema	Albumin edema	Freund's adjuvant arthritis
6MFA	40.5	18.6	14.4
Indo-methacin	18.0	13.8	8.4

The LD₅₀ of 6MFA was 890 (692-1,157) mg/kg (ip) and indomethacin data presented here are our own unpublished laboratory findings.

DISCUSSION

6MFA* has been shown to possess an ability to induce interferon in rats (3,19). Reviewing past literature, we found that interferon inducers, viz., poly I/C,

*Physico-chemical information on 6MFA

Chemical composition of 6MFA	% (w/w)
Components:	
Polysaccharide	90 - 92
Virus like particle (Nucleo protein)	8 - 10
ds RNA ¹⁾	0.27 to 0.34
RNA ²⁾	6.0 to 7.0

1): Expressed as % of 6MFA.

2): % of virus like particle (nucleo protein), five molecular fragments (0.5 to 1.5×10^6 daltons).

The polysaccharide component is inert but appears to contribute to the stability of 6MFA. The thermal melting point of ds RNA is 91.6 C in sodium citrate saline (Dr. B.M. Gupta's personal communication).

statolon and tilorone, inhibited experimentally induced inflammation in animals (7,8,11,20). The AI activity of interferon inducers was found to be not mediated through glucocorticoids liberation or prostaglandin synthetase inhibition (7,8,20). Furthermore, it was observed in the clinics that patients receiving interferon therapy showed a significant improvement in grip strength and motility of joints (12).

In the present study, it was observed that 6MFA showed a significant anti-inflammatory activity both in acute and subchronic animal models of inflammation. Moreover, 6MFA was equally effective in inhibiting both exudative and granu-lative phases of inflammatory processes. Furthermore, 6MFA treatment elicited a marked suppression of leukocyte migration to the site of inflammation.

Even today, the mode(s) of action of anti-inflammatory drugs in inflammation remains obscure or at least controversial (21). However, available reports indicate that non-steroidal anti-inflammatory drugs interfere with variety of cell functions e.g., generation and/or action on prostaglandin or other mediators (22-25). It was observed in our present study that 6MFA not only potentiated AI activity of indomethacin in carrageenin edema but also suppressed significantly cellular migration into the exudate of the granuloma pouch. Furthermore, Singh et al. (26), have reported that 6MFA treatment potentiated significantly the indomethacin toxicity in mice. These observations along with our present findings indicate that 6MFA by itself or induced interferons may affect the prostaglandin system either directly or indirectly and may elicit AI activity. Alternatively, it is possible that 6MFA like tilorone may activate endogenous AI substances from plasma (20).

However, it would be important to determine - firstly, whether interferon induces or activates the production of natural endogenous AI substances such as orgotenin, ceruloplasmin, etc., during the course of interferon induction? Secondly, whether the intermediate products formed during interferon induction are triggering anti-inflammatory processes? Thirdly, whether AI activity of 6MFA could be attributed to the combined effect of endogenous anti-inflammatory substances and interferons?

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