EFFECTS OF ARECOLINE ON TUMOR

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PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any university.

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SYED SHARABUDDIN

INTRODUCTION

The oral and oropharyngeal cancers predominate in the morbid scene of cancer in India. Their incidence varies from 4.4% to 47.0%. The high incidence of oral cancer in India has been associated with the chewing and smoking habits (Khanolkar, 1944, 1959; Sanghvi et al., 1955; Shanta and Krishnamurthy, 1963; Paymaster, 1971; Wahi, 1968; Reddy et al., 1975; Khanna et al., 1975). Prevalence data is available on oral cancer from the Mainpur district in Uttar Pradesh (Wahi, 1968), from Bhavnagar in Gujrat; from Poona in Maharashtra; from Ernakulam in Kerala; from Srikakulam in Andhra Pradesh and from Singbhum and Darbhanga in Bihar (Mehta et al., 1972; Pindborg et al., 1968, 1970 and 1971). Data on oral cancer has also been collected from a group of textile mill workers in Ahmedabad in Gujrat and from rural population in Alibagh Taluka in the Colaba district of Maharashtra (Jussawala, 1976). After an extensive survey of different parts of India by Mehta and others, it was found that oral cancer is more prevalent in the southern states of the country than elsewhere.

Among other etiological factors incriminated to explain the high incidence of oral cancer are vitamin deficiency, bad oral hygeine, excessive use of spices

and consumption of alcoholic beverages. It is also believed that the appearance of leukoplakia, pigmentation (melanoplakia) and the prevalence of submucus fibrosis may forecast the onset of neoplastic transformation in the oral cavity (Paymaster, 1956; Padmavati and Reddy, 1960; Shanta and Krishnamurthy, 1963; Martin and Koop, 1942). Jussawala and Deshpande (1971) made a retrospective study of cancer at high risk sites of the Bombay Registry and found sufficient evidence to indict chewing and smoking of tobacco as factors to great importance in the etiology of oral, pharyngeal, laryngeal and oesophageal cancer. The risk of developing cancer in the buccal mucosa was found to be 7.7 times higher in chewers than in non-chewers. Another interesting fact is deciphered from this study is that the oropharynx and oesophagus also show high risks in those who chew the betel quid without tobacco, thus indicating possible carcinogenic/ co-carcinogenic effect of other ingredients of the quid.

Betel chewing

The chewing of betel, a habit of great antiquity is very common among the inhabitants of Ceylon, India, Burma, Siam, Indo China, Malaya, Singapore, Indonesia, Phillipines, New Guinea, New Britain, New Island, Formosa and China. This habit has also prevailed in Zanzibar coast between

AD 1200-1400 and mention is made in Dutch archives of 1664 of an imposed duty on betel leaf imported from India into Malaca (Muir and Kirk, 1960).

Tobacco is generally mixed, flaked, with cracked, powdered or sliced dried betel nut (the fruit of the betel palm, Arecha catechu) and slaked stone or shell lime. The whole is wrapped in the leaf of the betel vine (Piper betel) on which catechu (an aqueous extract of the heartwood of the Acaciea Acacia catechu or Acacia suma) has been smeared. Spices such as cardamon and cloves and aniseeds may be added for additional flavour (Sanghvi, 1955). This quid is put in the gingivo-buccal fold and chewed for hours (Shanta and Krishnamurthy, 1959). In Thailand turmeric, the ground root of Curcuma aromatica, is usually added to chew (Ellis, 1921).

The whole betel quid is kept in the mouth for a few minutes to several hours and the regular chewers keep having it one after the other. The habit is so deep rooted in some people that they often sleep retaining the quid overnight in the gingivo-buccal gutter. It is kept generally on one side of the buccal pouch which may eventually become susceptible to preneoplastic or neoplastic growth.

In recent years intensive research has been underway as regards the association of betel quid chewing habits and oral cancer. Experimental studies are in progress to assess the role of each ingredient that constitute the total betel quid.

The involvement of the main constituents of "Pan"- betel leaf (Piper vine), betel nut (Areca catechu), tobacco (Nicotiana tobaccum), slaked shell or stone lime (slaked calcium hydroxide) and catechu (Acacia catechu) have been considered individually or in combination (Mody and Ranadive, 1959; Muir and Kirk, 1960; Dunham, Muir and Hamner, 1966). The role of lime appears rather controvertial, though some reports do suggest its correlation with the occurrance of oral cancer. Acanthosis of the oral epithilium was reported (Sirsat and Kandarkar, 1968) only in animals exposed to lime and reflected deep injury to the cellular growth pattern and also a chronically altered epithilium connective tissue interrelationship. Hence, it could be lime which is invaribly present in the "betel quid", acts thus by virtus of tissue reaction it provokes, as the co-carcinogenic agent (Atkinson et al.,

Tobacco has been considered the ingredient of the betel quid most likely to cause changes in the oral mucusa that eventuate in the development of oral cancer (Khanolkar, 1951; Sanghvi, Rao and Khanolkar, 1955; Shanta and

Krishnamurthy, 1963). Wahi et al. (1965) reported that a large proportion (66.5%) of the controls but only 9.62% of cancer cases were not using tobacco. Those who were both chewers and smokers figured at 37.88% in cancer cases and only 6.4% in controls. The chewing (tabacco) habit alone was found in 35.44% of patients and 5.9% of control subjects. This analysis brings an important relation of tobacco use with the prevalence of oral and oropharyngeal cancers. In experimental studies, Muir and Kirk (1960) have induced tumors in the skin of two of twelve mice at the site of daily painting with an aqueous extract of a typical whole Singapore betel quid. However, Dunham and Herrold (1962) could not induce tumors in the hamster cheek pouch treated by beeswax pellets that contained betel quid ingredients from several Asian countries. Suri et al. (1971) had conducted an experiment on hamsters using a North Indian variety of tobacco and betel nut. Tobacco caused only leukoplakial changes, DMSO extract of betel nut induced tumors in hamster cheek pouch at as high a rate as 38%. DMSO extract of betel nut plus tobacco caused cheek pouch tumors in 76% of animals. Recent reports by Ranadive et al. (1976) give some positive results which qualitatively though not quantitatively support the findings of Suri et al. On subcutaneous administration of betel nut extract (in water and DMSO),

60% of Swiss mice developed transplantable fibrosarcomas at the site of injection. Skin application of DMSO extracts of tobacco and of betel nut separately did not result in skin lesions; but when mixed DMSO extract of tobacco and betel nut was used, skin papilloma and epidemoid carcinoma developed in some animals. Similarly, hamster cheek pouches painted with a DMSO extract of betel nut showed only malignant changes whereas the result was negative when extract of tobacco was used. However, DMSO extract of a mixture of tobacco and betel nut positively increased the incidence of early malignant changes in the hamster cheek pouch, indicating the enhancing effect of betel nut in carcinogenesis.

Another ingredient of the betel quid, gambier, which is an extract from the vine, Uncaria gambir, is a suspected cancinogen by virtue of its tannin content (Korpassy and Mosonyi, 1950; Kirby, 1960).

Physiological effects of Areca nut

Areca nut is highly acidic and astringent to taste. It is neutrilized by the addition of lime. Besides this lime promotes the appearance of a red dye. Apart from other ingredients of betel quid, areca nut promotes intense salivation (Eisen, 1946), mild exhilaration and to a certain extent sleeplessness. By and large it has some effect on the central nervous system(CNS).

Role of different components of areca nut in oral cancer

Tannin, one of the components of areca nut (Table 1) is a powerful hepatotoxic agent when administered topically or by injection. In one set of experiments, about 50% of rats surviving tannic acid injection for longer than 100 days developed liver tumors. Moreover, simultaneous dosing with tannic acid and acetylaminofluorene induced more liver tumors and a greater degree of malignancy than did the synthetic carcinogen alone (Kosonyi and Korpassy, 1953). This component did not evoke local tumors on simple applications on the skin (Korpassy and Kosonyi, 1950). Other reports also show induction of tumors by tannin extract (Kirby, 1960). Tumors were induced in rats and mice by subcutaneous injections of various tannin extracts. Condensed tannins evoked Sarcomata at the site of injection as well as liver tumors, but liver tumors only were produced by extracts of hydrolysable tannins.

Literature is not available on carcinogenic property of other components of areca nut especially alkaloids (Table 1). Arecoline one of these alkaloids is the most important constituent of areca nut. The dried nut contains about 0.1% of arecoline.

Properties of Arecoline

1,2,5,6-Tetrahydro-1-methyl-3-pyridine carboxylic

acid methylester; methyl 1,2,5,6-tetrahydro-1-methyl nicotinate; methyl 1-methyl- $\Delta^{3,4}$-tetrahydro-3-pyridine carboxylate; methyl N-methyl tetra hydronicotinate.

$C_8H_{13}NO_2$; mol.wt. 155.9 (C 61.91%, H 8.44%, N 9.03%, O 20.62%).

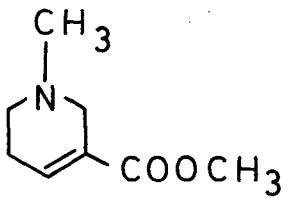


Fig.1: STRUCTURE OF ARECOLINE

It is extracted from the seeds of the betel nut palm - Areca catechu L., Palmaceae (catechu).

Physical properties

It is an oily liquid having b.p. 209°C, $b.p._7$ 92-93°C, $b.p._{12}$ 105°C. It is volatile with steam. A strong base with pk 6.84, k 1.5×10^{-7}, n_D^{20} 1.4302. d^{20} 1.0459. Miscible with water, alcohol and ether. It is soluble in chloroform. It is also found in amorphous form (synthesized) which is soluble in water.

Toxicity data : $LD_{s.c.}$ in mice : 100 mg/kg (see Merck Index).

Physiological effect

This alkaloid, like other parasympathomimitic drugs stimulates the brain. It has the actions like acetylcholine.

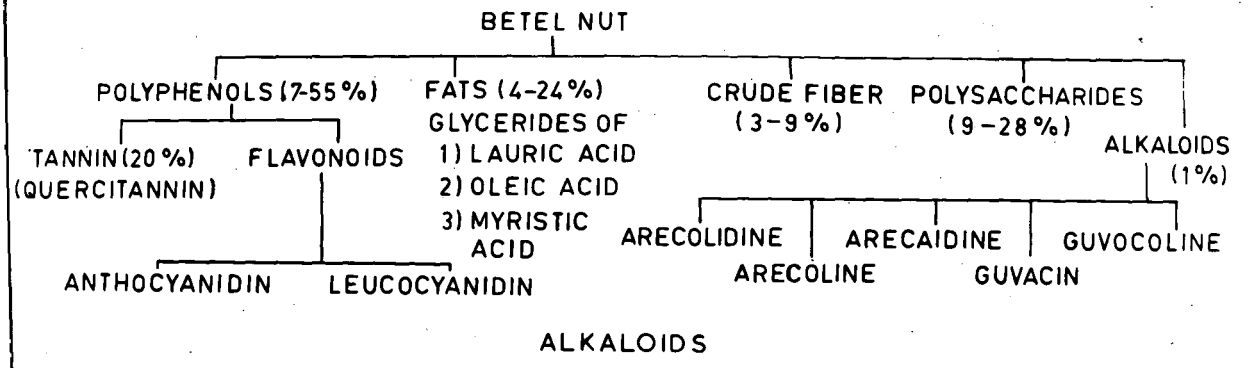
It is also called as cholinergic drug. Arecoline administered to rats has been shown to produce various effects on the central nervous system including, increased motor activity (Hertz, 1962) decreased conditioned avoidance responses (Hertz, 1962; Hertz and Yacoub, 1964; Pfeiffer and Jenny, 1957) activation of electroencephelogram (Riehl et al., 1962)and production of tremors (Holmstedt and Lundgren, 1966). In other words, it is cholinergic, exerting a sialogogue and diaphoretic action in normal dosage. All of these effects, however, could be antagonized by administration of the CNS m-cholinergic agent, atropine. It may exert a deleterious effect on the dental enamel (Riker, 1958). It is used in veterinary medicine against tapeworms.

Present study deals with the effect of arecoline, a major component of areca nut on transplantable mouse fibrosarcoma. The purpose is to evaluate whether this alkaloid has any tumor promoting or tumor inhibiting property. Indeed, certain plant alkaloids like Vincristine, Vinblastine and Colchicin do display the dual role carcinostatic as well as carcinogenic properties (Cutts, 1961; Vaitkevicius et al., 1962; Carbone et al., 1960; Cardinali and Enein, 1960; Palmar, 1963). Since arecoline is a plant alkaloid, it appeared worthwhile to investigate its effects on immuneresponses. Hence, additional

experiments have been performed to study the effects of arecoline on antibody formation and delayed hypersensitivity in mice.

TABLE-I

DIFFERENT COMPONENTS OF ARECA NUT



ALKALOIDS

ARECOLINE : CH_3^- ester of arecaidine

ARECAIDINE : NCH_3 Guvacine

GUVACOLINE : CH_3 ester of Guvacine

GUVACINE : Δ^3 tetrahydropyridine-3-carboxylic acid

ARECOLIDINE : 3:4 dimethoxy 1-CH_3 1:2 dihydropyridine

MATERIALS AND METHODS

A. Animals

Swiss albino mice (both inbred and outbred) were used in these experiements. Young adult animals (7-10 weeks old) were obtained from Cancer Research Institute (CRI), Bombay, India and Central Drug Research Institute (CDRI), Lucknow, India. The animals were housed in metal cages with rice husk bedding and maintained in an airconditioned animal house. They were provided with food and water ad libitum and their weights were recorded at regular intervals.

B. Tumors

Animals inoculated with 0.1 ml of Mouse Fibro-Sarcoma (MFS) cell suspension were obtained from CRI, Bombay. After 10-12 days of inoculation the developed tumor was transplanted in normal animals for the maintainance of the cell line. Animals bearing well developed tumor were sacrificed and their tumors were scrapped out. Hair and other fibrous tissue were removed. After washing in Hank's solution, the tumors were minced. Cell suspension was made in Hank's solution with the help of curved scissors. 0.2 ml of this cell suspension was injected in the right thigh of the mice (inbred and outbred) subcutaneously. 24-36 hrs after the inoculation the total

CHART - I

PARTICULARS OF THE EXPERIMENTAL DESIGN

Experiment	Strain and sex of the animal used	No. of control animals	No. of Exptl. animals	Animals died during Experimentation
Expt. I	Out bred ♂♀	9 (C_1-C_9)*	8+ (E_1-E_8)*	C_9-on the 27th day E_8- on the 24th day
Expt.II	Out bred ♂♀	6 (C_{10} - C_{15})*	6++ (E_9-E_{14})*	-
Expt.III	In bred ♂♂	5 (C_{16}-C_{20})*	5⊕ (E_{15}-E_{19})*	C_{16} & C_{19}-on the 18th day C_{17}.C_{18} & C_{20} - on the 23rd day
Expt.IV	In bred ♂♂	5 (C_{21}-C_{25})*	5⊕ (E_{20}-E_{24})*	C_{22} - on the 19th day C_{21}.C_{23}.C_{24} & E_{25} - on the 22nd day

*The figure in the parenthesis indicate the label of the individual animals.

+Arecoline treatment started on the 1st day of inoculation.

++Arecoline treatment started on the 7th day of inoculation.

⊕Arecoline treatment started on the 5th day of inoculation.

animals were divided into two groups by an accepted random method - one having experimental label whereas the other being control.

C. Arecoline injection

Arecoline was obtained from Sigma Company, U.S.A. This alkaloid was dissolved in physiological saline at the concentration 5 mg/ml. Fresh solution was used every time. Care was taken not to expose the solution to light.

After observing palpability of the tumor, the experimental animals were injected 0.1 ml of the fresh solution containing 0.5 mg of arecoline. Control animals received the injection of the vehicle only. These injections were given in the left thigh by subcutaneous route. The injections were given twice a day with the gap of 12-14 hrs between the two shots.

D. Weight of the animals

All the animals of experiments I & II were weighed before they were inoculated with tumor cells. Body weight was taken regularly after a particular interval (Table 3 and 4).

E. Size of the tumors

After the inoculation of KPS cells on the right

thigh, the site start swelling and after a week or so a prominent tumor is evident. All the tumors were measured in cm's with the help of slide calliper. Length and breadth of the tumors were noted. Measurements were taken for a month or so after every 4-6 days gap. Approximate "size" of the tumors was calculated by multiplying the length with breadth. This is a usual procedure used in several laboratories.

F. Haematological study

About twenty eight days after the start of the experiments I and II the animals both experimental as well as controls were bled. Blood was taken by puncturing the tail vein. Two experiments were carried out with the collected blood.

i. Enumeration of RBC.

ii. Determination of packed cell volume(VPRBC).

These two experiments were performed to assess the change in amount of RBC in the experimental and control animals with and without the treatment of arecoline. These experiments were planed after observing a steep fall in weight in the animals treated with arecoline.

i. Enumeration of RBC: As the number of red cells present in one cubic millimetre of blood is in millions the blood had to be diluted, so that the red cells would be seen

distinctly and directly. Therefore, a known volume of blood was diluted 200 times with a fluid which was isotonic with blood and would prevent its coagulation. Red cells in known volume of diluted blood were counted in a special counting chamber and the number of RBC, per c.m.m of the undiluted blood was calculated therefrom.

Apparatus and reagents used

Haemocytometer - This is a simple assembly consisting of RBC pipette, a slide and a thick cover slip.

Diluting fluid - The diluting fluid which was used is called Hayem's solution. Its composition is as follows:

Sodium chloride	0.5 gm
Sodium sulfate	2.5 gm
Corrosive sublimate	0.25 gm
Distilled water	100 ml

The fluid is isotonic with blood. It (), therefore, prevented haemolysis and rouleaux formation. Sodium sulfate acted as an anti-coagulant and corrosive sublimate prevented bacterial growth. After diluting the blood 200 times, a drop of it was taken on the slide and the cells were counted under a microscope.

ii. Packed cell volume

The packed cell volume was determined by centrifuging a sample of blood which had been made incoagulable by a

suitable anti coagulant heparin, until the corpuscles were packed to a constant volume.

Apparatus and reagents used

(i) Clean and dry Wintrobe's haematocrit tubes. (ii) Paraffin to block one end of the tube (iii) Heparin. (iv) Syringe. (v) Centrifuge.

Tail vein of the animals were punctured. 0.05 ml blood was drawn into a syringe having some heparin. After mixing properly, blood was filled in the haematocrit tube. One end of the tube was blocked with parafin. The tube so filled with blood was centrifuged in JANETZKI TH 12 centrifuge at the speed of 12,000 rpm for 30 min. After the centrifugation, the tubes were put in a scale which gave percentage of the volume of the packed cells.

G. Experiments to show effect of arecoline on the immune system of mice

Animals - 60 female out bred Swiss albino mice obtained from CDRI, Lucknow were used in these experiments.

Immunizer - Crystalline BSA from Sigma Co., U.S.A.

Adjuvant - Complete freund's adjuvent (CFA))- Difco Lab. Michigan, U.S.A.

Preparation of different concentrations of BSA

10 mg of BSA was dissolved in 5 ml of physiological saline. Three different concentrations were made from

this stock solution.

a. 100 microgram : 1.5 ml of stock solution having 3,000 microgram BSA was mixed with 1.5 ml of CFA. An emulsion was made by continuous jetting through a hypodermic needle. 0.1 ml of the emulsion contained 100 microgram of BSA.

b. 50 microgram : 0.75 ml of BSA stock was diluted with 0.75 ml saline and the total volume was mixed with 1.5 ml CFA to get 3 ml of emulsion having 1500 microgram BSA. 0.1 ml of this contained 50 microgram of BSA.

c. 10 microgram : 0.2 ml of BSA stock was diluted with 1.8 ml of saline and the total volume was mixed with 2 ml of CFA to get 4 ml of emulsion having 400 microgram BSA. 0.1 ml of this contained 10 microgram of BSA.

Administration of BSA - CFA emulsion

Administration of BSA-CFA emulsion was done in the following way:

100 microgram	20 animals
50 microgram	20 animals
10 microgram	20 animals

All these 60 animals were assorted into twelve groups of 5 animals each (Table 2). Booster injection of BSA was given in all the animals in the same dosage after 27 days of the primary immunization.

TABLE 2

GROUPING OF THE TREATED AND UNTREATED ANIMALS FOR THE STUDY OF HI AND CMI RESPONSES

Antigenic dose		Immune response	No.of animals used	Operation performed
10 µg	Control	HI	5	A
		CMI	5	B
	Exptl.	HI	5	A
		CMI	5	B
50 µg	Control	HI	5	A
		CMI	5	B
	Exptl.	HI	5	A
		CMI	5	B
100 µg	Control	HI	5	A
		CMI	5	B
	Exptl.	HI	5	A
		CMI	5	B

HI - Humoral Immunity; CMI - Cell Mediated Immunity.

A - Bled on 8th, 24th and 32nd days of immunization.

B - Given antigenic challenge of BSA dose according to the first dose in the right foot pad after 27 days of primary immunization.

Arecoline injection

Experimental animals were given 0.1 ml of arecoline (5 mg/ml) twice a day. Indirect Haemagglutination Test was performed for Humoral immune response whereas Foot Pad Injection Method was used for Cell Mediated Immunity.

Indirect Haemagglutination Test:

1. Mouse blood serum - Animals from different groups were bled after 8, 24 and 32 days of immunisation. Bleeding was done through the infraorbital vein with the help of capillary tubes. Blood was collected in small tubes and kept in cold for coagguletion. Sera were separated out after twenty-four hours and they were collected in another clean tube. These sera were centrifuged at 1200 rpm for 10 minutes (temp. - $4^{\circ}C$) and stored in cold. Before the experiment each serum was diluted 10 times.

2. Normal rabbit serum (NRS) - Normal rabbits were bled through the ear vein. Sera were extracted out after centrifugation. Most suitable serum (one free from antibodies against BSA) was retained for the experiment.

Before this serum could be used, it was decomplemented by heating at $56^{\circ}C$ for 30 minutes in a water bath. Then it was stored in the cold room. 1% NRS was prepared in PBS pH 7.2 before use. This solution was used as a diluent for antisera, antigen or cells during the assay.

3. Sheep red blood cornuscles (SRBC) - Sheep blood was taken out and was mixed with an equal volume of

Alsever's solution and stored at $4^{o}C$. It could be used upto 2-3 weeks as a source of SRBC if no contamination or haemolysis occurred.

4. Phosphate buffer saline (PBS) - 0.2 M solution of Na_2HPO_4 and NaH_2PO_4 were prepared separately. They were mixed in the following proportions to get stock buffer solution. Adjusted the pH with appropriate solution (0.1N HCl/0.1 N NaOH).

pH	0.2M NaH_2PO_4	0.2 M Na_2HPO_4
7.2	28.0 ml	72.0 ml
6.4	73.6 ml	26.4 ml

The stock buffer solution was diluted 20 times with 0.15M sodium chloride solution before use.

5. Tannic acid - Tannic acid method was followed in the present study in which tannic acid was used in the fixing or coating of antigen on SRBC. The binding is electrovalent interaction.

1 mg/ml solution of Tannic acid was prepared in PBS pH 7.2 and dilution was made by further addition of PBS. Total dilution was 1:40,000.

6. Antigen solution - An antigen solution of 20 mg BSA was prepared in 10 ml of PBS (pH 6.4).

Preparation of antigen coated SRBC

1. Alsever's solution was discarded after centrifuging SRBC at 3500 rpm (750 g) for 10 minutes. Pellet was washed 3 times at 750 g for 10 minutes. After final washing the cells were packed at 750 g for 15 minutes.

2. 0.2 ml of packed cells were taken in each of the 2 test tubes and suspended each in 10 ml PBS (pH 7.2) to give 2% SRBC suspension.

3. 10 ml of Tannic acid solution (1:40 dilution) was added to both the tubes. They were mixed well and incubated at 37° for 15 minutes.

4. After incubation both the tubes were centrifuged for 5 minutes at 750 g. Supernatant was removed and the cells were washed thrice in about 20 ml of PBS (pH 6.4). Finally it was resuspended in 10 ml of PBS (pH 6.4) to get 4% suspension of tanned SRBC.

5. To one of the tubes 10 ml of PBS (pH 6.4) was added. This was taken as control. To the other tube 10 ml of PBS (pH 6.4) containing 20 mg BSA (BSA was taken as an antigen) was added. The tubes were incubated at 37°C for 30 minutes. The cells treated with antigen (BSA) were labelled as coated SRBC and the other as control SRBC.

6. Centrifuged and washed the cells with 10 ml of 1% NRS in PBS (pH 7.2).
7. Finally the cells were suspended in 20 ml of 1% NRS in PBS. This gave 1% suspension of tanned SRBC of either type ready for use in the tube.

Preparation of standard anti BSA

0.2 ml of standard anti BSA was taken and mixed with 0.18 ml of PBS (pH 7.4).

Titration of antisera :

Titration of antisera was carried out in test tubes (small size of 10 x 75 mm).

1. 0.1 ml of 1% NRS was added in every tube.
2. 0.1 ml of the test serum was added in the first tube and serial dilutions were made with the help of 0.1 ml pipette.
3. 0.1 ml of coated SRBC was added to first eight tubes containing antiserum dilution.
4. In the last 4 tubes 0.1 ml of control SRBC was added.
5. Following additional controls were set up:
   a. 0.1 ml of coated SRBC + 0.1 ml of 1% NRS
   b. 0.1 ml of control SRBC + 0.1 ml of 1% NRS
   c. 0.1 ml of 0.1% NRS + serial dilution of standard antiserum + coated SRBC.
   d. 0.1 ml of 0.1 NRS + serial dilution of standard antiserum + control SRBC.

6. All the tubes were mixed gently by tapping the test tube stand having the tubes. All the tubes were covered with aluminium foil and kept overnight at room temperature.

Titres at different intervals were recorded for different antisera from different animals having different dosage of the antigen.

Foot Pad Injection Method

After 34 days of the first and 7 days of the second immunization 10 microgram, 50 microgram and 100 microgram of BSA were injected in the right foot pad of the animals according to the first immunizing dose. On the left foot pad equal volume of saline was injected. Thickness of the foot pad was measured with the help of schnilltester - callipers after 24 and 48 hours of antigenic challenge.

RESULTS

A. Mortality and body weight loss

No mortality of the animals was observed during the experimental period except in experiments III and IV where the control animals started dying after 18th day of inoculation with tumor cells possibly due to mass infection (see Chart I).

There was a continuous fall in the body weight of the animals treated with arecoline. The animals looked quite weak and were dull in their movements. There was less consumption of food in the experimental animals as compared to control. However, the animals regained their weight after the treatment was stopped. This is evident from Tables 3 and 4. This observation was made only in the animals of experiments I and II.

B. Haematological changes

Test for haematological changes in control and experimental animals of experiments I and II were performed. Both RBC count as well as percentage volume packed RBC showed loss in number of RBC in animals treated with arecoline. This is shown in Table 5. The maximum number of RBC in control animals was noted to be 949,0000 where as it was 721,0000 in the experimental animals. The minium number of RBC in control was 522,0000 where as that of experimental was 111,0000.

The volume packed RBC was 30% maximum in control where as that of experimental was 18%. The minimum mark in control was 15% whereas that in experimental was 11%.

TABLE 3

THE EFFECT OF ARECOLINE ON THE BODY WEIGHT OF THE ANIMALS INOCULATED WITH MFS CELLS. ARECOLINE TREATMENT STARTED ON THE IST DAY AND STOPPED ON THE 31ST DAY OF INOCULATION.

Animal No.	0 day	19th day	38th day	67th day
C_1	19.5	19.5	19.5	20.5
C_2	18.0	18.0	18.0	18.1
C_3	23.8	24.0	24.0	24.6
C_4	19.5	20.0	20.6	19.6
C_5	19.5	20.0	20.6	20.8
C_6	21.0	21.5	21.8	21.8
C_7	20.4	20.4	20.5	21.0
C_8	20.8	21.0	21.0	21.6
C_9	21.0	21.5	**	**
E_1	17.4	14.8	15.3	17.1
E_2	20.5	17.0	17.4	19.1
E_3	26.2	21.6	22.0	24.0
E_4	23.0	18.6	20.2	22.3
E_5	27.1	21.6	22.5	27.0
E_6	24.1	19.2	19.8	23.5
E_7	26.5	21.0	21.4	27.2
E_8	24.0	23.1	**	**

C - Control; E - Experimental.

** - Animal died.

TABLE 4

THE EFFECT OF ARECOLINE TREATMENT ON THE BODY WEIGHT OF THE ANIMALS INOCULATED WITH MPS CELLS. THE TREATMENT STARTED ON THE 7TH AND STOPPED ON THE 33RD DAY OF INOCULATION.

Animal No.	0. day	20th day	37th day	75th day
C_{10}	20.0	22.8	21.0	21.8
C_{11}	20.9	22.8	21.6	22.0
C_{12}	23.2	25.7	24.1	25.2
C_{13}	27.2	31.0	28.2	28.2
C_{14}	19.2	20.0	20.1	20.1
C_{15}	24.1	25.0	25.5	25.7
E_9	19.4	18.0	20.6	20.8
E_{10}	21.6	20.4	20.6	22.0
E_{11}	20.9	19.0	20.8	22.8
E_{12}	21.9	20.3	21.2	22.3
E_{13}	17.4	16.1	17.2	17.9
E_{14}	23.5	22.4	23.3	23.8

C - Control; E - Experimental.

TABLE 5

EFFECT OF CONTINUOUS TREATMENT OF ARECOLINE ON THE TOTAL RBC COUNT OF THE ANIMALS BEARING TRANSPLANTABLE TUMORS AFTER 28TH DAY OF INOCULATION.†

Animal No.	No.of RBC/c.m.m.	Animal No.	No.of RBC/c.m.m.
C_1	770.0000	E_1	721.0000
C_2	684.0000	E_2	211.0000
C_3	725.0000	E_3	111.0000
C_4	829.0000	E_4	596.0000
C_5	621.0000	E_5	570.0000
C_6	522.0000	E_6	453.0000
C_7	769.0000	E_7	552.0000
C_8	717.0000	E_8	**
C_9	**		
C_{10}	749.0000	E_9	429.0000
C_{11}	949.0000	E_{10}	650.0000
C_{12}	784.0000	E_{11}	476.0000
C_{13}	873.0000	E_{12}	507.0000
C_{14}	895.0000	E_{13}	434.0000
C_{15}	807.0000	E_{14}	614.0000

C - Control; E - Experimental.

** - Animal died.

† Statistical Analysis:

Control Mean + SE	Experimental Mean + SE	p
7.64×10^6 $\pm 1.01 \times 10^6$	4.86×10^6 $\pm 1.65 \times 10^6$	< 0.001

TABLE 6

EFFECT OF CONTINUOUS ARECOLINE TREATMENT ON THE PERCENTAGE VOLUME PACKED RBC OF THE ANIMALS BEARING TRANSPLANTABLE TUMORS AFTER 28TH DAYS OF INOCULATION†

Animal No.	% VPRBC	Animal No.	% VPRBC
C_1	20	E_1	14
C_2	22	E_2	15
C_3	28	E_3	17
C_4	15	E_4	15
C_5	20	E_5	14
C_6	20	E_6	15
C_7	27	E_7	15
C_8	25	E_8	**
C_9	**		
C_{10}	21	E_9	11
C_{11}	19	E_{10}	12
C_{12}	22	E_{11}	15
C_{13}	30	E_{12}	12
C_{14}	25	E_{13}	18
C_{15}	22	E_{14}	13

C - Control; E - Experimental.

** - Animal died.

†Statistical Analysis:

Control Mean ± SE	Experimental Mean ± SE	P
22.57 ± 3.87	14.30 ± 1.95	< 0.001

C. "Size" of the tumors

In the first experiment wherein out bred females were used as the recipients of the mouse fibrosarcoma, the tumor grew progressively in the animals treated with arecoline. The untreated animals failed to show any sign of tumor development which could be measured. One animal out of 9 controls did show appreciable growth in the beginning. This animal, however, died on the 27th day of inoculation (see Fig. 2). There was general regression in the size of the tumors of the experimental animals in the later stages.

The second experiment was also done on the out-bred female Swiss albino mice. But this gave slightly different result from the first experiment. Here both the treated and the untreated animals showed development of tumors (see Fig. 3). Like the first experiment, the treated animals showed a fast growth at the early stage. In the untreated animals there was increase in the size of the tumors but the growth rate was slower than that of the tumors of the treated animals. The rate of increase in the size was significantly high in the experimental animals as compared to control during 13th to 18th days of inoculation. Like the first experiment, regression in the size of the tumors was observed here also. Both control and experimental animals showed regression in the tumor size. But regression in the tumors of experimental animals was faster as compared to that of control. The difference in regression rate was significant.

The third and the fourth experiments were done on the in-bred male Swiss albino mice. Both these experiments had the

Fig. 2 : Effect of continuous treatment of arecoline on the size of the tumors in mice.

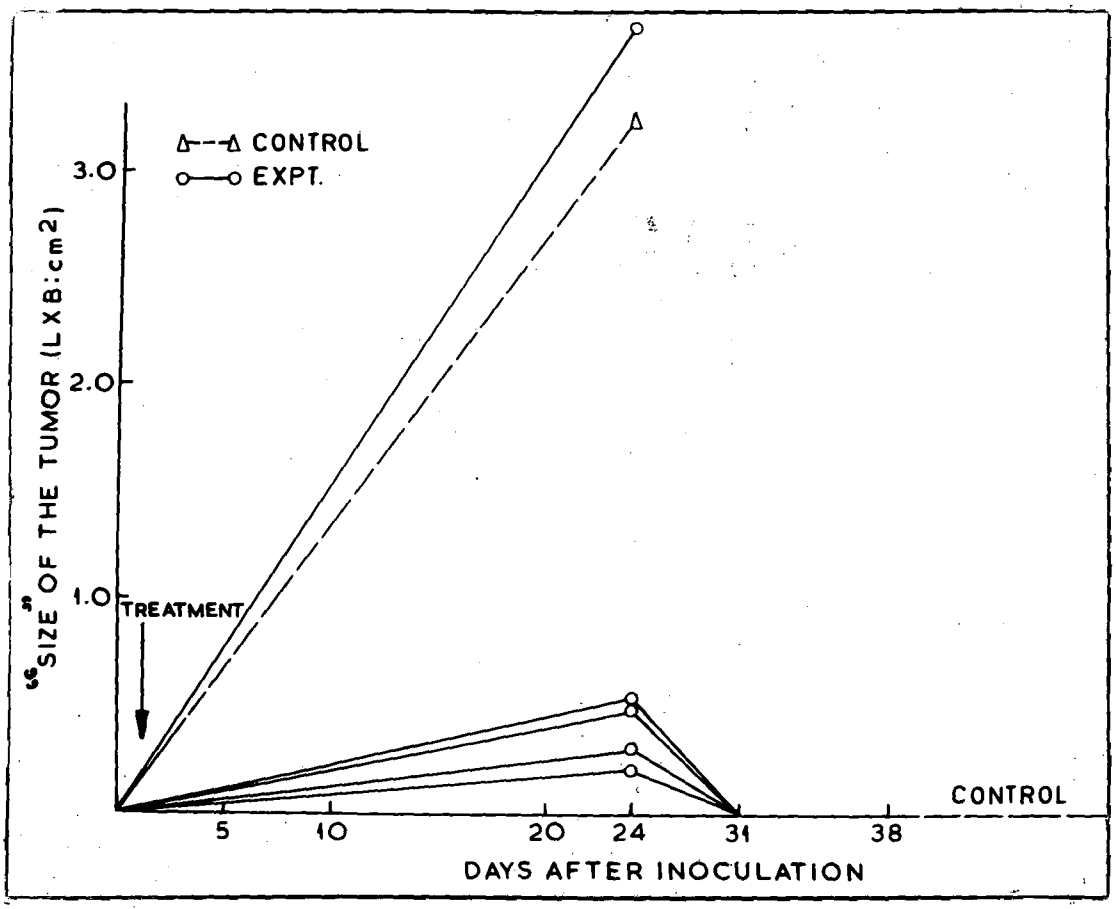

Δ---Δ CONTROL
o—o EXPT.
3.0
2.0
1.0
"SIZE" OF THE TUMOR (L X B: cm2)
TREATMENT
CONTROL
5
10
20
24
31
38
DAYS AFTER INOCULATION

Fig. 3 : Effect of continuous treatment of oracoline on the size of the tumors in mice.

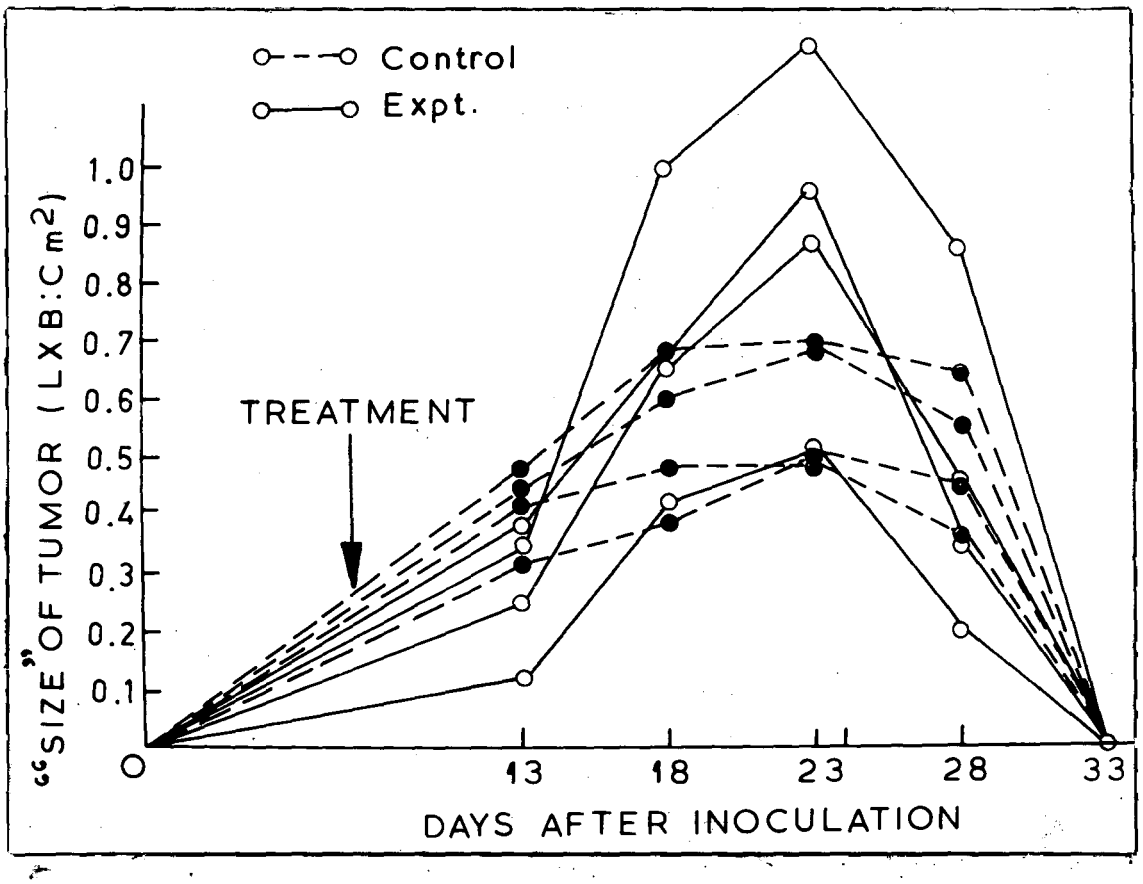

Control
Expt.
TREATMENT
"SIZE" OF TUMOR (LXB:C m²)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O
13
18
23
28
33
DAYS AFTER INOCULATION

Fig. 4 : Effect of continuous treatment of arecoline on the size of the tumors in mice.

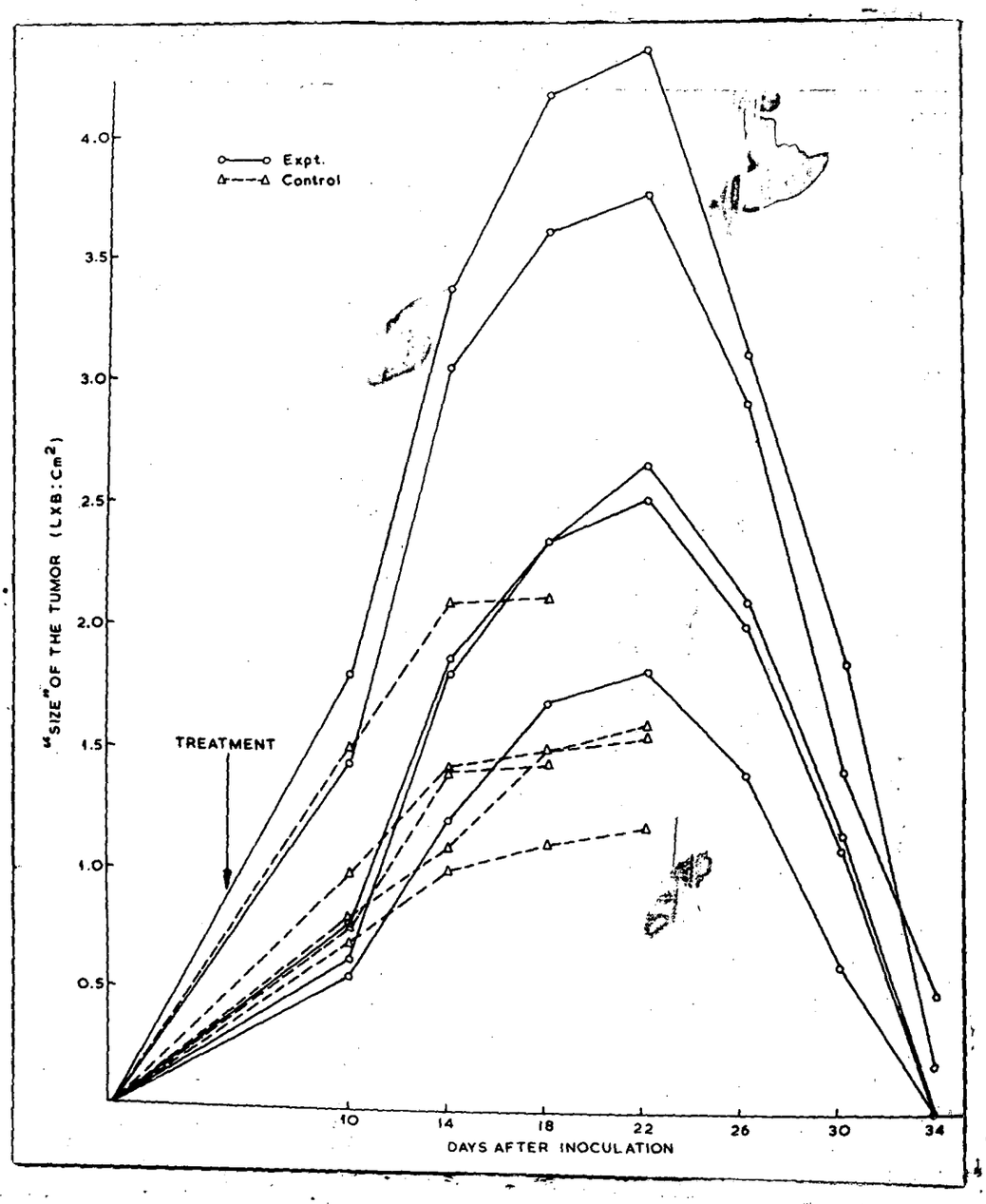
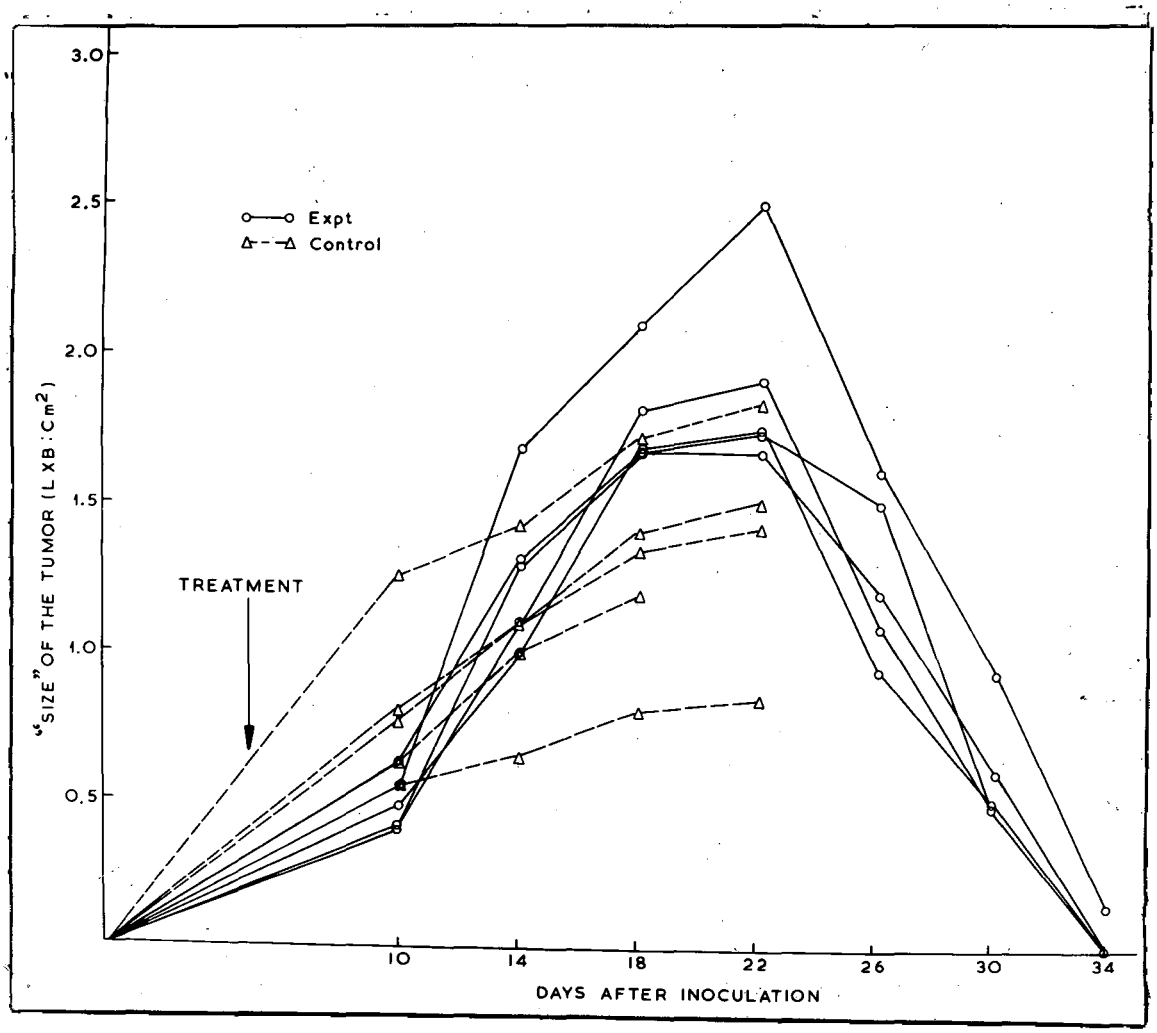

Expt.
Control
TREATMENT
"SIZE" OF THE TUMOR (LXB: Cm²)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
10
14
18
22
26
30
34
DAYS AFTER INOCULATION

Fig. 5 : Effect of continuous treatment of arecoline on the size of the tumors in mice.


3.0
2.5
2.0
1.5
1.0
0.5
Expt
Control
TREATMENT
"SIZE" OF THE TUMOR (L XB : Cm²)
10
14
18
22
26
30
34
DAYS AFTER INOCULATION

similar pattern as the second experiment. But in these two experiments the untreated animals started dying after 18th day of inoculation (Figs. 4 and 5). During the period available for comparison the treated animals showed significantly faster tumor growth than that of untreated control in early stages. In these two experiments also experimental animals showed regression in the tumor size. The rate of regression cannot be compared since all the control animals died during the experiment.

D. Effect of arecoline on immune response of mice

Haemagglutination experiments showed a low titre value in animals treated with arecoline as compared to that in untreated animals. The titre values in both the cases increased with time. That is titre values on 24th and 32nd days were more than that on 8th day. Challenge dose of 50 µg and 100 µg of BSA had a higher value as compared to that of 10 µg. Booster injections brought almost 4 fold increase in the haemagglutination titre but still its values were considerably less than that of the control. The difference in the titre value of the experimental and the control was almost 4-8 folds (Table 7).

Delayed type hypersensitivity response was determined by increase in foot pad thickness as measured by Schnilltester microcallipers. The response was higher at 48 hrs as compared to 24 hrs. There was considerable amount of difference in the thickness of the foot pads of the animals treated with and without arecoline. And this difference was more after 48 hrs of antigenic challenge (Tables 8 and 9).

TABLE 7

HAEMAGGLUTINATION TITRES ON THE 8TH, 24TH and 32ND DAYS OF IMMUNIZATION WITH BSA

Antigenic dose		8th day	24th day	32nd day
10 µg	Control	1 : 80	1:640	1 : 1280
	Exptl.	1 : 20	1 : 80	1 : 160
50 µg	Control	1 : 160	1 : 1280	1 : 2560
	Exptl.	1.: 20	1 : 80	1 : 320
100 µg	Control	1 : 160	1 : 1280	1 : 2560
	Exptl.	1 : 20	1.: 160	1 : 640

TABLE 8

DELAYED TYPE HYPERSENSITIVITY RESPONSE IN MICE AFTER 24 HRS OF THE ANTIGENIC CHALLENGE

Antigenic dose		Right foot pad thickness Mean ± S.E.	Left foot pad thickness Mean ± S.E.	Difference Mean ± S.E.	t
10 µg	Control	1.95 ± 0.18	1.45 ±0.20	0.525 ± 0.27	t 2.52⁻
	Exptl.	1.6 ± 0	1.5 ± 0.08	0.1 ± 0.08	
50 µg	Control	2.06 ± 0.10	1.6 ± 0.06	0.46 ± 0.10	t 3.20*
	Exptl.	1.86 ± 0.23	1.7 ± 0.08	0.16 ± 0.17	
100 µg	Control	2.025 ± 0.10	1.62 ± 0.08	0.4 ± 0.07	t 7.83**
	Exptl.	1.55 ± 0.11	1.475 ± 0.08	0.075 ± 0.04	

- : Not significant; * : Significant at 5%; **: Significant at 1%.

TABLE 9

DELAYED TYPE HYPERSENSITIVITY RESPONSE IN MICE AFTER 48 HRS OF THE ANTIGENIC CHALLENGE

Antigenic dose		Right foot pad thickness Mean ± S.E.	Left foot pad thickness Mean ± S.E.	Difference Mean ± S.E.	t
10 µg	Control	2.4 ± 0.25	1.5 ± 0.23	0.9 ± 0.18	t 6.82**
	Exptl.	1.63 ± 0.1	1.53 ± 0.09	0.1 ± 0.08	
50 µg	Control	2.72 ± 0.11	1.64 ± 0.04	1.08 ± 0.11	t 8.99**
	Exptl.	1.933 ± 0.26	1.8 ± 0.75	0.133 ± 0.18	
100 µg	Control	2.6 ± 0.15	1.675 ± 0.04	0.925 ± 0.12	t 12.57**
	Exptl.	1.6 ± 0.07	1.55 ± 0.11	0.05 ± 0.05	

** : Significant at 1%.

DISCUSSION

In the present study arecoline showed some interesting effects on the mouse fibrosarcoma as well as the host. Animals getting regular dose of arecoline showed loss in the body weight and became anaemic. Pilot experiments on normal animals also showed the same effect after arecoline treatment. This may be because of the toxic effect built during the continuous feeding of the compound (arecoline). This was indicated also by the low RBC count and the low percentage of RBC packed volume in the treated animals. All these changes may be considered as the side effects of arecoline. As it is evident from the previous reports, some other plant alkaloids do have similar type of side effects. Vinblastine shows a depressant effect of certain functions of bone marrow (Noble et al., 1958). Its other side effects consist in sharp leucopenia, not accompanied by considerable thrombocytopenia (Johnson et al., 1963; DeConti and Creasey, 1975). A fall in the haemoglobin level is also noted. Vincristine also shows certain side effects like constipation, nausea, vomiting, reduced reflexes and weakness (Johnson et al., 1963; DeConti and Creasey, 1975). According to Lovisetto et al. (1950, 1952) and Veronesi (1952) plant resinuous products, like Podophyllotoxin and the Peltantines cause in rats depression of bone marrow function, marked leucopenia and also anaemia.

Colchicine was found to give a number of toxin manifestations - diarrhea, depression of haemopoiesis, polyneuritis (Ludford, 1945). Colchamine causes fall in body weight, diarrhoea, leucopenia and anaemia and at high doses involvement of the nervous system. In the blood the greatest fall occurs in the number of neutrophils (Sharapov, 1956).

Besides these changes, arecoline brought about some other changes also. The most important to be noticed was the fast increase in the size of the tumor in the early stage. Control animals did show growth of the tumors size but to a lesser extent as compared to experimental animals. The early augmentation of tumor proliferation may be due to immunosupression elicited by arecoline administration. This may be the reason why in the first experiment, experimental animals only showed growth of considerable size of the tumor.

Immunological tests done indeed support this proposition. Haemagglutination titres and delayed type hypersensitivity skin responses using BSA as an immunizing agent in mice were used to show the effect of arecoline on the immune response of mice. Three dosages namely 10, 50 and 100 µg of BSA with CFA were used for immunization. The antibody response with 50 and 100 µg dosages was, however, better than 10 µg. A minimum amount of antibodies was raised even on the 8th day of immunization. The agglutination titres increase on the 24th day. A booster enhanced the

titre and the response could be seen on the 4th day of the secondary immunization. In all the cases the response in the arecoline injected animals was significantly less as compared to control immaterial of the dosages used.

Delayed type hypersensitivity (DTH) skin responses are a measure of *in vivo* cell mediated immunity status. In mice the DTH is measured by increase in the foot pad thickness after 24 and 48 hours of antigenic challenge. However, a significant increase in the foot pad thickness is demonstrable in the control animals whereas there was hardly any response in the animals given arecoline.

From these studies, it is not possible to comment on the mode of action of arecoline on the immune system. It might be acting directly and interfering with the metabolism of B-cells or T-cells or the effect could be by interference of the T-helper cells or might be generating the supressor cells which in turn effect the antibody production and cell mediated immune response. Similar type of findings have been reported by some workers using other plant alkaloids like vincristine, vinblastine and colchicine. The vinca alkaloids inhibited both the formation of antibodies to serum albumin and delayed hypersensitivity in rats (Aisenberg, 1963), although these drugs are not among the more effective immunosuppressive

agents. Both colchicine and vincristine inhibit the secretion of IgM antibody (Teplitz et al., 1975), but in contrast to cytochalasins, neither these antimitotics nor colcemid influenced antibody-dependent cytotoxicity measured by lysis of antibody-coated target cells (Gelfand et al., 1975). Besides, these alkaloids chemical suppression of immunological responses has been achieved by several classes of compounds. In particular, the alkylating agents (Hektoen and Corper, 1921), the antipurines (Schwertz, Eisner and Dameshek, 1959) and the folic acid antagonists (Uphoff, 1958) have been effective chemicals. However, since none of these drugs has offered complete suppression under condition of clinical trial, the discovery of new classes of chemicals which inhibit immune reactions remains an important objective. Intensive work on arecoline has to be done before it could be employed as a potent immunosuppresent in clinical field.

Continuous injection of arecoline brought about regression in the size of the tumors at later intervals. Slight regression was also observed in the controls but in this case the changes were slow. Appreciable degree of regression in the experimental animals may be due to general toxic effect, of the long term treatment of arecoline which

resulted in the loss of body weight and anaemia and secondly necrosis of the tumors which facilitated the shrinking of the tumors. In controls whatever degree of regression was seen could be attributed to necrosis in the central region of certain tumors. It is implicated that the immuno suppressive action of arecoline may facilitate the progression of oral cancer suspectedly induced by some agent present either in betel nut itself or in the other ingredients of betel quid.

CONCLUSIONS

1. Arecoline at the given dose level in the present experiment does not elicit mortality in mice.
2. Continuous administration of arecoline results in the progressive loss of body weight of experimental animals. Animals also suffer from anaemia as indicated by such haematological changes as fall in RBC count and low percentage of RBC packed volume. However, withdrawal of arecoline treatment eventuated gain in the body weight and general health.
3. Arecoline treatment enhances the growth of transplanted fibrosarcoma in mice during the early intervals. This may be due to immunosuppressive action of arecoline. Rapid regression in the size of the tumor was seen at the later intervals of arecoline treatment. The regression of tumors at the later intervals could be primarily due to the toxic effects of the alkaloid which had made the animals suffer from loss of body weight and anaemia. Whatever contribution in tumor regression made by central necrosis was common to both control and experimental animals.
4. Specific experiments conducted on Swiss albino mice clearly indicate immunosuppressive action of arecoline. Arecoline inhibits both the formation of antibodies to Bovine serum albumin and delayed hypersensitivity reaction.

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