Br. J. Pharmac. (1973), **48**, 88–96.

Pharmacological analysis of the acute inflammatory process induced in the rat's paw by local injection of carrageenin and by heating

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Summary

1. Local injection of carrageenin in the rat's paw produced oedema and leakage of dye which had been administered previously by the intravenous route. A net dissociation between both parameters was observed: while oedema developed slowly, maximal intensity being attained after 4–5 h, dye-leakage was maximum after 1 hour.

2. Anti-inflammatory drugs such as acetylsalicylic acid and indomethacin were effective in reducing oedema and dye-leakage when given before the injection of carrageenin, but much less effective when given 30 or 60 min after carrageenin. Hexadimethrine bromide was effective in reducing dye-leakage also when given 1 h after the injection of carrageenin.

3. Combined administration of benadryl and methysergide, before the injection of carrageenin caused only a slight reduction in oedema and dye-leakage.

4. When the paws were heated (55° C for 30 s) as a noxious stimulus the dissociation between maximal oedema and maximal dye-leakage was not observed, both phenomena running parallel. Pre-treatment of the animals with indomethacin did not afford any protection.

5. These results suggest that the inflammatory reaction to mild stimuli (carrageenin in our experiments) develops through different phases: initially the increased vascular permeability involves extravasation of plasma proteins and that phase is followed by an increased permeability mainly to water. Stronger stimuli (heating in our experiments) produce an overlapping of both phases, probably by inflicting severe damage to the vascular bed of the affected area.

6. The anti-inflammatory drugs employed affected chiefly the initial phase of the response.

Introduction

Dyes injected intravenously localize in areas of developing inflammatory reactions. Using xylene as an irritant applied to the skin of rabbits, Rigdon (1939, 1940) was able to show that Trypan blue and Indian ink concentrate in the injured areas only when injected into the circulation immediately after or within a period of less than 3–5 h following the application of xylene, though after that time these skin areas exhibited all the macroscopic and microscopic alterations associated with the inflammatory response. Rigdon concluded that increased vascular permeability is of brief duration following injury where xylene is applied.

On the other hand, oedema resulting from local injections of carrageenin and cellulose-sulphate develops rather slowly. In the rat's paw such oedema, as measured by the increase in volume of the paws, usually reaches maximal values around 4–5 h after the injection of the irritants (Van Arman, Begany, Miller & Pless, 1965 ; Garcia Leme, Schapoval & Rocha e Silva, 1967 ; Crunkhorn & Meacock, 1971).

It seemed of interest to investigate the relation between increased vascular permeability induced by noxious stimuli and the resulting oedema. In the present paper an attempt has been made to analyse the mechanisms through which inflammation develops, as well as the effect of drugs on such development by measuring dye-leakage and swelling induced in the rat's paw either by local injection of carrageenin or by heating.

Methods

Male adult Wistar rats (250–300 g) anaesthetized with pentobarbitone sodium (30–40 mg/kg intraperitoneally) were used. Not less than five animals were used in each experimental group.

Production of oedema in the rat paws

Carrageenin was dissolved in 0·9% w/v NaCl solution (saline) and 0·1 ml of the solutions (0·25, 0·50 and 1·0 mg/ml) injected into the subplantar area of one of the hind paws, the other being injected with an equal volume of saline. When heating was used as a noxious stimulus one of the hind paws was immersed in water at 55° C for 30 seconds. The volumes of the paws, up to the tibio-tarsal articulation were determined by plethysmography, at varying time intervals with a modification of the apparatus described by Winder, Wax & Been (1957). Results are expressed as percentage increase in relation to the initial volume of the paw. In most cases control values (saline-injected paws) were subtracted from values obtained in carrageenin-injected paws.

Double coaxial perfusion of the rat paws

Simultaneous perfusions of the subcutaneous space of both hind paws of the rat were made according to Garcia Leme, Hamamura & Rocha e Silva (1970). Through a small incision high in the limbs, polyethylene tubing (3 mm external and 2 mm internal diameter) was introduced into the subcutaneous space of the paws to their distal portion. A narrower tube, connected to a reservoir containing saline heated to 37° C, was introduced into the larger one, in such a way that the saline solution reached the subcutaneous space through the inner tubing and was collected through the outer one, the flow being adjusted to 1 ml/10 minutes. The perfusions were carried out for 40 minutes. Five minutes before starting the perfusion 20 mg/kg Evans blue dye in a 1% aqueous solution was injected intravenously. Swelling produced in the paws was determined before the introduction of the polyethylene tubing.

Assay of the perfusates

The perfusion fluid (4 ml) coming out from the subcutaneous space of the paws was assayed to determine the amount of dye present. A Coleman Jr. Spectrophotometer was used and absorption estimated at 600 nm. Results are expressed

as μg of dye per ml of perfusate and in most cases control values were subtracted from values obtained in carrageenin-injected paws.

Drugs and treatments

Pentobarbitone sodium (Nembutal), Abbott; acetylsalicylic acid, Bayer; 1-*p*-chlorobenzyl-2-methyl-5-methoxy-3-indolyacetic acid (indomethacin), Merck, Sharp & Dohme; diphenhydramine hydrochloride (Benadryl), Parke Davis; methysergide (Deserila), Sandoz; hexadimethrine bromide (Polybrene), Abbott. The drugs were dissolved in distilled water. To dissolve indomethacin, NaOH was added to pH 9 and the solution neutralized afterwards by the addition of acid. With the exception of acetylsalicylic acid which was given orally all the drugs mentioned above were injected intraperitoneally. Carrageenin was used as the sodium salt (M.W. 60,000–100,000), RENJ 3150, Marine Colloids, Inc.; Evans blue was from Merck Darmstadt.

Results

Increased vascular permeability and swelling induced by carrageenin in the rat paws

When various doses of carrageenin, from 25 to 100 μg, were injected into the rat paws a swelling developed which increased from 1 to 4 h after the injection and was proportional to the dose employed. In contrast, the amount of dye present in the perfusates from the same paws, though also proportional to the dose of the irritant, attained a maximum 1 h after the injection of carrageenin, decreasing thereafter (Table 1, Fig. 1).

TABLE 1. *Swelling and corresponding amount of Evans blue dye present in perfusates of rat paws after injection of carrageenin*

Carrageenin (μg injected)	Swelling (% increase in volume) 1 h	2 h	4 h	Amount of dye (μg/ml) 1 h	2 h	4 h
25	17·4±0·50 (5) ($P<0·01$)*	21·1±0·68 (5) ($P<0·01$)	25·6±0·68 (5) ($P<0·01$)	0·97±0·01 (5) ($P<0·01$)	0·83±0·01 (5) ($P<0·01$)	0·60±0·06 (5) ($P>0·1$)
50	22·0±0·56 (6) ($P<0·01$)	29·3±1·24 (6) ($P<0·01$)	39·8±1·13 (5) ($P<0·01$)	1·88±0·03 (6) ($P<0·01$)	1·33±0·03 (6) ($P<0·01$)	0·46±0·06 (5) ($P>0·1$)
100	40·4±1·12 (6) ($P<0·01$)	50·8±1·22 (5) ($P<0·01$)	75·4±2·20 (5) ($P<0·01$)	3·89±0·05 (6) ($P<0·01$)	2·62±0·12 (5) ($P<0·01$)	0·95±0·02† (5) ($P>0·1$)
Controls (saline injected paws)	10·0±0·51 (17)	7·9±0·62 (16)	6·0±2·40 (15)	0·61±0·05 (17)	0·60±0·04 (16)	0·51±0·06 (15)

One of the hind paws was injected with carrageenin and the other with equal volume of saline (controls). Estimations were made 1, 2 and 4 h after injection of carrageenin. Results are mean ± S.E. Figures within parentheses indicate number of experiments in each group. * Student's *t* test, by comparison with control values. † Paired controls (5 experiments) gave a value of 0·71 μg/ml.

In control paws, in which saline was used instead of carrageenin, exudation of dye and swelling were of minor significance, as compared to the corresponding inflamed paws, and were probably due to the introduction of the tubing for perfusion.

These results indicate that under such conditions a net dissociation exists between maximal extravasation of protein-dye complexes and maximal swelling.

Effect of anti-inflammatory drugs upon increased vascular permeability and swelling induced by carrageenin in the rat paws

When acetylsalicylic acid (150 mg/kg, orally) was given to rats 30 min before the subplantar injection of carrageenin, a marked reduction in the amount of Evans blue extravasated to the subcutaneous space of the paws and a partial inhibition of the swelling were observed, as measured 1–5 h after the injection of carrageenin. However, if the same dose of acetylsalicylic acid was administered 30 min after carrageenin, only a slight reduction, if any, occurred, either in exudation of dye or swelling; when given 60 min after carrageenin, exudation and swelling were indistinguishable from control values. See Tables 2 and 3.

Indomethacin 10 mg/kg injected intraperitoneally, was more effective than acetylsalicylic acid in reducing dye-leakage and swelling of the paws. This effect was more clearly seen when the drug was given 30 min before injection of carrageenin, and much less pronounced when administered 30 or 60 min after the carrageenin (Tables 2 and 3).

Hexadimethrine bromide, injected intraperitoneally (20 mg/kg) was also more potent than acetylsalicylic acid in reducing extravasation of dye and oedema. As in the preceding groups, the effect was more pronounced when the drug was administered before carrageenin. It is to be noted however, from Tables 2 and 3, that if carrageenin was allowed to act for one hour and then hexadimethrine injected, dye-leakage was reduced but the oedema developed almost to the same extent as in control animals.

TABLE 2. *Effects of acetylsalicylic acid (ASA), indomethacin and hexadimethrine on the amount of Evans blue dye present in perfusates of rat paws injected with carrageenin*

Treatment	Amount of dye (μg/ml) present in perfusates of rat paws (hours after carrageenin) 1 h	2 h	3 h	5 h
ASA 150 mg/kg (oral)				
(a) 0·5 h before carrageenin	0·30±0·04 ($P<0·01$)*	0·67±0·04 ($P<0·01$)	0·48±0·02 ($P<0·01$)	0·05±0·01 ($P>0·05$)
(b) 0·5 h after carrageenin	0·87±0·05 ($P<0·01$)	0·90±0·07 ($P>0·05$)	0·48±0·03 ($P>0·05$)	0·08±0·01 ($P>0·05$)
(c) 1 h after carrageenin		0·65±0·01 ($P>0·05$)	0·44±0·03 ($P>0·05$)	0·12±0·03 ($P>0·05$)
Indomethacin 10 mg/kg (i.p.)				
(a) 0·5 h before carrageenin	0·26±0·01 ($P<0·01$)	0·28±0·02 ($P<0·01$)	0·21±0·03 ($P<0·01$)	0·16±0·03 ($P>0·05$)
(b) 0·5 h after carrageenin	0·63±0·05 ($P<0·01$)	0·71±0·06 ($P<0·05$)	0·32±0·02 ($P<0·05$)	0·02±0·01 ($P>0·05$)
(c) 1 h after carrageenin		0·79±0·02 ($P>0·05$)	0·35±0·02 ($P>0·05$)	0·02±0·01 ($P>0·05$)
Hexadimethrine 20 mg/kg (i.p.)				
(a) 0·5 h before carrageenin	0·21±0·03 ($P<0·01$)	0·07±0·02 ($P<0·01$)	0·06±0·01 ($P<0·01$)	0·0
(b) 0·5 h after carrageenin	0·33±0·03 ($P<0·01$)	0·13±0·02 ($P<0·01$)	0·0	0·0
(c) 1 h after carrageenin		0·16±0·02 ($P<0·01$)	0·06±0·01 ($P<0·01$)	0·0
Controls (no treatment)	1·31±0·02	0·82±0·04	0·38±0·04	0·05±0·01

One of the hind paws was injected with 50 μg carrageenin and the other with equal volume of saline. Dye was injected i.v. Results are mean ± S.E. from at least 5 rats and are expressed as the difference between values obtained for carrageenin-injected and saline-injected paws. * Student's *t* test, by comparison with control values.

TABLE 3. *Effects of acetylsalicylic acid (ASA), indomethacin and hexadimethrine on swelling of rat paws induced by carrageenin as estimated by plethysmography*

Treatment	Swelling (% increase in volume) of the paws (hours after carrageenin)			
	1 h	2 h	3 h	5 h
ASA 150 mg/kg (oral)				
(a) 0·5 h before carrageenin	10·5±0·95 ($P<0·01$)*	17·5±0·60 ($P<0·01$)	23·0±0·33 ($P>0·1$)	29·5±0·98 ($P>0·1$)
(b) 0·5 h after carrageenin	14·0±1·10 ($P>0·1$)	22·0±0·62 ($P>0·1$)	27·5±1·03 ($P>0·1$)	33·0±1·20 ($P>0·1$)
(c) 1 h after carrageenin		21·0±1·00 ($P>0·1$)	29·0±1·00 ($P>0·1$)	38·0±0·54 ($P>0·1$)
Indomethacin 10 mg/kg (i.p.)				
(a) 0·5 h before carrageenin	5·4±1·3 ($P<0·01$)	11·8±0·73 ($P<0·01$)	16·8±0·68 ($P<0·01$)	23·6±0·60 ($P<0·01$)
(b) 0·5 h after carrageenin	11·6±0·51 ($P<0·01$)	18·4±1·03 ($P<0·01$)	21·4±0·86 ($P>0·1$)	31·2±1·15 ($P>0·1$)
(c) 1 h after carrageenin		19·8±1·39 ($P>0·1$)	28·4±0·73 ($P>0·1$)	34·4±0·81 ($P>0·1$)
Hexadimethrine 20 mg/kg (i.p.)				
(a) 0·5 h before carrageenin	5·2±0·58 ($P<0·01$)	9·5±0·54 ($P<0·01$)	10·7±0·69 ($P<0·01$)	3·5±0·44 ($P<0·01$)
(b) 0·5 h after carrageenin	11·1±0·28 ($P<0·01$)	12·0±0·63 ($P<0·01$)	14·5±1·48 ($P<0·01$)	17·1±0·71 ($P<0·01$)
(c) 1 h after carrageenin		17·2±0·19 ($P<0·01$)	22·2±1·01 ($P<0·01$)	26·0±0·65 ($P<0·01$)
Controls (no treatment)	14·5±1·23	22·9±0·64	30·0±0·65	35·5±1·40

One of the hind paws was injected with 50 μg carrageenin and the other with an equal volume of saline. Results are mean ± S.E. from at least 5 rats and are expressed as the difference between values obtained for carrageenin-injected and saline-injected paws. * Student's *t* test, by comparison with control values.

These results show that the three anti-inflammatory drugs in the doses employed are able to reduce the symptoms of the inflammatory reaction mainly when given before the application of carrageenin. If the irritant was injected before the administration of acetylsalicylic acid or indomethacin the inhibition was less marked, or absent, especially if an interval of one hour was observed between the injections. Hexadimethrine, however, was effective in reducing dye-leakage even when given one hour after carrageenin.

Effect of anti-histamine and anti-5-hydroxytryptamine drugs on increased vascular permeability and swelling induced by carrageenin in the rat paws

Combined pre-treatment of the animals by the intraperitoneal route, with benadryl (25 mg/kg) and methysergide (1 mg/kg), 30 min before the injection of carrageenin, caused a delay and a slight reduction both in the extravasation of dye and in the swelling of the paws. As can be seen in Fig. 1, the graphs representing exudation of dye are shifted to the right on the time scale, and the values attained are lower than those observed in control (untreated) animals. Animals examined 6 h after carrageenin received a second dose of benadryl (10 mg/kg) and methysergide (0·5 mg/kg), 3·5 h after the first one.

Increased vascular permeability and swelling induced by heating. Pre-treatment with indomethacin

When one of the hind paws of the rat was immersed for 30 s in a water bath heated to 55° C a marked swelling occurred 1 to 4 h afterwards with an increase

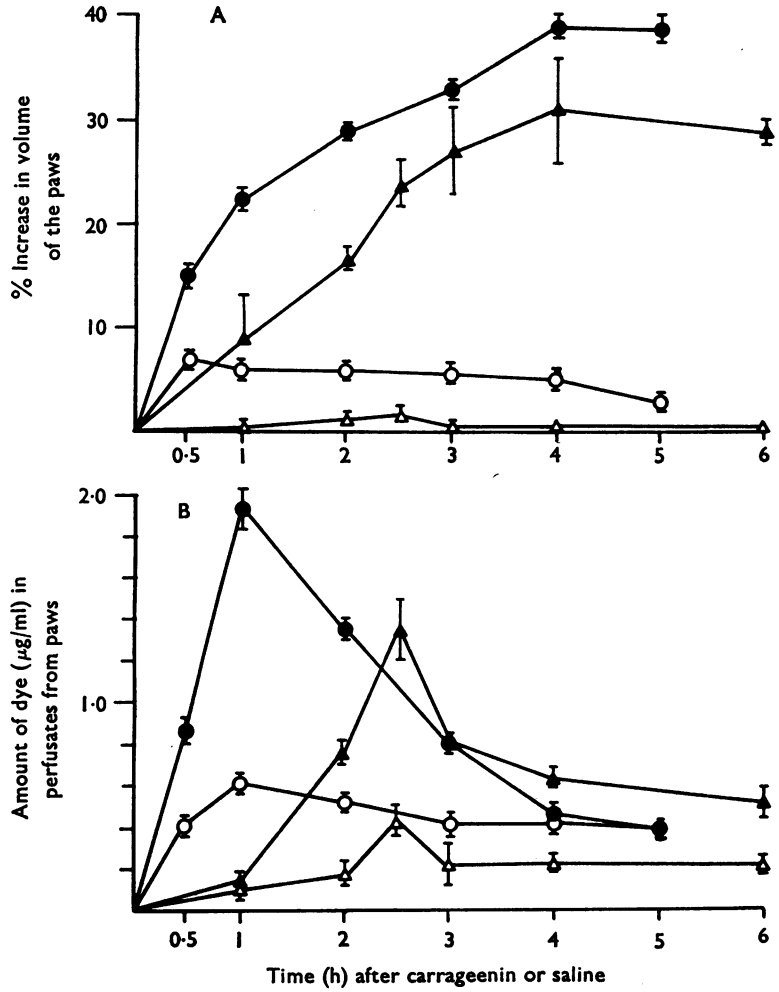

FIG. 1. Effect of combined i.p. administration of benadryl (25 mg/kg) and methysergide (1 mg/kg) on the oedema (A) and the amount of dye (B) present in perfusates from rat paws. Legends: ●-control animals, paws injected with 50 μg carrageenin; ○-control animals, paws injected with saline; ▲-treated animals, paws injected with 50 μg carrageenin; △-treated animals, paws injected with saline. Oedema was estimated as the percentage increase in relation to the initial volume of the paws and the amount of leaked dye by a perfusion technique. Results are mean ± S.E. of 5 experiments.

TABLE 4. *Percentage increase in volume of the rat hind paw and corresponding amount of Evans blue dye present in the perfusates 1 and 4 h after immersion of paw in water at 55° C for 30 seconds*

	% Increase in volume		Amount of dye (μg/ml)	
	1 h	4 h	1 h	4 h
Heated paws	58·8 ± 4·31	88·2 ± 5·36	5·67 ± 0·29 ($P<0·01$)*	3·18 ± 0·29 ($P<0·01$)
Non-heated paws	0·0	0·0	0·40 ± 0·09	0·19 ± 0·12

Results are mean ± S.E. of 5 experiments. * Student's *t* test, by comparison with control values (non-heated paws).

of dye-leakage (Table 4). Comparison of results in Table 4 with those in Table 1 show that the dissociation between maximal extravasation of dye and maximal swelling observed when carrageenin (50 μg) was used as an irritant does not occur

when the paw was heated. Pre-treatment of the animals with 10 mg/kg indomethacin i.p., 30 min before the application of heat afforded no protective effect towards the developing swelling: 1 h after heating the paws, their volumes had increased to a mean value of 74·4% ±9·90 and 4 h after to more than 100%.

Discussion

When carrageenin was injected into the paw, the extravasation of Evans blue reached its maximum around 60 min after the injection of the irritant, decreasing thereafter. The accompanying swelling developed slowly, the maximum being reached about 4–5 h after the injection of the irritant, a time when permeability to the protein-bound dye in carrageenin-treated paws was as low as that observed in control paws. Therefore, in these conditions, a clear dissociation exists between maximal extravasation of protein-dye complexes and maximal swelling. This finding agrees with that of Rigdon (1939, 1940) that increased vascular permeability to dyes in the rabbit skin following xylene application is restricted to the initial phase of the phenomenon.

When a thermal stimulus (55° C for 30 s) was applied to the rat's paw a persistent increase in vascular permeability to dye-protein complexes accompanied the development of the oedema, which was much more marked in this group than in rats treated with carrageenin. Sevitt (1958) also observed that the injection of dye into guinea-pigs 12 h after burning an abdominal area at 60° C for 30 s produced blue subdermal oedema and local colouring of the panniculus, which indicated that the permeability effect was still present.

These seem to be distinct reactions, most probably dependent on the intensity of the noxious stimulus applied and/or on the qualitative nature of the reaction.

It seems that two stages can be distinguished in the acute response to mild injury: initially the increased vascular permeability involves the extravasation of plasma proteins and that phase is followed by an increased permeability mainly to water. Extravasation of proteins to the interstitial spaces would be the factor that determines the succeeding exudation of water. This would explain the dissociation between maximal protein-dye-leakage and maximal swelling, as observed in the rat's paw injected with carrageenin. Stronger stimuli, such as heating, would lead to a severe damage of the area affected with a continuous loss of material from circulation.

By the use of anti-inflammatory drugs (acetylsalicylic acid, indomethacin, hexadimethrine bromide) in carrageenin-treated animals a reduction was observed either in dye-leakage or swelling, mainly when such drugs were given before the irritant. When injected 30 or 60 min after carrageenin their effects were much less pronounced. On the other hand, pre-treatment of the animals with indomethacin followed by the application of heat was of no value in counteracting the oedema which developed. These facts suggest that the anti-inflammatory agents employed mainly decrease the early vascular response, i.e., the increased permeability to large molecules. The later leakage of water seems to be less influenced by the anti-inflammatory drugs.

Pre-treatment of the animals with a combination of anti-histamine and anti-5-hydroxytryptamine drugs produced a delay and a partial reduction either in the amount of dye extravasated or in the swelling induced by carrageenin. This fact

strengthens the view that histamine and 5-hydroxytryptamine play a role in the early phases of some types of acute inflammatory responses.

The activation of the kinin system, either through the intervention of released histamine as suggested by Edery & Lewis (1963) or directly by the action of kininogens present in plasma and in the subcutaneous tissues would maintain the early vascular response (increased permeability to large molecules) to the point where the amount of extravasated proteins would be sufficient to induce the leakage of water through the vessel walls. In this respect, hexadimethrine bromide besides acting as an anti-inflammatory agent (Kellet, 1965 ; Garcia Leme *et al.*, 1967) and having a marked inhibitory effect upon the activation of the kinin system (Armstrong & Stewart, 1962 ; Garcia Leme *et al.*, 1970) proved in the present experiments to be a potent agent in counteracting the development of oedema and dye-leakage. It is to be noted that hexadimethrine bromide inhibits dye leakage even when given one hour after carrageenin. This might indicate that kinins are relevant factors in determining exudation of large molecules following injury.

Therefore inflammatory reactions following the injection of carrageenin seem to be mediated by several agents, e.g., histamine, 5-hydroxytryptamine, kinins. These substances are probably mainly restricted to the early phases of the process, promoting exudation of large molecules. Later, during the subsequent exudation of water, they may have a less important role. With strong stimuli this pattern of reaction would be altered due to the extensive damage inflicted to tissue and would represent a rather unspecific reaction.

Finally prostaglandins may play a role during that part of the reaction inhibited by aspirin and indomethacin since Vane (1971) and Ferreira, Moncada & Vane (1971) have shown that these anti-inflammatory agents inhibit synthesis of prostaglandins.

This work was supported in part by grants from The Fundação de Amparo à Pesquisa of the State of São Paulo (FAPESP).

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(*Received November* 13, 1972)