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THE EFFECT OF LOCALLY INJECTED ANTI-INFLAMMATORY DRUGS ON THE CARRAGEENIN GRANULOMA IN RATS

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Abstract—Granuloma was induced by injecting 2% carrageenin solution s.c. on the dorsum of rats. The granuloma pouch reached a peak in wet weight at day 5, then production of collagen in the pouch was accelerated, while accumulation of the pouch fluid took place mainly during 5–9 days. The total weight of granuloma pouch and its fluid amounted to about 20 per cent of total body weight.

The effect of anti-inflammatory drugs on the carrageenin granuloma was investigated in two ways. In the first experiment, drugs were tested for the inhibitory action on the granuloma formation by means of daily subcutaneous injections for 5 days beginning on the day of carrageenin injection. The second experiment was to test the ability of the drugs to reduce the preformed granuloma, in which drugs were daily applied directly into the pouch wall and its collagen content was determined.

Drugs employed were betamethasone as a steroidal drug, indomethacin, phenylbutazone and salicyclic acid as non-steroidals. A steroidal drug revealed an ability both to inhbit the formation of granuloma and to reduce the pre-existing granuloma, while non-steroidal drugs failed to reduce the pre-existing granuloma, though they had some effects on the granuloma formation. Some differences were noted in the mode of action among these non-steroidal drugs.

Diet limitation which resulted in marked body weight loss in young rats had little influence on both the formation and the maintenance of the carrageenin granuloma.

DURING the last 20 yr, corticoids have been mainly used in the treatment of the inflammatory diseases. But recently, because of undesirable side effects of steroids, a number of non-steroidal compounds have been intensively studied for the anti-inflammatory activity. Some of these new compounds are now commonly used in the clinical fields.

In parallel with the findings of new anti-inflammatory drugs, many experimental methods have been developed for evaluating the anti-inflammatory activity of drugs.[1–4] However, it was reported that an agent which was inhibitory in some tests was ineffective in others.[5–7] So far, these experimental methods are not successful for accurately predicting the anti-inflammatory effect of drugs in human diseases. The difficulty is caused by several factors involving the complexity of the inflammatory process and the diversity of the inflammatory diseases. Unfortunately few of the present experimental models have a close relation to the chronic inflammatory diseases of unknown causes and the mode of action of the anti-inflammatory drugs has not been sufficiently described. In fact, most of the experimental methods which have been used for the evaluation of the potency of the drugs hardly reveal any

difference in the mode of action between steroidal and non-steroidal drugs, while some differences are seen clinically.

In recent years carrageenin granuloma[8] induced s.c. in guinea pigs has been widely used in connective tissue studies.[9–11, 15–23] Carrageenin is also commonly used as an excellent phlogistic agent for inducing experimental edema.[3] In the present investigation carrageenin granuloma was produced on the dorsum of rats and the effect of the anti-inflammatory drugs on the granuloma was studied. This paper deals with some differences in the mode of action among the extensively used steroidal and non-steroidal drugs.

EXPERIMENTAL

Granuloma pouch was induced by the modification of Selye's method[12] using carrageenin as a phlogistic agent. Male rats of Donryu strain of 42 ± 3 days of age, ranging in body weight at the time of carrageenin injection from 92 to 140 g, were employed. Two per cent (w/v) solution of carrageenin (TS-36, kindly supplied by Taisho Pharmaceut. Co., Ltd., Tokyo) in 0·9% NaCl was sterilized by heating at 110° for 30 min. Antibiotics were added before injecting the solution (0·1 mg penicillin G potassium and 0·1 mg dihydrostreptomycin sulfate per ml of the solution). Six ml of air were injected s.c. on the dorsum of animals 1 day before the injection of the carrageenin solution. Then 4 ml of the solution were injected into the air sac already formed. The solution was warmed just before injecting it, because it coagulates at room temperature.

The effect of the anti-inflammatory drugs on the carrageenin granuloma was observed by two procedures. In the first procedure, the inhibitory action on the formation of the granuloma was investigated by giving drugs daily for the first 5 days. On the day of carrageenin injection (the day 0), the drugs were given being suspended in the carrageenin solution, thereafter were daily injected subcutaneously into the carrageenin sac. On day 5, the animals were killed and the entire fluid in the granuloma pouch was harvested, then the capsule of the granulomatous tissue was removed carefully. The volume of the fluid, designated as "pouch fluid", and the wet weight of the capsule, "pouch wall", were determined. "Net body weight" was calculated by subtracting "pouch fluid" and "pouch wall" from the gross body weight. Collagen was extracted as gelatin by heating the pouch wall in an autoclave at 110° for 3 hr. Extraction was done three times. The pooled extract was hydrolyzed in 6N HCl in a sealed tube at 140° for 4 hr and the hydrolyzate was assayed for hydroxyproline by Stegemann's method.[13] The amount of collagen was expressed in terms of the quantity of hydroxyproline.

In the second procedure, the effect of the drugs on the preformed granuloma was observed by administering the drugs directly into the granuloma pouch daily from day 5 up to day 8. Animals were killed on day 9. Besides the control killed on day 9 (9 day control), a group of rats were killed on day 5 just before the treatment of drugs was started (5 day control). All the assays were done as already described.

Drugs tested were betamethasone disodium phosphate (Glaxo Lab. Ltd., Greenford, England), indomethacin (Nippon Merck & Ban-yu Co. Ltd., Tokyo, Japan), Phenylbutazone (Fujisawa Pharmaceut. Co. Ltd., Ohsaka, Japan) and salicylic acid (Koso Chem. Co. Ltd., Tokyo). Two graded doses of each drug were applied as shown in tables. Drugs were given as suspensions of 0·1 or 0·2 ml in 0·5% (w/v) carboxy-

methyl cellulose solution once a day except salicylic acid of which daily doses were divided in two and applied to the animals twice a day because of the practical difficulty involved in injecting large doses and because of its relatively rapid metabolism.

An additional experiment was designed to observe the influence of limitation of the body weight increase on the granuloma, as the drugs often caused retardation of growth and body weight loss. Two groups of animals were pair-fed in individual cages to synchronize their body weight changes with those of drug-treated groups.

RESULTS

The injected carrageenin induced local swelling in the subcutaneous tissue, and then migration of phagocytic cells took place with increasing accumulation of fluid in the carrageeenin layer. In control animals, the accumulation of fluid in the pouches mainly occurred during 5–9 days with about 2-fold increase, and the volume remained nearly constant for a few days and then declined slowly. When the fluids were harvested on the days 5 and 9, clusters of dead cells, mostly consisting of leucocytes, combined with fibrin were found in the fluids in most animals.

A capsule of newly formed granulomatous tissue arose within a few days after the carrageenin injection from the subcutaneous tissue surrounding the carrageenin layer. On day 4, this newly formed tissue reached a development to be separated from the surrounding tissue. To the capsule of the granuloma, mainly on the part near the tail of rats, gelatinous translucent mass was attached in some cases. Such masses were dissected off before weighing the granuloma. Wet weight of the granuloma pouch reached a maximum at day 5 and then decreased slowly. On the other hand, the production of collagen in the tissue underwent further increase till it leveled off around day 14.

Betamethasone disodium phosphate revealed a strong inhibitory action on the development of the granulomas as shown in Table 1. In the animals received 1 mg daily for 5 days starting on the day of carrageenin injection (day 0), there was no evidence of the fluid accumulation and the pouch formation throughout the experimental period. In this group, a significant loss in body weight was observed. The treated group lost 17·5 g of body weight during 5 days, while the control group gained 10·3 g. Daily administration of 0·1 mg of betamethasone disodium phosphate markedly inhibited the fluid accumulation and significantly decreased both the formation of the granuloma pouch and the accumulation of collagen in the granuloma. Moderate body weight loss (— 7·3 g) was seen.

Indomethacin was as potent as betamethasone in inhibiting the formation of the granuloma. Its effect on the development of the carrageenin granuloma was investigated with the daily doses of 1 mg and 0·1 mg. In the group with the high dose, 4 out of the 7 animals died on the last day, but the formation of the pouch wall and the fluid accumulation were both negligible in the surviving three. The low dose of indomethacin was also effective, though not so potent as in the high dose, for inhibiting both the fluid accumulation and the formation of the granuloma pouch wall. The body weight gain was 12·9 g in the low dose group, 1·5 g in the high dose group, while the control gained 17·3 g.

Phenylbutazone, 20 mg daily for 5 days, inhibited the accumulation of the fluid at the first stage of the granuloma development without significant decreases in the wet weight of the pouch wall and in its collagen content. Slight loss of body weight

TABLE 1. EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE FORMATION OF CARRAGEENIN GRANULOMA IN RATS

Daily dose	No. of animals	Net body wt. (Initial)	Net body wt. (Final)	Pouch fluid (per cent inhibition)	(P)	Pouch wall (per cent inhibition)	(P)	Hypro, total (per cent inhibition)	(P)
		g	g	ml ± S.E.		g ± S.E.		mg ± S.E.	
Betamethasone									
5 day control	14	120·4	130·7	8·40 ± 0·71		7·70 ± 0·32		6·22 ± 0·38	
0·1 mg/rat	8	115·6	108·3	0·22 ± 0·10 (97·4%)	< 0·01	2·17 ± 0·58 (71·8%)	< 0·01	1·06 ± 0·26 (83·0%)	< 0·01
1 mg/rat	6	120·5	103·0	Negligible (99·9%)	< 0·01	Negligible (99·9%)	< 0·01	Negligible (99·9%)	< 0·01
Indomethacin									
5 day control	7	119·8	137·1	8·24 ± 1·29		6·56 ± 0·61		4·76 ± 0·71	
0·1 mg/rat	7	116·3	129·2	1·58 ± 0·28 (80·8%)	< 0·01	3·53 ± 0·49 (46·1%)	< 0·01	2·70 ± 0·56 (43·3%)	< 0·05
1 mg/rat	7 (4)*	119·0	120·5	0·36 ± 0·19 (95·6%)	< 0·01	0·16 ± 0·16 (97·6%)	< 0·01	0·63 ± 0·63 (86·8%)	< 0·01
Phenylbutazone									
5 day control	7	118·5	127·5	8·62 ± 0·98		7·74 ± 0·49		5·51 ± 0·43	
20 mg/rat	12	119·2	115·3	0·94 ± 0·26 (89·1%)	< 0·01	6·63 ± 0·37 (14·3%)		4·45 ± 0·29 (19·2%)	
Salicyclic acid									
5 day control	7	119·8	137·1	8·24 ± 1·29		6·56 ± 0·61		4·76 ± 0·71	
30 mg/rat	7	119·8	124·9	1·84 ± 0·41 (77·7%)	< 0·01	3·20 ± 0·53 (51·2%)	< 0·01	2·61 ± 0·39 (45·2%)	< 0·05
100 mg/rat	7 (3)*	121·2	129·3	0·41 ± 0·29 (95·1%)	< 0·01	2·69 ± 0·48 (59·0%)	< 0·01	2·52 ± 0·45 (47·1%)	< 0·05

* Figures in parenthesis are the number of animals which died during the treatment.

(— 3·9 g) was observed in the treated group. In order to make the effect of phenylbutazone more distinct, a daily dose of 40 mg was administered. However, all of the 7 animals used were dead in 24 hr after the single injection.

Salicylic acid showed an inhibitory effect on the formation of the granuloma. Accumulation of fluid was markedly inhibited in the 30 mg group and almost completely blocked in the 100 mg group. Pouch wall formation and collagen accumulation were reduced in both treated groups to about a half of those of the controls. Three out of the 7 animals in the 100 mg group died on the last day. Some reduction of body weight increase in both treated groups was seen.

In the second experiment, marked differences were observed among the drugs in the ability of reducing the preformed granuloma as shown in Table 2. When 1 mg of betamethasone was applied daily to the preformed granuloma for 4 days starting on the day 5, the fluid in the pouch almost disappeared and the weight of the pouch wall was reduced markedly. Accumulation of collagen in the pouch wall was completely suppressed although the body weight loss was observed.

Indomethacin showed substantially no effect on the pre-formed granuloma. A daily dose of 1 mg of indomethacin failed to produce any statistically significant change

Phenylbutazone proved to be ineffective on the preformed granuloma.

In the preformed granuloma salicylic acid at a daily dose of 100 mg revealed an inhibitory activity on the accumulation of the fluid, while the weight of the granuloma pouch wall was not affected significantly. However, it should be mentioned that there occurred no accumulation of collagen in the pouch wall during the treatment with salicylic acid of preformed granuloma. In this experiment, 2 out of the 8 treated animals died during the treatment.

As mentioned above, most of the treated animals showed more or less retardation of body weight increase and in some cases even body weight loss was observed. In order to evaluate the possible effect, if any, of growth retardation and body weight loss upon the development of the granuloma and on its maintenance, the pair-feeding experiment was performed, the results of which are tabulated in Table 3. Severe diet limitation which caused 6·8 g body weight loss in 5 days displayed a weak inhibitory action on the development of the granuloma in the same degree as milder limitation did. In the latter group, body weight was just maintained at the initial level in the course of the experimental period. Collagen content of the pouch wall was not affected in both groups, while the wet weight of the wall decreased by 24·2 per cent in the severely limited group and by 22·2 per cent in the mildly limited group (both significant at $P < 0{\cdot}05$). As to the amount of the pouch fluid, no inhibition was associated with the effect of diet limitation on the body weight. In the preformed granuloma, much more body weight loss (21·6 g and 9·2 g) were associated with much less, if any, inhibitory effect, as compared with its effect on the granuloma development. None of the apparent changes obtained was statistically significant on the preformed granuloma.

DISCUSSION

At the present time, various types of experimental inflammations are used for evaluating the anti-inflammatory activity of drugs. Among these methods, granuloma induced by various irritants is one of the most widely used types of inflammation. Experimental granuloma is more or less a model of the granulomatous connective

TABLE 2. EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE PRE-FORMED CARRAGEENIN GRANULOMA IN RATS

Daily dose	No. of animals	Net body wt. (Final)	Pouch fluid (per cent inhibition	(P)	Pouch wall (per cent inhibition)	(P)	Hypro, total (per cent inhibition)	(P)
		g	ml ± S.E.		g ± S.E.		mg ± S.E.	
Betamethasone								
5 day control*	7	133·8	8·19 ± 1·27		7·66 ± 0·43		6·92 ± 0·55	
9 day control	6	144·0	20·85 ± 1·67		6·81 ± 0·43		13·93 ± 1·25	
1 mg/rat	7	114·1	Negligible (99·9%)	< 0·01	2·64 ± 0·62 (61·2%)	< 0·01	5·96 ± 0·48 (57·2%)	< 0·01
Indomethacin								
5 day control	7	124·2	10·49 ± 0·91		4·81 ± 0·42		3·90 ± 0·56	
9 day control	7	137·6	14·64 ± 1·21		3·89 ± 0·19		7·11 ± 0·67	
1 mg/rat	7	130·0	15·70 ± 1·21 (0%)		3·65 ± 0·26 (6·2%)		6·14 ± 0·41 (13·6%)	
Phenylbutazone								
5 day control	7	158·6	11·73 ± 1·94		4·43 ± 0·45		4·98 ± 0·64	
9 day control	7	168·8	21·43 ± 2·75		4·37 ± 0·41		8·60 ± 0·81	
18 mg/rat	7	171·1	18·96 ± 2·32 (11·5%)		4·32 ± 0·23 (1·1%)		8·23 ± 0·95 (4·3%)	
Salicyclic acid								
5 day control	7	137·1	8·24 ± 1·29		6·56 ± 0·61		4·76 ± 0·71	
9 day control	8	129·7	18·31 ± 1·94		4·73 ± 0·39		7·85 ± 0·60	
100 mg/rat	8 (2)†	114·7	11·93 ± 2·17 (34·8%)	< 0·05	4·19 ± 0·57 (11·4%)		4·93 ± 0·59 (37·2%)	< 0·01

* Control taken before the treatment of drugs was started.

† Figures in parenthesis are the number of animals which died during the treatment.

TABLE 3. EFFECT OF DIET LIMITATION ON THE CARRAGEENIN GRANULOMA IN RATS

	No. of animals	Net body wt. (Initial)	Net body wt. (Final)	Pouch fluid (per cent inhibition)	(P)	Pouch wall (per cent inhibition)	(P)	Hypro, total (per cent inhibition)	(P)
On the granuloma formation									
		g	g	ml ± S.E.		g ± S.E.		mg ± S.E.	
5 day control	7	118·5	127·5	8·62 ± 0·98		7·74 ± 0·50		5·51 ± 0·43	
Diet limitation									
(mild)	8	121·0	121·8	7·51 ± 1·68 (12·9%)		6·02 ± 0·39 (22·2%)	< 0·05	4·51 ± 0·45 (18·1%)	
(severe)	5	113·6	106·8	13·30 ± 1·41		5·87 ± 0·61 (24·2%)	< 0·05	4·64 ± 0·22 (15·8%)	
On the pre-formed granuloma									
5 day control*	7		146·8	12·52 ± 1·31		4·43 ± 0·18		4·24 ± 0·25	
9 day control	8		154·2	18·02 ± 2·53		3·80 ± 0·21		8·20 ± 0·52	
Diet limitation									
(mild)	7		137·4	17·04 ± 1·27		3·54 ± 0·18 (6·8%)		7·61 ± 0·22 (7·2%)	
(severe)	8		125·1	19·06 ± 1·50		3·10 ± 0·30 (18·4%)		7·28 ± 0·37 (11·2%)	

* Control taken before the diet limitation was started.

tissue diseases involved in rheumatoid arthritis. The cotton pellet method[14] and the croton oil granuloma pouch method[12] are most commonly used for this purpose. However, inflammation is a very complex process consisting of several unit reactions involving the vascular permeability increase, the migration of granulocytes and various mononuclear cells, the proliferation and the maintenance of granulation tissue, etc. From this point of view, so-called anti-inflammatory activity of a drug should be regarded as an integrated result of some unit actions. Therefore, an analytical assay by which the action of anti-inflammatory drugs can be evaluated in terms of unit reactions of inflammation seems to be useful for clarifying the mode of action of drugs. By using the present carrageenin granuloma method, some unit processes involving the increased vascular permeability, the formation of granuloma and its maintenance and the accumulation of collagen in the granuloma can be separately measured. The carrageenin method is better than the croton oil method which often produces necrosis of the pouch wall.

To the author's knowledge most of the works so far dealt with the activity of drugs in terms of their effects on the granuloma formation, and few works were performed in respect to the activity of the drugs on the pre-existing granuloma. In the present study, the effect of drugs on the involution of the pre-existing granuloma, as well as on the inhibition of the granuloma formation was investigated. The effect of drugs on the pre-existing granuloma, if the doses used are proper, is considered to have a closer relationship with their potential use in clinical medicine, because there exists preformed granuloma in most of the chronic inflammatory diseases. When applied to the preformed granuloma, betamethasone was the only agent effective in reducing the weight of the pouch wall, the volume of the fluid and the content of collagen in the granuloma, though the dose used much exceeds that in clinical medicine. But unpublished data showed that hydrocortisone acetate (2 mg daily) also reduced the weight of the granuloma to a half of the control value with mild growth retardation. On the other hand, none of the non-steroidal drugs revealed any activity to reduce the preformed granuloma, even if they were treated at a dose near to lethal (Table 2). It may be concluded, therefore, that non-steroidal drugs are much less potent in their activity on the preformed carrageenin granuloma than steroids. However, it is worth noting that salicylic acid depressed collagen content without any influence on the wet of the granuloma pouch, though the mechanism of its action remains to be elucidated.

Turning to the effect of the drugs in the earlier stage of the granuloma, all the drugs revealed the strong inhibitory action on the accumulation of fluids (Table 1). The potency of the drugs along this line was very close with their potency for inhibiting carrageenin edema.[3, 4] The results with betamethasone in the earlier stage of the granuloma formation are in good accordance with other observations reporting the marked inhibitory action of steroidal drugs in reducing the wet weight of granuloma[23–27] and in inhibiting the collagen synthesis.[28–30] The results with indomethacin are also very similar to those obtained by other investigators.[6] However, phenylbutazone was ineffective in inhibiting the formation of the pouch wall of the granuloma in spite of a large dose near to lethal. Half of the dose tabulated in the Table 1 was also tested without any inhibitory effect. These results were inconsistent with the finding by Winter *et al.*[6] One possible explanation of this discrepancy is that in their cotton pellet method the reduction was determined by weighing the capsular granuloma together with the cotton pellet contaminated with exudate and pus, while in the

present study the activity of the drugs on the exudate and the pouch wall was determined separately. From such a view-point the present method is considered to be better than the classic cotton pellet assay. It is thus suggested that phenylbutazone does not inhibit inflammatory proliferation, though it exhibits a strong inhibition on the fluid exudation. Another explanation may be given by the fact that anti-inflammatory action of drugs is sometimes reversed by several factors involving the quality of the irritants,[5-7] the species and strains of animals used, and the phases of inflammation when drugs are administered.

Some of the toxic effects of the drugs may apparently act as an anti-inflammatory activity. As seen with phenylbutazone and salicylic acid, large doses of drugs may impare the function of some organs or, as seen with most of the drugs, such a toxic effect may produce the growth retardation and, consequently, may inhibit the exudation or the granuloma formation. However, a diet limitation inducing body weight loss was found to display little influence on the carrageenin granuloma in rats. The data accumulated in this laboratory revealed that even if the animals forming or carrying granuloma were fed with a protein-free diet or were starved for 3–4 days, the formation and the maintenance of the granuloma proceeded with only a slight decline (5–20 per cent decrease).

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