Effects of gold salts and prednisolone on inflammatory cells

II. Suppression of inflammation and phagocytosis in the rat

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In the preceding paper (Jessop, Vernon-Roberts, and Harris, 1973) we reported our findings in a study of carbon uptake by inflammatory cells in 'skin-window' exudates in rheumatoid patients receiving gold and prednisolone therapy. The findings suggested that both these anti-inflammatory agents suppressed the elevated phagocytic activity observed in rheumatoid patients not receiving gold or prednisolone. Unlike prednisolone, gold appeared to have a progressive and prolonged inhibitory effect on phagocytosis and on rheumatoid disease activity. In this paper we present our findings obtained in rat experiments designed to assist the interpretation of our clinical studies of inflammatory cell activity in rheumatoid patients and to investigate the apparent differences in the mechanisms whereby gold salts and corticosteroids suppress inflammation.

Material and methods

Adult male Sprague-Dawley rats were used throughout these experiments. They were bred in the animal house of The London Hospital and weighed between 350 and 400 g. when first injected.

ASSESSMENT OF PHAGOCYTOSIS

This was determined using a modification of the 'skin-window' coverslip technique introduced by Rebeck and Crowley (1955). Hair was removed from an area on each side of the dorsal thorax using electric clippers and the skin was shaved and cleaned. The surface of each shaved area was then abraded with a scalpel blade over an area 1 cm. in diameter until tiny bleeding points appeared. Circular glass coverslips, 8 mm. in diameter, previously coated with a dried layer of colloidal carbon (Gunther Wagner C11/1431a) were then applied to the abraded areas (Fig. 1), covered with a gauze dressing, and held in place with a plaster-of-Paris cast completely encircling the thoracic region of the animal (Fig. 2). The coverslips were removed 24 hrs after application, and the adherent inflammatory

FIG. 1 Two carbon-coated glass coverslips applied to abraded skin in the anaesthetized rat

FIG. 2 Plaster-of-Paris cast holding coverslips in place

cells were air-dried, fixed in absolute methyl alcohol, stained with Giemsa, and mounted with the cell-free surface uppermost. The percentage of macrophages and neutrophil polymorphs containing visible endocytosed carbon was assessed by randomly selecting and examining a minimum of 300 cells of each type on each coverslip using an oil-immersion objective (total magnification 1,000 $\times$).

ASSESSMENT OF INFLAMMATORY FLUID EXUDATE
This was determined by the method of Nicol, Quantock, and Vernon-Roberts (1967). Cotton dental pellets (John-

son and Johnson) were dried, individually weighed, steam autoclaved, and re-dried. Two pellets were then implanted subcutaneously into each animal, one in each flank, through a dorsal midline incision under ether anaesthesia using sterile technique. The animals of each dose-response group received pellets of identical weight. Before implantation the dried pellets weighed 8 to 10 mg. 4 days after implantation the pellets were removed. No attempt was made to dissect out the surrounding granulomatous reaction since this procedure reduces the quantitative efficiency of the assay, and the pellets are only slightly adherent to the surrounding tissues at this time. The pellets were then dried at 60°C. for 48 hrs and weighed again. The difference between the initial and final weight of each pellet is the 'granuloma weight'. This is a measure of the local inflammatory response and comprises the protein content of the fluid part of the exudate plus the cellular ingrowth. The fifth day was chosen as the optimum time to remove the pellets since previous studies had shown that there is least scatter about the mean weight gain of the pellets in untreated animals at this time, and also the changes in pellet weight at this time are due to changes in the protein content of the fluid part of the exudate and are not significantly affected by alterations in the cellular content (Nicol and others, 1967).

ASSESSMENT OF INFLAMMATORY CELLULAR EXUDATE

This was determined using the method of Nicol and others (1967). On the fifth day of implantation of cotton pellets, the pellets complete with overlying skin and underlying body wall were removed, fixed in formol-saline, and embedded in methacrylate. After suitable re-orientation of the embedded specimen, 5 μ . sections were cut through the central part of each pellet and were stained with haematoxylin and eosin (Fig. 3). Differential cell counts were made using a graticule eyepiece containing forty squares arranged in a rectangle (4 $\times$ 10 squares). The narrow edge of the rectangle was aligned along the edge of the pellet and a differential cell count was made in an area of pellet 0.08 $\times$ 0.2 mm. Six of these fields were counted in each section, three on the superficial surface

FIG. 3 Section of cotton pellet 4 days after implantation in untreated rat. Darker areas (arrowed) indicate extent of cellular infiltration. Haematoxylin and eosin. $\times 10$

and three on the deep surface. The number of cells in the six areas counted in each section were added together so that the final cell count for each pellet section represented the number seen in an area of 0.096 sq. mm.

ASSESSMENT OF MACROPHAGE MIGRATION

Gold-treated and control rats were given a single intraperitoneal injection of 20 ml. Brewer's thioglycollate medium. The resulting exudate cells (comprising about 90 per cent. macrophages) were harvested 48 hrs later by washing out the peritoneal cavity with medium TC 199 (Wellcome) containing 200 units penicillin/ml. and 100 μ g streptomycin/ml. together with 5 units heparin/ml. The cells were washed and re-suspended in the same medium with 10 per cent. foetal calf serum (Difco) which had previously been heated to 57°C. for 1 hr. Heparinized microhaematocrit tubes (Gelman Hawksley) were filled with cell suspension, sealed at one end with 'Cristaseal' (Gelman Hawksley), and centrifuged at 100 g for 5 min. Each tube was then divided at the cell-supernatant interface and the cell-containing portion placed in the well of a leucocyte migration plate (Sterilin). A minimum of ten tubes was used for each control and gold-treated test animal. After incubation of the chambers for 24 hrs at 37°C., the areas of migration of the cells from the capillary tubes was projected on graph paper using a microprojector, and the outlined areas of paper were cut out and weighed. The weights of the projected areas were used to calculate the means for each group. The percentage inhibition of migration of test groups relative to controls was calculated using the formula.

Percentage inhibition =

$$100 - \frac{\text{mean weight of projected area (test)} \times 100}{\text{mean weight of projected area (control)}}$$

ESTIMATION OF SERUM GOLD LEVELS

Immediately before each animal was killed at the end of each experiment, blood samples were obtained by incising the right axillary vessels under anaesthesia. To 2-ml. aliquots of serum from gold-treated rats was added 1 ml. de-ionized distilled water and 1 ml. 2 per cent. dodecyl sulphate solution. Internal standard gold solutions were prepared by taking 2-ml. aliquots of serum from control rats (not treated with gold) and adding 1 ml. aqueous gold chloride solution (500 μ g./100 ml.) and 1 ml. 2 per cent. dodecyl sulphate solution. The absorbance of paired unknown and standard samples was measured in a Pye Unicam Model SP 90 atomic absorptiometer at a wavelength of 243 m μ ., and the gold concentration in the unknown samples was calculated according to the method of Lorber, Cohen, Chin Chang, and Anderson (1968).

DRUG ADMINISTRATION

Sodium aurothiomalate ('Myocrisin', May and Baker Ltd.) was prepared at various concentrations, so that each dose was contained in 0.5 ml. 0.9 per cent. saline, and was administered by subcutaneous injection. Controls for the gold-treated groups were injected at the same time with 0.5 ml. 0.9 per cent. saline. Prednisolone (Organon Laboratories Ltd.) was prepared at various concentrations so that each dose was contained in 0.2 ml. arachis oil in experiments in which repeated doses were given, or in 0.2 ml. 2.5 per cent. ethyl alcohol when a single dose alone was given. Doses were given by subcutaneous injection,

and controls for the prednisolone-treated groups were injected at the same time with 0.2 ml. arachis oil or 2.5 per cent. ethyl alcohol.

Results

EFFECT OF SODIUM AUROTHIOMALATE AND PREDNISOLONE ON THE PHAGOCYTIC ACTIVITY ON INFLAMMATORY MACROPHAGES AND POLYMORPHS (Table I; Fig. 4)

Groups of rats (ten in each group) were given various doses (Table I) of sodium aurothiomalate or prednisolone on alternate days for 16 days. 24 hrs before they were killed, carbon-coated coverslips were applied for the assessment of phagocytic activity. Examination of stained coverslips revealed that the cell population consisted of approximately 75 per cent. macrophages and 25 per cent. neutrophil polymorphs. Differential cell counts did not reveal any significant differences between the cell populations of treated and control groups (Table I). In both saline-injected and oil-injected controls, about 40 per cent. of the macrophages and 20 per cent. of the polymorphs contained visible aggregates of phagocytosed carbon. When

compared with controls, a significant and progressive reduction in the percentage of macrophages and polymorphs containing carbon was observed as the doses of sodium aurothiomalate and prednisolone were increased (Table I). The reduction was more marked in the case of prednisolone. Analysis of the results showed that there is a linear relationship between the dose of sodium aurothiomalate and prednisolone and the percentage of macrophages and polymorphs containing carbon (Fig. 4). At the end of the experiment there was a significant degree of correlation between the total dose of sodium aurothiomalate and the serum gold level ($r = 0.99$; $P = <0.05$). However, subsequent experiments in which treatment was continued over a longer period have shown that the correlation between total dose of sodium aurothiomalate and serum gold level is lost after 3 to 4 weeks when serum gold reaches a plateau level despite further suppression of phagocytic activity as treatment is continued.

SPEED OF ACTION AND DURATION OF EFFECT OF SINGLE DOSES OF SODIUM AUROTHIOMALATE AND PREDNISOLONE ON PHAGOCYTIC ACTIVITY OF INFLAMMATORY MACROPHAGES AND POLYMORPHS (Tables II and III; Figs 5 and 6, overleaf)

Rats were given a single injection of 5 mg. sodium aurothiomalate or 1 mg. prednisolone in aqueous solution, and controls were given vehicle alone. At the time of injection, and at various intervals thereafter, coverslips were applied for 24-hr periods to test and control groups (ten rats in each group).

In the animals given sodium aurothiomalate (Table II; Fig. 5), serum gold levels were very high 24 hrs after the injection but fell rapidly during the next 2 days and remained at a low level thereafter. The percentages of macrophages and polymorphs containing carbon were not significantly different from control levels 24 hrs after the injection of sodium aurothiomalate, but thereafter fell progressively to reach a minimum level on the sixth day. Whereas the percentage of polymorphs containing carbon had returned to control level by the 10th day, the percentage of macrophages containing carbon remained at a

FIG. 4 Linear relationship between dose of sodium aurothiomalate and prednisolone and percentage of macrophages and polymorphs containing carbon.

Table I Effect of sodium aurothiomalate and prednisolone on phagocytic activity of coverslip macrophages and neutrophil polymorphs in the rat

Treatment	Dose (mg.)		Serum gold ($\mu\text{g. per cent.} \pm \text{s.e.}$)	Percentage cells containing carbon (mean $\pm$ s.e.)		Differential cell distribution (per cent. $\pm$ s.e.)	
	On alternate days	Total		Macrophages	Neutrophils	Macrophages	Neutrophils
Sodium aurothiomalate	1.0	8.0	324 $\pm$ 16	27 $\pm$ 3†	14 $\pm$ 2*	76 $\pm$ 8	24 $\pm$ 6
	5.0	40.0	825 $\pm$ 17	16 $\pm$ 2†	8 $\pm$ 2†	77 $\pm$ 13	23 $\pm$ 8
	10.0	80.0	1025 $\pm$ 2	11 $\pm$ 2†	6 $\pm$ 1†	84 $\pm$ 4	16 $\pm$ 8
Saline controls	—	—	—	44 $\pm$ 3	21 $\pm$ 2	73 $\pm$ 9	27 $\pm$ 10
Prednisolone	0.1	0.8	—	28 $\pm$ 3†	13 $\pm$ 3*	75 $\pm$ 12	25 $\pm$ 9
	1.0	8.0	—	15 $\pm$ 2†	5 $\pm$ 2†	72 $\pm$ 10	28 $\pm$ 6
	5.0	40.0	—	5 $\pm$ 1†	3 $\pm$ 1†	70 $\pm$ 8	30 $\pm$ 3
Oil controls	—	—	—	41 $\pm$ 3	19 $\pm$ 2	81 $\pm$ 11	19 $\pm$ 5

* $P = <0.05$. † $P = <0.01$. ‡ $P = <0.001$.

Table II Duration of effect of a single dose (5 mg.) of sodium aurothiomalate on phagocytic activity of coverslip macrophages and neutrophil polymorphs in the rat

Days after injection	Serum gold ($\mu\text{g. per cent.} \pm \text{s.e.}$)	Percentage cells containing carbon (mean $\pm$ s.e.)			
		Gold-treated		Controls	
		Macrophages	Neutrophils	Macrophages	Neutrophils
1	2148 $\pm$ 170	34 $\pm$ 3	20 $\pm$ 2	42 $\pm$ 3	25 $\pm$ 3
2	1336 $\pm$ 190	31 $\pm$ 2†	17 $\pm$ 2*	43 $\pm$ 3	24 $\pm$ 2
3	230 $\pm$ 10	27 $\pm$ 2‡	16 $\pm$ 1‡	39 $\pm$ 3	27 $\pm$ 2
6	111 $\pm$ 5	20 $\pm$ 2‡	12 $\pm$ 2‡	44 $\pm$ 3	23 $\pm$ 2
10	56 $\pm$ 3	19 $\pm$ 2‡	21 $\pm$ 2	41 $\pm$ 2	24 $\pm$ 2
17	46 $\pm$ 2	25 $\pm$ 3‡	24 $\pm$ 2	45 $\pm$ 3	22 $\pm$ 2
24	29 $\pm$ 1	29 $\pm$ 3*	23 $\pm$ 3	39 $\pm$ 3	25 $\pm$ 3
31	24 $\pm$ 1	36 $\pm$ 3	26 $\pm$ 2	42 $\pm$ 2	23 $\pm$ 2

* $P = < 0.05$. † $P = < 0.01$. ‡ $P = < 0.001$.**Table III** Duration of effect of a single dose (1 mg.) of prednisolone on phagocytic activity of coverslip macrophages and neutrophil polymorphs in the rat

Days after injection	Percentage cells containing carbon (mean $\pm$ s.e.)			
	Prednisolone-treated		Controls	
	Macrophages	Neutrophils	Macrophages	Neutrophils
1	21 $\pm$ 2‡	8 $\pm$ 2‡	46 $\pm$ 3	24 $\pm$ 2
2	42 $\pm$ 5	19 $\pm$ 3	50 $\pm$ 4	27 $\pm$ 3
3	49 $\pm$ 4	26 $\pm$ 3	48 $\pm$ 5	23 $\pm$ 4
4	51 $\pm$ 3	25 $\pm$ 4	47 $\pm$ 3	28 $\pm$ 3

‡ $P = < 0.001$. **FIG. 5** Duration of effect of a single dose of sodium aurothiomalate on serum gold and percentage of macrophages and polymorphs containing carbon

minimum level until the 10th day and did not return to control level until 31 days after the injection.

In contrast to the effects of sodium aurothio-

FIG. 6 Duration of effect of a single dose of prednisolone on percentage of macrophages and polymorphs containing carbon

malate, 24 hrs after the injection of prednisolone the percentages of macrophages and polymorphs containing carbon were markedly reduced but returned to control level during the next 24 hrs (Table III; Fig. 6).

EFFECT OF SODIUM AUROTHIOMALATE AND PREDNISOLONE ON THE FLUID AND CELLULAR PHASES OF THE INFLAMMATORY RESPONSE (Table IV; Fig. 7)

Groups of rats (sixteen rats in each group) were given various doses (Table IV) of sodium aurothiomalate or prednisolone on alternate days for 16 days. Pellets were implanted 4 days before they were killed for the assessment of the inflammatory response. After implantation for 4 days the pellets gained about 8.5 mg. dry weight in both saline-injected and oil-injected controls (Table IV). A significant and progressive reduction in the dry weight gain was observed as the doses of sodium aurothiomalate and prednisolone were increased (Table IV). The reduction was more

FIG. 7 Linear relationship between dose of sodium aurothiomalate and prednisolone and gain in weight and cell count in implanted cotton pellets

Table IV Effect of sodium aurothiomalate and prednisolone on dry weight gain and cell counts in implanted cotton pellets in the rat

Treatment	Dose (mg.)		Serum gold (μ g per cent. $\pm$ s.e.)	Dry weight gain of pellet (mg. $\pm$ s.e.)	Total cell count (mean $\pm$ s.e.)	Differential cell distribution (means $\pm$ s.e.)					
	On alter- nate days	Total				Macrophages		Neutrophils		Fibroblasts	
						Total	%	Total	%	Total	%
Sodium aurothiomalate	1.0	8.0	238 $\pm$ 11	7.3 $\pm$ 0.2†	794 $\pm$ 26‡	739 $\pm$ 24‡	93 $\pm$ 1	53 $\pm$ 6	7 $\pm$ 1	2 $\pm$ 1	<1
	5.0	40.0	463 $\pm$ 13	6.4 $\pm$ 0.1‡	548 $\pm$ 21‡	512 $\pm$ 18‡	94 $\pm$ 1	33 $\pm$ 4*	6 $\pm$ 1	3 $\pm$ 1	<1
	10.0	8.00	547 $\pm$ 8	6.1 $\pm$ 0.1‡	326 $\pm$ 10‡	306 $\pm$ 10‡	94 $\pm$ 1	19 $\pm$ 3†	6 $\pm$ 1	1 $\pm$ 1	<1
Saline controls	—	—	—	8.4 $\pm$ 0.2	1105 $\pm$ 60	988 $\pm$ 44	90 $\pm$ 2	114 $\pm$ 31	10 $\pm$ 2	3 $\pm$ 1	<1
Prednisolone	0.1	0.8	—	6.8 $\pm$ 0.2‡	552 $\pm$ 16‡	509 $\pm$ 12‡	93 $\pm$ 2	42 $\pm$ 12‡	7 $\pm$ 2	1 $\pm$ 1	<1
	1.0	8.0	—	5.7 $\pm$ 0.2‡	336 $\pm$ 20‡	303 $\pm$ 18‡	90 $\pm$ 1	32 $\pm$ 3‡	10 $\pm$ 1	1 $\pm$ 1	<1
	5.0	40.0	—	4.9 $\pm$ 0.1‡	165 $\pm$ 14‡	147 $\pm$ 12‡	90 $\pm$ 1	17 $\pm$ 3‡	10 $\pm$ 1	1 $\pm$ 1	<1
Oil controls	—	—	—	8.5 $\pm$ 0.2	1132 $\pm$ 48	1009 $\pm$ 40	90 $\pm$ 1	121 $\pm$ 18	11 $\pm$ 1	2 $\pm$ 1	<1

* $P = < 0.05$. † $P = < 0.01$. ‡ $P = < 0.001$.

Table V *Capillary tube migration of peritoneal macrophages harvested from rats treated with sodium aurothiomalate*

<i>Gold dose (mg.)</i>		<i>Weight of projected area of migration (mg $\pm$ s.e.)</i>	<i>Significance of difference from controls</i>	<i>Per cent. inhibition of migration</i>
<i>On alternate days</i>	<i>Total</i>			
1.0	9.0	70 $\pm$ 11	P < 0.02	29
5.0	45.0	34 $\pm$ 4	P < 0.001	65
10.0	90.0	15 $\pm$ 4	P < 0.001	83
Controls		102 $\pm$ 6	—	0

tion. A significant and progressive reduction in the areas of macrophage migration was observed as the dose of sodium aurothiomalate was increased (Table V).

Discussion

Using a modified skin-window technique, we have previously shown that the phagocytic activity of inflammatory macrophages and polymorphs is raised in patients with rheumatoid arthritis and is suppressed during treatment with gold salts and prednisolone (Jessop and others, 1973). Using a similar skin-window technique to assess phagocytosis in rats, we have shown that, as in the human, gold salts and prednisolone are effective in inhibiting the phagocytic activity of macrophages and polymorphs migrating to inflammatory sites.

It is widely recognized that gold salts have to be administered for some length of time before suppression of inflammatory activity in joints can be detected clinically in rheumatoid patients, whereas prednisolone has a very rapid action. Also, in rheumatoid patients, cessation of treatment with prednisolone may be followed by a rapid recrudescence of inflammation, whereas, in the case of gold salts, suppression of inflammation may continue for a period of time after withdrawal of treatment. We have investigated the speed of action and duration of effect of single doses of gold salts and prednisolone on phagocytosis in rats using the skin-window technique. After a single injection of gold salts, after an initial 24 hrs delay there is suppression of phagocytic activity lasting 1 week in the case of polymorphs and 4 weeks in the case of macrophages. This difference in the response of polymorphs and macrophages to gold salts may be explained by the fact that mature polymorphs have a very short lifespan whereas macrophages are long-lived cells, and the 'recovery' in the phagocytic activity of the polymorphs after 1 week may be due to the appearance of a significant population of cells or their precursors which have not been in contact with high concentrations of gold in the serum. These findings also help to explain the variability in polymorph scores which we previously observed in rheumatoid patients receiving gold therapy. 24 hrs after a single injection of prednisolone, the phagocytic activity of both macrophages and polymorphs

is markedly reduced, but it has returned to normal levels after 48 hrs. This striking difference between the speed of action and duration of effect of gold salts and prednisolone could be explained on the basis that gold salts have a progressive saturating effect on phagocytic cells. This concept is supported by the finding of a progressive reduction in macrophage phagocytic scores as gold treatment was continued in twelve of sixteen patients (Jessop and others, 1973), the persistence of gold circulating in the blood for at least one month after gold injection in animals and man, and the persistence of demonstrable gold in the tissues long after the cessation of chrysotherapy (Vernon-Roberts and Jessop, 1971; Vernon-Roberts, Doré, and Jessop, 1973, in preparation). In contrast, the rapid and relatively brief suppression of phagocytosis by prednisolone is probably related to the short biological half-life of the corticosteroids resulting from the rapid metabolism of administered prednisolone to metabolites not possessing anti-inflammatory activity.

The mechanisms whereby gold salts and prednisolone inhibit phagocytic activity have not been fully elucidated, but various possibilities exist. The macrophages and polymorphs which appear at inflammatory sites are known to be largely derived from precursors situated in the bone marrow and to migrate from the blood into inflamed areas (Volkman and Gowans, 1965a, b) where they are active in phagocytosing and digesting 'foreign' matter and damaged host tissues. Drugs possessing anti-inflammatory activity may reduce the numbers of phagocytes at inflammatory sites by inhibiting the formation or release of monocytes (immature macrophages) and polymorphs in the bone marrow, and by inhibiting the migration of cells from the blood stream into the inflamed area. There is evidence that glucocorticoids inhibit the release of monocytes from the bone marrow pool (Thompson and van Furth, 1970), and that they interfere with the margination and sticking of leucocytes to vascular endothelium (Allison, Smith, and Wood, 1955) by preventing the changes in the surfaces of endothelial cells and leucocytes caused by substances released from the tissues after injury (Allison, 1965). The findings presented here confirm previous reports (Nicol and others, 1967) that the number of macrophages and polymorphs in cotton-

pellet granulomas are reduced by treatment with glucocorticoids, and show that gold salts similarly reduce the number of inflammatory cells in experimental granulomas. We have shown that treatment with gold salts inhibits the migratory activity of inflammatory macrophages *in vitro*, and others have shown that glucocorticoids similarly stabilize the cell membrane of macrophages and bring about an inactive state (Eyring and Dougherty, 1955; Dougherty, 1961). We have also shown that, like prednisolone, gold salts reduce the amount of inflammatory fluid exudate, presumably by inhibiting increased capillary permeability in some way.

There is evidence that, besides reducing the numbers of actively phagocytic cells present at inflammatory sites, prednisolone and gold salts may inhibit phagocytic and digestive activity by a direct effect on macrophages and polymorphs, not only by stabilizing the cell membranes and inducing a quiescent state, but also by stabilizing the membranes enclosing lysosomes and preventing the release or activation of their digestive enzymes (Allison, 1965; Weissmann, 1966; Persellin and Ziff, 1966; Ennis, Granda, and Posner, 1968).

While it is universally accepted that prednisolone

effectively suppresses inflammation under clinical and experimental conditions, recent studies have produced conflicting results concerning the effectiveness of gold salts in suppressing adjuvant arthritis in rats (Jessop and Currey, 1968; Walz, Di Martino, and Misher, 1971). We have here produced experimental evidence that gold salts are effective in suppressing the cellular and fluid phases of the early inflammatory response and the phagocytic activity of inflammatory cells, but differ from prednisolone in potency, speed of action, and duration of effect.

Summary

Sodium aurothiomalate ('Myocrisin') and prednisolone inhibit the cellular and fluid phases of the inflammatory response, and suppress the phagocytic activity of inflammatory macrophages and polymorphs. Sodium aurothiomalate also inhibits the migration of exudate macrophages. Sodium aurothiomalate differs from prednisolone in potency, speed of action, and duration of effect.

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