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Effects of salicylates on blood changes in mycoplasma arthritis in rats

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Summary

1. The polyarthritis produced in rats by i.v. inoculation with *Mycoplasma arthritidis* was made more severe by salicylates.

2. The infection increased the erythrocyte sedimentation rate (ESR), serum lysozyme, counts of total white blood corpuscles, polymorphonuclear cells and lymphocytes, haemolytic serum complement (CH 50) and its component C3. Salicylates enhanced the rise in ESR, CH 50 and C3, but suppressed the rise in lymphocytes and even induced a fall.

3. Salicylates did not interfere with the development and action of metabolic inhibition antibodies against *M. arthritidis*, and did not promote the growth of *M. arthritidis*.

4. Rats treated with salicylate during the first infection acquired the same immunity to reinfection as did infected controls.

5. Salicylates did not render rats susceptible to *M. fermentans* which is non-pathogenic to rats, but may be involved in human rheumatoid arthritis.

Introduction

Several species of *Mycoplasma* produce polyarthritis in various animals. In the fowl, arthritis is caused by *Mycoplasma synoviae* and *M. gallisepticum*, in pigs by *M. hyorhinis* and *M. granularum*, in goats and sheep by *M. agalactiae* and in mice by *M. pulmonis* and *M. arthrotropicus* (Sharp, 1970). Inoculation with *M. arthritidis* produces an acute polyarthritis in rats. The disease also occurs spontaneously (Jasmin, 1967; Collier, 1939; Ward & Cole, 1970; Hannan & Hughes, 1971). In addition to arthritis, infected rats develop urethritis, rhinitis, conjunctivitis, corneal opacities, and flaccid limb paralyses.

Involvement of Mycoplasmas in human rheumatoid arthritis is suggested by the reported isolation of *M. fermentans* (Williams, 1968) and *M. hominis* II (Jansson, Makisara, Vainio, Vainio, Snellman & Tuuri, 1971) from the tissues and exudates of rheumatoid joints. The *in vitro* migration of leucocytes in such patients is inhibited in the presence of *M. fermentans*, which suggests that specific delayed hypersensitivity to this micro-organism may develop in rheumatoid arthritis (Williams, Brostoff & Roitt, 1970).

In view of these findings, the effects of drugs on experimental Mycoplasma arthritis are of potential clinical interest. However, such investigations require a more precise assessment of the disease than is possible by the scoring of joints.

In the present study this was attempted by following blood changes associated with inflammation throughout the disease.

The effects of salicylates on the polyarthritis induced in rats by *M. arthritidis* are described

Methods

Inoculation of rats. Male Wistar rats of 150–200 g were used. *M. fermentans* (P.G. 18) was cultured as described by Williams *et al.* (1970). *M. arthritidis* (Jasmin 14124) was cultured in the same way, except that the medium contained 1 per cent of arginine instead of glucose. Rats were lightly anaesthetized with ether, and 0·3 or 0·5 ml of cultures containing 10^7 micro-organisms per ml were injected into the tail vein.

Collection of blood. No. 23 gauge (0·65 mm bore) cannulae, attached to 1·5 mm bore polythene tubing rinsed with heparin, were inserted into the tail vein, and 0·5 ml of blood withdrawn (Eisen & Loveday, 1971).

Evaluation of arthritis. The inflammatory changes in joints were scored on an arbitrary scale of 0 to 6 units. The scoring observer did not know which treatment rats were receiving.

Erythrocyte sedimentation rate (ESR). A column of approximately 100 mm of blood was sucked into haematocrit tubes (Gelman & Hawksley, Ltd.) and the tubes planted upright into a 'Seal-ease' tube sealer and holder (Clay-Adam Inc.) Sedimentation was read with a ruler after 120 min and expressed as a percentage of the total height of the blood column.

Blood cell counts. Blood was diluted in Barr's fluid and white blood cells counted in improved Neubauer chambers. For differential cell counts, blood smears were stained with Leishman's stain and a brief haemotoxylin counterstain.

Serum complement. Total haemolytic complement was measured as 50% haemolytic units (CH50) by the method of Osler Strauss & Mayer (1952). All volumes were reduced to one-fifth of those in the original method.

Complement component C3. Rat C3 (β_1C/β_1A) was adsorbed on to boiled Zymosan particles by the method of Steinbuch, Quentin and Pejaudier (1963), and the particles injected into rabbits to produce antiserum to rat C3 (Mardiney & Muller-Eberhard, 1965). The C3 levels in rat sera were measured by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965), and expressed as the percentage of the level in pooled rat serum.

Plasma lysozyme. Forty μl of rat plasma was mixed with 480 μl of a 0·3% suspension of *Micrococcus lysodeicticus* (Boehringer Corp.) in 0·05 M phosphate-saline buffer, pH 7, placed into microcells (light path 5 mm), and the clearing of the suspension followed at 25° C and at 436 nm (Shugar, 1952). A change in optical density of 0·0005 per min was regarded as 1 unit of activity.

Serum antibodies inhibiting Mycoplasma *growth*

The metabolic inhibition test of Purcell, Taylor-Robinson, Wong & Chanock (1966) was used. The amount of complement was doubled so as to overcome the anti-complementary action of *M. arthritidis* in rat serum.

Effect of salicylates on M. arthritidis *growth.* Serial twofold dilutions of *M. arthritidis* cultures were incubated for 18 h with a constant concentration of sodium

salicylate. Each well was then serially diluted in medium and the growth assessed by the method of Purcell *et al.* (1966).

Results

As observed by Hannan & Hughes (personal communication), the polyarthritis produced in the rat by *M. arthritidis* is, in contrast to adjuvant arthritis, made more severe by salicylates. Figure 1 shows the joint score of infected rats, and the effects of sodium salicylate 200 mg/kg given by mouth daily and twice weekly.

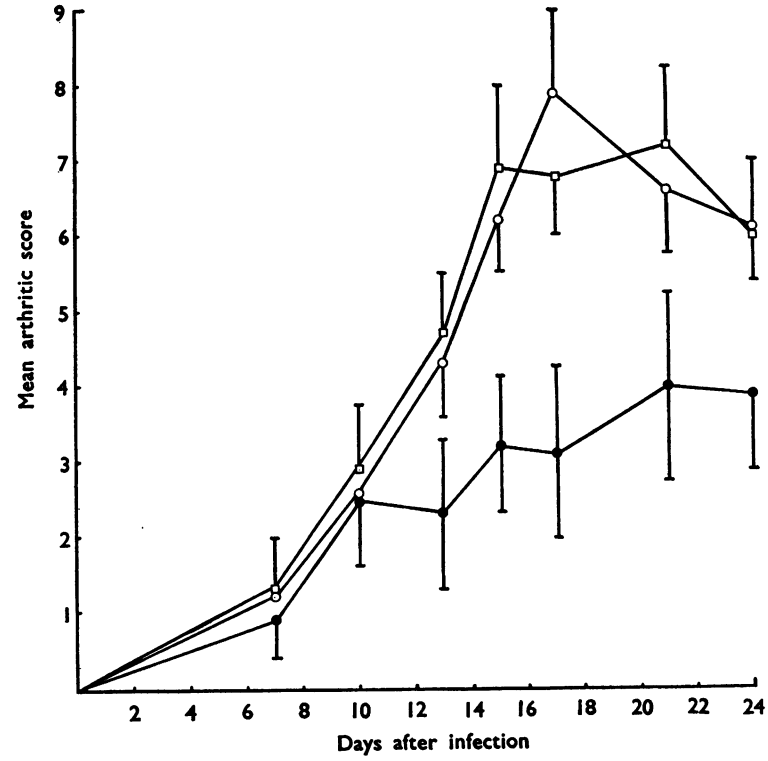

FIG. 1. The polyarthritis produced by 5×10^6 *M. arthritidis* i.v. (●) was rendered more severe by sodium salicylate 200 mg/kg given by mouth daily (□) or twice weekly (○) from the day of inoculation (day 0). Groups of ten rats were used. Mean scores ± S.E.M. are shown.

Since all blood changes were followed in individual rats, it was possible to test by paired *t* tests the significance of the changes observed in several experiments (Table 1). The table also shows that salicylates significantly influenced the reaction to the infection as expressed by ESR, total white blood cell (w.b.c.) counts, and complement levels.

The time-course of the blood changes was fairly well correlated with the development of the polyarthritis. This is illustrated in Fig. 2 which shows the changes in ESR and lysozyme observed in one experiment.

There was some variation in the effect of salicylate on total w.b.c. counts. The characteristic rise in the counts was suppressed by salicylate either during the

TABLE 1. *Effects on blood of infection with* Mycoplasma arthritidis (*Group* 1) *and of the same infection plus sodium salicylate* (*Group IS*)

		Days after infection						
		0	3	7	11	14	19	28
ESR	I (10)	5·03±0·67	28·0±3·17‡	—	18·82±2·83‡	6·13±0·96	3·77±0·62	5·24±1·52
	IS (10)	5·17±0·63	30·5±3·33‡	—	29·83±3·83†	13·98±2·54†	8·32±3·04	4·60±1·30
	DM	NS	NS		<0·05	<0·05	NS	NS
Plasma lysozyme	I (10)	69·12±6·34	169·5±20·98‡	136·7±16·20†	140·0±13·46‡	140·2±16·22†	117·5±8·82†	125·7±7·59†
	IS (10)	47·66±5·03	163·4±13·51‡	142·8± 9·14‡	130·2±10·11‡	153·5± 9·81‡	115·5±7·14‡	113·4±6·73‡
	DM	NS	NS	NS	NS	NS	NS	NS
Total wbc count	I (20)	9800±309	11700±2166*	17267±3176‡	14285±2777†	—	12785±1465*	11785±4810
	IS (20)	9550±283	11300± 895*	9777±1027	9850±3283	—	10100± 862	8714± 662
	DM	NS	NS	<0·01	<0·001	—	NS	NS
Polymorph count	I (20)	1672±162	4285±590‡	4937±902†	3168±437†	—	2467±220*	2887±468
	IS (20)	1791± 89	4880±541‡	4124±563‡	3813±530†	—	3272±318†	1954±220
	DM	NS	NS	NS	NS	—	NS	NS
Lymphocyte count	I (20)	7895±284	7232±680	12000±2341*	10900±867*	—	10128±1086	8614±613
	IS (20)	7605±261	6148±717	5397± 572*	5800±542†	—	6602± 618	6164±594
	DM	NS	NS	<0·01	<0·001	—	<0·05	NS
Total serum CH50	I (9)	36·10±2·27	28·31±2·11†	63·65±4·01‡	64·50±5·37‡	45·56±3·93†	45·92±3·16	37·87±2·93
	IS (9)	37·55±2·52	38·52±2·5	67·16±5·25‡	84·55±7·25‡	54·22±4·42†	50·12±2·05†	44·81±1·59*
	DM	NS	<0·02	NS	<0·02	NS	NS	NS
Serum C3	I (9)	84·3 ±1·53	82·6±3·86	101·2±2·36†	105·4±2·81‡	103·8±2·03‡	102·2±3·68‡	90·14±1·37
	IS (9)	87·37±1·30	88·5±2·53	104·2±1·93‡	114·3±2·1‡	110·0±2·59‡	108·3±1·90‡	98·91±1·73‡
	DM	NS	NS	NS	<0·01	<0·01	<0·05	<0·01

Means ± S.E.M. are given. Figures in brackets after I and IS show number of rats in group. DM=significance of difference (*P*) between means of I and IS groups; NS=not significant. *=Means significantly different with $P<0·05$ (paired *t* test) from means before infection (day 0). †=As * but with $P<0·01$. ‡=As * but with $P<0·001$.

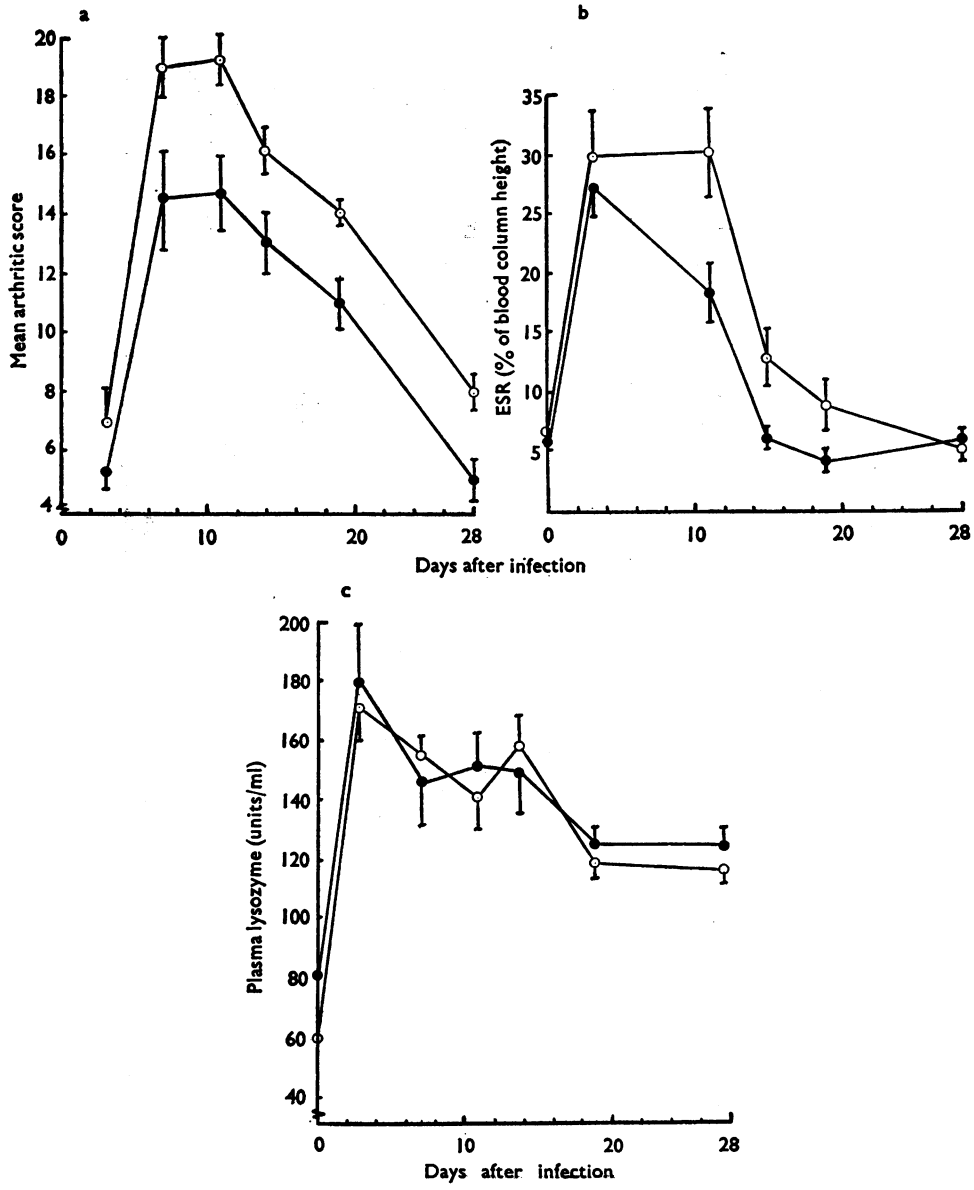

FIG. 2. During the polyarthritis (a) produced by 5×10^6 *M. arthritidis* i.v. (●), ESR (b) and plasma lysozyme (c) were raised. The enhancement of this polyarthritis produced by sodium salicylate 200 mg/kg daily (○), was accompanied by higher ESR (b) but not by higher plasma lysozyme levels (c). Groups of ten rats were used.

entire course of the disease (Fig. 3a), or only during the first part of it (Fig. 3b). The suppression was not due to an effect on polymorphonuclear cells, since the increase in these cells was not affected by salicylate. In contrast salicylate clearly interfered with the rise in lymphocytes. Whilst the counts rose in infected control rats, most salicylate-treated rats showed falls in counts particularly at 7 and 11 days. At this time, the difference between the groups was very definite ($P \ll 0{\cdot}01$), in spite of the considerable scatter of individual counts as illustrated by the experiment shown in Figure 4.

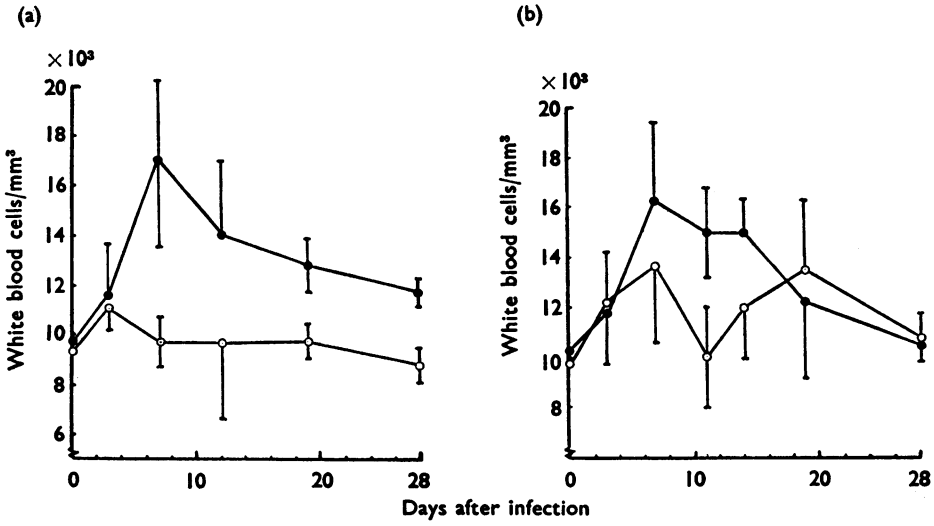

FIG. 3. The rise in total w.b.c. counts seen in rats infected with 5×10^6 *M. arthritidis* i.v. (●) was suppressed by sodium salicylate 200 mg/kg twice weekly (○) either during the entire course of the disease (a) or during the first part of it (b). Groups of ten rats were used. Means ± S.E.M. are shown.

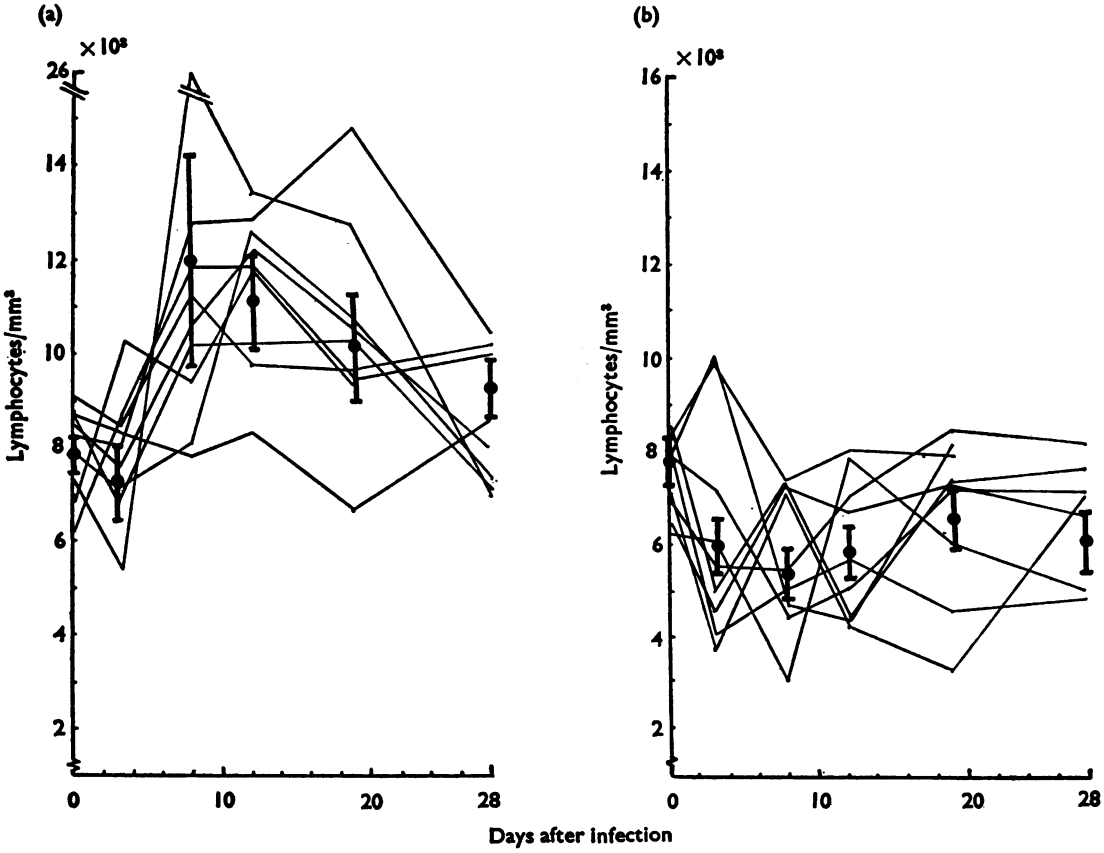

FIG. 4. The rise in lymphocytes induced in rats by infection with 5×10^6 *M. arthritidis* i.v. (a) was suppressed, and even converted into a fall, by sodium salicylate 200 mg/kg twice weekly (b). The sequential counts in individual rats, and group means ± S.E.M. are shown.

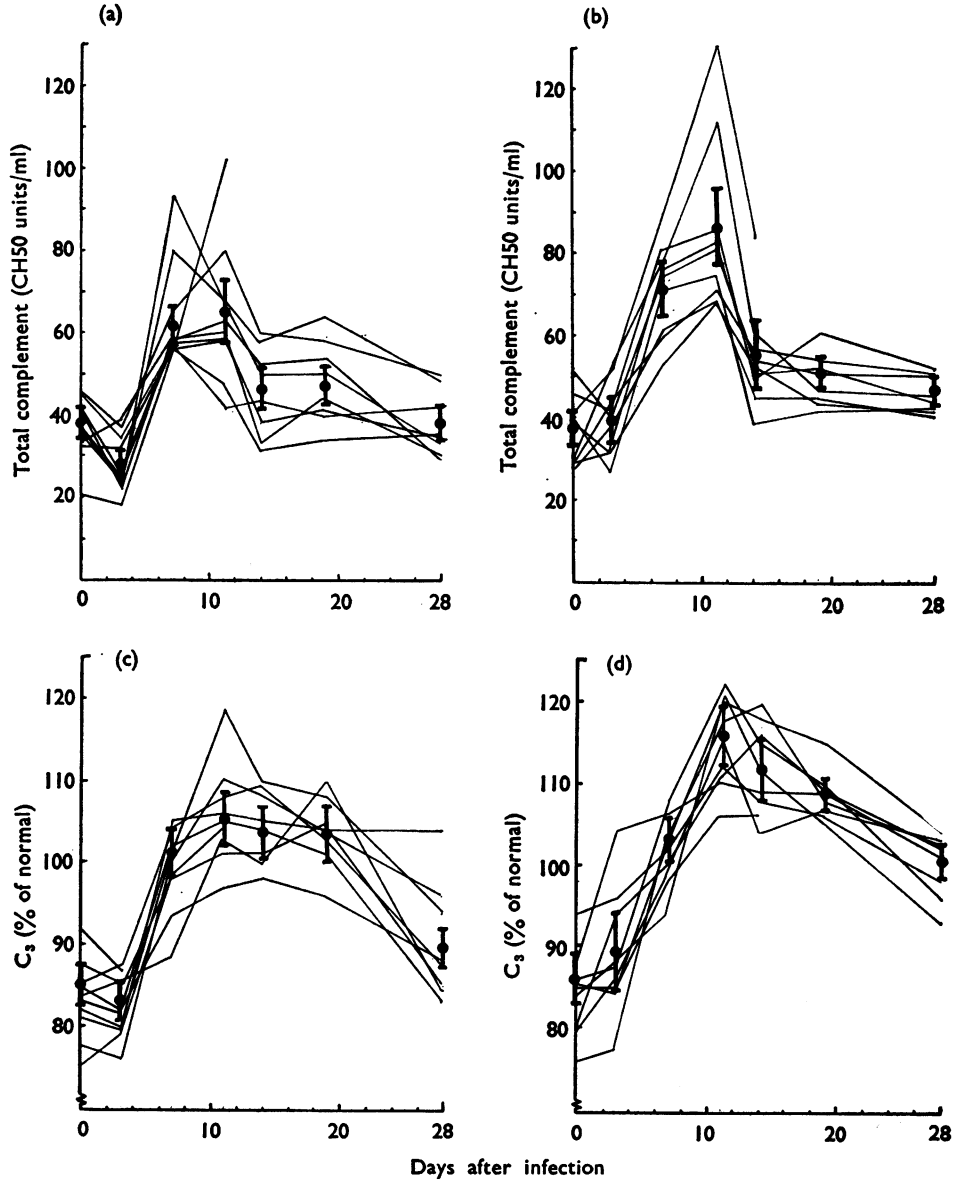

FIG. 5. Infection with 5×10^6 *M. arthritidis* i.v. first reduced and then increased total haemolytic complement (CH50) titres (a); sodium salicylate 200 mg/kg twice weekly abolished the fall and enhanced the rise (b). Levels of component C3 (shown as percentage of level in pooled normal rat serum) were increased by the infection (c); this was enhanced by salicylate (d). Sequential values in individual rats and group means ± S.E.M. are shown.

Complement changes followed a more consistent pattern (Fig. 5a, b). After a fall during the first three days following infection, CH50 titres rose sharply. In the salicylate-treated group the initial fall was less frequent at the three day interval; however, the subsequent increase was significantly potentiated. C3 levels changed in a similar way (Fig. 5c, d). The rise in levels was even more pronounced, and was considerably enhanced by salicylates.

TABLE 2. *Titres of metabolic inhibition antibodies in rat sera at* 19 *and* 28 *days after infection with* Mycoplasma arthritidis

Day	Treatment	Reciprocal of titre 8	16	32	64	128
19	No salicylate	1	6	3		
	Salicylate	1	8	1		
28	No salicylate		1	4	5	
	Salicylate		2	3	4	1

The number of rats with a given titre is shown.

No evidence was detected that sodium salicylate 200 mg/kg twice weekly affected the development in infected rats of antibodies which inhibit the metabolism of *M. arthritidis* (Table 2).

Added *in vitro*, 3–125 mM salicylate (5–200 μg/ml) did not interfere with the action of metabolic inhibition antibodies on the growth of *M. arthritidis* cultures. The growth of *M. arthritidis* and *M. fermentans* in medium cultures was not promoted by salicylates.

M. fermentans which is possibly involved in the pathogenesis of human rheumatoid arthritis (Williams, 1968; Williams *et al.*, 1970) has no known pathogenic action in the rat. Attempts to render rats susceptible to an intravenous inoculation with *M. fermentans* by treating them with sodium salicylate 200 mg/kg twice weekly for 10 weeks, were not successful.

Acetylsalicylic acid 200 mg/kg daily or twice weekly resembled sodium salicylate in its exacerbating effect on the arthritis produced in rats by *M. arthritidis*.

Reinfection combined with salicylates

Two groups of rats were infected with *M. arthritidis*, or infected and treated with sodium salicylate 200 mg/kg daily. Six weeks later, after recovery, both groups were reinfected and treated with salicylate. Neither group developed arthritis. The results suggested that salicylate interfered neither with the development of immunity, nor with the functioning of the established immune mechanisms.

Discussion

The evidence that Mycoplasma or other micro-organisms (Williams, 1968; Sharp, 1970; Duthie, 1971)—acting as pathogens or as antigens—may be amongst the causative factors of human rheumatoid arthritis, poses two questions for investigation:

1. Do any of the drugs used in rheumatoid arthritis act by suppressing some of the suspected micro-organisms?
2. Do drugs effective against these micro-organisms have a beneficial effect in rheumatoid arthritis?

The first question was studied here with salicylates, and the results did not reveal any effect against *M. arthritidis in vitro* or *in vivo*. In fact, salicylates exacerbated the experimental disease. The mechanism of this effect requires further study; a contributory factor could be the prevention by salicylates of the increase in circulating lymphocytes occurring normally during the infection. Muirden & Mills (1971) have reported evidence that a copious infiltration of the synovium by lymphocytes protects the rheumatoid joint against the characteristic damage of the cartilage and bone. The degree of this infiltration was not correlated with salicylate treatment

of the patients. It is however possible that in the higher doses applied in the rats, salicylates may reduce the increase in lymphocytes not only in blood but also in synovial tissues. In patients with rheumatic fever, salicylates depress the leucocytosis by an unknown mechanism (Goodman & Gilman, 1970). The effect is associated with, and may depend on the general depression of all inflammatory changes in rheumatic fever. In contrast, the suppression of the lymphocytosis in the infected rats was accompanied by enhanced arthritis.

Although salicylates suppressed the lymphocytosis in infected rats, this did not lead to lower levels of metabolic inhibition antibodies. When added *in vitro,* even high concentrations of salicylate did not reduce the action of these antibodies on *M. arthritidis* growth. The interaction of metabolic inhibition antibody with *M. arthritidis* antigen appears to differ in this respect from the precipitation reaction of egg albumen or of type III pneumococcus polysaccharide with rabbit antiserum ; these reactions are potently inhibited by salicylate (Coburn & Kapp, 1943 ; Friend, 1953 ; Austen, 1963).

The present results therefore do not support the view that salicylate exacerbated the Mycoplasma arthritis in rats by impairing the production or effectiveness of antibodies.

The higher complement levels induced by salicylate could conceivably improve the efficiency of the immune defences. On the other hand, this increase may contribute to the more severe polyarthritis, because active complement generates several factors which promote inflammation (Lachmann, 1969). The initial fall in complement appears to be due to a direct reaction between *M. arthritidis* and factors in rat serum, since the fall can be demonstrated *in vitro* (unpublished observation). The amount of complement bound and/or consumed by this reaction proved to be so large that additional complement had to be supplied for measurements of metabolic inhibition antibodies. This phenomenon is being further studied.

It remains to be investigated whether salicylates exacerbate first infections by impairing defence activities of cells, such as the prompt uptake of Mycoplasmas by leucocytes (Zucker-Franklin, Davidson & Thomas, 1966a, b) or cell-mediated immune reactions.

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