Assessment of anti-inflammatory drugs in the rat using subcutaneous implants of polyurethane foam impregnated with dead tubercle bacilli

A. K. CLARKE, B. VERNON-ROBERTS, AND H. L. F. CURREY

From the Bone and Joint Research Unit, The London Hospital Medical College, Turner Street, London, E1 2AD

Clarke, A. K., Vernon-Roberts, B., and Currey, H. L. F. (1975). *Annals of the Rheumatic Diseases*, 34, 326-331. Assessment of anti-inflammatory drugs in the rat using subcutaneous implants of polyurethane foam impregnated with dead tubercle bacilli. The fluid and cellular phases of inflammatory response have been measured using a technique employing subcutaneous implantation of polyurethane foam cubes impregnated with heat-killed *Mycobacterium tuberculosis*. Phenylbutazone, azathioprine, aspirin, cyclophosphamide, and prednisolone suppressed fluid response, whereas sodium aurothiomalate, hydroxychloroquine, and D-penicillamine had no effect. All the drugs used suppressed the infiltration of inflammatory cells into the cubes.

Laboratory tests of the anti-inflammatory action of drugs are based upon the suppression of some feature of the inflammatory response such as the migration of leucocytes, changes in blood flow, and vascular permeability, or the formation of granulation tissue and oedema. The weight of granulation tissue formed and the effects of drugs in suppressing it are the basis of the granuloma pouch test (Selye, 1953) and techniques employing the implantation of cotton pellets (Meier, Schuler, and Desaulles, 1950), polyvinyl sponges (Boucek and Noble, 1955), and other irritant materials. These techniques, and those which rely on the measurement of oedematous tissue swelling after the injection of irritants such as carrageenan, dextran, ovalbumin, formalin, etc., fail to separate the relative contributions of fluid and cellular exudation to the inflammatory reaction.

We have attempted to obtain this important information by implanting subcutaneously in the rat polyurethane foam cubes impregnated with an irritant. Such implants can be recovered after various intervals and processed in different ways for separate analysis of the fluid exudate and the infiltrating cells. In previous studies in which pieces of foam have been implanted subcutaneously in experimental animals it has been the irritant nature of the foam itself which has provoked an inflammatory response and the interest

in these models has been largely centred upon the later, fibrotic, stages of the inflammatory reaction (Bole and Heath, 1967; Kaltiala and Heikkinen, 1971). This report describes the effects of a variety of anti-inflammatory agents, commonly used in the treatment of rheumatic diseases, on the cellular and fluid phases of the inflammatory response to polyurethane cubes impregnated with heat-killed *Mycobacterium tuberculosis*.

Materials and methods

ANIMALS

Female Wistar or Sprague-Dawley rats weighing 150 to 250 g were used. Unless otherwise specified, each test group contained 8 animals.

PREPARATION OF CUBES

Polyurethane foam (Dunlopillo grade D12, Dunlop) was cut into cubes measuring slightly less than 1 cm³ and weighing 17 mg. Some of the cubes were then impregnated with heat-killed *M. tuberculosis* (TBC) (Tuberculin Section, Ministry of Agriculture, Fisheries and Food) by immersing and compressing them in an aqueous suspension of TBC (0.5 mg/ml) followed by drying overnight at 37°C (to constant weight). Each cube then contained approximately 0.33 mg dry TBC, and microscopical examination of suitably stained sections showed this to be distributed evenly through the cube. Before implantation cubes were

sterilized in ethylene oxide. Control (nonimpregnated) cubes were processed similarly except that the TBC were omitted.

IMPLANTATION OF CUBES

Under ether anaesthesia and using a sterile technique, cubes were implanted (one into each flank) through a dorsal midline incision. Each animal received an intraperitoneal injection of 20 mg chloramphenicol to prevent bacterial infection.

ASSESSMENT OF INFLAMMATORY FLUID EXUDATE

When cubes were removed, care was taken not to squeeze out any of the contained fluid. Cubes were then dried at 37°C overnight (to constant weight). The difference between the initial and final dry weight of each cube was recorded as the 'dry weight gain'. Preliminary studies in which rats were implanted with a TBC-impregnated cube in one flank and a nonimpregnated cube on the opposite side established that there was a reproducible and progressive daily increase in the dry weight gain of the irritant implant, while the dry weight gain of the nonirritant cubes remained relatively constant for the first 6 days (Fig. 1). This consideration led to the choice of 5 days for the duration of the experiments described below. Further preliminary studies employing trypsinization and separate analysis of the cellular and fluid contents had established that on day +5 cells accounted for only about 12.5% of the dry weight gain in the impregnated cube, and for about 2.5% in the nonirritant implants.

ASSESSMENT OF INFLAMMATORY CELLULAR EXUDATE

On the fifth day after implantation, cubes complete with overlying skin and underlying body wall were removed, fixed in formol-saline, and embedded in wax. 5 µm sections were cut through the central part of each cube and were stained with haematoxylin and eosin. A calibrated eyepiece was used to measure the depth of cellular invasion from the edge of the cube at three points on the superficial surface, three points on the deep surface, and two points on each of two remaining surfaces. The mean of these ten measurements gave the 'depth of cell invasion' for each cube. Preliminary experiments, similar to those described above, had indicated the progressive changes in this measurement after implantation (Fig. 2) and had shown that there was a marked difference between the control

FIG. 1 Dry weight gain (mean $\pm$ 2 SE) of polyurethane foam cubes impregnated with tubercle bacilli compared with dry weight gain of nonimpregnated cubes

FIG. 2 Comparison of depth of cellular invasion into cubes impregnated with tubercle bacilli and into nonimpregnated cubes examined at various intervals after implantation

and impregnated cubes on day +5. Preliminary studies had also established that this method of assessing the cellular infiltration correlated well with results obtained by actually counting the numbers of cells in chosen fields.

ADMINISTRATION OF DRUGS

Sodium aurothiomalate (Myocrisin, May and Baker) was given by intramuscular injection on alternate days. Prednisolone suspended in arachis oil was given by intraperitoneal injection. The remaining drugs were administered as a single daily dose via oral tube. The following preparations were employed.

Drug	Preparation	Vehicle
Hydroxychloroquine	Plaquenil, Winthrop	Distilled water
D-Penicillamine	Distamine, Dista	Deionized water
Phenylbutazone	Butazolidin, Geigy	0.25% carboxymethyl-cellulose
Azathioprine	Imuran, Wellcome	0.25% carboxymethyl-cellulose
Aspirin	Aspirin, B.P.	Distilled water
Cyclophosphamide	Endoxana, Ward	Distilled water
Nil (controls)	Nil	0.25% carboxymethyl-cellulose

Results

EFFECT OF VARIOUS DRUGS ON DRY WEIGHT GAIN OF CUBES IMPREGNATED WITH *M. TUBERCULOSIS* (TABLES I AND II; FIG. 3)

FIG. 3 Graph illustrating relative suppression of dry weight gain of tubercle-impregnated cubes by increasing doses of prednisolone (PRED), cyclophosphamide (CY), azathioprine (AZ), phenylbutazone (PBZ), and aspirin (ASP)

Table I *Effect of various anti-inflammatory agents on dry weight gain of polyurethane cubes impregnated with heat-killed M. tuberculosis*

<i>Drug</i>	<i>Route of administration</i>	<i>Frequency of administration (day 0 = day of cube implantation)</i>	<i>Daily dose (mg/kg)</i>	<i>Dry weight gain of cubes (mg + SE)</i>
Sodium aurothiomalate	Intramuscular	Alternate days -10 to +4	12.5	84.7 ± 3.2
			25	85.6 ± 2.6
			50	84.8 ± 2.7
			100	85.3 ± 3.3
Hydroxychloroquine	Oral	Daily -10 to +4	6.25	87.0 ± 3.7
			12.5	86.9 ± 2.5
			25	80.1 ± 1.8
			50	86.5 ± 2.6
D-Penicillamine	Oral	Daily -10 to +4	25	78.5 ± 2.0
			50	79.3 ± 2.2
			100	69.7 ± 3.5*
			200	75.6 ± 3.6
Phenylbutazone	Oral	Daily 0 to +4	37.5	69.3 ± 1.9†
			75	64.9 ± 1.9†
			150	63.5 ± 1.9†
			300	60.8 ± 3.0†
Azathioprine	Oral	Daily 0 to +4	25	80.5 ± 2.0
			50	75.4 ± 3.9
			100	60.0 ± 3.6†
			200	54.9 ± 6.1†
Aspirin	Oral	Daily 0 to +4	100	78.9 ± 2.0
			200	70.3 ± 2.0†
			400	63.9 ± 2.2†
			800	53.6 ± 0.9†
Cyclophosphamide	Oral	Daily 0 to +4	6.25	77.2 ± 2.1
			12.5	63.1 ± 2.0†
			25	54.0 ± 1.3†
			50	49.0 ± 2.4†
Prednisolone	Intra-peritoneal	Daily 0 to +4	6.25	66.1 ± 1.6†
			12.5	52.4 ± 2.3†
			25	39.0 ± 1.3†
			50	41.4 ± 2.5†
Controls	—	—	—	82.1 ± 2.7

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

Groups of rats (8 in each group) were given one of the drugs in the range of doses shown in Table I. Each rat was implanted on day 0 with an impregnated cube into either flank and all cubes were removed on day +5. Considerations regarding the duration of treatment that may be necessary to produce an anti-inflammatory effect with some of these drugs led us to administer sodium aurothiomalate, hydroxychloroquine, and D-penicillamine from day -10 to day +4, and the remaining drugs from day 0 to day +4.

Table I and Fig. 3 show the results. The magnitude of the anti-inflammatory effect achieved is indicated by comparing these figures with those in Fig. 1. Control (nonimpregnated) cubes were not included in this experiment, but preliminary testing had

established that neither azathioprine nor phenylbutazone given in daily doses of 200 mg/kg produced significant reduction in the weight of nonimpregnated cubes. In the doses tested, sodium aurothiomalate and hydroxychloroquine had no effect on dry weight gain, while D-penicillamine produced a significant reduction only at a daily dose of 100 mg/kg (with no significant effect at doses above and below this level). By contrast, phenylbutazone, azathioprine, aspirin, cyclophosphamide, and prednisolone all caused a progressive reduction in dry weight gain with increasing daily doses.

Table II shows the correlation and regression coefficients relating logarithmic daily dose of the effective drugs to dry weight gain of the cubes.

Table II Correlation and regression coefficients relating doses of various anti-inflammatory agents to dry weight gain of polyurethane cubes impregnated with heat-killed *M. tuberculosis*

Treatment	Correlation coefficient <i>r</i>	Significance of <i>r</i>	Regression coefficient <i>m</i>	Significance of <i>m</i>
Phenylbutazone	0.98	$P < 0.02$	-8.96	$P < 0.001$
Azathioprine	0.98	$P < 0.01$	-30.75	$P < 0.001$
Aspirin	0.99	$P < 0.01$	-27.25	$P < 0.001$
Cyclophosphamide	0.98	$P < 0.05$	-30.91	$P < 0.001$
Prednisolone (all doses)	0.91	$P < 0.1$	-29.03	$P < 0.001$
Prednisolone (excluding highest dose)	1.00	$P < 0.001$	-45.16	$P < 0.001$

Examination of the correlation coefficients shows that there is a linear relationship between the dose of phenylbutazone, azathioprine, aspirin, and cyclophosphamide and dry weight gain. This is not the case when all four doses of prednisolone are used to calculate the correlation coefficient for this drug ($r = 0.91$; $P < 0.1$). However, approximately equal suppression of weight gain was achieved by the two higher doses of prednisolone, and exclusion of the highest dose from the calculation reveals that perfect linear correlation exists between the dose of prednisolone and weight gain (Table II, Fig. 3). The regression coefficients reveal parallelism of the dose-response 'line of best fit' for azathioprine, aspirin, and cyclophosphamide; by contrast, phenylbutazone has a much shallower dose-response line and prednisolone a

steeper line. Since there was a marked difference between the regression coefficient for phenylbutazone (-8.96) and the regression coefficients for azathioprine, aspirin, and cyclophosphamide (all in the region of -30), the effect of phenylbutazone was investigated again using a different range of daily dose (5, 10, 20, and 40 mg/kg). This second experiment revealed an almost identical regression coefficient of -8.72. Some measure of the relative potency of the effective drugs, when tested in this way, can be obtained by studying Fig. 3.

EFFECT OF VARIOUS DRUGS ON INFLUX OF INFLAMMATORY CELLS INTO CUBES IMPREGNATED WITH *M. TUBERCULOSIS*
Groups of rats (5 in each group) had TBC impreg-

FIG. 4 Section through tubercle-impregnated cube removed on day +5 from an untreated rat. Shows clearly demarcated band of inflammatory cells which have extended into the cube from its periphery. The cellular infiltrate appears black in the photomicrograph. Haematoxylin-eosin $\times 8$

FIG. 5 Section through nonimpregnated cube removed on day +5 from an untreated rat. Shows absence of inflammatory cells within cube. Haematoxylin-eosin $\times 8$

nated cubes implanted, one in either flank, and received the highest doses of the drugs according to the regimen shown in Table I. Five days after implantation sections of each cube (2 from each animal), together with the surrounding tissues, were prepared for microscopical examination and the mean depth of penetration of invading cells was assessed (see Methods).

Fig. 4 shows the appearance of a TBC-impregnated cube on day +5 from a control rat which had not received any drug. Over 95% of the infiltrating cells

consist of neutrophil polymorphs. This ingrowth of cells is absent from a similarly treated but non-impregnated cube (Fig. 5). This experiment thus tested the ability of the various drugs to suppress the cellular component of an acute inflammatory reaction attributable to the impregnated irritant (TBC).

Table III and Fig. 6 show the results. Compared with the untreated controls, all the treated groups showed a significant reduction in the depth of cell infiltration. This includes three drugs, sodium aurothiomalate, hydroxychloroquine, and D-penicillamine, which (at the maximum dosage tested) failed to influence the fluid phase of this inflammatory

Table III Effect of various anti-inflammatory agents on depth of cellular invasion into cubes impregnated with heat-killed *M. tuberculosis*. (Frequency of drug administration as in Table I)

Treatment	Dose (mg/kg)	Depth of cell invasion (mm $\pm$ SE)
Azathioprine	200	0.72 $\pm$ 0.11*
D-Penicillamine	200	0.57 $\pm$ 0.10†
Aspirin	800	0.57 $\pm$ 0.14†
Sodium aurothiomalate	100	0.51 $\pm$ 0.05†
Hydroxychloroquine	50	0.50 $\pm$ 0.06†
Phenylbutazone	300	0.32 $\pm$ 0.05†
Cyclophosphamide	50	0.22 $\pm$ 0.08†
Prednisolone	50	0.12 $\pm$ 0.01†
Controls	—	1.12 $\pm$ 0.06

* $P < 0.01$; † $P < 0.001$.

FIG. 6 Histogram illustrating comparative depth of cellular invasion (mean $\pm$ SE) into tubercle-impregnated cubes removed on day +5 from untreated control rats and rats treated with various anti-inflammatory drugs

response (see above). Further, among the other drugs tested, the orders of ranking for degree of inhibition of cellular infiltration (Fig. 5) and that for inhibition of dry weight gain (maximum doses in Fig. 3) show clear differences.

Discussion

The particular type of polyurethane foam used in these experiments is biologically relatively inert. After subcutaneous implantation in the rat it fills with 'wound juice', then (judged by the measures applied here) the implant provokes no inflammatory reaction until the 7th day, when granulation tissue begins to grow into it. In a sense, therefore, these cubes provide an inert matrix within which an inflammatory response can be provoked. Our continuing interest in this model is based on preliminary evidence that it provides a practical means of separately harvesting the fluid and cellular components of the inflammatory reaction for detailed studies.

The present report concerns only two relatively crude measures applied to the model—dry weight gain and depth of cellular infiltration—selected because of their simplicity and reproducibility. Studies on fluid expressed from 'wet' cubes have established that the dry weight gain is largely a measure of protein in the fluid exudate, while the depth of infiltrating cells has been shown to reflect the numbers of cells actually in the cube. The inflammatory response studied here was acute and the very high proportion of neutrophil polymorphs present made differential cell counts uninformative.

The results reported have shown that the fluid phase of this acute inflammatory response to the presence of dead TBC is reduced by treatment with phenylbutazone, azathioprine, aspirin, cyclophosphamide, and prednisolone, but is not affected by the administration of sodium aurothiomalate, hydroxychloroquine, or D-penicillamine (apart from one dosage level with this latter drug). By contrast, all these drugs, including sodium aurothiomalate, hydroxychloroquine, and D-penicillamine, suppress

the invasion of inflammatory cells into the sponges. These findings support the concept that the factors which mediate the fluid phase of the inflammatory exudate are probably independent of the factors which are concerned with the number of cells which ultimately appear in the inflammatory lesion; they also indicate that the degree of suppression of the fluid phase of inflammation by a particular anti-inflammatory drug may not be paralleled by the degree of suppression of the cellular phase. These observations stress the importance of using techniques which can separately measure the fluid and cellular phases of inflammation when carrying out assays of anti-inflammatory drugs.

In a previous study, Vernon-Roberts, Jessop, and Doré (1973) found that sodium aurothiomalate suppressed both the fluid and cellular phases of the inflammatory response studied by the cotton pellet technique, whereas in the present study sodium aurothiomalate suppressed the cellular exudate alone. The disparity in these findings may relate to the relatively mild inflammatory response to the non-impregnated cotton pellet, evidenced by the predominance of macrophages (over 90%) in the exudate 4 days after implantation; by contrast, the present studies concerned a marked inflammatory reaction to a more potent irritant, evidenced by the predominance of neutrophil polymorphs (over 95%). Further studies may therefore show that it is necessary to use inflammatory stimuli of varying intensities when assessing the actions of anti-inflammatory drugs.

The regression coefficients relating the doses of azathioprine, aspirin, and cyclophosphamide to dry weight gain of the cubes revealed parallelism of the dose-response relationships of these drugs and could suggest a close similarity in their mechanism of action in suppressing the fluid phase of inflammation. By contrast, the different coefficients for phenylbutazone and prednisolone indicate that the mechanism of action of these two drugs may differ from each other and from that of the other drugs investigated.

This study was supported by the Arthritis and Rheumatism Council.

References

- BOLE, G. G., AND HEATH, L. E. (1967) *Arth. and Rheum.*, **10**, 377 (The effect of 6-mercaptopurine on the inflammatory response stimulated by subcutaneous implantation of polyvinyl sponge)
- BOUCEK, R. J., AND NOBLE, N. L. (1955) *Arch. Path.*, **59**, 553 (Connective tissue: a technique for its isolation and study)
- KALTIALA, E. H., AND HEIKKINEN, E. S. (1971) *Acta chir. scand.*, **137**, 639 (Influence of phenylbutazone and salicylic acid on the histamine content of rat experimental granuloma)
- MEIER, R., SCHULER, W., AND DESAULLES, P. A. (1950) *Experientia (Basel)*, **6**, 469 (Zur Frage des Mechanismus der Hemmung des Bindegewebswachstums durch Cortisone)
- SELYE, H. (1953) *Proc. Soc. exp. Biol. (N.Y.)*, **82**, 328 (Use of 'granuloma pouch' technique in the study of antiphlogistic corticoids)
- VERNON-ROBERTS, B., JESSOP, J. D., AND DORÉ, J. (1973) *Ann. Rheum. Dis.*, **32**, 301 (Effects of gold salts and prednisolone on inflammatory cells. II. Suppression of inflammation and phagocytosis in the rat)