# The aggregation of rat neutrophils by arachidonic acid: a possible bioassay for lipoxygenase activity

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Polymorphonuclear leucocytes (PMNs) metabolize arachidonic acid by cyclooxygenase and lipoxygenase enzyme systems leading to the production of either prostaglandins and thromboxanes or hydroxy fatty acids (Borgeat et al 1976). Hydroxy fatty acids have been proposed as potential mediators of inflammation because of their chemotactic activity towards PMNs (Goetzl & Sun 1979). Substances reported to be chemotactic for PMNs also cause transient aggregation of these cells in suspension (O'Flaherty et al 1978). Arachidonic acid (AA), which does not possess intrinsic chemotactic properties (Turner et al 1975; Goetzl et al 1977), has been shown to aggregate human PMNs (O'Flaherty et al 1979a, b). These authors have shown that such aggregation is not induced by structurally similar fatty acids, that the response is enhanced by cytochalasin B and is blocked by indomethacin and 5,8,11,14-eicosatetraynoic acid (TYA), substances known to inhibit arachidonic acid metabolism. It was suggested that AA-induced aggregation is caused by intermediates formed either via the cyclooxygenase or by the lipoxygenase pathways. The present study was designed to distinguish whether products of one or both pathways are responsible for the aggregatory response.

Cell suspensions (>85% PMNs) were prepared from peritoneal exudates obtained 24 h after the injection of 6% sodium caseinate into 200–300 g male Wistar rats (Cunningham et al 1979). Aggregation assays were carried out in a Payton aggregometer (Cunningham Shipley & Smith in press), cells being suspended at a concentration of $3 \times 10^7$ cells $ml^{-1}$ in Medium 199 buffered to pH 7·4 with 25 mM *N'*-2-hydroxy-ethyl-piperazine-*N'*-2-ethane sulphonic acid (HEPES). Stock solutions of the following drugs, indomethacin, TYA and nordihydroguaiaretic acid (NDGA), were made up in ethanol, cytochalasin B was dissolved in dimethyl-sulphoxide (10 mg $ml^{-1}$) and diluted down to a concentration of 10 $\mu$g $ml^{-1}$ in medium and AA was made up in ethanol at a concentration of 1 mg $ml^{-1}$. The experiment was initiated by placing 0·5 ml aliquots of the cell suspension in the aggregometer and then adding 5 $\mu$l of drug solution or ethanol as control. After 1 min, 25 $\mu$l of cytochalasin B stock solution was added, followed 4 min later by 1·5 $\mu$l of the AA stock solution. The effects of the drugs were expressed as the mean % change of the control aggregation response ($n > 15$).

The effects of the three drugs on thromboxane $B_2$ ($TXB_2$) production was investigated by incubating 0·5 ml aliquots of the cell suspension for 30 min in a shaking water bath at 37 °C with the concentrations of the drugs, cytochalasin B and AA described above. The cells were removed by centrifugation and $TXB_2$ in the supernatant was estimated by antibody-dilution radioimmunoassay as follows. Supernatants were diluted 1 : 10 and 0·1 ml aliquots were incubated with 0·1 ml of 10 $\mu$g $ml^{-1}$ rabbit anti$TXB_2$/bovine serum albumin conjugate antisera and 0·1 ml of $^3$H-$TXB_2$ (40 nCi $ml^{-1}$). After equilibration, free $TXB_2$ was precipitated with charcoal/dextran and samples of the supernatant, containing bound $TXB_2$, were counted. $TXB_2$ concentrations in the samples were calculated from a standard curve (range 10–5000 pg $ml^{-1}$). From these data, inhibitory constant values (IC50) were determined for the drugs on $TXB_2$ production.

The results of the present work confirm previous reports that AA can induce a transient aggregation response in human PMN suspensions and show that the response also occurs in the rat. The optimal AA concentrations and the enhancing effects of cytochalasin B in the rat were similar to those found for the human cells (O'Flaherty et al 1979a, b).

The IC50 values for inhibition of the aggregation response (Table 1) derived from data in Fig. 1, showed no correlation with the IC50 values calculated for $TXB_2$ production. Thus, indomethacin, a powerful inhibitor of cyclooxygenase activity and a weak inhibitor of lipoxygenase activity (Hamberg & Samuelsson 1974), was also a weak inhibitor of the aggregation response. In contrast, NDGA, which is a known inhibitor of lipoxygenase activity (Tappel et al 1953), proved to be a potent inhibitor of the aggregation response. TYA, which is known to inhibit both pathways (Hamberg & Samuelsson 1974), showed a similar IC50 for both $TXB_2$ production and the aggregation response. It therefore appears that inhibition of AA-induced PMN aggregation by these compounds results

Table 1. IC50 values for the effects of indomethacin, TYA and NDGA on thromboxane $B_2$ production and arachidonic acid induced aggregation of rat polymorphonuclear leucocytes.

| Drug | IC50 for the production of thromboxane $B_2$ ($\mu$M) | IC50 for arachidonic acid-induced aggregation($\mu$M) |
|---|---|---|
| Indomethacin | 0·4 | 15 |
| 5,8,11,14-Eicosa-tetraynoic acid (TYA) | 3 | 8 |
| Nordihydroguaiaretic acid (NDGA) | 25 | 0·9 |

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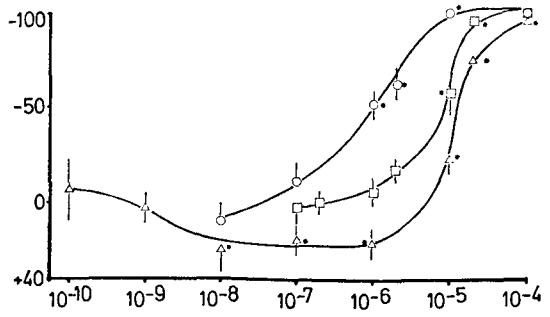


FIG. 1. The effects of indomethacin (△), 5,8,11,14-eicosatetraynoic acid (□) and nordihydroguaiaretic acid (○) on arachidonic acid-induced polymorphonuclear leucocyte aggregation. Values represent the mean of between 6 and 16 determinations and are shown with standard errors. $*P < 0.05$ (Student's *t*-test). Ordinate: % changes compared with arachidonic acid control. Abscissa: molarity.

preferentially from an interference with lipoxygenase enzymes. PMNs may aggregate in response to AA as a result of production of biologically active products of lipoxygenase enzymes (Goetzl & Sun 1979), such as hydroperoxy or epoxide intermediates, or hydroxy fatty acids, e.g. 5-hydroxyeicosatetraenoic acid. These metabolites may play important roles in the biological effects resulting from the metabolism of AA by mammalian cells. The enhancement of the aggregatory response observed with low doses of indomethacin may be due to enhancement of lipoxygenase pathway activity as has been previously reported for this drug (Hamberg & Samuelsson 1974). AA-induced aggregation of PMNs may prove to be a simple and reproducible bioassay for lipoxygenase enzyme activity.

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# Sustained release of sulphamethizole from agar beads

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A possible use of natural polymers such as konjac, a glucomannan, in dosage form design for sustained release has been examined (Nakano et al 1978). Some of the polysaccharides are known to form gels in which drugs may be incorporated. Agar has been used as culture media in microbiology and agarose, which is a a purified form of agar, has been employed extensively in separation and purification in biochemistry (Hjertén 1964). Agarose beads have recently been examined for possible use in haemoperfusion (Lösgen et al 1978).

In the present study, a possible use of agar for sustained release of sulphamethizole has been examined. The choice of agar rather than agarose is largely due to economic reasons. Agarose is still expensive as an ingredient in dosage forms for commercial production, but it was also studied in order to compare the release characteristics with those of agar. The choice of sulphamethizole as a drug is based on its short plasma half-life and small extent of metabolism (Triggs et al 1975) as well as the availability of reliable analytical methods.

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Sulphamethizole has been used as a urinary disinfectant because most of the dose is excreted in urine in unchanged form. Because of its short half-life in plasma, however, it has to be repeatedly administered in order to maintain the effective concentration in urine. Therefore, a sustained release dosage form may be beneficial.

*Materials.* Powdered agar was of first grade, Japanese Industrial Standard, purchased from Wako Pure Chemical Industries, Osaka. Water content and total ash as determined according to the test procedures in Japanese Pharmacopoeia were 16·7% and 2·45% (dry weight basis), respectively. Agarose with a labelled gel strength of 600 g $cm^{-2}$ was obtained from Dojin Lab., Kumamoto. Sulphamethizole was of Japanese Pharmacopoeia grade from Eisai Co., Tokyo. Diethyl ether, cyclohexane, light petroleum (b.p. 30–60 °C), hydrochloric acid, potassium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate were of reagent grade and used as received whereas ethyl acetate, acetone, dioxane, and ethanol were distilled before use.