J. Pharm. Pharmac., 1970, **22**, 86–92 *Received August* 4, 1969

The inhibitory effect of non-steroidal anti-inflammatory agents on aggregation of red cells *in vitro*

P. GÖRÖG* AND IREN B. KOVÁCS†

* *Pharmacological Department of United Pharmaceutical Works, Budapest, X, Kereszturi ut* 38, *and* †*Otto Korvin Hospital, Budapest, Hungary*

Aggregation of red cells of rat in saline suspension was induced by gelatin, high molecular weight dextran and fibrinogen. Except chloroquine, non-steroidal anti-inflammatory agents (NAIA) inhibited macromolecule-induced red cell aggregation *in vitro*. Glucocorticoids failed to inhibit red cell aggregation even in large concentrations. Of many drugs examined, only the NAIA and certain antihistamines, having experimental anti-inflammatory effects proved to be effective inhibitors of gelatin-induced red cell aggregation, *in vitro*.

Thrombosis in small vessels plays a significant role in the development of various inflammatory and necrotic reactions. The adhesive nature of platelets is a decisive factor in the development of a thrombus. *In vitro* aggregation of platelets induced by collagen particles is inhibited by certain non-steroidal anti-inflammatory agents (NAIA) (O'Brien, 1968; Görög & Kovács, 1968).

Though there can be no doubt that platelets are more prone to aggregate in response to various stimuli than other cells, most cells are liable to aggregate under appropriate conditions. The intra-arteriolar aggregation of erythroyctes (sludge phenomenon) is of pathophysiological significance. The red cell aggregates, transiently plug small vessels and can cause microcirculatory failure and thereby organ dysfunction. According to Bloch (1956) and Knisely (1963), intra-arteriolar aggregation of erythrocytes can never be demonstrated in the retinal vessels of healthy persons; when observable, it always indicates the presence of systemic disease.

In our studies, red cell aggregation induced by various macromolecules *in vitro* was significantly inhibited by NAIA. The investigation of many compounds revealed inhibition of gelatin-induced red cell aggregation to be highly specific to NAIA. Therefore, in addition to *in vivo* tests, this quick and specific *in vitro* method is recommended for use in the investigation of the anti-inflammatory effects of new compounds.

EXPERIMENTAL

Materials

Compounds were dissolved in 0·9% saline and the pH adjusted to 7·2–7·8 by addition of either NaOH or HCl. Aggregation of red cells was induced by gelatin (Knox Gelatin Inc., Johnstown, New York), by dextran (Koch-Light Labs., Ltd., Colnbrook, Bucks, England, M: 5–40 $\times$ 10^6) or by bovine fibrinogen (Phylaxia, Budapest, Hungary). The aggregating agents were dissolved in 0·9% saline. The

anti-inflammatory agents used were: salicylic acid, acetylsalicylic acid, phenylbutazone, sulphinpyrazone, mefenamic acid, flufenamic acid, cinchophen, indomethacin, chloroquine phosphate, benzydamine, ibufenac (4-isobutylphenylacetic acid), ibuprofen [2-(4-isobutylphenyl)propionic acid], BDH 7538 [4-(p-biphenylyl)-3-hydroxybutyric acid], ICI 54 450 [2-(4-chlorphenyl)thiazol-4-ylacetic acid, fenclozic acid], glyvenol (ethyl-3,5,6-tri-*o*-benzyl-D-glucofuranoside), prednisolone hemisuccinate sodium and dexamethasone 21-phosphate.

Methods

Adult rats of either sex were anaesthetized with pentobarbitone (45 mg/kg, intraperitoneally). Blood was allowed to flow from the abdominal aorta through a polyethylene cannula into a centrifuge tube containing heparin to give 20 U/ml final concentration. Of the blood collected from several animals in about 15–20 min, 3·0 ml samples were pipetted into Wassermann tubes. The blood was centrifuged for 20 min at 2000 rev/min, after which the plasma, leucocytes and platelets were removed and discarded. To the packed red cells, 2·0 ml of the solution prepared from the substance to be tested was added. In each series, two test and two control tubes were used. The tubes were sealed with rubber stoppers and the cells were cautiously suspended by ten inversions of the tube. After 5 min standing at 20°, 2·0 ml of 2% solutions of the aggregation-inducing agents were added to the tubes and the contents mixed by another ten inversions. The measure of aggregation was estimated by the erythrocyte sedimentation rate (ESR) which could be continually and accurately defined under the existing conditions. The tubes were held before an opal bulb to facilitate reading. Microscopic examination showed that increased ESR always implied enhanced cell-aggregation. 30 mm sedimentation was assigned a value of 100% for the control sample (no drugs). At the same time, sedimentation of test samples were determined, expressed as a percentage of the control and subtracted from 100% to give "Inhibition of aggregation (%)".

For *in vivo* experiments, male Wistar rats, 150–180 g, were treated subcutaneously with the substance to be tested and 1 h later the animals were exsanguinated under pentobarbitone anaesthesia. Samples of 3·5 ml of the heparinized blood were pipetted into Wassermann tubes; the aggregating solution (1·5 ml 2% gelatin) was added to each tube, the contents mixed and the ESR determined. The time for 30 mm sedimentation was measured and compared with the values obtained from saline-treated control rats. Two parallel readings were made for each animal.

RESULTS

Erythrocytes of the rat in heparinized whole blood or rat erythrocytes suspended in physiological saline show practically no sedimentation (ESR < 2 mm/h). On the addition of gelatin, swift aggregation of red cells is followed by rapid sedimentation (Coulson & Chalmers, 1964). Cellular agglomeration is looser on dextran or fibrinogen therefore the sedimentation is slower than on gelatin. As demonstrated in Fig. 1, the ESR is linear for the times between 5 and 35 mm values. Changes of temperature between 20–35° and of pH between 6·0–8·2 caused no alteration in the ESR. The sedimentation rate of erythrocyte suspensions can be determined accurately but not that of whole blood, therefore for the *in vitro* studies erythrocyte suspensions were used.

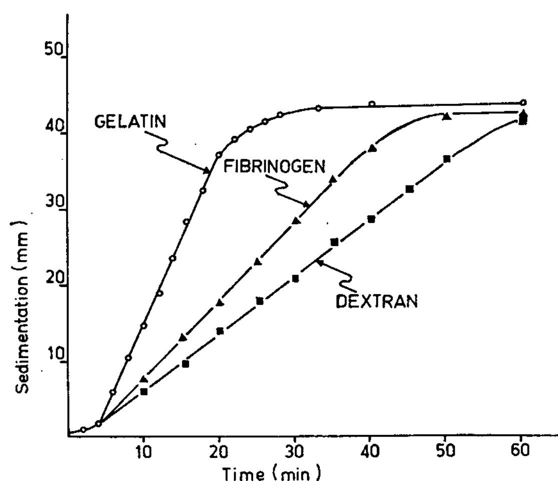

FIG. 1. Time-sedimentation curve of red cell aggregation induced by various macromolecules. Inhibitory effect of drugs (%) were calculated on the basis of 30 mm sedimentation of control sample for gelatin and fibrinogen and 20 mm sedimentation for dextran.

Table 1. *Effect of anti-inflammatory agents on red cell aggreggations induced by various macromolecules*

Drug (5×10^{-4}M)	Inhibition of aggregation (%)* induced by		
	Gelatin	Dextran	Fibrinogen
Salicylic acid	13·4	2·3	12·4
Acetylsalicylic acid	37·5	23·2	27·4
Phenylbutazone	100·0	89·4	76·3
Flufenamic acid	100·0	78·6	69·4
Mefenamic acid	96·0	68·2	57·7
Indomethacin	81·7	47·4	35·2
Cinchophen	89·2	39·7	37·1
Chloroquine	3·8	2·7	5·4
Benzydamine	63·4	23·4	18·8
Prednisolone	4·5	5·7	1·2
Dexamethasone	7·1	2·4	2·2

* = Calculated on the basis of readings when the ESR of control was 30 mm for gelatin and fibrinogen, and at control ESR = 20 mm for dextran.

Table 1 demonstrates that red cell aggregation induced by macromolecules is inhibited by NAIA. Of the three aggregating agents the effect of gelatin proved to be the most susceptible to inhibition with drugs, thus the gelatin-induced aggregation of red cell suspension was investigated further.

The effect of known anti-inflammatory agents is presented in Table 2. Aggregation was inhibited by all the compounds investigated except chloroquine and water-soluble glucocorticoids. The effect of the well-known compounds diminished in the order: phenylbutazone = flufenamic acid > mefenamic acid > indomethacin > ibufenac > acetylsalicylic acid > salicylic acid. Numerous compounds were investigated to decide whether the inhibition on gelatin-induced red cell aggregation is specific to NAIA or not. The effect of various compounds having different therapeutic use but all of them exerting a common antihistamine action—is presented in Table 3. The inhibitory potency of the effective compounds diminished in the order:

Table 2. *Effect of anti-inflammatory agents on gelatin-induced red cell aggregation* in vitro

Drug	Inhibition of aggregation (%)* at final concentration (M)							R†
	1×10^{-3}	5×10^{-4}	2×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-6}	
Salicylic acid‡	15·0	13·4						358·9
Acetylsalicylic acid	49·0	37·5	24·2					108·7
Phenylbutazone				95·0	89·4	51·7	26·2	1·0
Sulphinpyrazone			84·5	40·7	13·0			12·2
Mefenamic acid				89·7	55·4	27·3	8·6	4·6
Flufenamic acid					90·7	46·7	23·4	1·0
Cinchophen		89·2	30·8	14·5				32·6
Indomethacin		81·7	70·3	60·7	31·7	6·7		8·9
Chloroquine	2·4	3·8						—
Benzydamine	82·5	63·4	36·4	18·2				38·0
Ibufenac	91·0	65·0	44·8	17·5				28·2
Ibuprofen	90·0	64·8	37·9	15·0				30·4
BDH 7538		100·0	80·6	55·0	23·2			9·1
ICI 54 450	96·0	65·0	38·4	15·0				35·9
Glyvenol	68·7	40·3	20·2					73·9
Prednisolone	3·1	4·5		2·2				—
Dexamethasone	4·3	7·1		0·5				—

* = Inhibition $<15\%$ is significant ($P > 0{\cdot}001$).
† = Relative potency (phenylbutazone = 1·0), calculated on the basis of 50% inhibitory concentrations.
‡ = Salicylic acid produced 50% inhibition in $3{\cdot}3 \times 10^{-2}$M concentration.

Table 3. *Effect of antihistamines on gelatin-induced red cell aggregation* in vitro

Drug	Inhibition of aggregation (%)* at final concentration (M)			Antagonism of i.v. histamine† (oral ED50 mg/kg)
	5×10^{-4}	2×10^{-4}	1×10^{-4}	
Diphenhydramine	4·8			1·02 ± 0·5
Chloropyramine	5·0			
Diethazin	4·5			
Tripelenamine	2·4			0·50 ± 0·08
Amitryptiline	10·4			0·21 ± 0·03
Promethazine	18·0	4·1		
Imipramine	42·7	27·3	4·7	7·43 ± 1·60
Desipramine	30·0	17·4	2·2	
Chlorpromazine	49·2	25·0	10·2	4·10 ± 2·80
Cyproheptadine	39·2	18·2	2·2	0·08 ± 0·02

* = Inhibition $>15\%$ is significant ($P < 0{\cdot}001$).
† = All data are obtained from the paper of Lish, Robbins & Peters (1966).

chlorpromazine > imipramine > cyproheptadine > promethazine. The aggregation-inhibitory effects of antihistamines is much weaker than that exhibited by the NAIA. The inhibitory effect on aggregation is independent of antihistamine potency. It is noteworthy that experimentally induced inflammations have been inhibited by chlorpromazine and promethazine (Lish, Alberts & others, 1960; Brown & Robson, 1964), by imipramine (Tangri, Saxena & others, 1966) and by cyproheptadine (Selye & Somogyi, 1967), while evidence that the experimental anti-inflammatory effects of antihistamines do not influence red cell aggregation is not available. Some 100 drugs in widely differing categories were found inactive.

The efficacy of several NAIA *in vivo* is illustrated in Fig. 2. Gelatin-induced red cell aggregation was significantly inhibited by pretreatment of the rats with non-toxic doses of phenylbutazone or acetylsalicylic acid, while flufenamic acid or indomethacin have inhibitory effects only in toxic doses. Unlike acetylsalicylic acid, sodium salicylate was ineffectual *in vivo*.

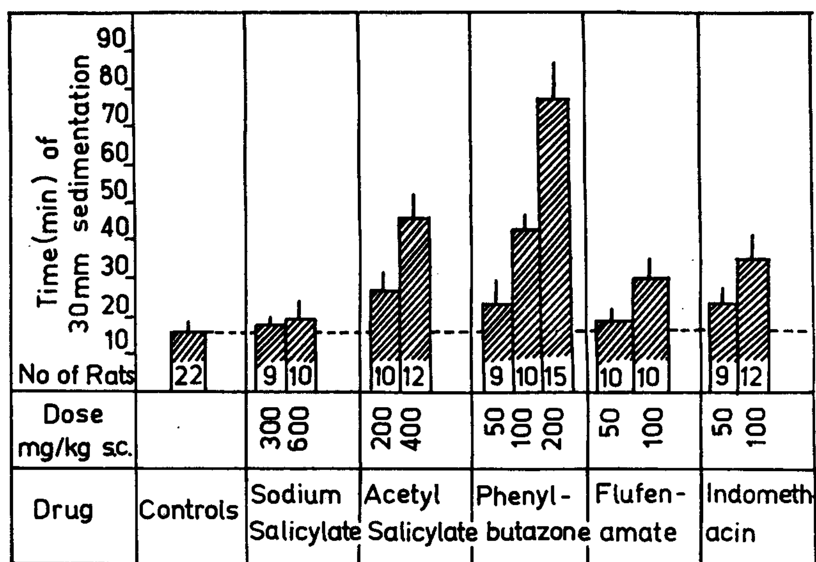

FIG. 2. *In vivo* effect of anti-inflammatory drugs on gelatin-induced red cell aggregation. Vertical lines show the standard errors. According to a separate experiment, the drugs in the applied doses caused no death within 6 h. After 48 h, flufenamate, 100 mg/kg dose, was lethal to 3 of 10 rats, indomethacin in 50 and 100 mg/kg doses was lethal to 9 and 10 of 10 rats, respectively.

DISCUSSION

The NAIA cause several *in vitro* effects which can be used to investigate the mechanism of action of new anti-inflammatory agents. NAIA can inhibit heat-induced denaturation of proteins (Mizushima & Kobayashi, 1968), the heat-induced haemolysis of erythrocytes (Brown, Mackey & Riggilo, 1967), the biosynthesis of mucopolysaccharides in the connective tissue (Whitehouse & Boström, 1962). In these tests numerous compounds have been effective which have not shown any anti-inflammatory action either under experimental conditions or in the therapy.

As in all the above tests, as well as in our method, the effect of drugs was observed in plasma protein-free medium, it was justified to compare the effective concentrations. Of the *in vitro* methods listed, that based on the inhibitory effect of NAIA on gelatin-induced red cell aggregation proved to be the most sensitive to the influence of NAIA.

Despite the essential differences in sensitivity and selectivity of these *in vitro* methods, the order of diminishing potency proved by every test to be: flufenamic acid $\geqq$ phenylbutazone $>$ indomethacin $>$ ibufenac $>$ acetylsalicylic acid $>$ salicylic acid. Of the anti-inflammatory agents investigated, glucocorticoids, even in large concentrations, failed to inhibit red cell aggregation. This is an essential difference from the method based on inhibition of heat-induced haemolysis of erythrocytes in which various steroids are active (Brown & Mackey, 1968). Of the NAIA, chloroquine alone failed to inhibit red cell aggregation. This is unexpected as it is the only drug to be recommended as a "desludging" agent on the basis of its therapeutic action (Sandler, Ilaki & Lawson, 1963). On the other hand, chloroquine is the only NAIA the efficacy of which has not been fully confirmed by acute experiments.

According to Knisely, Bloch & others (1950) sludging is probably unrelated to the rouleaux formation associated with the ESR, the increase in which is thought to be due to a much looser aggregation of red cells. This may be the cause of differences between our results based on erythrocyte aggregation and the results of Ruhenstroth-Bauer, Brittinger & others (1960) relating to the inhibition of increased ESR. The latter authors concluded that the increased ESR is caused by specific plasma proteins called agglomerines and their effect can be inhibited partially or completely. According to the authors, complete blockers include salicylic acid, gentisic acid, phenylbutazone, and cinchophen, while partial blockers are cortisone, antazoline, and chloroquine. These findings show the wide differences existing between the inhibition of increased ESR and the macromolecule-induced red cell aggregation.

However, the susceptibility of red cell aggregation to inhibition and the inhibition of collagen-induced aggregation of platelets, *in vitro* have some similarities. Glucocorticoids have proved to be ineffective in both tests and the order of effectiveness of NAIA is also very similar. The only exception is chloroquine which, according to our experiments, inhibits the collagen-induced platelet aggregation but fails in the gelatin-induced red cell aggregation.

For the most effective NAIA, the inhibitory concentrations on the aggregation of red cells *in vitro* were those usually attained in patients after oral medication. Nevertheless only phenylbutazone and acetylsalicylic acid were effective in the *in vivo* experiments. Flufenamic acid, the most effective compound *in vitro* had the minimal *in vivo* effect. This discrepancy resembles the uncoupling of oxidative phosphorylation by flufenamic acid *in vitro* and *in vivo* (Whitehouse, 1965). According to Whitehouse, the ineffectiveness of anthranilates in biochemical tests *in vivo* is due to the excessively lipophil nature of these compounds barring the development of effective blood levels.

On the evidence we have derived about the mechanism responsible for the inhibitory effect of NAIA on red cell aggregation, the site of action is most probably on actomyosin-like contractile protein having ATPase activity and situated on the outer surface of the erythrocyte. This contractile protein plays an important role in maintaining the form of the erythrocyte and the distribution of the surface charge on the outer membrane. Effective NAIA bind to this contractile protein and inhibit its ATPase and contractile property. The results of our biochemical findings which have furnished the basis of the above hypothesis is to be published later. Presumably, adenosinetriphosphate (ATP) utilization is inhibited by NAIA through a similar mechanism not only in the red cell membrane (and most probably in the membrane of platelets as well), but also in the true target of these drugs: in the connective tissue. At all events, the highly selective inhibition of red cell aggregation by NAIA *in vitro* suggests the existence of a relation between the effect in the connective tissues and that exerted on the erythrocyte membranes.

With most *in vitro* biochemical tests (including that in the present paper) the order of efficacy of the effective compounds differs from that registered in *in vivo* tests for anti-inflammatory action (carrageenan-induced oedema test). For this reason Glenn (1969) disclaims any relation between various *in vitro* biochemical effects and anti-inflammatory action, and regards the former as manifestations of other side-effects of NAIA, for instance those exerted on coagulations.

We think that every new *in vitro* effect of NAIA takes us nearer to understanding the mode of action of these compounds. At the same time we emphasize the

importance of elucidating other factors (drug-metabolism, lipid-solubility, binding to plasma proteins, pK_a) that are responsible for any discrepancy between *in vitro* biochemical effects and *in vivo* anti-inflammatory potency.

Acknowledgements

We would like to thank the following people for kindly supplying compounds: Dr S. S. Adams, Boots Pure Drug Co., Ltd., Nottingham, for ibufenac and ibuprofen; Dr D. I. Barron, BDH Ltd., Godalming, Surrey (U.K.) for BDH 7538; Dr B. B. Newbould, ICI Ltd., Macclesfield, Cheshire, for ICI 54 450.

REFERENCES

BLOCH, E. H. (1956). *Ergebn. Anat. EntwGesch.*, **35**, 1–8.

BROWN, J. H., MACKEY, H. K. & RIGGILO, D. A. (1967). *Proc. Soc. exp. Biol. Med.*, **125**, 837–843.

BROWN, J. H. & MACKEY, H. K. (1968). *Ibid.*, **128**, 504–509.

BROWN, D. M. & ROBSON, R. D. (1964). *Nature, Lond.*, **202**, 812–813.

COULSON, A. S. & CHALMERS, D. B. (1964). *Lancet*, **1**, 468.

GLENN, M. E. (1969). *Proc. Soc. exp. Biol. Med.*, **130**, 1327–1332.

GÖRÖG, P. & KOVÁCS, I. B. (1968). *Int. Symp. on Inflammation Biochem. and Drug Interaction*, Como, 1968. Excerpta Medica, in the press.

KNISELY, M. H., BLOCH, E. H., BROOKS, F. & WARNER, L. (1950). *Am. J. med. Sci.*, **219**, 249.

KNISELY, M. H. (1963). *Sludged-blood.* II. Europ. Conf. on Microcirculation, Pavia, 1962. White Plains, New York. Editors: Harders, H., Kargar A. G.

LISH, P. M., ALBERT, J. R., PETERS, E. L. & ALLEN, L. E. (1960). *Archs int. Pharmacodyn. Thér.*, **129**, 77–107.

LISH, P. M., ROBBINS, S. I. & PETERS, E. L. (1966). *J. Pharmac. exp. Ther.*, **153**, 538–543.

MIZUSHIMA, Y. & KOBAYASHI, M. (1968). *J. Pharm. Pharmac.*, **20**, 169–173.

O'BRIEN, J. R. (1968). *Experientia*, **1**, 894–895.

RUHENSTROTH-BAUER, G., BRITTINGER, G., GANZER, E. & NASS, G. (1960). *Dt. med. Wschr.*, **85**, 808–814.

SANDLER, G., ILAKI, M. A. & LAWSON, C. W. (1963). *Angiology*, **14**, 319–321.

SELYE, H. & SOMOGYI, A. (1967). *Medna. Pharmac. exp.*, **17**, 255–263.

TANGRI, K. K., SAXENA, P. R., SETH, P. K. & BHARGAVA, K. P. (1966). *Biochem. Pharmac.*, **15**, 825–831.

WHITEHOUSE, M. W. & BOSTRÖM, H. (1962). *Ibid.*, **11**, 1175–1201.

WHITEHOUSE, M. W. (1965). *Properties of anti-inflammatory drugs. Progress in Drug Research*, **8**, 321–429. Editor: Jucker, E. Basel: Birkhauser.