Platelets, Prostaglandins, Red Cells, Sedimentation Rates, Serum and Tissue Proteins and Non-Steroidal Anti-Inflammatory Drugs (36893)

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The erythrocyte sedimentation rate (ESR) is a useful index for the study of certain types of tissue damage (1). Some non-steroidal anti-inflammatory drugs inhibit the accelerated ESR produced by the addition *in vitro* of carrageenan, dextran, gelatin, polyadenylic acid and others (2, 3). These same ESR-acceleratory materials cause acute inflammation when inoculated locally *in vivo* (2). These latter reactions are inhibited by treatment of animals with appropriate non-steroidal anti-inflammatory drugs, especially when these are administered before inoculation of the phlogistin (2).

It appears pertinent to the whole problem of inflammation therapy to determine if red-cell aggregation occurs in response to substances suspected to be present, activated, or released during inflammation in the whole organism and, if so, to determine if it is inhibited by selected non-steroidal anti-inflammatory drugs.

The purpose of the following studies is to show that substances likely to occur in severe tissue damage *in vivo* (hyaluronic acid, chondroitin sulfate, etc.) produce accelerated ESR's when added *in vitro*. They produce inflammation and elevate the ESR when inoculated locally *in vivo*. These exaggerated reactions are inhibited by certain non-steroidal anti-inflammatory drugs.

Platelet release or synthesis of $PGF_{2\alpha}$ occurs also upon the addition *in vitro* of these and many other naturally occuring substances. Many of these latter reactions are suppressed by the addition of anti-inflammatory drugs like indomethacin.

The general applicability of these types of studies will be demonstrated, further, by showing that certain serum proteins, elevated in the rat inflammatory disease, arthritis, have inhibitory effects towward acute inflam-

TABLE I. Effect of Various Enzymes on Red Cell Sedimentation Rate *In Vitro*.

Enzyme (units/mg)[a]	mg/ml	ESR (mm/hr)	Comment
Vehicle only	—	0	—
Alpha-amylase (?)	25	70	
Acid phosphatase, Type II (?)	1	71	
Alpha-chymotrypsin (42)	25	13	
Trypsin (10,000)	25	0	
Neuraminidase (0.08)	25	0	Lysis
Cholinesterase (?)	25	0	
Deoxyribonuclease (?)	25	0	
Histidase (?)	25	0	
Lipase (?)	25	63	Lysis
Peptidase (0.016)	25	0	
Ribonuclease (56)	25	0	
Phospholipase A (?)	0.5	0	Total lysis
Lysozyme muramidase (30,000)	25	0	
Hyaluronidase (350)	50	0	
Collagenase (225)	25	10	
Beta-glucosidase (3.5)	50	0	
Beta-glucuronidase (130)	50	0	
Sulfatase (5)	50	0	
Acetyl cholinesterase (3.2)	50	40	
Alpha-glucosidase (4)	25	22	
Beta-galactosidase (0.32)	50	0	
Dextran (control)	10	70	

[a] Enzymes added to duplicate tubes. The above numbers represent the highest concentrations studied. All enzymes have been investigated at several other lower concentrations. (?) Activity of enzyme preparation unknown or undetermined.

TABLE II. Various Substances and Their Ability to Accelerate the ESR *In Vitro*.

Addition (mg/ml)		ESR (mm/hr)[a]	Comment
Collagen	(2.5)	0	Allowed to react for 20 hr
	(5.0)	0	
	(10.0)	0	
	(20.0)	0	Total lysis
Ribonucleic acid	(12.5)	0	Untreated
(Type XI from Baker's yeast)	(25.0)	0	
	(50.0)	65	
Ribonucleic acid	(12.5)	0	
(Type XI from Baker's yeast)	(25.0)	0	Heated 100°, 5 min
	(50.0)	65	
Deoxyribonucleic acid	(0.6)	15	Untreated
(Type I from calf thymus)	(1.2)	35	
	(2.5)	65	
	(5.0)	—	Total lysis
Deoxyribonucleic acid	(0.6)	0	Heated 100°, 5 min
(Type I from calf thymus)	(1.2)	0	
	(2.5)	0	
	(5.0)	0	
Dextran	(10.0)	70	Control
Gelatin	(10.0)	72	Control
Hyaluronic acid	(0.5)	17	
	(1.0)	38	
	(2.0)	59	
	(2.5)	70	
Chondroitin sulfate	(5.0)	22	
	(10.0)	50	
	(15.0)	70	
	(20.0)	70	
Carrageenan	(1.5)	72	Control

[a] Above numbers the mean of duplicates. The values for RNA and DNA are for 3 hr; rather than one hour shown in all other instances.

mation *in vivo* and the ESR *in vitro*.

Materials and Methods. The aggregation of red cells and subsequent measurement of the ESR in response to various materials has been described previously (2). $PGF_{2\alpha}$ is assessed by radioimmunoassay (4) and the platelet-rich plasma (PRP) is prepared by taking rat blood from the dorsal aorta and suspending in 10% v/v sodium citrate (3.8% in saline). Blood is centrifuged (1000 rpm × 15 min) and the PRP obtained by the usual laboratory methods. Various materials are added to either washed red cells or the PRP in isosmotic sodium phosphate buffer (pH = 7.0). The "platelet button" is resuspended in distilled water and freeze–thawed repeatedly for at least 4 times in a dry ice and acetone bath to determine the total content or availability of materials in platelets. Other methods are described in the appropriate tables and text. For *in vivo* studies, 5–10 rats per group are used routinely.

Enzymes, hyaluronic acid (Grade I from human umbilical cord), chondroitin sulfate (Grade III, sodium salt from whale cartilage) and collagen (from bovine tendon), among others, are all obtained as highest-purity products from SIGMA Chemical Com-

TABLE III. Anti-Inflammatory Drugs and Effects on Different Types of Artificially-Induced ESR.

Drug	mg/ml[a]	Type of ESR[b]	Percent inhibition
Phenylbutazone	0.1	Dextran-induced	80
		Gelatin-induced	100
		Chondroitin sulfate-induced	74
		Hyaluronic acid-induced	58
		RNA-induced (3 hr)	100
		DNA-induced (20 hr)	100
Ibuprofen	1.0	Dextran-induced	82
		Gelatin-induced	100
		Chondroitin sulfate-induced	100
		Hyaluronic acid-induced	100
		RNA-induced (3 hr)	88
		DNA-induced (20 hr)	78
Flufenamic acid	0.1	Dextran-induced	82
		Gelatin-induced	100
		Chondroitin sulfate-induced	100
		Hyaluronic acid-induced	95
		RNA-induced (3 hr)	100
		DNA-induced (20 hr)	82
Salicyclic acid	3.0	Dextran-induced	84
		Gelatin-induced	82
		Chondroitin sulfate-induced	88
		Hyaluronic acid-induced	64
		RNA-induced (3 hr)	91
		DNA-induced (20 hr)	85

[a] The above inhibitory effects are dose-related for all drugs. The above concentrations of each drug are those previously determined to have almost 100 percent inhibitory effects.

[b] The concentrations of accelerators are adjusted to give a ESR of 60–70 mm/hr in all cases, except RNA- and the DNA-induced fall, which is extremely slow.

pany and used without further purification. They are suspended or solubilized in the appropriate buffer and added to cell preparations in a manner similar to that used for the addition and study of drug effects (2, 5). Serum proteins of the rat and other species are obtained from Miles. Carrageenan (Viscarin) is obtained from Marine Colloids, Inc. Filipin is from The Upjohn Company.

Results. When twenty-one enzymes are added individually to washed red cells, a few accelerate the ESR (Table I). Among these active materials are alpha-amylase, acid phospatase, trypsin, lipase, collagenase, acetylcholinesterase and alpha-glucosidase. Beta-glucosidase is not effective. Lipase, neuraminidase and phospholipase A cause red cell lysis. None of the acceleratory enzymic ESR reactions is inhibited by the addition *in vitro* of the non-steroidal anti-inflammatory drugs, aspirin, indomethacin and ibuprofin. Of all the above acceleratory agents tested in rats with 30 ml air pouches, acid phosphatase and phospholipase A are the only substances causing severe chronic granulomatous inflammatory reactions, with significant fluid accumulation lasting for 2 or more weeks, when injected as single intra-pouch doses (25–100 mg/pouch). On the other hand, all of the acceleratory enzymes elicit significant dose-related acute hindpaw edema and inflammation when inoculated locally at 2.5–10 mg per paw. Substances like alpha-amvlase, for example, while capable of eliciting acute inflammatory reactions at 2.5–10 mg per paw in the rat do not produce chronic inflammation when inoculated alone as single intra-pouch dosages (100 mg/pouch) in rats and

TABLE IV. Effect of Various ESR Accelerators and Other Agents on the Release of $PGF_{2\alpha}$ from Platelets.

Addition to the PRP[a]	mg/ml	$PGF_{2\alpha}$ released (ng/ml)	LDH released[c] (units/ml)
None	2.5	0	0
Acid phosphatase	25.0	3.1	70
Phospholipase A[d]	0.05	40.0	0
Alpha-amylase	25.0	0.5	70
Dextran	25.0	1.3	75
Carrageenan	2.5	16.3	71
Gelatin	25.0	3.2	150
Polyadenylic acid	25.0	1.6	120
Chondroitin sulfate	25.0	2.2	75
Hyaluronic acid	5.0	4.0	340
Calcium (0.0275 *M*)[b]	—	44.4	1260
Arachidonic acid (10^{-3} *M*)		250.0	0
Supernatant only; no platelets	—	0	0
Platelet button lysed in distilled water and frozen-thawed 4 times	—	15.1	1200

[a] Additions made as shown and incubated at 37° for 1 hr prior to above determinations; except in those cases where calcium added or the platelet button frozen and thawed.

[b] Note that addition of calcium to PRP appears to cause increased "production" of $PGF_{2\alpha}$ but this is uncertain. This $PGF_{2\alpha}$ "release" is inhibited 100% by the addition of indomethacin at 1×10^{-4} *M*.

[c] All additions and determination are duplicate.

[d] Note that phospholipase A causes an increase in net synthesis of $PGF_{2\alpha}$ and no release of LDH. This increased $PGF_{2\alpha}$ synthesis is inhibited completely by indomethacin and flufenamic acid at 10^{-5} *M*.

inflammation assessed 2 weeks later.

Hyaluronic acid, chondroitin sulfate, ribonucleic acid and deoxy-ribonucleic acid are accelerators of the ESR when added to rat red cells *in vitro* (Table II). Heating the nucleic acids at 100° for 5 min inactivates the acceleratory effects of DNA but fails to alter the response to RNA. Although the collagen used in these studies is ineffective toward accelerating the ESR when added *in vitro,* it causes lysis of red cells when added at concentrations of 20 mg/ml.

With the exception of ESR's caused by enzymes, all of the acceleratory sedimentation reactions elicited by other pre-selected biologic substances are inhibited by the addition of certain non-steroidal anti-inflammatory drugs (Table III).

Certain of these acceleratory materials are capable also of releasing small amounts of $PGF_{2\alpha}$ and the enzyme, lactic dehydrogenase, when these are added to platelet rich plasma (Table IV). Carrageenan is the most effective agent toward causing platelet release of $PGF_{2\alpha}$ and hyaluronic acid the most effective toward LDH release.

It is easier, however, to demonstrate these release reactions with other substances. When added to platelet rich plasma (PRP) at concentrations of 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} *M*, indomethacin produces dose-related inhibition of calcium and phospholipase-A-induced production or release (see bottom of Table IV) and also has dose-related inhibitory effects on $PGF_{2\alpha}$ release or production when the PRP is either frozen–thawed four times or when sonicated 60 min after drug addition (Table V). Sodium fluoride elicits a biphasic response on platelet $PGF_{2\alpha}$ release and the ascending part of the effect is inhibited also by indomethacin (Table V). Both calcium and fluoride lyse platelets at 1 mg/ml and therefore elicit maximal release of all the available lactic dehydrogenase of the PRP. Magnesium ions behave similarly. Potassium ions are ineffective. Digitonin and Filipin (80

TABLE V. Effect of Indomethacin on Release of $PGF_{2\alpha}$ from Frozen–Thawed, Sodium Fluoride-Treated or Sonicated Platelets.

Type of treatment[a]	Final molarity of indomethacin	$PGF_{2\alpha}$ (ng/ml)
None	0	1.0
None	2×10^{-4}	1.0
Freeze–thawed	0	17.2
Freeze–thawed	2×10^{-5}	1.2
Sonicated	0	17.1
Sonicated	2×10^{-5}	0.9
Sodium fluoride-treated (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg/ml)	0	13.1, 16.2, 22.8, 6.20, 1.7, 1.9
Sodium fluoride-treated (1 mg/ml)	0	19.6
Sodium fluoride-treated (1 mg/ml)	2×10^{-5}	0
Calcium chloride-treated (0.5, 1.0, 2.0, 4.0, 8.0 mg/ml)	0	8.0, 23.3, 15.4, 8.1, 1.4
Calcium chloride-treated (1 mg/ml)	2×10^{-5}	1.2
Rabbit anti-rat platelet antibody serum (0.2 ml/ml)[b]	0	12.8
Rabbit anti-rat platelet antibody serum (0.2 ml/ml)	2×10^{-5}	4.2

[a] Drug and PRP incubated at 37° for 1 hr prior to sonication and freeze–thawing. All samples in duplicate. After treatments, cell debris centrifuged out and discarded. Analyses made on supernatants.

[b] Antibody raised according to methods described in Ref. 5.

μg/ml) elicit the release of all the available $PGF_{2\alpha}$ and LDH. A net increase in total synthesis of $PGF_{2\alpha}$ occurs, however, in response to these latter agents.

All of these artificially induced $PGF_{2\alpha}$ release reactions occurring in response to various agents are inhibited completely by the addition of indomethacin at 10^{-5} *M*. LDH release is unaffected by the addition of drug. Phospholipase A, digitonin, Filipin, NaF and $CaCl_2$ all elicit dose-related acute inflammatory edema, lasting for at least 8 hr, when inoculated into the hindpaws of rats at 20–1000 μg/paw.

Indomethacin, given orally, decreases serum concentrations of $PGF_{2\alpha}$, having little or no effect on the lower plasma concentrations (Table VI). The effect occurs rapidly, within one hour, and persists for at least 16 hr as shown here. When the PRP from these same drug-treated animals is frozen and thawed, however, the platelets release the prostaglandin. In the case of drug-treated

TABLE VI. Effect of Orally Administered Indomethacin on Serum and Plasma Concentrations of $PGF_{2\alpha}$.

Type of preparation[a]	Pre-treatment[b]	$PGF_{2\alpha}$ (ng/ml)[c]
Serum	0	30.0 ± 2.3
Serum	+	2.0 ± 1.1
Plasma (platelet-free)	0	1.2 ± 0.2
Plasma (platelet-free)	+	1.0 ± 0.2
PRP/frozen–thawed[d]	0	35.0 ± 4.0
PRP/frozen–thawed[d]	+	25.0 ± 3.2

[a] Whole bloods (serum) or citrated bloods (plasma) removed from dorsal aortas of 5 rats/group.

[b] Single dose, 4 mg/kg, po, 16 hr prior to blood removal. Serum concentrations of $PGF_{2\alpha}$ fall within one hour, however, after dosing with drug.

[c] Numbers represent amounts of $PGF_{2\alpha}$ with SEM.

[d] These samples are the same as the previous plasma samples, except the platelet button from both control and indomethacin-treated rats resuspended in distilled water and frozen–thawed quickly on acetone and dry ice four times.

TABLE VII. Production of Inflammation (Hindpaw Edema) and Accelerated ESR's when Compounds Inoculated Locally into the Hindpaw of Rats.

Compound[a]	Mg both hindpaws 0.2 ml saline	Mg edema/100 gm body wt	ESR: (mm/hr)[b]
Saline	—	0	0
Acid phosphatase	20	900 ± 25	16 ± 5
Carrageenan	1	450 ± 18	12 ± 3
Dextran	20	175 ± 11	3 ± 0.5
Alpha-amylase	20	338 ± 12	5 ± 0.3
Polyadenylic acid	5	350 ± 18	12 ± 3
Hyaluronic acid	5	375 ± 12	4 ± 0.5
Chondroitin sulfate	20	600 ± 33	15 ± 2

[a] Compounds inoculated into both hindpaws and both heparinized bloods and paws removed from 10 rats/group 16 hr later.

[b] Values expressed are those from 10 rats/group with SEM.

rats, less of the material appears in the plasma or supernatant fraction. This correlates well with results shown previously in Table V.

What we have shown, so far, is that some agents are capable of eliciting increased ESR's and platelet release reactions when added to the appropriate cells *in vitro*. These rather non-specific reactions are inhibited by certain non-steroidal anti-inflammatory drugs. One can account for the inhibitory effects of certain drugs on the serum concentrations of $PGF_{2\alpha}$, almost totally, by their direct inhibitory effects *in vivo–in vitro* on platelet release or synthesis. Preliminary studies indicate that red cells do not contain $PGF_{2\alpha}$. It has been shown, further, that either the release or synthesis of $PGF_{2\alpha}$ from sonicated, sodium fluoride or calcium, phospholipase A-treated and freeze–thawed platelets is inhibited by the addition of representative anti-inflammatory drugs. Other non-steroidal drugs have been studied with similar results. These studies are merely more detailed extensions of previous work done by others (6).

To proceed, however, agents capable of eliciting ESR's and platelet release reactions *in vitro*, are effective inducers of accelerated ESR's and inflammation *in vivo* (Table VII).

The suggestive appropriateness and relatedness of some of the above information is shown, indirectly, in the following studies. It has been known for a long time that the alpha- and beta-globulin fractions of rat blood increase in response to various types of tissue damage (7). The plasma albumin decreases, gamma globulin decreases or remains the same and fibrinogen concentrations are elevated in rats with the inflammatory disease, adjuvant-induced arthritis (7). When these various serum proteins of the rat are added to dextran-treated red cells, in which the ESR is artificially elevated, the alpha and beta globulins decrease the effect. Rat gamma-globulin has a slight inhibitory effect. Albumin is ineffective (Table VIII).

When given intravenously to rats with carrageenan-induced hindpaw edema and the inflammation assessed shortly thereafter, rat alpha- and beta-globulins behave as effective anti-inflammatory proteins (Table IX). Gamma-globulin elicits less inhibition. Albumin is almost devoid of significant anti-inflammatory activity. The correlation persists.

Discussion and Summary. In a circumscribed series of somewhat indirectly interrelated investigations, it has been shown that some substances (hyaluronic acid, chondroitin sulfate, ribonucleic acid, deoxyribonucleic acid and certain enzymes, among others) accelerate erythrocyte sedimentation rates when added or inoculated *in vitro* and *in vivo*. It has been shown, too, that some of these same agents; as well as others like digitonin, Filipin, and phospholipase A, cause varying amounts of release or an increase in synthesis of $PGF_{2\alpha}$ when added to platelets.

TABLE VIII. Effect of Various Serum Proteins on the Dextran-induced ESR *In Vitro*.

Serum protein (mg/ml)		Species	Percent inhibition of the rat ESR[a]
None	—	—	0
Albumin	(20)	Human	0
Alpha-globulin	(2.5)	Human	100
Beta-globulin	(2.5)	Human	100
Gamma-globulin	(10)	Human	0
Albumin	(20)	Rabbit	0
Alpha-globulin	(10)	Rabbit	100
Beta-globulin	(2.5)	Rabbit	100
Gamma-globulin	(20)	Rabbit	0
Albumin	(20)	Sheep	0
Alpha-globulin	(2.5)	Sheep	100
Beta-globulin	(2.5)	Sheep	100
Gamma-globulin	(20)	Sheep	0
Albumin	(20)	Rat	0
Alpha-globulin	(2.5)	Rat	100
Beta-globulin	(2.5)	Rat	100
Gamma-globulin	(20)	Rat	25

[a] Control ESR's were 72 mm/hr. Samples in triplicate.

Certain of the above reactions (the accelerated ESR and platelet release of $PGF_{2\alpha}$) are inhibited by the addition or administration of appropriate anti-inflammatory drugs *in vivo* and *in vitro*. Freeze–thawing and sonication, as well as addition of calcium and NaF to platelets, also elicits increases in $PGF_{2\alpha}$ concentrations in the surrounding plasma. This $PGF_{2\alpha}$ release (presumably synthesis) is inhibited by adding the appropriate anti-inflammatory drugs. LDH release is unaffected.

It appears that the non-enzymic-induced ESR, as well as certain artificially induced platelet $PGF_{2\alpha}$ release reactions, may be secondary manifestations of primary alterations on cell membranes. They may be interrelated. The two processes are inhibited by similar types of drugs, the platelet reaction being far more sensitive, and they may have significance to initiation and maintenance of inflammatory reactions in animals and man. These ideas gain more merit, perhaps, when it is shown that certain serum proteins of the rat (the alpha- and beta-globulins) increase in response to some types of inflammation, that artificially induced inflammatory reactions in the rat are inhibited by the intravenous administration of these particular rat proteins and that, less importantly perhaps, the same proteins prevent the accelerated ESR *in vitro* in response to the addition of dextran. Their effects on platelets have not been studied.

It may be surmised, then, that certain serum protein changes in response to tissue damage may be protective, primarily, and that they, like non-steroidal anti-inflammatory drugs, modulate characteristic responses to changes in the cellular environment.

Although observations with red cells, platelets, certain drugs, and tissue or plasma proteins appear logical, pertinent and interrelated, these artificially derived laboratory results may be fortuitous. More work needs doing, for example, in the specific areas of cells and, even more importantly, in the greater general areas of inflammatory disease. The most pressing problem concerns the probable application of these isolated observations to the treatment of man and his diseases—inflammation, vascular disease, transplant complications, and a variety of others in which cell accumulation, aggregation and synthesis-release of tissue reactants like prostaglandins may play important contributory physiologic-pathologic roles.

TABLE IX. Effect of Rat Serum Proteins on Acute Inflammation in the Rat.

Protein[a]	mg/kg, iv	Edema: mg/100 g of bw	% Inhibition
Vehicle	—	266 ± 26	—
Alpha-globulin	11	264 ± 33	0
	22	175 ± 16	34
	44	167 ± 13	37
	88	151 ± 29	43
	176	128 ± 15	52
	352	108 ± 19	59
Beta-globulin	11	256 ± 35	0
	22	214 ± 26	20
	44	181 ± 17	32
	88	128 ± 13	52
	176	92 ± 11	65
	352	69 ± 14	74
Gamma-globulin	11	258 ± 16	0
	22	285 ± 43	0
	44	288 ± 27	0
	88	201 ± 11	24
	176	201 ± 21	24
	352	127 ± 13	52
Albumin	11	284 ± 11	0
	22	296 ± 20	0
	44	283 ± 22	0
	88	299 ± 31	0
	176	270 ± 30	0
	352	237 ± 17	11

[a] 5 rats/group. Injections via tail vein 4 hr prior to removal of hindpaws previously injected with 0.5 mg carrageenan immediately after intravenous injections of proteins.

Perhaps $PGF_{2\alpha}$ plays a supportive role in inflammation; perhaps not (8). At any rate, it represents a convenient assay method for the assessment of more important facets of cellular release mechanisms, alterations, or distortions, in general, and the action of nonsteroidal anti-inflammatory drugs, specifically. The serum response of this material to drugs like indomethacin occurs rapidly; within one hour, following a single oral dose in the rat, and persists for many hours.

Finally, from these and other studies in our laboratories, it appears that there is no dearth of so-called "mediators" to assure that inflammation will proceed in the almost total absence of any one of them. Perhaps the final role of the prostaglandins and the control of their synthesis, may reside in the area of physiologic homeostasis rather than in that of pathologic phenomena, as reflected by various inflammatory reactions.

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