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INHIBITION OF SUPEROXIDE ANION PRODUCTION IN NON-STIMULATED GUINEA PIG PERITONEAL EXUDATE CELLS BY ANTI-INFLAMMATORY DRUGS

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Abstract—Inhibitory effects of anti-inflammatory drugs on the production of superoxide anion ($\cdot O_2^-$) by isolated non-treated guinea pig peritoneal exudate cells (PEC) was studied spectro-photometrically using NADH and lactate dehydrogenase (LDH). Values of ID_{50} were; diclofenac sodium (2×10^{-5}M), indomethacin (3×10^{-5}M), oxyphenbutazone (8×10^{-5}M), mefenamic acid (1×10^{-4}M), ibuprofen (1×10^{-4}M), benzydamine (3×10^{-4}M), aspirin (10^{-3}M <) and dexamethasone (10^{-3}M <). The mechanism of inhibition seemed to block plasma membrane associated NAD(P)H oxidase(s) activity which produces $\cdot O_2^-$. ID_{50} values of other drugs; superoxide dismutase (SOD, 2×10^{-8}M), cytochalasin B (1×10^{-7}M) and NEM (6×10^{-6}M). D-Mannitol (hydroxyl radical scavenger), 1,3-diphenyl-isobenzofuran (singlet oxygen scavenger) and sodium azide (mitochondrial electron transport inhibitor and singlet oxygen scavenger) were negative.

Superoxide radical itself or oxygen-centered radical(s) derived from $\cdot O_2^-$ is supposed recently as a rate-limiting factor for prostaglandin (PG) synthetase. Whether the inhibition of non-steroidal anti-inflammatory drug (NSAID) on $\cdot O_2^-$ production is linked directly to PG biosynthesis or not, $\cdot O_2^-$ was already demonstrated in our laboratory to make a role for the development of rat carageenan foot oedema. It may serve as a new *in vitro* sceening method of NSAID, to check the inhibitory potency of a compound on $\cdot O_2^-$ production by guinea pig PEC.

Superoxide radical ($\cdot O_2^-$) has been reported to be produced by granulocytes and to serve as bactericidal agent [1–3]. Host over defence by producing excess $\cdot O_2^-$ results the inflammation in my concept. Repeated intravenous injections of superoxide dismutase (SOD, 0.5–2.0 mg/kg) suppressed the prostaglandin phase swelling of rat carrageenan foot-oedema [4]. NSAIDs inhibited $\cdot O_2^-$ productions of paraffin oil induced macrophages but not those of xanthine oxidase system [5] suggesting that NSAIDs block $\cdot O_2^-$ productions and do not work as $\cdot O_2^-$ scavenger. Pyrophosphate (PPi) anion activated the macrophage $\cdot O_2^-$ production [6] and this anion is reported to induce inflammation in the experimental animals [6–8]. PPi is also elevated in synovial fluids of patients suffering from rheumatoid arthritis etc. [9, 10]. Adenosine nucleotides are potent stimulators of prostaglandin (PG) biosynthesis in many tissues and stimulated the macrophage $\cdot O_2^-$ production [6, 11]. With all these results, the participation of $\cdot O_2^-$ on the inflammation development, claims adequacy.

Nevertheless, there has been a point to be clarified. Our *in vitro* screening method was based on the artificially stimulated macrophages. A possibility that NSAID inhibit only the part of artificially stimulated $\cdot O_2^-$ production by paraffin oil, has not been excluded [5]. Normal guinea pig peritoneal exudate cells (PEC) were little in quantity, but it is now possible to measure the $\cdot O_2^-$ production from them by concentrating cells and amplifing the capacity of spectrophotometor. In this paper, it is proved that the representative NSAID inhibited also the $\cdot O_2^-$ productions by non-stimulated PEC indicating that they inhibit the physiologically producing $\cdot O_2^-$.

MATERIALS AND METHODS

Peritoneal exudate cells. Male Hartley guinea pig (250–350 g) were sacrificed by decapitation and the cells were washed out from the peritoneal cavity with about 50 ml of fresh medium (9.3 g/l Eagle MEM "Nissui 3" for cell culture without phenol red and 0.292 g L-glutamic acid, adjusted to pH 6.5 with 10% sodium bicarbonate). Cells from 20 to 30 animals were centrifuged separately (500 *g* for 10 min) at room temperature and once washed. Preparations contaminated heavily with red cells, were discarded. Precipitates were mixed and suspended to an appropriate concentration with always the same medium.

Types of non-treated guinea pig peritoneal exudate cells, was examined by Giemsa staining on the cells obtained with guinea pig blood plasma, and were

In my previous report *Biochem. Pharmac.* **25**, 1473, (1976), the concentration of LDH 1.2 U/ml must be read as 1.2×10^3 U/ml.

Abbreviations—NSAID = non-steroidal anti-inflammatory drug; PG = prostaglandin; PEC = peritoneal exudate cells; MEM = minimum essential medium for cell culture; LDH = lactate dehydrogenase; DMF = *N,N'*-dimethylformamide; SOD = superoxide dismutase; NEM = *N*-ethylmaleimide; PMN = polmorphonuclear leucocyte; HETE = 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; TXA_2 = thromboxane A_2; TXB_2 = thromboxane B_2; MPO = myeloperoxidase; Pi = phosphate, PPi = pyrophosphate.

identified as granulocytes, monocytes, lymphocytes and histiocytes (27, 3, 18 and 52 per cent respectively). As lymphocytes do not produce $\cdot O_2^-$, so they were not calculated as well as contaminated red cells. We have no information on the $\cdot O_2^-$ production of histiocytes, but they were counted as PEC for the convenience of routine cell counting. PEC number in this report, therefore, means the sum of larger cells only, namely granulocytes, monocytes and histiocytes (30, 4 and 66 per cent respectively).

Assay of superoxide anion production. The decrease of NADH absorption at 340 nm was recorded continuously with Shimadzu Multi-Purpose MPS-5000 spectrophotometer at 37°. Cuvette comtained 3 ml reaction mixture of 125 mM sodium phosphate buffer pH 6.5, 80 mM EDTA, 1.2×10^3 U/ml LDH (very high concentration required, because LDH is not used for enzymatic activity but as a kind of carrier protein of NADH), 160 μM NADH, PEC suspension and various concentration of drugs. This condition was determined in the previous report using macrophages [5]. DMF (final concentration, 0.1%) was used to dissolve drugs if necessary and the inhibitions were calculated based on 0.1% DMF containing control value. Samples containing drugs but no PEC were also measured to obtain basal value of NADH oxidation, and the percent inhibitions were calculated from the following equation:

$$1 - \left(\frac{\Delta A(\text{PEC} + \text{drug}) - \Delta A(\text{drug basal})}{\Delta A(\text{PEC only}) - \Delta A(\text{medium basal})} \right) \times 100$$

where ΔA is difference of absorption at 340 nm before and after the reaction. The details of experiments were described in the previous paper [5].

Chemicals. The main chemicals are LDH (from rabbit muscle, in 3.2 M ammonium sulfate 550 U/mg of Boehringer Co.), SOD (lyophilized powder, 3000 U/mg protein of Sigma Co.), β-NADH (disodium salt from yeast of Oriental Yeast Co.), diclofenac sodium (GP 45840, Ciba–Geigy), dexamethasone ("Decadoron" phosphate ester for injection use, Japan, Merck), 1,3-diphenyl-isobenzofuran (Aldrich Chem. Co.) and Cytochalasin B (Serva Feinbiochemica). All the other chemicals were obtained from the usual commerical sources.

RESULTS

$\cdot O_2^-$ production by different PEC concentration. Figure 1 shows the NADH oxidations by the increasing concentrations of three different PEC preparations. All NADH oxidations increased linearly at least up to 15 min. Sufficient amount (32 μg/mg) of SOD completely inhibited the NADH oxidation, demonstrating that the NADH was oxidized only by

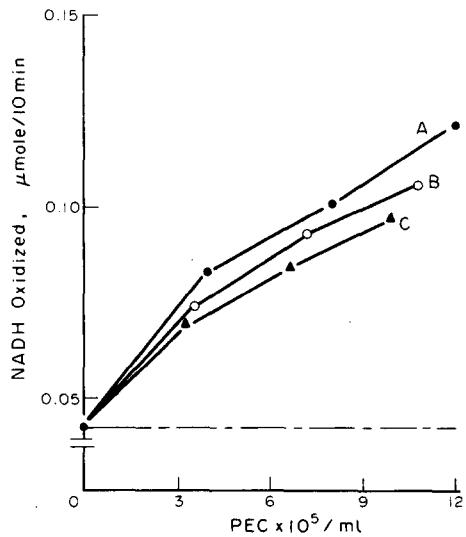

Fig. 1. Effect of peritoneal exudate cell (PEC) concentration on $\cdot O_2^-$ production at 10 min. Medium contains 125 mM phosphate buffer pH 6.5, 80 mM EDTA, 1.2×10^3 U/ml LDH and 160 μM NADH. A, B and C are different PEC preparations.

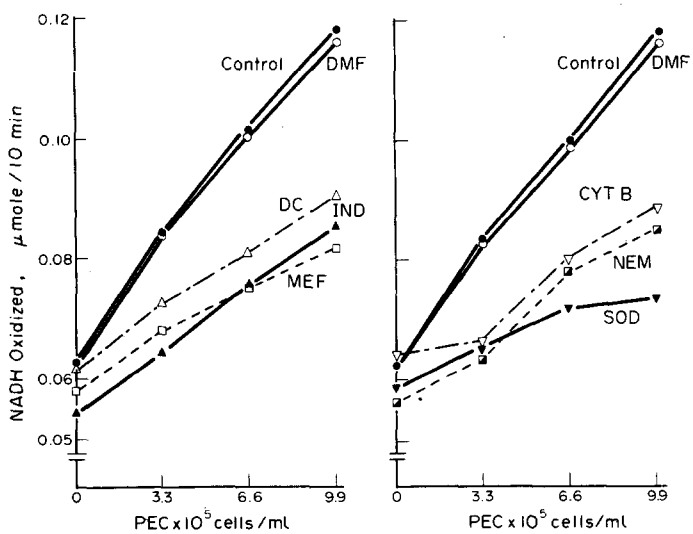

Fig. 2. Effect of PEC concentration on drug inhibitions. Medium and conditions are as in Fig. 1. Control (●), 0.1% DMF (○), 1.6×10^{-5}M diclofenac sodium (Dc, △), 6.4×10^{-5}M indomethacin (Ind, ▲), 2.5×10^{-4}M mefenamic acid (Mef, □), 2.5×10^{-7}M cytochalasin B (Cyt B, ▽), 6.4×10^{-5}M NEM (◪) and 1 μg/ml = 3×10^{-8}M superoxide dismutase (SOD, ▼).

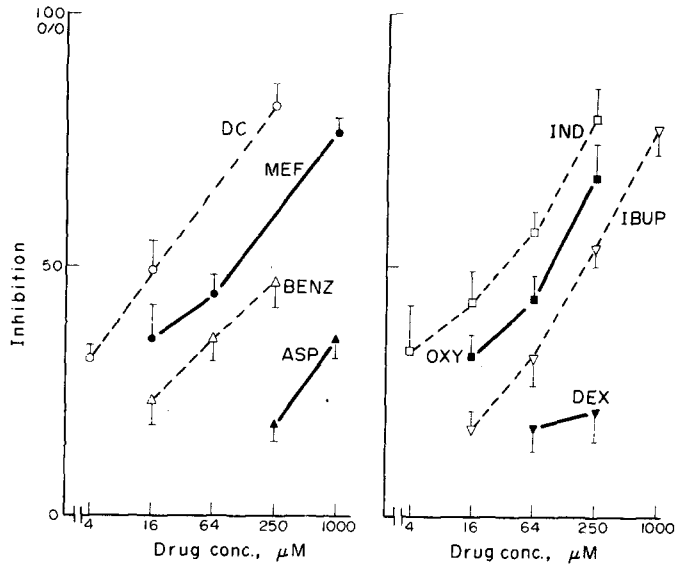

Fig. 3. Inhibition of superoxide anion production of PEC by antiinflammatory drugs. Incubations were for 10 min at 37°. Controls had 50–90 nmoles NADH oxidizing capacities per 10 min. DMF (0.1%) was used to dissolve diclofenac sodium (Dc, ○), mefenamic acid (Mef, ●), aspirin (Asp, ▲), indomethacin (Ind, □), oxyphenbutazone (Oxy, ■) and ibuprofen (Ibup, ▽). Benzydamine (Benz, △) and dexamethasone (Dex, ▼) were water-soluble. Vertical lines represent S.E.M. of the mean of five experiments.

$\cdot O_2^-$. In order to test the inhibition of drugs, 10 min incubation with 5–10 × 10^5 PEC/ml was chosen to be suitable.

Effect of PEC concentration on drug inhibition. The application of the solvent DMF facilitated the assay of many water insoluble drugs and 0.1% DMF had a negligible effect on the $\cdot O_2^-$ production (Fig. 2). Principal drugs were tested on the different PEC concentrations and found that they inhibited $\cdot O_2^-$ productions with nearly the same ratio except Cytochalasin B at 3.3 × 10^5 PEC/ml (Fig. 2). As far as 5–10 × 10^5 PEC/ml used, the inhibition rates of drugs including Cytochalasin B, seemed to be constantly measured at 10 min.

Inhibition by anti-inflammatory drugs. Eight anti-inflammatory drugs were tested on $\cdot O_2^-$ production by PEC (Fig. 3 and Table 1). Diclofenac sodium was the most potent inhibitor, but the concentration required to obtain ID_{50}, was higher with PEC assay than that by macrophage assay [5]. This tendency was also true about oxphenobutazone and aspirin. On the other hand, indomethacin, mefenamic acid, ibuprofen and benzydamine inhibited the $\cdot O_2^-$ productions by PEC as efficiently as that by macrophages. There is no possible explanation for the different characters of anti-inflammatory drugs between such as diclofenac sodium and indomethacin etc. In any way, NSAID tested were proved to inhibit rather

Table 1. ID_{50} of various drugs on $\cdot O_2^-$ production by peritoneal exudate cells (PEC) and by paraffin oil induced macrophages

Drugs		Inhibition ID_{50} PEC [P]	Macrophages [M]*	Ratio P/M
Diclofenac sodium	D†	2 × 10^{-5}M	4 × 10^{-7}M	50
Indomethacin	D	3 × 10^{-5}M	1 × 10^{-5}M	3
Oxyphenbutazone	D	8 × 10^{-5}M	1 × 10^{-6}M	80
Mefenamic acid	D	1 × 10^{-4}M	7 × 10^{-5}M	1
Ibuprofen	D	1 × 10^{-4}M	1 × 10^{-4}M	1
Benzydamine	W	3 × 10^{-4}M	2 × 10^{-4}M	1
Aspirin	D	>10^{-3}M	1.5 × 10^{-4}M	17‡
Dexamethazone (phosphate ester)	W	>10^{-3}M	>10^{-3}M	—
Superoxide dismutase	W	2 × 10^{-8}M	1.5 × 10^{-8}M	1
D-Mannitol	W	>10^{-3}M	>10^{-3}M	—
1,3-Diphenyl-isobenzofuran	D	>10^{-3}M	>10^{-3}M	—
Cytochalasin B	D	1 × 10^{-7}M	6 × 10^{-8}M	—
N-Ethylmaleimide	W	6 × 10^{-5}M	5 × 10^{-5}M	1
Sodium azide	W	>10^{-3}M	>10^{-3}M	—

* Cited from the previous report [5].
† Solvent used, D = 0.1% DMF, W = water.
‡ Calculated from ID_{30} values.

efficiently the $\cdot O_2^-$ productions by PEC as that by macrophages. A steroidal anti-inflammatory drug, dexamethasone was ineffective in both PEC and macrophage assay.

Inhibition by non anti-inflammatory drugs. SOD inhibited $\cdot O_2^-$ productions by PEC at very low concentration comparable to the result by macrophage assay (Fig. 4, Table 1). Hydroxyl radical ($\cdot OH$) scavenger, D-mannitol and singlet oxygen ($^1O_2$) scavenger,1,3-diphenyl-isobenzofuran failed to inhibit the $\cdot O_2^-$ production by PEC as well as by macrophages [12, 13]. Mitochondrial electron transport inhibitor, sodium azide was also ineffective suggesting that the $\cdot O_2^-$ production is not derived to mitochondrial function. Cytochalasin B was a potent inhibitor of $\cdot O_2^-$ production by PEC and by macrophages. This drug has been reported to modulate the organization of the plasma membrane and may influence the activity of $\cdot O_2^-$ producing enzyme(s) located on cell membrane [14]. This enzyme(s) may be SH-inhibitor sensitive because NEM was also effective to block the $\cdot O_2^-$ production.

DISCUSSION

The $\cdot O_2^-$ production from PEC was inhibited by NSAID. This result suggests that the inhibition of $\cdot O_2^-$ production by paraffin oil induced macrophages is not an artificial phenomenon. It may be possible to apply PEC assay as an *in vitro* screening method if the variety of non anti-inflammatory drugs are proved negative as already demonstrated in macrophage assay [5]. Some NSAID showed less potent inhibitory activity in PEC assay; for example, ID_{50} value of aspirin was over 10^{-3}M indicating the difficulty to pick up this drug. Nevertheless, if the target drug is limited to NSAID which is more potent than ibuprofen, PEC assay must be appreciated. This method is tedious and less troublesome than that by macrophages, because it is permissible to administer the paraffin oil and easy to handle the oil free cell preparation.

Summing up the results from our laboratory and from the others, the role of $\cdot O_2^-$ to inflammation development is presented in Fig. 5 only for facility of understanding. The $\cdot O_2^-$ producing enzyme(s) on granulocyte membrane is reported to be NAD(P)H oxidase(s) and NSAID may inhibit this enzyme directly or via the alteration of membrane structure [15, 16]. NAD(P)H oxidase(s) seems a SH-inhibitor sensitive enzyme and is suppressed by exogenously added cytochalasin B, perhaps through the alteration of membrane structure.

The formation of PGs have been reported to be limited only by free arachidonic acid content, but the other factor(s) is demonstrated to participate in this reaction [19]. $\cdot O_2^-$ tention contribute to PGs formation the involvement of a peroxidase and of hydroxyl like radical is presented in PGs biosynthesis [20–22]. Our previous investigation *in vivo* indicated that only PG phase swelling is inhibited by SOD injections [4]. $\cdot O_2^-$ may limit the formations of PGs rather than free arachidonic content. Directly participating radical(s) to PG biosynthesis is unknown, because other oxygen-centered radicals and $\cdot O_2^-$ transform rapidly with each other spontaneously or enzymaticaly by SOD or myeloperoxidae (MPO)-halide system etc. PGE_2 content is in good accordance with the degree of inflamation in many reports, yet this augmentation may not be the cause but the result of inflammation development in my hypothesis. PGE_2 and/or $PGF_{2\alpha}$ contents must serve as only signals to decrease $\cdot O_2^-$ production through the inhibition of NAD(P) H oxidase(s) and of lymphokine release. Exogenous PGE_2 or $PGF_{2\alpha}$ inhibited $\cdot O_2^-$ production by isolated guinea pig macrophages (ID_{50} = about 1.10^{-5}M, respectively) [23]. PGE_2 is also suppressor of lymphokine release from lymphocytes which might result less

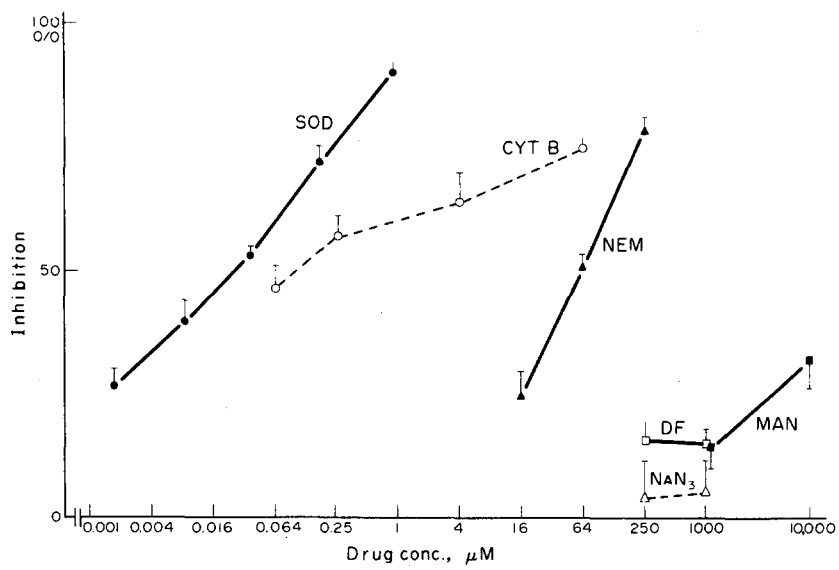

Fig. 4. Inhibition of superoxide anion production of PEC by non anti-inflammatory drugs. Conditions are as Fig. 3 DMF (0.1%) was used to dissolve cytochalasin B (Cyt B, ○) and 1,3-diphenyl-iso-benzofuran (Df, □). SOD (●), NEM (▲), sodium azide (NaN_3, △) and D-mannitol (Man, ■) were water-soluble. Vertical lines represent S.E.M. of the mean of five experiments.

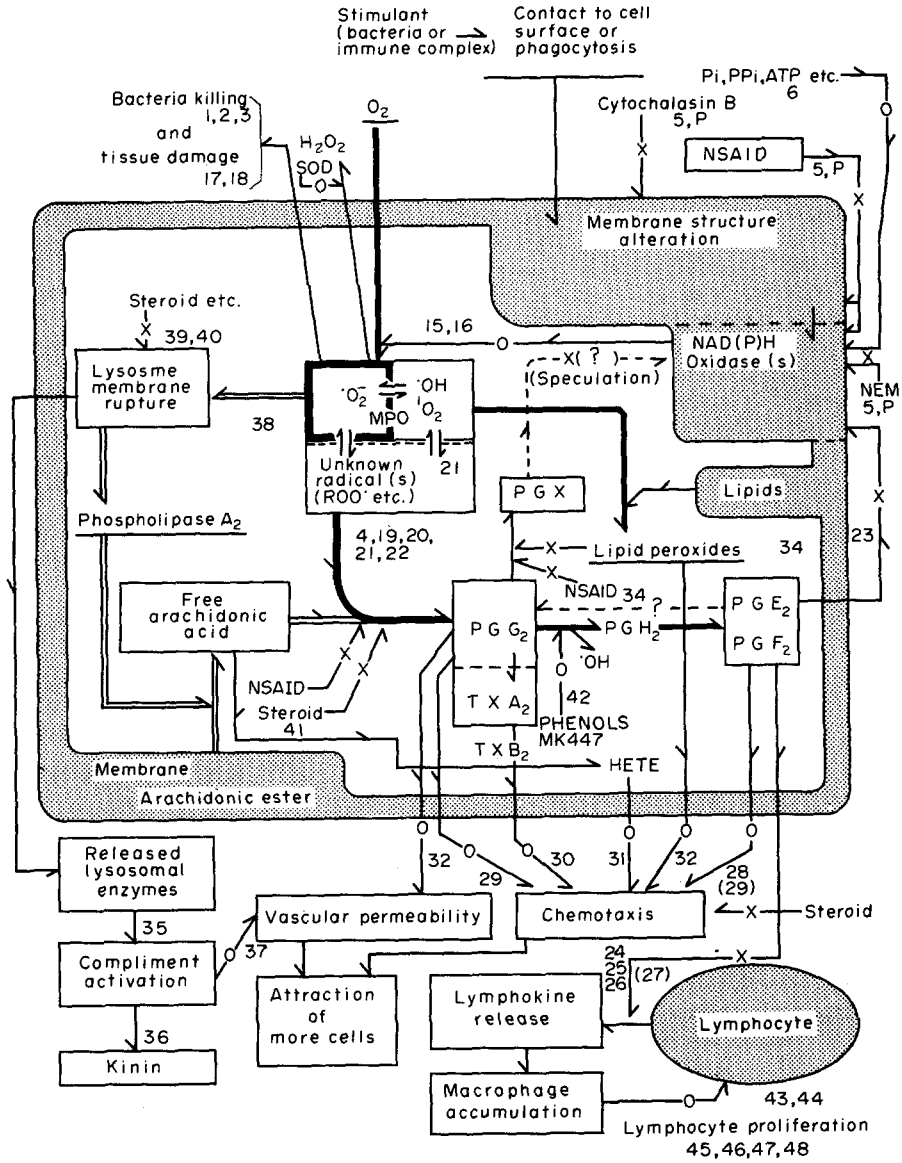

Fig. 5. A hypothetical role of $\cdot O_2^-$ produced by macrophages or by PMN leucocytes for the development of inflammation. Numbers indicate those of references (P = present report). The references for well known mechanism was not cited. Possible participations of cyclic nucleotides, platelet aggregation, histamine release etc. to this figure, was excluded. (—O→) shows stimulation and (— x →) inhibition. See also text. After preparation of this diagram, a report concerning platelet [J. G. White *et al.*, *Am. J. of Pathol.* **88**, 387 (1977)] appeared and demonstrated that oxidation mechanism is involved to make free arachidonic acid as activated state which is rate-limiting step for PGs synthesis. It is possible also in macrophages and leucocytes that $\cdot O_2^-$ or same radical(s) derived from $\cdot O_2^-$ participates for activating free arachidonic acid to produce PGs.

accumulation of macrophages in the inflammed site [24–26]. PGE_2 injections suppressed also adjuvant induced inflammation of rats [27]. In order to explain the induction of inflammation by PGE_2 in earlier investigations, it must be supposed a possibility that PGE_2 transfer partially to PGG_2 or related inflammation-inducing intermediate(s).

It is not sure whether PGE_2 is chemotactic or not in rat and human PMN. PGG_2, PXB_2, HETE and lipid peroxides are candidates as chemotaxin [28–32]. PGs can potentiate the increase of vascular permeability provoked by histamine, serotonin and kinin [33]. PGX (prostacycline) is studied mainly with relation to platelet aggregation and aorta contraction. The formation of this unstable PGX is inhibited by lipid peroxides which may be produced from $\cdot O_2^-$ and membrane lipid [34]. Aortaic microsomal PGX synthetase is inhibited by NSAID [34]. In addition to regulatory role of PGE_2 and $PGF_{2\alpha}$, a similiar mechanism is tentatively supposed concerning PGX to control NAD(P)H oxidase(s) activity and is interested as a future problem.

Released lysosomal enzymes work on removal of tissue fragments and may also activate the compliment [35]. The later action makes kinin formation and increases vascular permeability [36]. The supply of free arachidonic acid is naturally one rate-limiting factor of PGs formation, but phospholipase A_2 (a lysosomal enzyme which split the membrane arachidonic acid ester to free arachidonic acid) content itself is possible to be regulated by $\cdot O_2^-$ content. Hydroxy radical ($\cdot OH$) derived from $\cdot O_2^-$ attacks lysosomal membrane [38]. Stabilization of lysosomes by steroid and by some NSAID has been reported, but high concentration of drugs is generally required [39, 40].

The most accepted action of NSAID today, is inhibition of PG synthetase. Steroids are also informed to inhibit this step of rheumatoid synovia [41]. Nevertheless, many contradictory data including that of Kuehl *et al.* have been appearing [42]. These authors proposed a concept that the removal of $\cdot OH$ is another way of some anti-inflammatory drug's action as their MK 447. This compound and some phenols scavenge $\cdot OH$ and accelerate the transformation of PGG_2 (inflammatory) to PGH_2 (less-inflammatory). They supposed also the importance of 15-hydroxyperoxy PGE_2 which produces oxygen-centered free radical as inflammation promoting substance. On the other hand, my concept is based on the inhibition of $\cdot O_2^-$ production by NSAID and may make it possible to amalgamate PG biosynthesis theory, lysosomal damage theory and chemotaxisis theory with more detailed experiments. Lymphocyte–macrophage interaction is informed to be required for the maximum efficiency of lymphocyte proliferation. $\cdot O_2^-$ might play some role for regulation of this binding, because the oxidation of sialic acid-containing plasma membranes of these cells by sodium periodate or by galactose oxidase [45, 46], results the increase of lymphocyte-macrophage interaction and of lymphocyte proliferation. Membrane sialic acid is, on the other hand, essential for $\cdot O_2^-$ production of human PMN [47]. $PGF_{2\alpha}$ may be a mediator of T-T lymphocytes and T-B lymphocytes interaction [48].

In this report, nevertheless, I would like only to propose a new simple *in vitro* screening method for NSAID using PEC of non-treated guinea-pigs which may be supported with reasonable physiological or pathological background as mentioned above.

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