COMPARATIVE STUDY BETWEEN STEROIDAL AND NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON THE MODE OF THEIR ACTIONS ON VASCULAR PERMEABILITY IN RAT CARRAGEENIN-AIR-POUCH INFLAMMATION

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(Received February 2, 1980)

In an attempt to clarify mechanisms of anti-inflammatory actions of steroidal and nonsteroidal anti-inflammatory drugs with special reference to the possible role of E type prostaglandins in the inflammatory process, mode of actions of these drugs was investigated by using acute exudative stage and chronic proliferative stage of carrageenin-air-pouch inflammation in rats. This inflammation was induced on the back of rats by subcutaneous injection with air and carrageenin solution. Plasma exudation in the inflammation locus was determined with the aid of radioiodinated serum albumin. In the acute exudative stage of this inflammation, a single oral administration of dexamethasone, indomethacin, phenylbutazone or aspirin all exerted potent anti-exudative effect, while in the chronic proliferative stage, only dexamethasone was effective in inhibiting the exudation of the labeled albumin. Prostaglandin E level in the exudate of the acute stage inflammation was suppressed by either dexamethasone or indomethacin in accordance with the inhibition of the vascular permeability. These results suggest (1) significant role of prostaglandin E in the acute exudative inflammation, (2) similarity between steroidal and nonsteroidal anti-inflammatory drugs in the mechanism of their anti-exudative actions in the acute inflammation, (3) independency of the exudative process in the chronic proliferative inflammation from prostaglandins and (4) role of extra-prostaglandin mechanism(s) for the anti-exudative action of the anti-inflammatory steroids.

Keywords—dexamethasone; nonsteroidal anti-inflammatory drug; prostaglandin E; acute exudative inflammation; chronic proliferative inflammation; vascular permeability

It is generally accepted that non-steroidal anti-inflammatory drugs such as indomethacin and aspirin exert their anti-inflammatory effect through inhibition of prostaglandin synthesis.¹⁾ Steroidal anti-inflammatory drugs also inhibit prostaglandin production in a variety of tissues and cultured cells.²⁻⁵⁾ These findings appear to indicate that steroidal and nonsteroidal anti-inflammatory drugs share a common mechanism underlying their anti-inflammatory actions. However, we have observed a distinct difference in their actions between steroidal and nonsteroidal drugs, when these drugs were applied in the

chronic proliferative stage of carrageenin-induced inflammation in rats.⁶⁾ Volume of the exudate in the locus of chronic proliferative inflammation was not reduced by treating animals with indomethacin or phenylbutazone, whereas betamethasone treatment reduced markedly the exudate volume. In these experiments the drugs were administered once a day over the period of four consecutive days. Such consecutive treatments with anti-inflammatory steroids may exert anti-exudative effect through a secondary mechanism such as inhibition of the vascularization in granulation tissues.⁷⁾ Therefore, primary

action of a drug should be investigated after a single administration of the drug.

The present experiments were designed to determine whether there was any difference between steroidal and nonsteroidal anti-inflammatory drugs in respect to their primary actions on the vascular permeability in the acute exudative or chronic proliferative inflammation or both. We used carrageenin-air-pouch inflammation in rats, which had been developed in this laboratory as an experimental model suitable to investigate entire process of inflammation ranging from acute exudative stage to chronic proliferative stage.^{6,8)}

MATERIALS AND METHODS

Drugs and Chemicals — Seakem No. 202 carrageenin was purchased from Marine Colloid Inc., Springfield, N.J., U.S.A., ¹³¹I-human-serum-albumin (¹³¹I-HSA, 5 μ Ci/mg) from Dainabot Co., Tokyo, ¹²⁵I-human-serum-albumin (¹²⁵I-HSA, 2.5 μ Ci/mg) from Kaken Kagaku Co., Ltd., aspirin from Iwaki Pharmaceutical Co., Tokyo and dexamethasone from Sigma Chemical Co., St. Louis, M.O., U.S.A. Naproxen was a gift from Tanabe Pharmaceutical Co., Osaka, phenylbutazone from Fujisawa Pharmaceutical Co., Osaka and indomethacin from Nippon Merck Ban-yu Co., Tokyo.

As commercial preparations of ¹³¹I- and ¹²⁵I-human-serum-albumins contain a significant amount small molecular radiochemical impurities, purification through Sephadex G-100 gel filtration was carried out before use as described in a previous paper.⁹⁾

Induction of Carrageenin-Air-Pouch Inflammation — Carrageenin-air-pouch inflammation was induced according to the procedure already reported.⁸⁾ Male rats of Donryu strain and Sprague-Dawley strain of 6 weeks old were used. No fundamental difference was observed between two strains in the sensitivity to the drugs used in the present study. The rats were injected subcutaneously with 8 ml of air on the dorsum to make an air sac and 24 hr later 4 ml of 2% (w/v) solution of carrageenin in 0.9% NaCl was injected into the air sac already formed. The carrageenin

solution was sterilized by autoclaving at 120° for 15 min and injected after cooling to a temperature of 40–45°. Immediately before the injection, penicillin and streptomycin were added to the carrageenin solution to the final concentration of 0.1 mg/ml each. The day of carrageenin injection was designated as day 0.

Acute Exudative and Chronic Proliferative Stages of Carrageenin-Air-Pouch Inflammation — As reported in our previous paper,⁸⁾ carrageenin-air-pouch inflammation is an appropriate experimental model to investigate inflammatory process not only in acute exudative stage but also in chronic proliferative stage of the inflammation. Vascular permeability response in the acute exudative stage of this inflammation model is most prominent in the period of 2 to 8 hr after the injection of carrageenin. After that, the vascular permeability response in the acute stage declines gradually with the passage of time and subsides substantially by 24 hr. The state of subsidence, however, does not continue beyond day 3, as vascular permeability of the local tissues is forced to increase in accordance with the proliferation of granulomatous tissue in chronic proliferative stage of the inflammation. Maximal level in the vascular permeability in the chronic proliferative stage is commonly observed around day 7.

Evaluation of Anti-Inflammatory Effect of Drugs on Vascular Permeability in the Acute Exudative Stage — Nonsteroidal anti-inflammatory drugs, indomethacin, naproxen, phenylbutazone and aspirin, were administered 1 hr before the injection of carrageenin through a stomach tube as suspensions in 0.5% (w/v) carboxymethyl cellulose solution. A steroidal drug, dexamethasone, dissolved in 10% ethanol in 0.9% NaCl, was also administered through a stomach tube 2 hr before the injection of carrageenin. Control animals were treated with each vehicle. At 2.5 hr after the injection of carrageenin, animals were injected intravenously with 1 μ Ci of ¹³¹I-HSA in 0.2 ml 0.9% NaCl. After 30 min, animals were sacrificed by cutting the carotid arteries under ether anesthesia. Entire exudate fluid in the inflammation locus was collected and volume of the fluid

was measured. An aliquot (1.0 ml) of the exudate was taken and its radioactivity was measured in a well type scintillation counter Aloka JDC-751 (Aloka Co., Mitaka, Tokyo). Total radioactivity of the entire exudate fluid was calculated and expressed in terms of percent of the radioactivity injected and used as an index of the vascular permeability in the inflammation locus.

Evaluation of Anti-Inflammatory Effect of Drugs on Vascular Permeability in the Chronic Proliferative Stage — Steroidal and nonsteroidal anti-inflammatory drugs were all administered in a similar manner as in the case of acute stage of inflammation with the exception of time of the drug treatments. The drug treatments were performed on day 7. Effects of the drugs were assayed as described in our previous paper⁹⁾ by double tracer method using ^{125}I - and ^{131}I -HSAs.

In brief, vascular permeability of each inflammation locus was measured twice, firstly with ^{125}I -HSA immediately before the administration of test drugs and secondary with ^{131}I -HSA at appropriate times after the drug treatments. In the vehicle-treated animals, the ratio of the permeability values, ^{131}I -HSA/ ^{125}I -HSA, commonly comes to a value near 1.0 as already reported,⁹⁾ since the vehicle exerts no significant influence on vascular permeability. This double tracer method has been proved to be efficient in reducing experimental error arising from statistical variation among individual animals.⁹⁾ Time intervals between drug treatments and vascular permeability measurements were selected to be 4 hr in case of nonsteroidal drugs and 5 hr in case of dexamethasone in agreement with those in the acute stage experiments.

Determination of Prostaglandin E Level in the Inflammatory Exudates — Assay of prostaglandin E in the exudate was carried out by the method reported in a previous paper.¹⁰⁾ In brief, an aliquot (1.0 ml) of the exudate was extracted with 3.0 ml of a mixture of ethyl acetate/isopropanol/0.1 N HCl (3:3:1, by vol.) and phase was separated by the centrifugation. The organic solvent phase was dried under N_2 stream at 45° . The crude residue obtained was dissolved in a mixture of solvent 1

(benzene/ethyl acetate/methanol, 6:4:1, by vol.) and solvent 2 (benzene/ethyl acetate, 6:4, by vol.), 1:4, by vol. The solution was applied on the mini-column of silicic acid (100 mesh, Mallinckrodt Co., St. Louis, M.O., U.S.A.) which had been prepared by feeding activated silicic acid (0.5 g) suspension in the solvent 2 (5 ml). The column was washed with solvent 3 (benzene/ethyl acetate/methanol, 6:4:2, by vol.) and then with solvent 2 before application of the sample solution. After applying the sample and draining the solvent, a mixture of benzene/ethyl acetate/methanol, 6:4:0.2, by vol., were allowed to drain the fraction containing E type prostaglandins. The fraction was dried under N_2 stream at 45° . The resultant residue was dissolved in 250 μl of isogel/Tris buffer (0.01 M Tris buffer containing 0.1% gelatin, 0.14 M NaCl, $1.6 \cdot 10^{-3}\text{M}$ MgSO_4 and $1.6 \cdot 10^{-4}\text{M}$ CaCl_2 , pH 7.4) and 200 μl of this solution was subjected to alkaline dehydration for conversion of PG E to PG B. The immunoassay reaction mixture consisted of 50 μl of the standard PG B₁ solution or 50 μl of the alkaline dehydrated samples, 0.01 μCi of [^3H]PG B₁ (Clinical Assays Inc., Cambridge, M.A., U.S.A.), 100 μl of appropriately diluted rabbit anti-PG B₁ serum (Clinical Assays, Inc.) and 1.0 ml of the isogel/Tris buffer. This reaction mixture was incubated at 37° for 60 min. After the incubation was completed, normal rabbit serum and goat anti-rabbit serum were added, well mixed then incubated at 4° for 20 hr. Antiserum-bound PG B was precipitated by centrifugation, and the precipitate was dissolved in 1.0 ml of 1M NaOH. An aliquot (0.5 ml) of the resultant solution was mixed with 5 ml of triton-toluene scintillation fluid (PPO 7g, POPOP 0.3g, in toluene 667 ml and Triton X-100 333 ml) and the radioactivity was measured in a liquid scintillation counter, Aloka LSC-671R. The calibration curve was obtained by plotting the percent of the binding of [^3H]PG B₁ at each concentration of PG B₁.

RESULTS

Effects of Nonsteroidal Anti-Inflammatory Drugs on Vascular Permeability in Carrageenin-Air-Pouch In-

flammation

Anti-exudative effect of nonsteroidal drugs, indomethacin, naproxen, phenylbutazone and aspirin were tested in both the acute exudative and chronic proliferative stages of the carrageenin-air-pouch inflammation. Each drug was administered 1 hr before the injection of carrageenin in experiments for the acute stage and on day 7 for the chronic proliferative stage. Measurement of the vascular permeability was always done 4 hr after the administration of the drugs. The time interval of 4 hr between administration

of the drugs and measurement of the vascular permeability was decided from the data of the drug kinetics in the literature.¹¹⁾ The results are summarized in Fig. 1. In the acute stage experiments, at the doses of 10, 30 and 100 mg/kg of naproxen, the vascular permeability was inhibited by 27, 43 ($p < 0.01$) and 39% ($p < 0.025$), respectively; phenylbutazone at the doses of 20, 60 and 200 mg/kg inhibited the vascular permeability by 24 ($p < 0.01$), 48 ($p < 0.005$) and 58% ($p < 0.005$), respectively; aspirin at the doses of 60, 200 and 600 mg/kg by 36 ($p < 0.01$), 46 ($p < 0.005$) and

FIG. 1. Effects of Naproxen (A), Phenylbutazone (B), Aspirin (C) and Indomethacin (D) on Vascular Permeability in Carrageenin-Air-Pouch Inflammation

The vertical bars indicate standard errors of the means. Statistically significant difference was indicated by marking with † ($p < 0.005$), * ($p < 0.01$) and § ($p < 0.025$). Numbers in open columns represent number of rats used for the assay of vascular permeability.

54% ($p < 0.005$), respectively; indomethacin at the doses of 1, 3 and 10 mg/kg by 33 ($p < 0.005$), 51 ($p < 0.005$) and 51% ($p < 0.005$), respectively. On the other hand, in the chronic proliferative stage, none of the nonsteroidal drugs tested was effective in suppressing the vascular permeability even at the highest dose, 100 mg/kg of naproxen, 200 mg/kg of phenylbutazone, 600 mg/kg of aspirin or 30 mg/kg of indomethacin.

Effect of Dexamethasone on Vascular Permeability in Carrageenin-Air-Pouch Inflammation

Anti-exudative effect of a steroidal drug dexamethasone was also tested similarly as in the case of nonsteroidal drugs. Dexamethasone was administered 2 hr before the injection of carrageenin in experiments for the acute stage and on day 7 for the chronic stage. Vascular permeability was assayed 5 hr after the administration of dexamethasone. A time interval of 5 hr between administration of the drug and measurement of the vascular permeability was decided for maximum effect in accordance with our previous observations.¹²⁾ The results are summarized in Fig. 2. In the acute stage, dexamethasone at doses of 0.1, 0.3 and 1.0 mg/kg suppressed vascular permeability

FIG. 2. Effect of Dexamethasone on Vascular Permeability in Carrageenin-Air-Pouch Inflammation

The vertical bars indicate standard errors of the means. Statistically significant difference was indicated by marking with [†] ($p < 0.005$). Numbers in open columns represent number of rats used for the assay of vascular permeability.

FIG. 3. Effects of Dexamethasone (A) and Indomethacin (B) on Prostaglandin E Level in the Exudate of the Acute Stage Inflammation

■ : prostaglandin E level in the exudate, □ : vascular permeability as measured in terms of the quantity of leaked radioiodinated serum albumin and expressed as % of the injected radioactivity. The vertical bars indicate standard errors of the means. Statistically significant difference was indicated by marking [†] ($p < 0.005$). Numbers in closed and open columns represent number of the samples used for the assay of prostaglandin E and number of the rats used for the assay of vascular permeability, respectively.

by 31 ($p < 0.005$), 34 ($p < 0.005$) and 55% ($p < 0.005$), respectively. In contrast with non-steroidal anti-inflammatory drugs, dexamethasone was effective also in chronic proliferative stage at doses of 0.1, 0.3 and 1.0 mg/kg, brought about 35 ($p < 0.005$), 50 ($p < 0.005$) and 62% ($p < 0.005$) inhibition, respectively.

Effect of Dexamethasone and Indomethacin on Prostaglandin E Level in the Exudate of the Acute Stage Inflammation

It was demonstrated in our previous study that there was a close parallelism between time change of the exudate prostaglandin E level and time change of the vascular permeability response in the acute exudative stage of the carrageenin-air-pouch inflammation.⁸⁾ To our knowledge, however, it was still uncertain whether vascular permeability and E type prostaglandins changed in parallel with each other under the influence of steroidal and nonsteroidal anti-inflammatory drugs. In an attempt to answer this question, effect of anti-inflammatory drugs on prostaglandin E level in the exudates of the acute stage inflammation was investigated. In respect to dexamethasone the exudates of the above mentioned acute stage experiment were taken as samples for the investigation of prostaglandin E. Regarding indomethacin a supplemental experiment was performed: indomethacin was administered orally 4 hr after the injection of carrageenin, ¹³¹I-HSA was injected intravenously at 7.5 hr and exudates were taken 30 min later for the assay of radioactivity and prostaglandin E. The results are summarized in Fig. 3. Prostaglandin E and vascular permeability decreased together in dose-dependent manner under the influence of either dexamethasone or indomethacin.

DISCUSSION

Among a number of experimental models of the inflammatory lesions which have been developed up to date, carrageenin-induced paw edema in the rat has been most widely used in the field of experimental pharmacology. In fact, many nonsteroidal anti-inflammatory drugs have been developed on the basis of drug-screening

data depending on the carrageenin paw edema method.^{13,14)} Some strong nonsteroidal drugs such as indomethacin are well comparable with the steroids in respect to the potency for inhibiting carrageenin-induced paw edema. However, none of the nonsteroidal drugs is comparable with the steroids in the potency for relieving rheumatoid patients from inflammatory symptoms. These facts appear to suggest that carrageenin paw edema method is not suitable for an accurate estimation of anti-rheumatic efficacy of anti-inflammatory drugs. Since carrageenin-induced paw edema is a typical inflammation of acute exudative nature, this model may be inappropriate to mimic the proliferative phase of rheumatoid synovitis. From this point of view, chronic proliferative stage of the carrageenin-air-pouch inflammation, which has been developed in our laboratory, is considered to meet requirement for the experimental model for proliferative phase of rheumatoid synovitis. In the inflammatory lesions of rheumatoid arthritis, acute exudative inflammation as reflected on leucocyte emigration is frequently accompanied with the proliferative processes. Therefore, evaluation of anti-inflammatory effect of a drug on the acute phase inflammation model is also recommendable. Anti-inflammatory steroid dexamethasone was equally effective in both the acute exudative and the chronic proliferative stages of the carrageenin-air-pouch inflammation, though nonsteroidal anti-inflammatory drug, indomethacin, naproxen, phenylbutazone and aspirin were inhibitory only in the acute stage. Such difference between steroidal and nonsteroidal drugs in the mode of anti-inflammatory action may account for the difference in their efficacy against rheumatoid inflammation.

Concerning mechanism of anti-inflammatory action of nonsteroidal anti-inflammatory drugs, the concept that suppression of prostaglandin production through blockade to prostaglandin synthetase underlies their anti-inflammatory action is widely accepted.¹⁾ Moreover, it has been shown that anti-inflammatory steroids also inhibit prostaglandin production in various tissues

and cultured cells²⁻⁵⁾ probably through inhibition of arachidonic acid liberation.^{4,5)} In addition to these findings, Tam *et al.* indicated close correlation of the inhibitory potency of various glucocorticoids against production of E type prostaglandins by cultured cells with their anti-inflammatory potency as evaluated by carrageenin paw edema method.¹⁵⁾ These observations appear to indicate that both steroidal and nonsteroidal anti-inflammatory drugs exert their anti-inflammatory effect through inhibition of the production of proinflammatory prostaglandin E. In fact, in the acute exudative stage of the carrageenin-air-pouch inflammation, both steroidal and nonsteroidal anti-inflammatory drugs brought about dose-dependent inhibition of vascular permeability in parallel with inhibition of prostaglandin E level in the exudate (Fig. 3).

Role of E type prostaglandins in promoting vascular permeability response in acute inflammation has been frequently suggested on the basis of various lines of evidence.^{8,16-18)} Similarity of nonsteroidal anti-inflammatory drugs to anti-inflammatory steroids (Fig. 3) in respect to suppression of vascular permeability response and prostaglandin E level in the locus of acute inflammation serves, on the one hand, as strong evidence for the role of E type prostaglandins in the acute inflammation and suggest, on the other hand, similarity in the mechanisms responsible for their anti-inflammatory effects.

In chronic proliferative stage of the carrageenin-air-pouch inflammation, levels in the exudate of prostaglandins E, $F_{2\alpha}$ and 6-keto-PG $F_{1\alpha}$ and thromboxane B_2 all declined with the passage of time in contrast with the increase of the vascular permeability.^{8,19)} It is unlikely, therefore, that prostaglandins and thromboxanes play any significant role in the chronic proliferative stage of the carrageenin-air-pouch inflammation. In agreement with this concept, none of the nonsteroidal anti-inflammatory drugs (prostaglandin synthetase inhibitors) exerted any inhibitory effect on the vascular permeability in the chronic proliferative stage of the inflammation (Fig. 1). On the contrary, anti-inflammatory steroid dexa-

methasone was very effective in inhibiting the vascular permeability in the chronic proliferative stage as well as in the acute exudative stage (Fig. 2). Hydrocortisone was also effective in the chronic proliferative stage as already reported.¹²⁾ Therefore, anti-exudative action of the steroidal anti-inflammatory drugs in the chronic proliferative stage should be explained independently of their influence on prostaglandin production. From this point of view, effect of the steroid derived from suppression of lipoxygenase pathway *via* reduction of the liberation of starting materials such as arachidonic acid from membrane phospholipids^{5,20)} should be investigated. Alternately, steroid-induced direct antagonism to the contraction of vascular endothelial cells in the inflammation locus as suggested in our previous paper^{8,21,22)} should be considered as a possible mechanism.

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