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Preparation of immunoaffinity mini-columns for the analysis of platelet activating factor (PAF) in biological samples

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ABSTRACT

Using an antibody to BN 52719, an analogue of platelet activating factor (PAF), immunoaffinity mini-columns for the separation of PAF from biological samples were prepared. Rabbits were immunized with BN 52719 and immunoglobulin G (IgG) from the antiserum was coupled with Sepharose 4B. The resulting suspension of the IgG-coated Sepharose 4B in 25 m*M* phosphate buffer (pH 6.9) was poured into a plastic mini-column (bed volume 2.0 × 0.8 cm). Stepwise elution of the column with methanol revealed that lyso-PAF is eluted with 20–30% methanol in water whereas PAF is eluted with 50–80% methanol. For the determination of PAF in biological samples, it is recommended that lipids are extracted from the samples and the extract, reconstituted in 20% methanol, is loaded on the column. The column is then washed with 50% methanol followed by elution of PAF with 80% methanol. A small amount of [^{3}H]PAF

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is added to the samples for measurement of the recoveries of PAF during the procedures of extraction and elution. The PAF is then quantified by radioimmunoassay or bioassay. Employing the immunoaffinity mini-column and radioimmunoassay, the contents of PAF in macrophages and conditioned medium after stimulation with calcium ionophore A23187, or tumor promoters such as TPA and thapsigargin, were measured.

INTRODUCTION

Platelet activating factor (PAF) is a likely candidate as a mediator of inflammation as it has a variety of proinflammatory properties and is produced by a variety of inflammatory cells such as neutrophils, monocytes, macrophages, platelets and vascular endothelial cells after a number of stimuli [1–4]. In order to clarify roles of PAF in inflammation [5,6], it is necessary to determine PAF in the inflammatory locus.

For the determination of PAF in biological samples, several methods have been developed, such as bioassay [7], radioimmunoassay (Kits are commercially available from New England Nuclear, Boston, MA, USA, and Amersham International Amersham, UK) and gas chromatography–mass spectrometry (GC–MS) [8]. Bioassay and radioimmunoassay cannot avoid cross-response or cross-reaction with endogenous substances and GC–MS requires a tedious purification process prior to analysis. Consequently, it is desired to establish a simple and quantitative clean-up method for the determination of PAF in biological samples. Recently, a selective method for the purification of a prostacyclin derivative using an immobilized antibody was reported [9]. Similarly, using an immobilized antibody to PAF, we have developed immunoaffinity mini-columns for the determination of PAF in biological samples. In this paper, we describe the preparation of the immunoaffinity mini-columns and their applications for the assay of PAF in macrophages in combination with the radioimmunoassay method.

EXPERIMENTAL

Preparation of the BN 52179–bovine serum albumin conjugate for immunization

The structure of the PAF analogue BN 52179 is shown in Fig. 1. The hapten was designated not to be metabolized by acetyl hydrolase. An amino group of BN 52179 was used to form an amide bond with a carboxyl function of succinylated bovine serum albumin in the presence of carbodiimide [10]. To 20 μmol of BN 52179 were added 20 mg of a succinylated bovine serum albumin dissolved in 0.1 *M* sodium phosphate buffer (pH 7.4), followed by the addition of 23 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Bio-Rad Labs., Richmond, CA, U.S.A.). The reaction mixture was incubated at room temperature overnight, then dialyzed against isotonic 0.01 *M* Tris buffer (pH 7.4). To the dialyzed conjugate (24 mg of succinylated bovine serum albumin, a negatively charged immunogen) were added 2.4 mg methylated bovine serum albumin (a positively charged macromolecule) to form an insoluble salt complex [10,11], to which 4 ml of complete Freund's adjuvant were added and emulsified. The emulsion was injected into five rabbits intradermally and subcutaneously. For booster injections, the same doses were used. Rabbit blood was collected 2, 3 and 4 weeks after each course of injection and antibody titers to BN 52179 were measured.

Iodination of BN 52179

After the benzene had been evaporated from 1.3 mCi of ^{125}I-labeled Bolton–Hunter reagent (New England Nuclear) under a gentle stream of nitrogen, 10 μl of BN 52179 (2 mg/ml in dimethyl sulfoxide) and 10 μl of 0.1 *M* sodium phosphate buffer (pH 8.0) were added and the mixture was stirred at room temperature for 20 min. The reaction was stopped by addition of 250 μl of 0.4 *M* glycine in 0.1 *M* sodium phosphate buffer (pH 8.0). After 5 min, 250 μl of a 0.1% (w/v) solution of bovine serum albumin were added and the reaction mixture was applied to a Sephadex G-10 column (26 × 1 cm

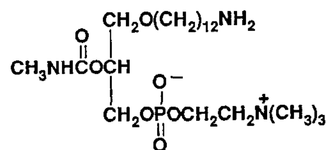

Fig. 1. Structure of BN 52179.

I.D.). The column was eluted with 0.05 *M* sodium phosphate buffer (pH 7.5) containing 0.1% (w/v) of bovine serum albumin. The radioactivity in each fraction was measured and peak fractions were collected and used.

Measurement of antibody titer in rabbit serum

The diluent for all reagents was 0.01 *M* Tris buffer (pH 7.4) containing 0.14 *M* NaCl and bovine serum albumin (10 mg/ml). One hundred-fold diluted rabbit antiserum was incubated at 37°C for 60 min with [^{125}I]BN 52179. Antigen–antibody complexes were precipitated with goat anti-rabbit immunoglobulin G (IgG) and radioactivities in the precipitate were measured.

Preparation of IgG

A 40% (w/v) $(NH_4)_2SO_4$-insoluble fraction of the rabbit antiserum to BN 52179 that was obtained after the third course of immunization was dialyzed against 0.1 *M* sodium phosphate buffer (pH 8.0). The dialyzed IgG was applied to a protein A-Sepharose (Sigma, St. Louis, MO, USA) column that had been equilibrated with the same buffer. The protein A-Sepharose column was then eluted with 0.1 *M* sodium citrate buffer (pH 3.0) and the fractions containing IgG were collected and immediately neutralized with 0.1 *M* sodium phosphate buffer (pH 8.0).

Cross-reactivity of the IgG against BN 52179 was examined using [^{3}H]PAF (1-O-[octadecyl-9,10-^{3}H(N)]-2-acetyl-*sn*-glyceryl-3-phosphocholine, 120 Ci/mmol; New England Nuclear) and unlabeled samples such as BN 52179, L-PAF (Sigma), D-PAF (Sigma) and D,L-lyso-PAF (Sigma).

Preparation of the immunoaffinity mini-column

The IgG thus obtained was coupled with Sepharose 4B [9] and the resultant suspension of the IgG-coated Sepharose 4B in 25 m*M* sodium phosphate buffer (pH 6.9) was poured into a plastic mini-tube to make an extraction column (bed volume 2.0 × 0.8 cm). The column was conditioned with 10 ml of 25 m*M* sodium phosphate buffer (pH 6.9) before use.

In order to determine the retention capacity of the column for PAF and lyso-PAF, a mixture of [^{3}H]PAF and [^{3}H]lyso-PAF (1-O-[alkyl-1′,2′-^{3}H]-*sn*-glyceryl-3-phosphocholine, 50 Ci/mmol, New England Nuclear) dissolved in 20% (v/v) methanol solution in 25 m*M* sodium phosphate buffer (pH 6.9) was applied to the column and eluted with each 3 ml of water containing increasing amounts of methanol. The radioactivity in each fraction was measured.

Macrophage culture and sample preparation

Rat peritoneal macrophages were harvested 4 days after intraperitoneal injection of a solution containing soluble starch (Wako, Tokyo, Japan) and bacto peptone (Difco, Detroit, MI, USA), 5% each [12]. The macrophages were seeded at $6 \cdot 10^6$ cells per 60-mm plastic tissue culture dish in 4 ml of Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% (v/v) calf serum (Flow Labs., North Rydge, NSW, Australia), penicillin G potassium (18 μg/ml) and streptomycin sulfate (50 μg/ml), (Meiji Seika, Tokyo, Japan) and incubated for 2 h at 37°C. After incubation, the dishes were washed three times with the medium to wash out non-adherent cells. The adherent cells were further incubated for 20 h in 4 ml of the medium. After washing three times with the medium containing no calf serum but 0.1% (w/v) of bovine serum albumin (BSA, essentially fatty acid-free; Sigma), the cells were incubated for 1 h in 4 ml of the medium containing 0.1% (w/v) of BSA and various drugs. A TPA-type tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma), a non-TPA-type tumor promoter thapsigargin (Funakoshi, Tokyo, Japan) and calcium ionophore A23187 (Sigma) were used as drugs. After incubation, the conditioned medium was centrifuged at 1600 *g* for 5 min at 4°C and the supernatant fraction was collected. The dishes were washed three times, then 2.5 ml of a 2% (v/v) solution of acetic acid in methanol were added to the dish and the cells were scraped out with a "rubber policeman". For the measurement of recovery, $3.0 \cdot 10^{-3}$ μCi of [^{3}H]PAF were added to both fractions, the conditioned medium and the cells. Total lipids were then extracted from both fractions and reconstituted in 25 m*M* sodium phosphate buffer (pH 6.9) containing 20% (v/v) of methanol.

Clean-up of the samples for measurement of PAF

The reconstituted solution was loaded on the immunoaffinity mini-column, then the column was

washed with 5 ml of 50% (v/v) methanol in water and PAF was eluted with 5 ml of 80% (v/v) methanol in water. The eluate was evaporated to dryness and the residue was reconstituted in 50 m*M* sodium phosphate buffer (pH 6.3) containing 0.05% (v/v) of Tween 20, and a portion of the solution was used for radioimmunoassay. The remainder of the solution was used for the measurement of the radioactivity of [^{3}H]PAF, and the recovery of PAF was calculated.

Radioimmunoassay of PAF

Radioimmunoassay of PAF was performed using the commercially available kit (Platelet Activating Factor [^{125}I]-RIA kit; New England Nuclear), in which a calibration graph was constructed using cold PAF as a mixture of 1-O-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (C_{18}-PAF) and 1-O-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (C_{16}-PAF) (1:1, w/w). Cross-reactivity of the antibody is described in the Instruction Manual accompanying the kit. Further details of the radioimmunoassay procedure are also described in the Instruction Manual.

RESULTS AND DISCUSSION

Three out of five rabbits produced antibodies that bound [^{125}I]BN 52179. The titer was the highest after the third course of immunization, when the IgG was prepared using the protein A-Sepharose column.

An IgG used for the immunoaffinity mini-column cross-reacted with [^{3}H]L-PAF. Such cross-reaction binding could be displaced by unlabeled L-PAF in addition to the homologous hapten BN 52179. About 10 pmol of L-PAF were required for 50% displacement and maximum displacement varied from 70 to 90%. The homologous BN 52179 displaced the cross-reaction binding completely. However, with unlabeled D-PAF or lyso-forms of D- and L-PAF, such cross-reaction binding was not displaced (Fig. 2). Consequently, after loading on the column, lyso-PAF was easily eluted from the column with 20–30% methanol in water (Fig. 3). In contrast, higher concentration of methanol were required to elute PAF from the column (Fig. 3).

The results shown in Fig. 3 indicate that substances other than PAF are eluted by washing the column with 50% methanol in water, and PAF is eluted by washing the column with 80% methanol in water. Consequently, in the following experiments, PAF was obtained from the column by elution with 5 ml of 80% methanol in water, after washing the column with 5 ml of 50% methanol.

The capacity of the column to retain PAF was measured by applying increasing amounts of PAF on the column. As shown in Fig. 4, the maximum capacity was *ca.* 7 pmol of PAF. Less than 7 pmol

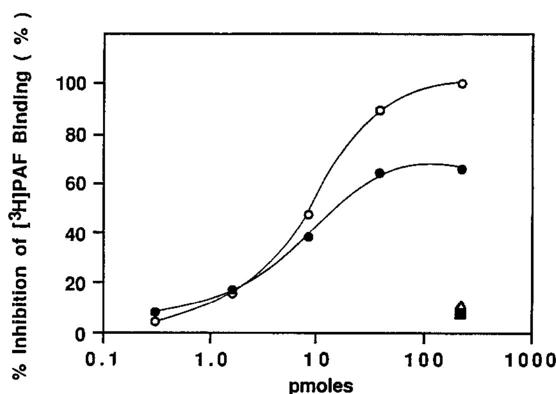

Fig. 2. Inhibition of [^{3}H]PAF binding to the IgG by (●) L-PAF, (○) BN 52179, (△) D-PAF and (■) D,L-lyso PAF. 16 000 cpm [^{3}H]PAF added; 4000 cpm specifically bound. One hundred-fold diluted IgG was used.

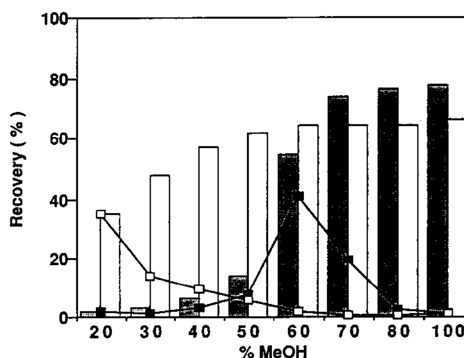

Fig. 3. Elution profiles of [^{3}H]PAF and [^{3}H]lyso-PAF. [^{3}H]PAF and [^{3}H]lyso-PAF were dissolved in 20% (v/v) methanol solution in 25 m*M* sodium phosphate buffer (pH 6.9) and were applied to the immunoaffinity mini-column, then the column was washed with 3-ml portions of water containing indicated concentrations of methanol. The radioactivity in each fraction was measured and recoveries of (■) [^{3}H]PAF and (□) [^{3}H]lyso-PAF were calculated. Shaded columns and open columns represent the cumulative recoveries of [^{3}H]PAF and [^{3}H]lyso-PAF, respectively.

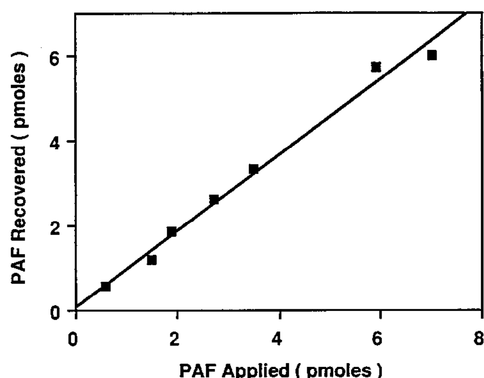

Fig. 4. Recoveries of PAF applied to the immunoaffinity mini-columns. Various amounts of [^{3}H]PAF were applied to the column. The column was washed with 5 ml of 50% (v/v) methanol in water and the eluate was discarded, then [^{3}H]PAF was eluted with 5 ml of 80% (v/v) methanol in water and the total radioactivity in the eluate was measured. The net amount of PAF was calculated from the specific radioactivity of [^{3}H]PAF.

of PAF was quantitatively obtained. Application of higher amounts of PAF decreased the recovery of PAF (data not shown). Consequently, for the determination of PAF in biological samples, it is desirable to add a small amount of [^{3}H]PAF to the sample and measure the recoveries of PAF during the procedure. Preliminary experiments revealed that the direct application of biological samples such as serum and inflammatory exudate on the column tended to plug the column and made it difficult to elute. Further, the recoveries of [^{3}H]PAF in 50–80% methanol fraction were decreased drastically, probably owing to the existence of interfering substances that inhibit PAF binding to the immobilized antibody. In order to overcome these problems, total lipids should be extracted from the samples by Bligh and Dyer's method [13], and applied to the column.

Employing the immunoaffinity mini-columns and radioimmunoassay method, PAF production by rat peritoneal macrophages was examined. The antibody to PAF used in the radioimmunoassay is specific to a mixture of C_{18}-PAF and C_{16}-PAF (1:1, w/w) and cross-reacts very little with triglyceride, sphingomyelin, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, lyso-PAF, arachidonic acid, testosterone and prostaglandins (Instruction Manual for Measurement of PAF provided with PAF radioimmunoassay kit from New England Nuclear). After macrophages had been incubated for 30 min in the medium containing 1 μM A23187, conditioned medium was collected and 40 pmol of PAF were added to both the conditioned medium and the macrophages then the PAF levels were measured in both fractions (Table I). Without stimulation, macrophages produced no detectable amount of PAF. However, A23187 treatment stimulated PAF production, and the levels of PAF were much higher in the macrophages than in the conditioned medium. A23187-stimulated production of PAF has been reported in human mononuclear phagocytes [14] and polymorphonuclear leukocytes [15] measured by incorporation of [^{3}H]acetate into [^{3}H]PAF and bioassay, respectively. As shown in Table I, the PAF levels in both fractions, the conditioned medium and the macrophages, after addition of 40 pmol of PAF to each were exactly reflected. These results suggest that the assay method employing the immunoaffinity mini-columns is accurate.

Thapsigargin is a hexaoxygenated tetraacylated sesquiterpene lactone isolated from the roots of *Thapsia garganica* L. (Apiaceae) [16] and is one of the non-TPA-type tumor promoters in two-stage carcinogenesis using mouse skin [17]. Previously, we reported [18] that thapsigargin stimulates arachi-

TABLE I

MEASUREMENT OF PLATELET ACTIVATING FACTOR (PAF) ADDED TO THE FRACTION OF MACROPHAGES AND CONDITIONED MEDIUM AFTER STIMULATION WITH A23187

Macrophages ($6 \cdot 10^6$ cells) were incubated at 37°C for 30 min in 4 ml of medium containing 0.1% (w/v) BSA with or without A23187 (1 μM). After the conditioned medium had been collected, 0 or 40 pmol of PAF were added to the cells and to the conditioned medium. The PAF levels in the cells and the conditioned medium were then radioimmunoassayed after clean-up of the samples by the immunoaffinity mini-column. Values are means ± standard errors (S.E.) from four dishes.

	PAF added (pmol)	PAF (pmol per $6 \cdot 10^6$ cells)	
		Cell-associated	Conditioned medium
Control	0	N.D.[a]	N.D.
A23187 (1 μM)	0	49.9 ± 3.2	14.0 ± 2.4
	40	89.6 ± 3.7	54.1 ± 3.3

[a] N.D., not detectable.

TABLE II

STIMULATION OF PLATELET ACTIVATING FACTOR (PAF) PRODUCTION BY TPA AND THAPSIGARGIN IN MACROPHAGES

Macrophages ($6 \cdot 10^6$ cells) were incubated at 37°C for 1 h in 4 ml of medium containing 0.1% (w/v) BSA and the indicated concentrations of A23187, TPA or thapsigargin. PAF levels in cells and the conditioned medium were radioimmunoassayed after clean-up the samples by the immunoaffinity mini-column. Values are means ± S.E. from four dishes.

	PAF (pmol per $6 \cdot 10^6$ cells)	
	Cell-associated	Conditioned medium
Control	N.D.[a]	N.D.
A23187 (1 *μM*)	41.5 ± 6.3	9.3 ± 1.6
TPA (10 ng/ml)	7.7 ± 0.8	N.D.
Thapsigargin (10 ng/ml)	25.9 ± 3.4	N.D.

[a] N.D., not detectable.

donic acid metabolism in rat peritoneal macrophages as well as TPA at very low concentrations. The stimulation of arachidonic acid metabolism by thapsigargin was suggested to be due to stimulation of phospholipase A_2 [18]. If phospholipase A_2 is activated, production of the lyso-form of PAF, a precursor of PAF, will be increased. It is expected that increased production of lyso-PAF might lead to increased production of PAF. The present investigation was intended to clarify whether PAF production is increased by treatment with thapsigargin. We found that thapsigargin stimulates PAF production more potently than TPA at the same concentration (Table II). Precise analysis of thapsigargin-stimulated production of PAF by macrophages will be described elsewhere.

In conclusion, the immunoaffinity mini-columns are useful for the preparation of PAF from biological samples for quantitative measurement by radioimmunoassay or bioassay.

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