STUDIES ON THE PATHOGENESIS OF FEVER

VII. Preliminary Chemical Characterization of Leucocytic Pyrogen*

By GALE W. RAFTER, Ph.D., ROBERT D. COLLINS,‡ M.D., and W. BARRY WOOD, Jr., M.D.

(*From the Department of Microbiology, The Johns Hopkins University School of Medicine and School of Hygiene and Public Health and the Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore*)

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Polymorphonuclear leucocytes isolated from acute inflammatory exudates contain a pyrogen which is indistinguishable in its effects from the endogenous pyrogenic factors demonstrable in the circulation of rabbits during experimental fevers (1, 2). The present study deals with the chemical properties of the leucocytic pyrogen (3). Although it has not as yet been isolated in a homogeneous state, it has been shown: (*a*) to contain an essential protein moiety and (*b*) to be chemically distinguishable from bacterial endotoxins (4), Menkin's pyrexin (5), and the pyrogenic tissue polysaccharides of Landy and Shear (6).

Methods

Preparation of Leucocytic Extracts.—Acute granulocytic exudates were obtained from the peritoneal cavities of rabbits as previously reported (7). After a 6 ml. aliquot had been removed from the pooled exudate for culture and white cell count, each lot was centrifuged at 625 *g* for 20 minutes at 4°C. The supernatant fluid was discarded, and the cells were resuspended in pyrogen-free saline so that each 10 ml. contained 3.5×10^8 cells. The saline-cell mixture was then incubated at 37°C. for 24 hours with occasional agitation. After separation by centrifugation in the cold (625 *g* for 20 minutes) the cells were discarded, and the supernatant fluid containing the pyrogen was stored at 4°C. The pyrogenicity of each lot of supernatant fluid was determined by testing an aliquot of 10 to 20 ml.

Tests for Pyrogenicity were performed by injecting 10 to 20 ml. samples intravenously into normal rabbits. When the test material had been previously concentrated, smaller volumes were injected. The purified pyrogen (*vide infra*) was also tested in endotoxin-tolerant rabbits (8). The methods used in handling the rabbits, taking their temperatures and avoiding the introduction of extraneous pyrogens from glassware, etc., have been described in previous publications (1, 9). All chemical reagents (including trypsin and pepsin) were tested for pyro-

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‡ Senior Research Fellow, United States Public Health Service. Present address: Department of Pathology, Vanderbilt University School of Medicine, Nashville.

genicity, and only non-pyrogenic preparations were used. Precipitates obtained during chemical fractionation were dissolved in normal saline, and each soluble fraction was dialyzed against normal saline before being assayed for pyrogenicity. Control saline effluents from the ion-exchange columns used to purify the pyrogen were also tested for thermogenic contaminants.

Methods of Chemical Assay.—Protein concentrations were measured either by the Lowry phenol technique (10) or by determining optical densities at 280 mμ with a Beckman D.U. spectrophotometer (11). In the former method, crystalline serum albumin was used as the protein standard; in the latter, 1 mg. of protein per ml. was assumed to equal an optical density of 1.0. Tests for carbohydrate were performed by the orcinol method (12).

Electrophoretic Analysis.—Preliminary characterization of the protein component of the crude leucocytic extracts was determined by paper electrophoresis (13).

Procedures Used for Characterization of Leucocyte Pyrogen.—The following methods were used to obtain additional information concerning the chemical nature of the active pyrogen.

1. Proteolysis Experiments.—The trypsin reaction mixture employed contained 3 mg. of trypsin,[1] leucocytic extract concentrated 5- to 10-fold by dialysis against 25 per cent dextran,[2] 4×10^{-3} M Ca^{++},[3] and sufficient 0.15 M tris(hydroxymethyl)aminomethane buffer (pH 8) to make a total volume of 11 ml. All samples were incubated for 4 hours at 37°C. before being dialyzed with saline and tested for pyrogenicity.

The pepsin reaction mixture contained 2 mg. of pepsin,[4] concentrated leucocytic extract, and 0.02 M sodium acetate buffer (pH 4.0) in a final volume of 10 ml. Each sample was incubated for 2 hours at 37°C.

Control samples containing (*a*) no trypsin (or no pepsin) and (*b*) no leucocytic pyrogen were also tested for pyrogenicity.

2. Phenol Extraction.—At room temperature, enough phenol was added to 10 ml. of leucocytic extract to make a final concentration (*w/v*) of 90 per cent (15). The mixture was shaken and the supernatant aqueous layer was removed, dialyzed against normal saline, and tested for pyrogenicity.

3. Periodate Oxidation.—Two ml. of concentrated leucocytic extract were mixed with 1 ml. of 0.033 M potassium periodate and 8 ml. of 0.01 M sodium acetate buffer (pH 5.2) (16). The mixture was allowed to stand for 5 hours at 0°C., was dialyzed against saline, and tested for pyrogenicity. A control sample was similarly treated, except that the periodate was omitted from the mixture.

4. Treatment with p-Chloromercuribenzoate.—Eight ml. of 0.01 M tris(hydroxymethyl)-aminomethane (pH 8.3) containing 0.6 mg. *p*-chloromercuribenzoate (17) were added to 2 ml. of concentrated leucocytic extract. After the mixture had been allowed to stand for 30 minutes at 0°C., it was dialyzed overnight against saline, and its pyrogenicity was compared with that of an appropriate control sample of the same extract.

Concentration and Purification of Leucocytic Pyrogen.—Separation of the active material was first attempted with saturated ammonium sulfate (18), which was added stepwise to samples of concentrated extract at pH 7 to make the final solutions 33, 50, and 70 per cent saturated. The precipitates which formed at each of the salt concentrations were redissolved in saline. These and the corresponding supernatant fluids were then tested for pyrogenicity.

The fractionation achieved with ammonium sulfate was neither sharp nor sufficiently re-

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[1] Worthington Chemical Company, Freehold, New Jersey.

[2] Dextran (6 per cent *w/v*), purchased from Wyeth Laboratories, Philadelphia, was concentrated to 25 per cent by boiling. A fresh concentrate was prepared after each sixth dialysis.

[3] To prevent autodigestion of the trypsin (14).

[4] Kindly provided by Dr. Roger Herriott of the Department of Biochemistry of the Johns Hopkins School of Hygiene and Public Health.

producible. The procedure outlined in Fig. 1 was finally adopted for isolating the active pyrogen from the crude extracts of leucocytes. All operations were performed at 0–4°C., unless otherwise stated. The methods used were as follows:—

(*a*) *Saline Dialysis.*—Leucocytic extracts placed in cellophane tubes were dialyzed against 20 volumes of normal saline for 18 hours. Several changes of saline were made during the period of dialysis.

(*b*) *Butanol Extraction.*—The dialyzed extract was concentrated 5- to 10-fold by dialysis against 25 per cent dextran. Butanol was then added to make a final concentration of 20 per cent (*v/v*) (19). The resulting mixture was shaken intermittently for 15 minutes and centrifuged at 900 *g* (10 minutes). The butanol layer and the lipid-containing precipitate at the butanol-saline interface were both removed with a pipette and discarded.

(*c*) *Methanol Precipitation.*—To the remaining aqueous layer an equal volume of methanol was slowly added with vigorous stirring (18). When the mixture had been allowed to stand for 20 minutes, the precipitate which formed was separated by centrifugation (900 *g* for 15 minutes) and was discarded, after it had been shown to be non-pyrogenic. The supernatant fluid was dialyzed against saline to remove the methanol.

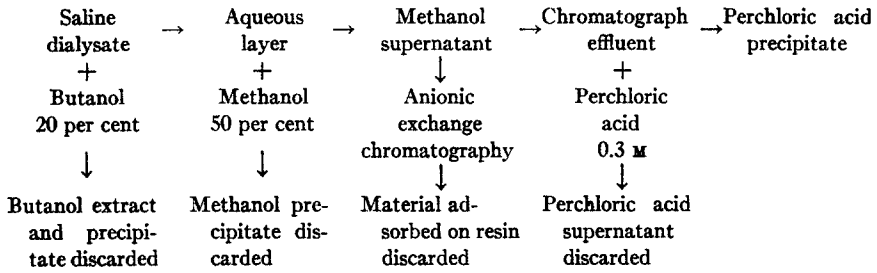

FIG. 1. Method of partially purifying leucocytic pyrogen. (Concentration and dialysis steps not included—see Methods.)

(*d*) *Anionic Exchange Chromatography.*—The dialyzed methanol supernatant fluid was concentrated 10-fold by dialysis against dextran and was then dialyzed against 20 volumes of 0.01 M Na_2HPO_4 (pH 8) for 5 hours during which several changes of the phosphate solution were made. The concentrated supernatant fluid was applied to a packed column, 2 cm. × 0.8 cm., of diethylaminoethyl cellulose (DEAE)[5] (20), which had just been washed with 500 ml. of 0.01 M Na_2HPO_4 (pH 8) solution. The flow rate through the column was maintained at 3 ml. per minute by applied pressure. The effluent, or "break-through" fraction, was collected and stored at 4°C. The adsorbed material remaining on the column was removed by 20 ml. of 0.4 M KH_2PO_4 (pH 5).

(*e*) *Precipitation with Perchloric Acid.*—The effluent from the DEAE column was concentrated by dialysis against 25 per cent dextran to give a final protein concentration of approximately 2 mg. per ml. Sixty per cent perchloric acid was added drop by drop to the concentrated effluent with constant stirring, until the final concentration was 0.3 M (21). The precipitate which formed was separated after 15 minutes by centrifugation (900 *g* for 18 minutes), dissolved in saline, and neutralized with 1 N NaOH.

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[5] DEAE (diethylaminoethyl cellulose) was purchased from Eastman Kodak Company, Rochester, New York. Before being used, it was washed successively with 95 per cent ethyl alcohol, 0.6 N NaOH, and 0.05 M Na_2HPO_4 (pH 8). After its pH had been adjusted to 8.0, the washed resin was stored at 4°C.

Immunochemical Test for Homogeneity.—Antisera were prepared by repeatedly inoculating guinea pigs subcutaneously at 8-day intervals with 2 ml. amounts of a 1 to 1 mixture of concentrated leucocytic extract and Freund's adjuvant[6] (22). The serum obtained was stored at −20°C. The tests for antigen-antibody interaction were performed by the Ouchterlony technique (23). Petri dishes containing 0.7 per cent agar with 0.01 per cent merthiolate were prepared in the usual manner. The circular wells cut in the agar were 6 mm. in diameter and 1.5 cm. apart as measured from center to center. The volume of fluid placed in each well was 0.03 ml. All plates were sealed and allowed to stand at room temperature for at least 72 hours before being read. The preparation of purified pyrogen being tested for homogeneity was added to the antigen well, after having been concentrated to 10 mg. per cent of protein.

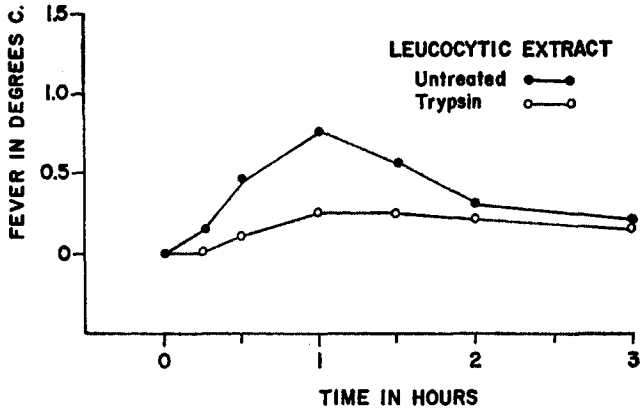

FIG. 2. Fever responses produced in normal rabbits by equal quantities (20 ml.) of a single sample of leucocytic extract, one aliquot of which was untreated (upper curve) and the other subjected to proteolysis with trypsin (lower curve).

RESULTS

The acute inflammatory exudates harvested from rabbits with saline-induced peritonitis were bacteriologically sterile and of approximately neutral pH. Ten to 20 ml. aliquots of the saline extracts of cells from these exudates produced a brisk monophasic fever. When heated to 90°C. for 30 minutes, they lost their pyrogenicity (2).

Upon chemical analysis, the leucocytic extracts were found to contain protein, carbohydrate, lipid, and a component with an absorption peak at 260 mμ. The latter, presumably containing nucleotides, was readily removed by saline dialysis without affecting the pyrogenicity of the extract. Treatment with butanol, to separate the lipid component,[7] also failed to decrease pyrogenicity. Likewise, full pyrogenicity was retained following treatment with periodate. Since periodate, in the concentration employed, oxidizes most carbohydrates,

[6] Bacto-adjuvant complete (Freund), Difco Laboratories, Detroit.

[7] A lipid-containing precipitate regularly formed at the butanol-water interface and was removed with the butanol from the aqueous layer.

it was tentatively concluded that carbohydrate is not an essential component of the active pyrogen. Further evidence in support of this conclusion was obtained in experiments involving extraction with phenol. The latter procedure, which removes all of the protein, but leaves free polysaccharides in solution (15), completely inactivated the leucocytic extracts.

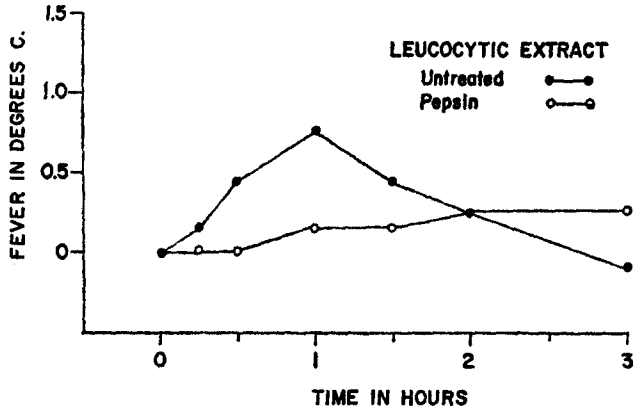

FIG. 3. Comparative febrile reactions of normal rabbits to equal intravenous doses (20 ml.) of untreated (upper curve) and pepsin-treated leucocytic extract (lower curve).

TABLE I

Relative Amounts of Total Protein Present in Various Fractions of Leucocytic Extract Recovered during Partial Purification of Pyrogen

Fraction	Protein in original fraction
	per cent
Original leucocytic extract	100
Methanol-soluble fraction	25
"Break-through" from DEAE column	5
Perchloric acid precipitate	2

The above findings strongly suggested that the pyrogenic activity of the extracts resides in the protein fraction. In keeping with this hypothesis was the finding that the pyrogen is precipitable with perchloric acid. In addition, it was found to be inactivated by proteolysis. As shown in Fig. 2, the pyrogenicity of 20 ml. of the extract was lost following treatment with trypsin. A similar result was obtained with pepsin (Fig. 3). Since the enzyme-treated preparations in these experiments were dialyzed against saline before being tested for pyrogenicity (see Methods), the possibility remained that the proteolysis had merely rendered the pyrogen dialyzable. Accordingly, the trypsin

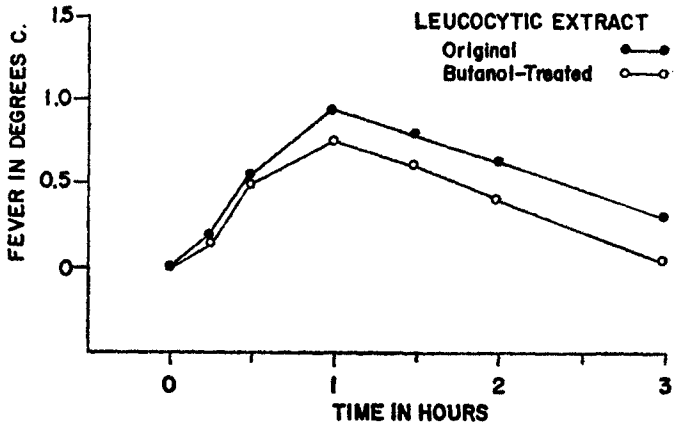

FIG. 4. Fever produced in normal rabbits by a pyrogenic aliquot (20 ml.) of leucocytic extract (upper curve) and an equal quantity of the same extract following treatment with butanol (lower curve).

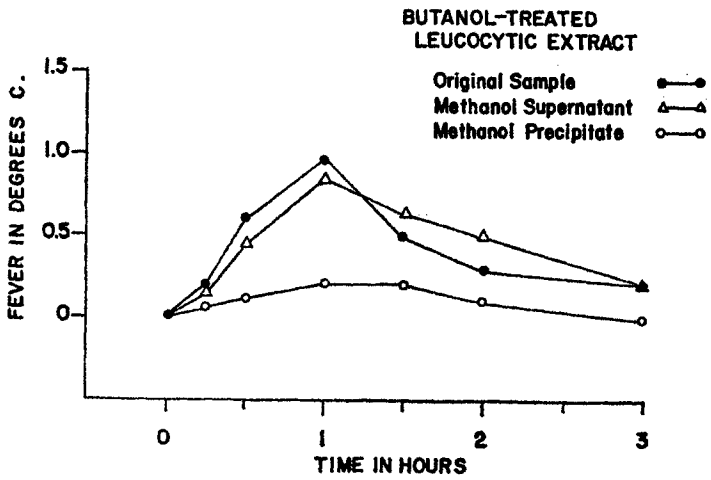

FIG. 5. Effect of methanol fractionation on pyrogenicity of butanol-treated leucocytic extract. Fevers produced by original (butanol-treated) extract (20 ml.) and by methanol supernatant are shown in upper curves, and lack of response to redissolved methanol precipitate is indicated by the lower curve.

experiment was repeated without dialysis. Again, the enzyme-treated extract was found to be non-pyrogenic, indicating that the pyrogen had been destroyed by the proteolysis. Thus, it was concluded that an essential component of the active pyrogen is a protein. Furthermore, since its activity was unaffected by

treatment with *p*-chloromercuribenzoate, its action was tentatively concluded to be independent of the presence of sulfhydryl groups (17).

The average total protein content of 20 ml. of the crude extract obtained from 7×10^8 leucocytes was 25 mg. At least 6 different components were demonstrable by paper electrophoresis. Fractionation of the proteins was first attempted with saturated ammonium sulfate. Although the precipitate which formed in the 33 per cent saturated mixture was non-pyrogenic, those which formed in the 50 per cent and 70 per cent saturated salt fractions were both thermogenically active. A more satisfactory fractionation was obtained by the

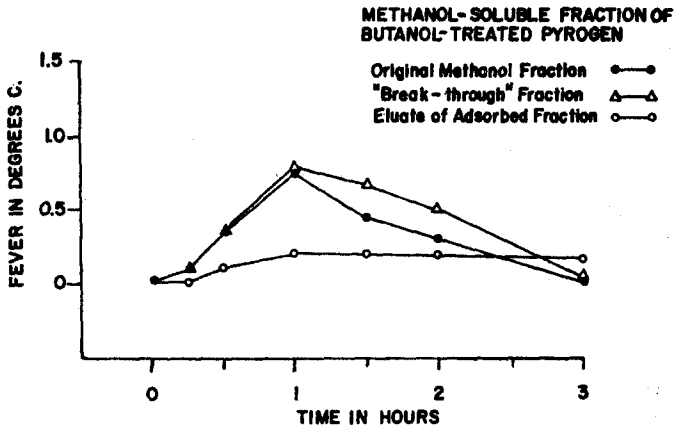

FIG. 6. Effect of anionic exchange chromatography on methanol-soluble fraction of leucocytic pyrogen obtained from 7×10^8 cells and previously treated with butanol. Fevers caused by original methanol fraction and by "break-through" fraction from DEAE column are recorded in upper curves. The fraction, which remained on the column and was later eluted from it, failed to produce a significant fever, as shown by the bottom curve.

procedure outlined in Fig. 1. As little as 0.5 mg. of the final perchloric acid precipitate caused a reproducible fever in normal rabbits. Thus, the degree of purification achieved was approximately 50-fold. The amount of protein recovered after each step of fractionation of 20 ml. of the crude extract is shown in Table I, and the febrile responses produced by the fractions obtained following the butanol, methanol, and chromatographic steps are recorded in Figs. 4, 5, and 6 respectively. The final perchloric acid precipitate contained less than 1 per cent carbohydrate. Despite the degree of purification achieved, however, the final product was shown by the Ouchterlony technique to contain at least two antigens.

The biological properties of the purified pyrogen were essentially indistinguishable from those of the original pyrogenic extract. Both preparations

were heat-labile, both produced similar fever responses in normal rabbits (compare Figs. 2 and 6), and both were equally active in normal and tolerant recipients (2, 3, 8).

DISCUSSION

The chemical and enzymatic data of the present study indicate that the fever-producing effect of the pyrogen derived from rabbit polymorphonuclear leucocytes (1, 3) is due to the presence of a protein. Not only is the active factor non-dialyzable through cellophane, precipitable with perchloric acid and inactivated by extraction with phenol, but its pyrogenicity is also destroyed by incubation with either trypsin or pepsin.[8] When partially purified by chemical means, including treatment with butanol and precipitation with perchloric acid, it contains less than 1 per cent carbohydrate and no readily extractable lipid. Its solubility in 33 per cent ammonium sulfate and 50 per cent methanol suggests an albumin-like molecule, and its failure to be adsorbed by the anionic-exchange resin (DEAE) when dissolved in 0.01 M Na_2HPO_4 is indicative of cationic properties at pH 8. Although perchloric acid precipitation is useful in separating it from mucoproteins, the procedure occasionally causes appreciable loss of pyrogenic activity, presumably due to denaturation of the active protein.

As reviewed elsewhere (2, 24), the biological properties of leucocytic pyrogen are quite different from those of pyrogenic bacterial endotoxins (25), Menkin's "pyrexin" (5), and the pyrogenic "tissue polysaccharides" described by Landy and Shear (6). The results of the present study indicate that these other pyrogenic substances are also chemically distinguishable from leucocytic pyrogen. Bacterial endotoxins are high molecular weight lipopolysaccharide-protein complexes which retain their pyrogenicity when subjected to proteolysis (4). Also, they are relatively heat-stable (2), and preliminary experiments indicate that the thermogenic activity of at least some of them is destroyed by treatment with butanol (26). Pyrexin, likewise, is heat-stable (2) and is inactivated by butanol treatment (26). Furthermore, in contrast to leucocytic pyrogen, pyrexin is precipitated by 33 per cent saturated ammonium sulfate (5). The Landy-Shear tissue pyrogens are polysaccharides which are prepared by treating cellular extracts with trypsin (6)—a procedure which destroys leucocytic pyrogen. It is evident, therefore, that none of these heat-stable thermogenic factors (2) is either biologically or chemically similar to leucocytic pyrogen.

The immunochemical studies thus far completed with the leucocytic extracts reveal at least two antigenically active components in the purified material which produces fever. Whether either of these is responsible for the pyrogenicity

[8] The finding that proteolysis destroys the activity of leucocytic pyrogen is contrary to that previously reported by Bennett and Beeson (3). Their failure to obtain inactivation with trypsin may have been due either to the nature of the preparations which they employed or to the conditions under which the proteolytic effect of the enzyme was tested.

is, as yet, not known. Experiments designed to answer this question are, at present, in progress. In addition, an attempt is being made, by further fractionation, to isolate the pyrogenic component as a single antigen. If such a homogeneous fraction can be obtained, it will not only be subjected to more rigorous chemical analysis but also will be used to prepare specific antiserum. The latter is needed particularly for immunochemical experiments designed: (*a*) to investigate the possible identity of the several "endogenous pyrogens" which have already been described in various forms of experimental fever (2, 24), and (*b*) to define more precisely the cellular origins and sites of action of the circulating pyrogen in the intact host.

SUMMARY

Study of the chemical properties of the pyrogenic component of rabbit polymorphonuclear leucocytes reveals it to contain an essential, non-dialyzable protein which: (*a*) is precipitated by perchloric acid, (*b*) is removed by extraction with phenol, (*c*) is soluble in 50 per cent methanol and 33 per cent saturated ammonium sulfate, and (*d*) is destroyed by the proteolytic action of both trypsin and pepsin.

By combined chemical and chromatographic techniques the leucocytic pyrogen has been purified approximately 50-fold. The partially purified material contains less than 1 per cent carbohydrate, is resistant to periodate oxidation, is unaffected by extraction with butanol and contains at least two immunologically active components when tested by the Ouchterlony gel-diffusion technique. Its chemical properties distinguish it from other known pyrogenic substances which have been implicated in the pathogenesis of fever.

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