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PREPARATION OF ADSORBENTS FOR PYROGEN ADSORPTION

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SUMMARY

Preparation of adsorbents having a higher affinity for "pyrogen" was investigated. Adenine, cytosine, histamine and histidine showed high affinities as ligands for pyrogen. Cellulose and agarose were the most suitable as matrices for the preparation of adsorbents having a high affinity for pyrogen. When the chain length of the spacer was 19.7–29.0 Å, the adsorbents showed the highest affinity for pyrogen. The adsorbent immobilized histamine to aminohexyl-Sepharose CL-4B with glutaraldehyde had the highest affinity for pyrogen originating from *Escherichia*, *Klebsiella* and *Salmonella* cells.

INTRODUCTION

Among pyrogenic substances, it is well known that lipopolysaccharides (LPS; synonyms "pyrogen", "endotoxins" and "O-antigen"), constituents of the cell wall of gram-negative bacteria, produce the highest fever in animals[1]. If a certain pharmacologically active substance is contaminated with pyrogen, its intravenous administration produces transient fever in homothermic animals. Therefore, it is necessary to remove pyrogen present in the substance.

The following methods have been used in attempts to remove the pyrogen present in certain substances: (a) physical adsorption on charcoal[2], ion-exchange resins[3,4], etc., (2) chemical decomposition with an acid, alkali[5] or oxidizing agent[6] and (3) filtration using an ultramembrane filter[4]. However, when pyrogen is removed from a substance by the above physical adsorption or chemical methods, in some instances the substance is also non-specifically adsorbed or decomposed. We therefore considered that it would be useful to prepare an adsorbent specific for pyrogens. To design this adsorbent, we chose compounds containing purine, pyrimidine or imidazole residues as the ligand.

In this paper, we describe the selection of ligands, matrices and chain length of the spacer suitable for the preparation of adsorbents with high affinities for pyrogen.

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EXPERIMENTAL

Materials

Endotoxin (*Klebsiella pneumoniae*) was prepared according to the method of Westphal *et al.*[7]. Other endotoxins were purchased from Difco Labs. (Detroit, MI, U.S.A.). Cyanogen bromide-activated Sepharose 4B and Sepharose CL-4B were purchased from Pharmacia (Uppsala, Sweden). Diaion WK-11, WK-20 (weak cation-exchange resin), CR-10 (chelate resin) and WA-21 (weak anion-exchange resin) and highly porous chloromethylpolystyrene resin were obtained from Mitsubishi (Tokyo, Japan). Toyopearl HW-55 and HW-65 (polyvinyl alcohol resin) were purchased from Toyo Soda (Tokyo, Japan). Filter pulp (No. 4) was purchased from Toyo Roshi (Tokyo, Japan). Pre Gel (*Limulus* amoebocyte lysate) was purchased from Seikagaku Kogyo (Tokyo, Japan). *tert.*-Butyloxycarbonyl-L-isoleucyl-L-glutamylglycyl-L-arginine-4-methylcoumaryl-7-amide (hydrochloride form) (Boc-Ile-Glu-Gly-Arg-MCA-HCl), dicyclohexylcarbodiimide and 1-hydroxybenztriazole were purchased from the Peptide Institute (Osaka, Japan). Adenine hydrochloride, cytosine, 5-methylcytosine, 2-amino-4-hydroxy-6-methylpyrimidine and 2-amino-4,6-dimethylpyrimidine were purchased from Sigma (St. Louis, MO, U.S.A.). Histidine was purchased from Ajinomoto (Tokyo, Japan). Ethylenediamine dihydrochloride, epichlorohydrin, hexamethylenediamine, histamine dihydrochloride, sodium borohydride and glutaraldehyde were purchased from Katayama Chemical Industries (Osaka, Japan). Other alkyldiamines were purchased from Nakarai Chemicals (Kyoto, Japan). Acrinol and pyrogen-free water were the products of Tanabe Seiyaku (Osaka, Japan). All other chemicals were of analytical-reagent grade.

Preparation of aminohexylcellulose

Aminohexylcellulose was prepared according to the method of Watanabe *et al.*[8].

Preparation of aminoalkylagaroses

Cyanogen bromide procedure. In 40 ml of 0.1 *M* sodium hydrogen carbonate–20 m*M* ethylenediamine (pH 8.0) or 40 ml of 0.1 *M* sodium hydrogen carbonate–20 m*M* putrescine (pH 10.0), 11.1 g (wet weight) of cyanogen bromide-activated Sepharose 4B were suspended. The suspension was gently stirred at 25°C for 24 h. After the reaction, the aminoalkylagarose was collected and washed with 1 *M* sodium chloride solution and water.

Epichlorohydrin procedure. In 270 ml of water, 180 g (wet weight) of Sepharose CL-4B were suspended. To the suspension, 117 ml of 2 *N* sodium hydroxide solution and 27 ml of epichlorohydrin were added and the mixture was shaken at 40°C for 2 h. The epichlorohydrin-activated Sepharose CL-4B was collected and washed with water. In 80 ml of water or 50% ethanol, 4.31 mmole of alkyldiamine were dissolved and then adjusted to pH 11.0 with 0.1 *N* sodium hydroxide solution or 0.1 *N* hydrochloric acid. In the solution, 20 g (wet weight) of the epichlorohydrin-activated Sepharose CL-4B were suspended. The suspension was shaken at 60°C for 2 h. The aminoalkylagarose was washed with water or 50% ethanol.

Preparation of aminohexyl synthetic resins

In 20 ml of dimethylformamide containing 105 mmole of dicyclohexylcarbodiimide, 30 ml of each resin were suspended, and then 20 ml of dimethylformamide containing 105 mmole of 1-hydroxybenztriazole and 60 ml of dimethylformamide containing 210 mmole of hexamethylenediamine were added to the suspension. The mixture was shaken at 30°C for 20 h. After the reaction, the aminohexyl resin was collected and washed with dimethylformamide, methanol and water.

Preparation of aminohexylpolystyrene

In a mixed solution of 15 ml of dichloromethane and 15 ml of methanol, 5 g (dry weight) of high porous chloromethylpolystyrene were suspended, and 1.42 ml of triethylamine were added to the suspension. The mixture was refluxed at 70–80°C for 4 h and the resin was collected and washed with water. In a mixed solvent of 30 ml of methanol and 20 ml of 2 *N* sodium hydroxide solution, 2.32 g of hexamethylenediamine were dissolved and the above-obtained resin was suspended in the resulting solution. The suspension was refluxed at 70–80°C for 4 h. The aminohexylpolystyrene was collected and washed with water.

Immobilization of ligands to matrices containing primary amino group

In 12 ml of 0.05 *M* phosphate buffer (pH 7.2), 8 ml of matrix were suspended and 12 ml of 25% glutaraldehyde were added to the suspension. The mixture was stirred at 25°C for 2 h. The resulting glutaraldehyde-activated matrix was collected and washed with 0.1 *M* phosphate buffer (pH 7.2), and then suspended in 19.5 ml of 0.1 *M* phosphate buffer containing 200–500 μmole of each ligand. The suspension was stirred at 25°C for 2 h. After the reaction, the immobilized ligand was collected and washed with 200 ml of 1 *M* sodium chloride solution and then suspended in 10 ml of 0.1 *M* phosphate buffer (pH 7.2). To the suspension, 100 mg of sodium borohydride were added and the mixture was occasionally stirred at 25°C for 4 h. After the reaction, the adsorbent was collected and washed with 1 *M* sodium chloride solution and water.

Immobilization of histamine to cyanogen bromide-activated Sepharose 4B

In 9.9 ml of 0.2 *M* sodium hydrogen carbonate containing 200 μmole of histamine, 6 g (wet weight) of cyanogen bromide-activated Sepharose 4B were suspended. The suspension was stirred at 25°C for 20 h. After the reaction, histamine-Sepharose 4B was collected and washed with 1 *M* sodium chloride solution and water.

Immobilization of histamine to epichlorohydrin-activated Sepharose CL-4B

Epichlorohydrin-activated Sepharose CL-4B was prepared by the same procedure as described above. In 40 ml of water, 184 mg (1 mmole) of histamine dihydrochloride were dissolved and the pH was adjusted to 13 with 1 *N* sodium hydroxide solution. In the solution, 10 g (wet weight) of the epichlorohydrin-activated Sepharose CL-4B were suspended and the suspension was shaken at 60°C for 2 h. After the reaction, the adsorbent was collected and washed with 200 ml of 1 *M* sodium chloride solution and water.

Determination of content of ligands immobilized to the matrix

Histamine and histidine were determined by the ninhydrin method[9], and the other compounds were determined by measuring the absorbance at 260–340 nm. The content of ligands immobilized to the matrix was calculated from the difference of their concentrations in the reaction mixture between, before and after the reaction with the matrix.

Measurement of affinity of each adsorbent for pyrogen

The affinity of each adsorbent for pyrogen was measured by the column method as follows. An 8-ml volume of adsorbent was washed with 200 ml of 1.5 *M* sodium chloride solution on a glass filter and packed in a sterilized column (6.0 × 1.3 cm). The column was washed with 300 ml of 1.5 *M* sodium chloride solution (pyrogen-free), 100 ml of water (pyrogen-free) and 100 ml of 0.05 *M* sodium chloride solution (pyrogen-free). Each pyrogen was dissolved in 0.05 *M* sodium chloride solution (pyrogen-free) at a concentration of 1000 ng/ml. The pyrogen solution was passed through the column at a flow-rate of 100 ml/h at 25°C.

Pyrogen assay method

Assay of pyrogen was carried out by the modified method of Harada *et al.*[10] using horseshoe crab clotting enzyme. Synthetic substrate (Boc-Ile-Glu-Gly-Arg-MCA-HCl) was dissolved in pyrogen-free water at a concentration of 2 m*M*. Tris–HCl buffer (0.4 *M*, pH 8.0) containing 0.04 *M* magnesium chloride was prepared using pyrogen-free water and autoclaved at 120°C for 2 h. One ampoule of Pre Gel was dissolved in 270 μl of the above buffer and 50 μl of substrate solution, 50 μl of Pre Gel solution and 100 μl of sample solution were incubated at 37°C for 10 min. After incubation, the reaction was stopped by adding 2.3 ml of 12.5% acetic acid and the fluorescence at 460 nm excited at 380 nm was measured. Pyrogen solution at a concentration of 1 ng/ml was used as a standard. Sample solutions were diluted successively with pyrogen-free 0.05 *M* sodium chloride solution.

TABLE I

SCREENING OF LIGANDS WITH AN AFFINITY FOR PYROGEN

Each ligand was immobilized to aminohexylcellulose with glutaraldehyde. Measurement of the affinity of each adsorbent for pyrogen was carried out by the method described in the text except for using 400 ml of pyrogen solution (*E. coli* O128:B12, LPS 1000 ng/ml).

Ligand	*Content of ligand (μmole/ml of adsorbent)*	*Concentration of pyrogen in effluent (ng/ml)*
Adenine	0.4	0.2
2-Amino-4,6-dimethyl-pyrimidine	7.8	1.6
2-Amino-4-hydroxy-6-methylpyrimidine	8.0	1.3
Cytosine	7.6	0.7
5-Methylcytosine	9.4	1.4
Ethacridine	2.1	3.7
Histamine	9.4	0.1
Histidine	1.8	0.2

RESULTS AND DISCUSSION

Screening of ligands

Kanoh *et al.*[11] reported that ribonucleic acid has a high affinity for pyrogen and it is very difficult to remove it from nucleic acids. Therefore, we considered that if the components of nucleic acid and related compounds are immobilized to water-insoluble matrices, the matrices obtained may be used as specific adsorbents for pyrogen.

Various heterocyclic compounds containing nitrogen were therefore covalently bound to aminohexylcellulose and their affinities for pyrogen were compared. The results are shown in Table I. These adsorbents were prepared under the same conditions, but the contents of their ligands differed. All compounds tested showed a high affinity for pyrogen, especially adenine, cytosine, histamine and histidine, for which the concentrations of pyrogen in effluents were below 1 ng/ml.

Contents of histamine in adsorbents on adsorption of pyrogen

The effect of the content of histamine as a ligand on the affinity of adsorbents for pyrogen was investigated. The results are shown in Table II. No difference was observed between contents of histamine in the adsorbent from 0.5 to 15.5 μmole/ml of adsorbent and the affinity for pyrogen.

Selection of matrices suitable for preparation of adsorbents

In order to select matrices suitable for the preparation of adsorbents adsorbing pyrogen, histamine was covalently bound to various matrices containing amino groups and the affinities of the adsorbents obtained for pyrogen were tested. The results are shown in Table III. Cellulose and agarose were suitable matrices for the preparation of adsorbents having a high affinity for pyrogen, but synthetic resins were not.

TABLE II

EFFECT OF CONTENT OF HISTAMINE IN ADSORBENTS ON ADSORPTION OF PYROGEN

Immobilization of histamine to aminohexylcellulose was carried out by the method described in the text except for employing 10–300 μmole of histamine. Measurement of the affinity of each adsorbent for pyrogen was carried out by the method described in the text except for using 100 ml of pyrogen solution (*E. coli* O128:B12, LPS 1000 ng/ml).

Content of histamine (μmole/ml of adsorbent)	*Concentration of pyrogen in effluent (ng/ml)*
0.5	0.15
1.5	0.79
2.6	0.08
3.4	0.25
6.1	0.06
9.5	0.11
15.5	0.10

TABLE III

SELECTION OF MATRICES SUITABLE FOR PREPARATION OF ADSORBENT BINDING HISTAMINE

Histamine was immobilized to each matrix with glutaraldehyde. Measurement of the affinity of each adsorbent for pyrogen was carried out by the method described in the text except for using 400 ml of pyrogen solution (*E. coli* O128:B12, LPS 1000 ng/ml).

Matrix	*Content of histamine (μmole/ml of adsorbent)*	*Concentration of pyrogen in effluent (ng/ml)*
AH-cellulose	9.4	0.1
AH-Sepharose 4B*	3.1	1.4
AH-Sepharose CL-4B**	4.9	0.7
AH-Diaion WK-11	5.5	1100
AH-Diaion WK-20	11.8	650
Diaion WA-21	52.5	710
AH-Diaion CR-10	32.5	850
AH-polystyrene	36.8	460
AH-Toyopearl HW-55	16.4	470
AH-Toyopearl HW-65	21.5	390

* Hexamethylenediamine was bound to cyanogen bromide-activated Sepharose 4B.
** Hexamethylenediamine was bound to epichlorohydrin-activated Sepharose CL-4B.

TABLE IV

EFFECT OF CHAIN LENGTH OF SPACER IN ADSORBENTS ON ADSORPTION OF PYROGEN

Histamine was immobilized to cyanogen bromide-activated Sepharose 4B or epichlorohydrin-activated Sepharose CL-4B directly or with each alkyldiamine and glutaraldehyde. Measurement of the affinity of each adsorbent for pyrogen was carried out by the method described in the text except for using 400 ml of pyrogen solution (*E. coli* O128:B12, LPS 1000 ng/ml).

Matrix	*Spacer*	*Chain length (Å)*	*Content of histamine (μmole/ml of adsorbent)*	*Concentration of pyrogen in effluent (ng/ml)*
CNBr-activated Sepharose 4B	$\geq C=N\,(CH_2)_2\,NH\,(CH_2)_5-$	13.6	3.0	88
	$\geq C=N\,(CH_2)_4\,NH\,(CH_2)_5-$	16.7	2.0	5.9
Epichlorohydrin-activated Sepharose CL-4B	$-CH_2CH\,(OH)\,CH_2-$	4.6	12.2	320
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_2\,NH\,(CH_2)_5-$	16.7	2.8	17
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_4\,NH\,(CH_2)_5-$	19.7	3.0	1.4
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_6\,NH\,(CH_2)_5-$	22.8	4.9	0.7
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_8\,NH\,(CH_2)_5-$	25.9	1.8	2.9
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_{10}NH\,(CH_2)_5-$	29.0	3.6	1.7
	$-CH_2CH\,(OH)\,CH_2NH\,(CH_2)_{12}NH\,(CH_2)_5-$	32.0	4.6	12

Chain length of spacer on adsorption of pyrogen

It is known from studies on affinity chromatography that the accessibility of macromolecules towards a ligand increases with extension of the spacer arm. Therefore, the effect of the chain length of the spacer on the affinity of adsorbents for pyrogen was investigated, and the results are shown in Table IV. The affinity increased with increase in the chain length of the spacer and resulted in a plateau when the chain length was 19.7–29.0 Å. The low affinity of adsorbents with short chain lengths may be due to steric hindrance of the matrix towards the pyrogen. However, the precise reason for the low affinity of adsorbents with longer chains is not known.

TABLE V

ADSORPTION OF PYROGEN ORIGINATING FROM VARIOUS MICROORGANISMS TO HISTAMINE-GA-AH-SEPHAROSE CL-4B

Hexamethylenediamine was bound to epichlorohydrin-activated Sepharose CL-4B, and then histamine was immobilized to this with glutaraldehyde. Each pyrogen was dissolved in 0.05 *M* sodium chloride solution at a concentration of 1000 ng/ml. Measurement of the affinity of adsorbent for each pyrogen was carried out by the method described in the text except for using 100 ml of the above pyrogen solution.

Pyrogen	*Concentration of pyrogen in effluent (ng/ml)*
Escherichia coli O128:B12	0.12
Klebsiella pneumoniae	0.02
Salmonella abortus equi	0.63
Salmonella enteritidis	0.64
Salmonella flexneri	0.04
Salmonella minnesota 9700	0.13

Adsorption of pyrogen originating from various microorganisms

In order to clarify the adsorption specificity of the adsorbent immobilized histamine to aminohexyl-Sepharose CL-4B with glutaraldehyde, pyrogen originating from various microorganisms was passed through a column packed with the adsorbent, and the affinity for each pyrogen was tested. The results are shown in Table V. The adsorbent had high affinity for pyrogen originating from all microorganisms tested.

From these results, this new adsorbent is considered to be useful as a tool for the selective removal of pyrogen present in certain substances. The adsorption characteristics and applications of this adsorbent for pyrogen will be described elsewhere.

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