[Chem. Pharm. Bull. 32(10)4124—4129(1984)]

Radioimmunoassay for N^{α}-(*N*-Acetylmuramyl-alanyl-D-isoglutaminyl)-N^{ε}-stearyl-lysine

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(Received June 6, 1984)

A radioimmunoassay for N^{α}-(*N*-acetylmuramyl-alanyl-D-isoglutaminyl)-N^{ε}-stearyl-lysine (MDP-Lys-L18) was established. Antibody to MDP-Lys-L18 was obtained from rabbits by immunization with MDP-Lys-L18 conjugated with bovine serum albumin (BSA). ^{3}H-MDP-Lys-L18 labelled at C_9 and C_{10} of the stearyl residue was used. The ammonium sulfate method was adopted for the separation of bound and free fractions. Though the protein binding of MDP-Lys-L18, which was due to the stearyl residue in the molecule, influenced the sensitivity of the radioimmunoassay, the lower limit for the quantitative determination of MDP-Lys-L18 in serum was estimated to be 500 pg/ml and the coefficient of variation of this assay was between 3 and 5%.

Keywords——radioimmunoassay; muramyl dipeptide derivative; immunogen; water-soluble carbodiimide; antiserum; bound fraction; free fraction; ammonium sulfate method

N^{α}-(*N*-Acetylmuramyl-alanyl-D-isoglutaminyl)-N^{ε}-stearyl-lysine (hereafter referred to as MDP-Lys-L18) is a derivative of muramyl dipeptide (MDP; *N*-acetylmuramyl-L-alanyl-D-isoglutamine), which has been reported[1] to have an immunomodulating effect in experimental animals. In order to develop a method for the quantitative analysis of MDP-Lys-L18, a radioimmunoassay was investigated. It has been reported[2-5] that MDP and several of its derivatives are not immunogenic, but when coupled to various carriers these compounds can act as haptens leading to anti-MDP or anti-MDP analogue production; however, little work has been done on immunological methods for quantitative determination of MDP or its analogues. In the present study we prepared three different immunogens by coupling MDP-Lys-L18 with bovine serum albumin (BSA), using water-soluble carbodiimide, and obtained anti-MDP-Lys-L18 from rabbits by immunization with these immunogens. A sensitive radioimmunoassay for MDP-Lys-L18 was developed using anti-MDP-Lys-L18 and ^{3}H-MDP-Lys-L18.

Materials and Methods

Reagents——MDP-Lys-L18, MDP, MDP-lysine (MDPL) and *N*-acetylmuramic acid (NAM) were provided by the Laboratory of Medicinal Chemistry, Research Institute, Daiichi Seiyaku Co., Ltd. ^{3}H-MDP-Lys-L18, labelled at C_9 and C_{10} of the stearyl residue, was synthesized by Daiichi Pure Chemical Co., Ltd. Its specific radioactivity was 6.9 Ci/mmol. Water-soluble carbodiimide (ethyl-CDI; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) was obtained from Kokusan Kagaku Co., Ltd. BSA, human serum albumin (HSA) and rabbit γ-globulin were purchased from Sigma Chemical Co. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were obtained from Difco Laboratories. For counting of radioactivity, AQUASOL-2 scintillator (New England Nuclear) was used. DEAE-cellulose was obtained from Seikagaku Kogyo Co., Ltd. Saturated ammonium sulfate solution was adjusted to pH 7.2 with ammonia solution. The other reagents used were of analytical grade.

Preparation of Immunogens (MDP-Lys-L18–BSA Conjugate)——MDP-Lys-L18 (Fig. 1) was conjugated with BSA by using ethyl-CDI according to the method of Goodfriend *et al.*[6] Three different immunogens, Imm. I (hapten 10—15/BSA), Imm. II (hepten 20—25/BSA) and Imm. III (hapten 30—35/BSA), were prepared in the following manner. BSA (100 mg) and MDP-Lys-L18 [15 mg (Imm. I), 29 mg (Imm. II), 42 mg (Imm. III)] were dissolved in 20 ml of phosphate-buffered saline (PBS, pH 7.2), and ethyl-CDI [10 mg (Imm. I), 20 mg (Imm. II), 30 mg (Imm. III)]

dissolved in 5 ml of PBS was added dropwise with stirring at room temperature. The mixture was continuously stirred overnight, then dialyzed against water at 4 °C. The resulting solution was lyophilized. The content of MDP-Lys-L18 in each immunogen was determined by the amino acid analysis method. The immunogen was sealed with 6 N HCl in a Pyrex-glass ampoule and heated at 120 °C for 20 h. The hydrolyzed sample was dried under a vacuum and dissolved in 0.2 N HCl. MDP-Lys-L18 and BSA were hydrolyzed in the same fashion. Amino acid composition was analyzed on a Hitachi type 835 amino acid analyzer.

The number of MDP-Lys-L18 residues per molecule of BSA was calculated by means of the following equation:

$$[\text{molar ratio of Ala/Leu (or Lys/Leu) of immunogen} - \text{molar ratio of Ala/Leu (or Lys/Leu) of BSA}] \times 61^{*}$$

*Number of leucine residue per molecule of BSA reported by Hughes[7]

Immunization——New Zealand White male rabbits ($n=30$) and male guinea pigs ($n=20$) were challenged with each immunogen in FCA or FICA. Immunogen (1 mg) dissolved in 0.5 ml of PBS and an equal volume of FCA or FICA was emulsified (w/o) in a Polytron homogenizer. Rabbits or guinea pigs received the emulsified immunogen intradermally into all four foot pads or subcutaneously at multiple dorsal sites. Otherwise, New Zealand White male rabbits ($n=15$) were challenged with immunogen (Imm. II or Imm. III) intravenously at a dose of 1 mg/body. Two or three days after each adaptive immunization, sera were obtained from rabbits or guinea pigs and the production of antibody to MDP-Lys-L18 was examined by assessing the binding (bound/total) with ^{3}H-MDP-Lys-L18. The assay procedure was as follows: 100 μl of diluted antiserum (1 : 500) was incubated with 20000 dpm of ^{3}H-MDP-Lys-L18 for 24 h at 4 °C, and the antibody fraction was separated by salting out with ammonium sulfate (1/2 saturation). The radioactivity of the centrifugal (3000 rpm × 15 min) precipitate was measured.

Antiserum——Rabbits which showed high production of antisera to MDP-Lys-L18 were sacrificed under anesthesia and the antisera were collected. Aliquots of these sera were diluted (1 : 300—1 : 1000) with PBS and incubated with ^{3}H-MDP-Lys-L18 (6.9×10^{-10} M–1.6×10^{-8} M) for 24 h at 4 °C. The association constant (K_a) for binding of antibody with MDP-Lys-L18 was determined. The separation of bound MDP-Lys-L18 from free MDP-Lys-L18 was accomplished by salting out with ammonium sulfate (1/2 saturation). Rabbit IgG was added as a carrier and the radioactivity of the centrifugal (3000 rpm × 15 min) precipitate was counted. The sera thus obtained were purified in the following manner to give IgG fraction. One milliliter of the antiserum was diluted to 3 ml with PBS containing 0.2% EDTA, then 5 mg of BSA was added, and the mixture was incubated for 72 h at 4 °C.

After the removal of immune complex by centrifugation (3000 rpm × 15 min), the IgG fraction was obtained by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography.[8] Namely the precipitated fraction at 1/5—1/2 saturation of ammonium sulfate was collected, dialyzed against PBS and further fractionated on a DEAE-cellulose column.

Radioimmunoassay——In the assay, 10 ml glass tubes (non-siliconized) were used. Dilution of each antiserum or IgG fraction of antiserum was done with PBS. Then 100 μl of blank serum or test sample, 100 μl of diluted antiserum or IgG fraction, 100 μl of ^{3}H-MDP-Lys-L18 (1500 dpm) in PBS containing 0.1% HSA, and 100 μl of unlabelled MDP-Lys-L18 or MDP-Lys-L18 analogue in PBS containing 0.1% HSA were added to each tube. The mixture was incubated for 50 h at 4 °C. To separate MDP-Lys-L18 bound to antibody from free MDP-Lys-L18, 500 μl of chilled saturated ammonium sulfate solution was added to the incubation mixture, and the whole was allowed to stand for 1 h, then centrifuged at 3000 rpm for 15 min below 4 °C. The supernatant was aspirated, and the precipitate was dissolved in 1 ml of PBS containing 1% normal rabbit serum and counted for radioactivity. In order to investigate the inhibition of the assay by compounds related to MDP-Lys-L18, MDP, MDPL and NAM were employed.

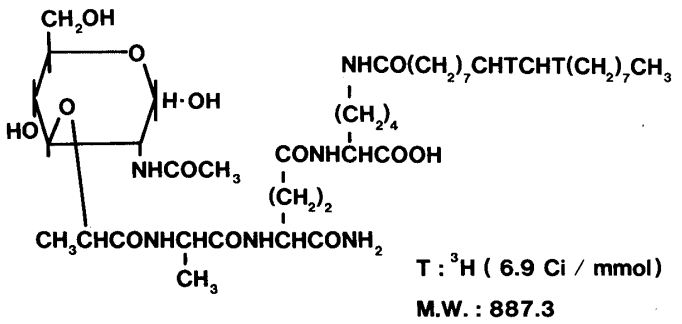

Fig. 1. Structure of MDP-Lys-L18 [N^{α}-(*N*-Acetylmuramyl-alanyl-D-isoglutaminyl)-N^{ε}-stearyl-lysine]

Results

Immunogen

All immunogens prepared were found to be very soluble in water. Table I shows the results of amino acid analysis of MDP-Lys-L18, BSA and immunogens. It appears that MDP-

TABLE I. Amino Acid Analysis of MDP-Lys-L18, BSA and Immunogens

Hydrolyte	Calculated	Observed
MDP-Lys-L18 (2 mg)	Ala 209 μg Lys 410 μg	Ala 226 μg Lys 418 μg

Hydrolyte	MDP-Lys-L18/BSA (Calculated)	–Ala– (Observed) Ala/Leu	–Ala– (Observed) MDP-Lys-L18/BSA	–Lys– (Observed) Lys/Leu	–Lys– (Observed) MDP-Lys-L18/BSA
Imm. I	9.87	0.95	$(0.95—0.79)^{a)} \times 61^{c)}$ 9.76	1.08	$(1.08—0.93)^{b)} \times 61^{c)}$ 9.15
Imm. II	23.16	1.14	$(1.14—0.79)^{a)} \times 61^{c)}$ 21.35	1.23	$(1.23—0.93)^{b)} \times 61^{c)}$ 18.30
Imm. III	35.18	1.34	$(1.34—0.79)^{a)} \times 61^{c)}$ 33.55	1.44	$(1.44—0.93)^{b)} \times 61^{c)}$ 31.11

a) Experimental datum (Ala/Leu) for BSA. *b*) Experimental datum (Lys/Leu) for BSA.
c) Reported number of Leu residues in BSA.

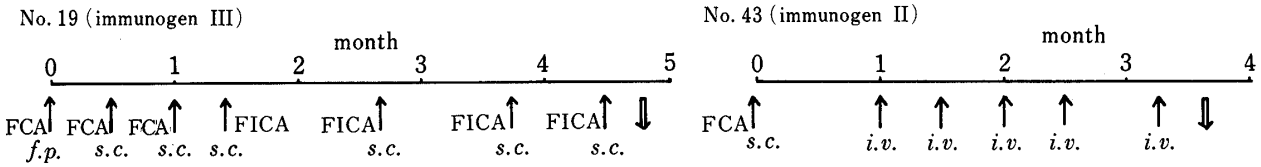

Fig. 2. Protocols for Immunization of Rabbit (New Zealand White)

↑, immunization; ⇩, collection of antiserum; FCA, Freund's complete adjuvant; FICA, Freund's incomplete adjuvant; *f.p.* intradermal immunization in foot pads: *s.c.*, subcutaneous immunization in dorsum; *i.v.*, immunization in vein.

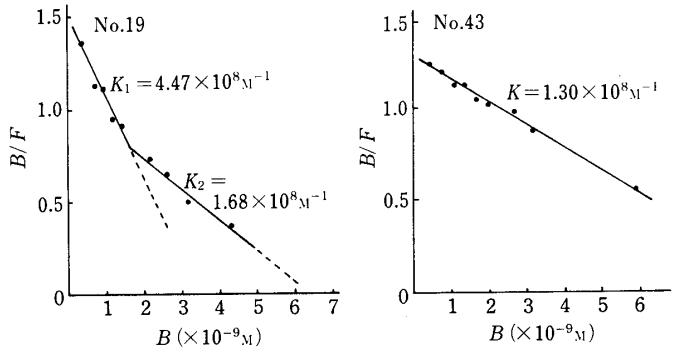

Fig. 3. Scatchard Plots of the Binding of ^{3}H-MDP-Lys-L18 to the Rabbit Antiserum (No. 19, × 1/300 Diluted) (No. 43, × 1/1000 Diluted)

B and *F* show the molar concentrations of bound and free ^{3}H-MDP-Lys-L18 at equilibrium, respectively.

Lys-L18 was hydrolyzed quantitatively to form alanine and lysine. The numbers of the haptens bound per BSA molecule were nearly equal to the values which were calculated on the basis of the amounts of MDP-Lys-L18 and BSA employed. Consequently the reaction yield was estimated to be approximately 100%.

Antiserum

In spite of various attempts to immunize guinea pigs and rabbits, the production of antibody to MDP-Lys-L18 that could be used for competitive binding assay was restricted to only two rabbits. One was the rabbit (No. 19) which was challenged with Imm. III intradermally at first, followed by six subcutaneous immunizations over less than five months. The other was the rabbit (No. 43) which received a subcutaneous challenge first and was then immunized with Imm. II intravenously in the ear vein five times over a little more than three months. Figure 2 shows the protocols for the immunization of these two rabbits. In the guinea pigs and in the rabbits which were immunized with Imm. I, no trace of antibody formation was found. Figure 3 shows the Scatchard plots[9] of ^{3}H-MDP-Lys-L18 with these antisera. The association constants of binding were calculated to be $4.47 \times 10^8\ \text{M}^{-1}$ and $1.68 \times 10^8\ \text{M}^{-1}$ for the antiserum from No. 19 and $1.30 \times 10^8\ \text{M}^{-1}$ for that from No. 43.

Immunoassay

MDP-Lys-L18 is easily adsorbed on the surface of a glass or plastic tube. This property, which might be due to the existence of a hydrophobic long aliphatic chain, *i.e.* the stearyl group, in the molecule, was undesirable for developing a sensitive quantification method. However, it was found that the adsorption of MDP-Lys-L18 on the surface of tubes could be prevented by adding albumin solution to the sysem, but was facilitated by previous siliconization of utensils. Therefore, although the coexistence of albumin in the system might not be favorable for the assay on other grounds, HSA was employed in order to avoid the adsorptive loss of MDP-Lys-L18 in this study, since no alternative is currently available. Figure 4 shows titration curves of antisera obtained from the rabbits (No. 19 and No. 43); the sera of No. 19 and No. 43 bound 50% of ^{3}H-MDP-Lys-L18 added (250 pg/ml) at dilutions of 1 : 800 and 1 : 3500, respectively. With further dilution, 15—20% of the added radioactivity

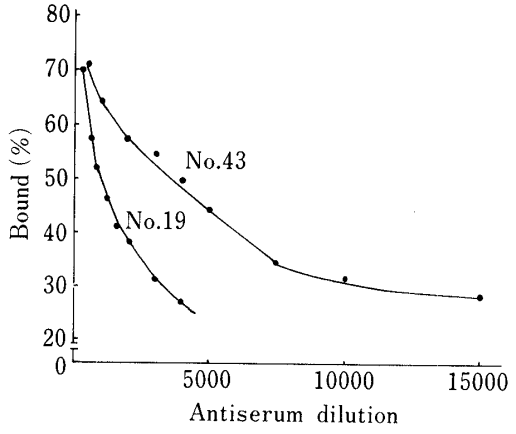

Fig. 4. Titration Curves of Anti-MDP-Lys-L18 Sera Obtained from Two Rabbits

^{3}H-MDP-Lys-L18 250 pg/ml, incubation for 50 h at 4 °C, separation by the ammonium sulfate method.

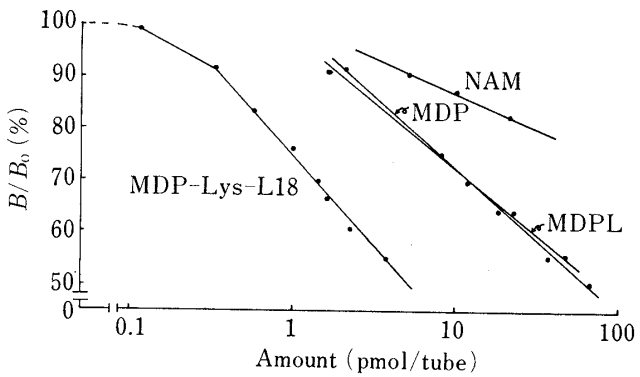

Fig. 5. Standard Curve of MDP-Lys-L18 and the Inhibitory Effect of NAM, MDP and MDPL on the RIA System Using the Purified IgG Fraction (Absorbance at 280 nm: 0.15) of the Antiserum (No. 19)

B and B_0 represent the amount of radioactivity bound in the presence and in the absence of the unlabelled MDP-Lys-L18 (or its derivatives), respectively.

was found in the salted-out protein fraction, possibly resulting from the protein binding of MDP-Lys-L18 and the coexistence of anti-BSA in the antiserum. In order to minimize the effect of anti-BSA on the competitive binding assay, the antiserum was purified to the IgG fraction after most of the anti-BSA had been removed as the immune complex with BSA. Figure 5 shows the standard curve of MDP-Lys-L18 and the inhibitory effects of MDP, MDPL and NAM on the assay using the purified IgG fraction of the antiserum (No. 19). When MDP-Lys-L18 was employed in the range from 50 pg to 3 ng per tube, the ratio B/B_0 decreased linearly. The lower limit for the quantitative determination of MDP-Lys-L18 in serum could be calculated to be 500 pg/ml. The intra-assay variation coefficient was between 3 and 5%. Analogues, MDP and MDPL, were found to be poor inhibitors of the assay of MDP-Lys-L18; they showed about 10% inhibition of the binding of MDP-Lys-L18. NAM was less inhibitory. With regard to the system using antiserum from No. 43, the inhibitory effects of MDP, MDPL and NAM on the assay were almost equal to those obtained using the other antiserum (No. 19).

Discussion

In the present study, we succeeded in obtaining anti-MDP-Lys-L18 antiserum, which enabled us to develop a radioimmunoassay system for MDP-Lys-L18. It is generally considered that, in order to make a nonantigenic compound immunogenic by coupling it to a carrier, rigidity of the hapten is indispensable. As MDP-Lys-L18 possesses a flexible long chain, the immunogens prepared in this study did not seem satisfactory in this respect. As regards establishing a highly specific immunoassay system for MDP-Lys-L18 it might be less favorable to couple it with the carrier directly at the MDP moiety, which could be an antibody recognition site. Therefore, a carboxyl group of the lysine residue was chosen as a coupling site to BSA, although lack of rigidity of the hapten could not be avoided.

It was reported that MDP or some of its derivatives possess adjuvant activity and, when coupled to various carriers, enhance the immunogenicity of the carrier molecules.[2,3] As MDP-Lys-L18 also has adjuvant activity,[1] the production of anti-BSA would be promoted when MDP-Lys-L18–BSA conjugate was employed for immunization. However, the effect of the adjuvant activity of MDP-Lys-L18 on the production of antiserum to itself was not clear. It is likely that the number of MDP-Lys-L18 molecules bound to BSA has an effect on the production of the antiserum to MDP-Lys-L18.

Bahr *et al.*[5] reported the production of antibody to MDP by intravenous immunization of MDP-carrier conjugate to rabbits. In our experiments, it was found that rabbits immunized intravenously produced anti-MDP-Lys-L18 with higher probability than those immunized intradermally and subcutaneously, though higher mortality was recognized in intravenously immunized rabbits. The reason why anti-MDP-Lys-L18 was not produced in guinea pigs is not known.

Since it is generally known that long chain fatty acids bind to protein, especially to albumin, it was expected that MDP-Lys-L18 would also bind to albumin. Although the association constant of binding between MDP-Lys-L18 and albumin might be small, the binding capacity of albumin with MDP-Lys-L18 should be large. On the other hand, the antiserum showed large association constants of the order of $10^8\ \mathrm{M}^{-1}$ for binding with MDP-Lys-L18, but its binding capacity would be small. Accordingly, albumin present in the system would have some influence on the sensitivity of the immunoassay for MDP-Lys-L18.

Moreover, it is likely that anti-BSA coexists in the antiserum to MDP-Lys-L18. If this is the case, the anti-BSA would react with the albumin to which MDP-Lys-L18 is bound and the immune complex formed would be co-precipitated with the MDP-Lys-L18-antibody complex at the time of B/F (bound/free) separation, resulting in an increase in non-specific binding of

MDP-Lys-L18 and a consequent decrease in the sensitivity of the assay. Therefore, removal of the anti-BSA in the antiserum was conducted.

With regard to separation of the bound and free fractions, methods using a membrane filter (MF), dextran-coated charcoal (DCC), double antibody and ammonium sulfate were investigated. The MF and DCC methods were found to be unsuitable because of the random binding of MDP-Lys-L18 to the membrane or charcoal, presumably through the stearyl group. The double antibody method was applicable, but we adapted the ammonium sulfate method on the grounds that it was easy, simple and cheap compared with the double antibody method. In the case of the ammonium sulfate method, the immunoglobulin fraction precipitated with 1/2 saturation would be contaminated with other proteins, consisting mainly of fibrin, but binding of MDP-Lys-L18 to these proteins might be negligible.

On examination of the inhibitory effect of analogues on the assay, MDP, MDPL and NAM were found to be inhibitors. However, when MDP-Lys-L18 was administered to animals, neither MDP nor MDPL was found as a metabolite (unpublished data). Therefore, it seems unlikely that these analogues interfere with the assay of MDP-Lys-L18 in biological samples. On the other hand, NAM was detected as a metabolite in mice (unpublished data), but its inhibitory effect was so weak and the amount formed as a metabolite was so minute that its influence on the assay should be negligible. Whether or not fatty acids, especially stearic acid, had any effect on the assay was not examined, because they are endogenous materials. Generally speaking, the production of anti-fatty acids is highly unlikely. If they were produced, they would have impeded the assay.

The radioimmunoassay method developed in the present study has quite high sensitivity and may be applicable to the practical quantitative analysis of MDP-Lys-L18, *e.g.*, in preclinical or clinical investigations on its pharmacokinetics or metabolic disposition.

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