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Sequencing and cloning of the cDNA of guinea pig eosinophil major basic protein

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Major basic protein (MBP) purified from guinea pig eosinophils elicited histamine release from rat peritoneal mast cells at concentrations higher than 3 μg/ml both in the presence and in the absence of extracellular Ca^{2+}. After reverse-phase high-performance liquid chromatography, it was revealed that MBP was composed of two different proteins with quite similar molecular weights and pI values, although the amino acid compositions were slightly different. The partial amino acid sequence of one of these MBPs was determined and the primers for the polymerase chain reaction (PCR) were synthesized according to the partial amino acid sequence. Using these primers and the cDNAs obtained from guinea pig eosinophils, the PCR was carried out in order to synthesize the hybridization probe of MBP for screening the cDNA library. After screening with 8×10^5 clones, a positive clone, which encoded a full length of pre-proMBP, was obtained. According to the sequencing data of this clone, it was revealed that pre-proMBP was composed of 3 domains; signal peptide, acidic domain and mature MBP. The predicted pI value of mature MBP was 11.7, though that of proMBP was 7.8. The homology in the amino acid sequence between guinea pig proMBP and human proMBP was 49.4%, while guinea pig mature MBP was more homologous (58%) to human mature MBP.

Guinea pig eosinophil; Major basic protein; cDNA cloning; Mast cell; Histamine release

1. INTRODUCTION

It is well known that eosinophils accumulate at the sites of allergic inflammations and that proinflammatory proteins such as eosinophil peroxidase, major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin are released from the activated eosinophils [1,2]. Among such proteins, it has been suggested that MBP plays some important roles in the allergic reactions and inflammations, since MBP is capable of releasing histamine from mast cells and damaging the epithelial cells of bronchial tubes [3,4]. Since the activated mast cells release the eosinophil-activating factors, such as leukotriene B_4, platelet activating factor, ECF-A tetrapeptides and histamine [5–7], it was assumed that in allergic reactions mast cells and eosinophils activate each other by releasing their own chemical mediators. Barker et al. reported the cDNA sequences of human MBP and ECP obtained from HL-60 cells [8,9]. However, the sequence of the MBP derived from experimental animals is not known. Since the guinea pig is one of the most widely used experimental animals in the study of allergic reaction and asthma, it may be of use to determine the cDNA sequence of MBP derived from guinea pig eosinophils. In the present study, MBP and a cDNA library were obtained from guinea pig eosinophils and the cDNA sequence of guinea pig MBP was determined.

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Abbreviations: MBP, major basic protein; ECP, eosinophil cationic protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PCR, polymerase chain reaction

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number *D90251*

2. MATERIALS AND METHODS

2.1. *Purification of guinea pig eosinophil MBP*

Male Hartley guinea pigs (300–350 g) were intraperitoneally injected once a week with 1 mg/animal of polymyxin B (Sigma) and 1 mg/animal of ascaris suum extract. Six weeks later, the guinea pigs were anesthetized with ether and the peritoneal exudated cells were collected by perfusing 50 ml of physiological buffered solution (in mM; NaCl 154, KCl 2.7, $CaCl_2$ 0.9, HEPES 5; pH 7.4; PBS). The eosinophils were purified by means of Percoll density gradient centrifugation to more than 95% purity. The mean yield was about 2×10^7 eosinophils/animal. The eosinophils were disrupted in a solution consisting of 0.25 M sucrose, 5 mM HEPES (pH 7.4) and 1 mg/ml of heparin and the granules were collected by centrifugation at 10000 × *g* for 10 min at 4°C. The eosinophil granules were dissolved with 0.1 N HCl and this solution was applied to a Sephadex G-50 column and eluted by a buffer containing 150 mM NaCl and 20 mM acetic acid–sodium acetate buffer (pH 4.3) [10]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were car-

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ried out by means of Phast system (Pharmacia). MBP fractions were collected and further purification was carried out by means of reverse-phase HPLC (Waters). In brief, 250 μl of MBP fraction dissolved in 0.1% trifluoroacetic acid (TFA) was injected onto a Vydac C-4 column (4.6 mm i.d. × 150 mm) and eluted at a constant rate of 1 ml/min. The elution buffer consisted of solvent A (0.1% TFA) and solvent B (CH_3CN/0.1% TFA = 9:1). The column was eluted with a linear gradient from solvent A to 30% solvent B for the first 5 min. Subsequently, the column was eluted with a linear gradient from 30% to 50% of solvent B.

2.2. *Histamine release from rat peritoneal mast cells*

Rat peritoneal mast cells were harvested from the abdominal cavity of male Wistar rats and purified by means of Percoll density gradient centrifugation. The cells were suspended in PBS both in the presence and in the absence of Ca^{2+}, and preincubated at 37°C for 5 min. Thereafter, MBP was added to the medium and incubated for 10 min at 37°C. The histamine released in the supernatant and the residual histamine in the cell pellet were determined separately by a fluorometric assay [11].

2.3. *Determination of amino acid composition and partial sequence*

Protein samples were hydrolyzed in 4 M methane sulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 130°C for 4 h. Hydrolyzates were analyzed on an amino acid analyzer (Hitachi) using o-phthalaldehyde as a coloring reagent. Edman degradations of MBP and its fragments obtained from digestion with lysylendopeptidase were carried out using a gas phase sequencer (Applied Biosystems, Forster City). Phenylthiohydantoin derivatives of amino acids from each cycle were analyzed by reverse phase HPLC.

2.4. *Construction of cDNA library of guinea pig eosinophils*

Purified guinea pig eosinophils were disrupted in 6 M guanidine-thiocyanate solution containing 5 mM sodium citrate, 0.1 M β-mercaptoethanol and 0.5% sodium sarkosyl (pH 7.0). The lysate was layered on cesium trifluoroacetic acid solution containing 0.1 M EDTA (pH 7.0, ρ = 1.51) and centrifuged at 85000 × g for 24 h at 25°C. The total RNA of guinea pig eosinophils at the bottom of the centrifugation tube was collected and poly(A)$^+$RNA was selected using oligo-dT latex beads (Oligo-Latex, Takara). The λgt10 cDNA library was constructed by means of cDNA synthesis system (Amersham) and cDNA cloning system-λgt10 (Amersham) using 1 μg of poly(A)$^+$RNA, and subsequently, more than 10^6 independent clones were obtained.

2.5. *Polymerase chain reaction (PCR) and synthesis of screening probes*

PCR primers were synthesized according to the partial amino acid sequence of MBP by means of an automated oligonucleotide synthesizer (Applied Biosystems), and PCR was carried out using the primers and cDNAs of guinea pig eosinophils to obtain a screening probe for MBP using GeneAmp DNA amplification reagent kit (Perkin-Elmer-Cetus) and DNA Thermal Cycler (Perkin-Elmer-Cetus). The PCR was run for 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 30 s); the extension period was prolonged by 4 s in each successive cycle. The amplified 92-bp fragment was ligated into plasmid pUC118. Thereafter, two types of screening probes were synthesized. To obtain a first screening probe of the cDNA library, the 92-bp fragment was labeled by PCR using 10 mM [α-^{32}P]dCTP instead of 200 mM dCTP in the amplification reaction mixture. In this reaction, the 92-bp fragment inserted pUC118 was used as a template and Takara M13 primers were employed; M13 primer M4 d(GTTTTCCCAGTCACGAC) and M13 primer RV d(CAGGAAACAGCTATGAC). The 92-bp fragment inserted into the plasmid pUC118 was also radiolabeled with [α-^{32}P]-dCTP by means of multiprimer DNA labeling system (Amersham) to obtain the second screening probe. The reaction mixtures were passed through a NICK column (Pharmacia) to separate the amplified DNA from unincorporated [α-^{32}P]dCTP.

2.6. *Screening of cDNA library and determination of the cDNA sequence*

The cDNA library was screened with the ^{32}P-labeled 92-bp fragment according to the method developed by Benton and Davis [12]. The hybridization solution was 6 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% SDS and 100 μg/ml denaturated salmon sperm DNA. Firstly, the filters were hybridized with PCR-amplified labeled probe overnight at 65°C, and the filters were washed in 2 × SSC and 0.1% SDS at 60°C. After being slightly air-dried, the filters were exposed to Kodak XAR5 film overnight at −70°C. A positive clone was obtained from the library, and the cDNA insert of about 1 kbp was subcloned into pUC118 in order to determine the sequence by dideoxy chain termination method of Sanger et al. [13].

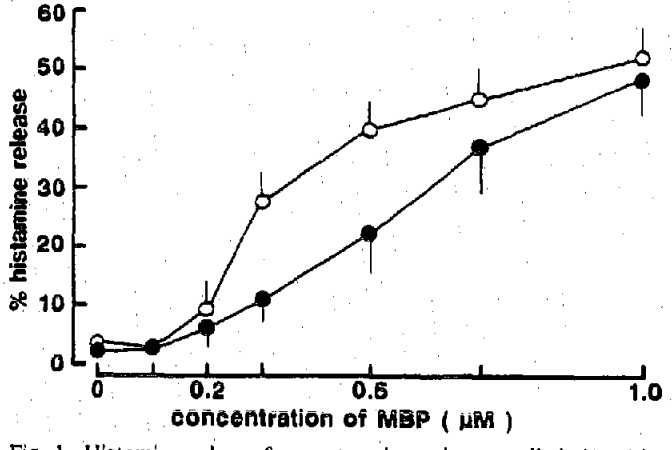

Fig. 1. Histamine release from rat peritoneal mast cells induced by guinea pig MBP in the presence and in the absence of extracellular Ca^{2+}. (●) in the presence of Ca^{2+} (0.9 mM); (○) in the absence of Ca^{2+} (supplemented with 0.1 mM of EGTA).

3. RESULTS AND DISCUSSION

After Sephadex G-50 column chromatography, the eluted MBP exhibited a single band on SDS-PAGE with

Table I

Amino acid composition of two fractions of guinea pig major basic protein

Amino acid	Amino acid composition (%)	
	GMBP1	GMBP2
Aspartic	7.26	8.71
Threonine	5.19	3.12
Serine	6.54	10.15
Glutamic	10.30	9.92
Glycine	15.41	14.61
Alanine	8.46	6.80
Valine	8.21	7.21
Methionine	0.72	0.48
Isoleucine	2.09	3.55
Leucine	4.88	6.71
Tyrosine	4.10	3.21
Phenylalanine	5.30	4.34
Lysine	2.88	4.30
Histidine	3.20	5.26
Arginine	15.45	11.26
Proline*	0	0
Cysteine*	0	0
Tryptophan*	0	0

*Proline, Cysteine and Tryptophan were not determined in the present method.

a molecular weight of about 11000. When IEF was carried out, MBP migrated to the cathode, suggesting that the pI value of MBP was higher than 10. As indicated in Fig. 1, MBP potently released histamine from rat peritoneal mast cells both in the presence and in the absence of extracellular Ca^{2+} at concentrations higher than 3 μg/ml. These results are in good agreement with those reported previously [3,10].

When the MBP was applied to reverse-phase HPLC, the protein exhibited two different peaks. The molecular weights and pI values of these two proteins are quite similar and both are effective in releasing histamine from rat peritoneal mast cells (data not shown). When the amino acid compositions of these proteins were determined, it became apparent that these two proteins are highly basic and that the similarity in amino acid composition is high, but these two proteins are apparently different as indicated in Table I. This result clearly indicates that MBP is composed of two kinds of proteins. In the present study, further in-

(a)

```
                                                                    30
h-MBP   T-G-R-Y-L-L-V-R-S-L-Q-T-F-S-Q-A-W-F-T-C-R-R-C-Y-R-G-N-L-V-S-

GMBP1   T-R-G-Y-V-M-V-G-S-A-R-T-F-N-E-A-Q-W-V-C-Q-R-C-Y-R-G-N-L-A-S-
                                  primer GMBP1-1
                                                                    60
h-MBP   I-H-N-F-N-I-N-Y-R-I-Q-C-S-V-S-A-L-N-Q-G-Q-V-W-I-G-G-R-I-T-G-

GMBP1   I-H-?-F-A-F-N-Y-Q-V-Q-C-T-?-A-G-L-N-
                      primer GMBP1-2
                                                                    90
h-MBP   S-G-R-C-R-R-F-Q-W-V-D-G-S-R-W-N-F-A-Y-W-A-A-H-Q-P-W-S-R-G-G-
                                                                  117
h-MBP   H-C-V-A-L-C-T-R-G-G-Y-W-R-R-A-H-C-L-R-R-L-P-F-I-C-S-Y
```

(b)

PCR primer GMBP1-1 (5'-primer)

```
            13                                  19
protein     P h e A s n G l u A l a G l n T r p V a l

mRNA   5'   U U U A A U G A A G C U C A A U G G G U U 3'
                C     C     G     C     G           C
                                  A                 A
                                  G                 G

probe  5'   T T T A A T G A A G C T C A A T G G G T  3'   complexity 64
                C     C     G     C     G
                                  A
                                  G
```

PCR primer GMBP1-2 (3'-primer)

```
            37                                  43
protein     A s n T y r G l n V a l G l n C y s T h r

mRNA   5'   A A U U A U C A A G U U C A A U G U A C U 3'
                C     C     G     C     G     C     C
                                  A                 A
                                  G                 G

probe  3'   T T A A T A G T T C A A G T T A C A T G  5'   complexity 128
                G     G     C     G     C     G
                                  T
                                  C
```

Fig. 2. Partial amino acid sequence of GMBP1 and nucleotide sequence of the synthesized PCR primers. (a) Partial amino acid sequence of GMBP1 in comparison with human MBP [8]; (b) Nucleotide sequence of the synthesized PCR primers in the underlined position in Fig. 2a.

vestigation was carried out using fraction No. 67 (guinea pig MBP-1; GMBP1).

The partial amino acid sequence of GMBP1 is indicated in Fig. 2a compared with that of human MBP. According to this partial sequence, PCR primers in the underlined positions were synthesized as indicated in Fig. 2b. Using these primers and cDNAs obtained from guinea pig eosinophils, PCR was carried out in order to amplify the partial sequence of cDNA of MBP. After PCR, the length of amplified DNA was 92 bp, which corresponds to the length estimated from the amino acid length shown in Fig. 2. The amplified DNA, which corresponds to the amino acid sequence determined in Fig. 2a, was used for the screening of a cDNA library of guinea pig eosinophils. After plaque hybridization, a positive clone was obtained from 8×10^5 independent clones. The determined sequence of cDNA is indicated in Fig. 3. Comparing the amino acid sequence with human MBP [8] and that obtained from GMBP1 protein, it became apparent that this cDNA corresponds to

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-74  GAGGACCCAACGACCTGCGTGGGGGCCTTTAGTGCTCACTGTCCTTGGTGCAGCTGGCTC

                                                              ↓
-14  TGGACAAGGTCGAGATGAAACTCCTGCTCCTCCTGGCTCTCCTTCTGGGGGCGGTGTCCA
                      M  K  L  L  L  L  L  A  L  L  L  G  A  V  S  T   16

 46  CCCGGCATCTCAAGGTGGACACGTCCAGCTTGCAGAGCCTGCGGGGAGAGGAGAGCTTGG
       R  H  L  K  V  D  T  S  S  L  Q  S  L  R  G  E  E  S  L  A   36

106  CCCAGGATGGGGAGACTGCAGAAGGGGCCACAAGGGAGGCCACTGCAGGGGCACTGATGC
       Q  D  G  E  T  A  E  G  A  T  R  E  A  T  A  G  A  L  M  P   56

166  CACTGCCTGAGGAGGAGGAGATGGAAGGAGCCTCTGGAAGTGAAGATGACCCTGAAGAGG
       L  P  E  E  E  E  E  M  E  G  A  S  G  S  E  D  D  P  E  E   76

226  AGGAGGAGGAGGAGGAGGAAGTGGAGTTCAGCTCAGAGCTGGATGTGAGCCCCGAGGATA
       E  E  E  E  E  E  V  E  F  S  S  E  L  D  V  S  P  E  D  I   96
                                                                 *
286  TCCAGTGTCCTAAGGAAGAGGACACAGTAAAATTCTTCAGCAGACCTGGATACAAAACCC
       Q  C  P  K  E  E  D  T  V  K  F  F  S  R  P  G  Y  K  T  R  116

346  GTGGTTATGTTATGGTGGGGTCTGCCAGGACATTTAATGAAGCTCAGTGGGTGTGCCAGA
       G  Y  V  M  V  G  S  A  R  T  F  N  E  A  Q  W  V  C  Q  R  136

406  GATGCTACAGGGGCAACCTTGCATCCATCCACAGTTTTGCCTTTAATTACCAAGTCCAGT
       C  Y  R  G  N  L  A  S  I  H  S  F  A  F  N  Y  Q  V  Q  C  156

466  GCACTTCCGCTGGACTCAATGTGGCCCAGGTCTGGATTGGAGGCCAACTCAGGGGCAAGG
       T  S  A  G  L  N  V  A  Q  V  W  I  G  G  Q  L  R  G  K  G  176

526  GTCGCTGCAGACGCTTTGTTTGGGTGGACAGAACCGTATGGAATTTTGCGTATTGGGCAC
       R  C  R  R  F  V  W  V  D  R  T  V  W  N  F  A  Y  W  A  R  196

586  GTGGGCAGCCCTGGGGAGGTCGTCAACGTGGCAGATGCGTGACCCTGTGTGCCCGAGGAG
       G  Q  P  W  G  G  R  Q  R  G  R  C  V  T  L  C  A  R  G  G  216

646  GTCACTGGCGCCGATCTCACTGTGGCAAGAGACGCCCCTTTGTCTGCACCTACTGAGTGT
       H  W  R  R  S  H  C  G  K  R  R  P  F  V  C  T  Y  *        233

706  ACATTGAGGCCTGGAGCTCCTCTCTGCTGCCCCACCCCTGCCTGCACCCCTCCCTGCCAC

766  ACCCTCCCTCCACTGCCCTACAATAAAATCGCTTTCCTGAAGTGAAAAAAAAAAAAAAAA
```

Fig. 3. Nucleotide sequence of GMBP1 cDNA. Nucleotides −74–825 and amino acid 1–233 are numbered on the left and right of the figure, respectively. An arrow indicates the putative signal peptide cleavage site. An asterisk indicates the first amino acid in eosinophil granule MBP.

```
guinea pig:    1'  MKLLLLLALLLGAVSTRHLKVDTSSLQSLRGEESLAQDGETAEGATREATAGALMPLPEE
                   *** ****** ****  **   **       *   *  * ** *     *       *    *
human MBP:     1"  MKLPLLLALLFGAVSALHLRSETSTFETPLGAKTLPEDEETPEQEMEETPCREL-----E

guinea pig:   61'  EEMEGASGSEDEPEEEEEEEEEEVEFSSELDVSPEDIQCPKEEDTVKFFSRPGYKTRGYVM
                   ** *  **** *         *         *        ** ******    **  *  *
human MBP:    56"  EEEEWGSGSE-DASKKDGAVESISVPDMVD---KNLTCPEEEDTVKVVGIPGCQTCRYLL

guinea pig:  121'  VGSARTFNEAQWVCQRCYRGNLASIHSFAFNYQVQCTSAGLNVAQVWIGGQLRGKGRCRR
                   * *  **  *   * *******  *** *  **  **     **  ******   * *****
human MBP:   112"  VRSLQTFSQAWFTCRRCYRGNLVSIHNFNINYRIQCSVSALNQGQVWIGGRITGSGRCRR

guinea pig:  181'  FVWVDRTVWNFAYWARGQPWGGRQRGRCVTLCARGGHWRRSHCGKRRPFVCTY
                   * ***   *******  ***     * ** ** *** *** **  * ** * *
human MBP:   172"  FQWVDGSRWNFAYWAAHQPW--SRGGIICVALCTRGGYWRRAHCLRRLPFICSY
```

Fig. 4. Comparison of the amino acid sequences of pre-proMBP in guinea pig (GMBP1) and human [8]. Identical residues are indicated by an asterisk. Gaps have been introduced to achieve maximum sequence homology.

the full length of the mRNA of MBP. It is indicated that this cDNA encoded pre-proMBP may be composed of a signal peptide, an acidic domain and mature MBP, as in the case of human MBP [8]. Although the domain of mature MBP is highly basic (calculated pI value is 11.7), pre-proMBP and proMBP are slightly acidic proteins (calculated pI values are 5.4 and 5.3, respectively) due to the existence of the acidic domain of proMBP. The calculated pI of the acidic domain was 3.8. As in the case of human MBP [8], it was suggested that MBP may be firstly synthesized as pre-proMBP in the cell from its mRNA and converted to MBP via proMBP by protease digestion. Since it is supposed that the high basicity of MBP may be the reason for its cytotoxicity, the acidic domain of proMBP may be effective in neutralizing the basicity and cytotoxicity of MBP inside the cell.

The calculated molecular weight of MBP is 13800. It has been indicated that the molecular weight of guinea pig MBP is 11000 as determined by SDS-PAGE [10]. The possible reason for the difference in the molecular weights between the calculated value and that determined on SDS-PAGE can be ascribed to the highly positive charges in MBP, which may influence the migration of SDS-bound proteins on SDS-PAGE to the position corresponding to its molecular weight.

Comparison of the amino acid sequence in human and guinea pig MBPs is indicated in Fig. 4. The homology in the amino acid sequences between human and guinea pig pre-proMBPs was 49.4%, though that of mature MBP was 58%. The basic region of guinea pig MBP was quite similar to that seen in human MBP. The amino acid sequence of the acidic domain of guinea pig proMBP bears little resemblance to that of humans, while the acidity in both proteins was quite similar.

It is known that many basic polymers, such as compound 48/80, poly-L-lysine, substance P and histone, are capable of releasing histamine from isolated rat peritoneal mast cells both in the presence and in the absence of extracellular Ca^{2+} [14–16]. In such cases, it has been shown that Ca^{2+} is released from the intracellular Ca store [16,17]. Since MBP also caused histamine release from mast cells both in the presence and in the absence of extracellular Ca^{2+}, it was supposed that both MBP and compound 48/80 share the basic mechanism in histamine release. It can be assumed that the active portion of MBP necessary for releasing histamine may be the basic region as in the case of substance P [15].

REFERENCES

[1] Ayars, G.H., Altman, L.C., Gleich, G.J., Loegering, D.A. and Baker, C.B. (1985) J. Allergy Clin. Immunol. 76, 595–604.
[2] Gleich, G.J., Ottesen, E.A., Leiferman, K.M. and Ackerman, S.J. (1989) Int. Arch. Allergy Appl. Immunol. 88, 59–62.
[3] Zheutlin, L.M., Ackerman, S.J., Gleich, G.J. and Thomas, L.L. (1984) J. Immunol. 133, 2180–2185.
[4] Gleich, G.J., Flavahan, N.A., Fujisawa, T. and Vanhoutte, P.M. (1988) J. Allergy Clin. Immunol. 81, 776–781.
[5] Pincus, S.H., DiNapoli, A.-N. and Schooley, W.R. (1982) J. Invest. Dermatol. 79, 53–57.
[6] Fechter, M., Egger, D., Auer, H. and Popper, H. (1986) Exp. Pathol. 29, 153–158.
[7] Wardlaw, A.J., Moqbel, R., Cromwell, O. and Kay, A.B. (1986) J. Clin. Invest. 78, 1701–1706.
[8] Barker, R.L., Gleich, G.J. and Pease, L.R. (1988) J. Exp. Med. 168, 1493–1498.
[9] Barker, R.L., Loegering, D.A., Ten, R.M. Hamann, K.J., Pease, L.R. and Gleich, G.J. (1989) J. Immunol. 143, 952–955.
[10] Gleich, G.J., Loegering, D.A., Kueppers, F.H., Bajaj, S.P. and Mann, K.G. (1974) J. Exp. Med. 140, 313–332.
[11] Siraganian, R.P. (1974) Anal. Biochem. 57, 383–394.
[12] Benton, W. and Davis, R. (1977) Science 196, 180–182.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Ennis, M., Atkinson, G. and Pearce, F.L. (1980) Agents Actions 10, 222–228.
[15] Fewtrell, C.M.S., Foreman, J.C., Jordan, C.C., Oehme, P., Renner, H. and Stewart, J.M. (1982) J. Physiol. 330, 393–411.
[16] Tasaka, K., Mio, M., Akagi, M. and Saito, T. (1990) Agents Actions 30, 114–117.
[17] Tasaka, K., Mio, M. and Okamoto, M. (1986) Ann. Allergy 56, 464–469.