FEBS Letters 412 (1997) 456–460 FEBS 18974

Monoclonal antibodies neutralizing the toxin II from *Androctonus australis hector* scorpion venom: usefulness of a synthetic, non-toxic analog

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Received 6 May 1997; revised version received 11 June 1997

Abstract Scorpion venom contains toxins that act on ion channels. Some are responsible for the noxious effects observed when people are stung by scorpions. The study of the neutralization of these molecules and the production of monoclonal antibodies (mAbs) should prove valuable. Toxin II from *Androctonus australis hector* scorpion (AahII) is one of the most potent toxins and has been well-characterized and studied. Producing mAbs against such molecules is often difficult due to their toxicity. We used a synthetic, non-toxic analog, $(Abu)_8$-AahII, to obtain mAbs which recognize and neutralize the native toxin AahII. Sets of peptides spanning the entire sequence of AahII were assayed to identify the binding sites of the mAbs. The various mAbs recognized only the largest peptides (12–17 residues). They recognized peptides corresponding to different parts of the AahII sequence, suggesting that several regions of the $(Abu)_8$-AahII sequence mimic AahII epitopes and then elicit mAbs directed against toxin.

Key words: Scorpion toxin; Analog; Chemical synthesis; Monoclonal antibody; Epitope; Neutralization

1. Introduction

Hybridoma technology [1] is widely used in clinical applications (diagnosis and therapy; [2,3]) and topographical analysis of antigens [4,5]. It can be useful to investigate the structure-activity relationships and the specificity of the neutralization of the lethal effect of infectious or toxic molecules. Numerous animal species cause mammalian envenomation. This phenomenon is mainly due to venom proteins which disturb vital functions. Producing monoclonal antibodies (mAbs) against these proteins may prove valuable, but the toxicity of the immunogen sometimes renders their production very difficult. This is the case for scorpion toxins, for which only a few mAbs have been reported [6,7]. Low-dose immunization or modification of the immunogen was necessary to obtain live immunized animals. The venom of the scorpion *Androctonus australis hector* contains several toxins that act on ion channels. The most potent, AahII, interacts with potential-dependent sodium channels and can kill mice (LD_{50} in BALB/c of 300 ng/20 g mice when injected subcutaneously). We protected mice against challenge by native toxin or venom using a non-toxic analog of AahII, the $(Abu)_8$-AahII, encompassing the entire sequence of the native toxin but without disulphide bridges [8].

In the present work, $(Abu)_8$-AahII was used as a non-toxic immunogen to generate mAbs. The mAbs obtained were analysed for their capacity to recognize and neutralize the native toxin. Delineation of antibody binding sites was performed using different sets of synthetic peptides.

2. Materials and methods

2.1. Peptide synthesis

Chemical synthesis and characterization of the immunogen, $(Abu)_8$-AahII, was as previously described [8]. Two series of overlapping peptides (12 to 17 residues) were tested for recognition by mAbs using ELISA. In one series, cysteine residues were blocked by acetamidomethyl group (Acm) and in the other cysteine residues were replaced by α-aminobutyric acid (Abu). Sequences of Acm-containing peptides were: 1 (residues 1–15), 2 (6–22), 3 (13–27), 4 (18–32), 5 (23–37), 6 (29–43), 7 (34–48), 8 (39–53), 9 (45–59) and 10 (49–64) and those of Abu-containing peptides were: 1 (1–13), 2 (8–19), 3 (15–27), 4 (23–34), 5 (30–43), 6 (38–50), 7 (45–57) and 8 (52–64). Details of synthesis will be published elsewhere (Mabrouk et al., in preparation). The 58 overlapping heptapeptides covering the entire 64-residue sequence of AaH II [9] were simultaneously synthesized by the Pepscan method [10], as described [11].

2.2. Immunization of mice

Six-week-old females BALB/c were injected intraperitoneally (i.p.) and subcutaneously (s.c.) with 36 μg of $(Abu)_8$-AahII in complete Freund adjuvant (CFA). Boosts of 20 μg in incomplete adjuvant (IFA) were given on days 24, 42, 66, 86 and 130. Sera from all bleedings were tested against AahII using ELISA. The sera were tested in a liquid-phase assay (RIA) and the mouse with the highest response was selected for fusion. Three boosts of 50, 75 and 75 μg of $(Abu)_8$-AahII in 0.9% NaCl were given at 1 h intervals (i.p., i.p. and intravenously (i.v.), respectively) three days before fusion.

2.3. Generation of mAbs

mAbs were produced as described by Galfré and Milstein [12]. Splenocytes from immune mouse (74×10^6 cells) were fused with X63-F cells (15×10^6) using 50% of polyethyleneglycol 1500 (Boehringer). Hybrids were selected in HAT-RPMI 1640 medium (Eurobio) containing 15% FCS (Boehringer). Antibody-secreting cells were expanded and cloned twice at limiting dilution using mouse peritoneal macrophages as feeder cells. Cloned cells were grown as ascites tumors in pristane-primed mice.

Antibodies were purified on protein-A Sepharose (Pharmacia) as described by Devaux et al. [13] using 0.1 M Na citrate pH 5 as the elution buffer. They were then dialysed in TBS. Purity was assessed using 12% SDS-PAGE and p*I* 3-9 IEF gel (Phast System, Pharmacia). The protein concentration of IgG solutions was measured by UV spectrophotometry using $A_{278} = 1.4$ cm^2/mg. Antigen-mediated ELISA was used to determine the heavy chain isotype (Sigma ISO-2 kit) or the light chain isotype (rat mAbs anti-mouse kappa or lambda chain from Sigma).

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PII S0014-5793(97)00826-0

2.4. Immunoassays

2.4.1. RIA. The binding of each mAb to ^{125}I-AahII was assessed. ^{125}I-AahII was prepared and purified as previously described [14]. Thirty microlitres of each dilution of different concentrations of purified IgG were mixed with 30 μl of ^{125}I-AahII (5×10^{-10} M) and 30 μl of PBS-0.1% BSA. The mixtures were incubated for 90 min at 37°C and then overnight at 4°C. Sheep anti-mouse precipitating antibody (0.5 ml; UCB Bioproducts, Belgium) was added. The mixtures were incubated for 30 min at 4°C, then centrifuged at $9000\times g$ for 10 min. Radioactivity was measured with a gamma counter (Packard Crystal II). All assays were duplicated.

2.4.2. ELISA. Microtiter plates (Maxisorb, NUNC) were coated with 100 μl/well of AahII (1 μg/ml) in PBS overnight at 4°C. Blocking solution (150 μl of 5% non-fat milk in PBS) was added to each well and the samples incubated for 1 h at 37°C. The plates were then washed with 0.9% NaCl containing 0.05% Tween 20 (NT). Cell supernatants or IgG were added (100 μl/well) for 90 min at 37°C. The plates were washed with NT and further incubated with 100 μl/well of a 1:1000 dilution of peroxidase-conjugated goat anti-mouse IgG (Jackson, France). The wells were washed again with NT. Peroxidase substrate solution (TMB, Kirkegaard and Perry Labs, MD) was added (100 μl/well) and the plates were left 10 min at room temperature. Absorbance at 620 nm was measured using a Titertek Multiskan photometer (Flow Labs). The assays were duplicated.

For epitope mapping, ELISAs were carried out as described above. The plates were coated with peptides at a concentration of 2 μg/ml and purified IgG were diluted to between 0.03 and 1 nmol/ml. The mean of three independent experiments is given. Dissociation constants (K_D) of toxin-antibody interactions were determined under the conditions described by Friguet et al. [15] using ELISA to measure the concentration of free antibody at equilibrium.

2.5. Neutralization assays

Receptor binding assay. Rat brain synaptosomal fraction was prepared using the method of Gray and Whittaker [16] described by Jover et al. [17]. Binding assays were performed in 140 mM choline chloride, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES and 0.1% BSA at pH 7.4. Thirty microlitres of ^{125}I-AahII (5×10^{-10}M) were incubated with 30 μl of each of a series of dilutions of purified IgG for 90 min at 37°C. Synaptosomal preparation was added (30 μl) and the incubation was continued for an additional 30 min at 37°C. The mixtures were centrifuged at $9000\times g$ and washed three times. The radioactivity of the pellets was measured. Assays were duplicated.

2.5.1. Assays in vivo

The neutralizing capacity of mAbs was tested by the intracerebroventricular (i.c.v.) route. Each of a series of amounts of AahII equal or higher than the value of the lethal dose (LD_{50} = 1.5 ng for C57BL/6 mice) were preincubated for 90 min at 37°C and 30 min at 4°C with un equal volume of protein-A Sepharose purified IgG from mAbs or from nonimmune serum. Female C57BL/6 mice (groups of six) were injected by the i.c.v. route. Surviving mice were recorded after 24 h.

3. Results

3.1. Production of hybridomas and monoclonal antibodies (mAbs)

Immunization of BALB/c mice with $(Abu)_8$-AahII is a non-toxic alternative to low-dose immunization with native toxin. $(Abu)_8$-AahII was injected into several mice and the mouse with the strongest response was selected by the ability of its antibodies to recognize AaH II on coated plates (ELISA) or ^{125}I-labelled AahII (RIA) (Fig. 1). Serum titers determined by ELISA were 1/50 000 with $(Abu)_8$-AahII and 1/5000 with AahII-coated plates. Hybrid cells grew in more than 50% of the wells (800) containing cell fusion mixture. Nine supernatants cross-reacted with AahII-coated plates. Cells from these wells were cloned twice. Three clones (denoted 23C6, 37D3, 26D8) developed suitably and were grown in mice. MAbs were purified from ascitic fluids by protein A-Sepharose chromatography. They all belong to the IgG1 subclass and their light chain is of kappa type.

3.2. General characteristics of mAbs

Purified mAbs were resolved as a single band in non-reducing 12% SDS-PAGE and in p*I* 5–9 IEF gel (data not shown). They had different p*I*s (Table 1). All mAbs recognized AahII in indirect ELISA, with titers between 5 and 100 nM IgG. The affinity of 23C6 and 37D3 mAbs for the native AahII toxin was measured by competitive ELISA at equilibrium. K_D values were 0.4 and 4 μM, respectively (Table 1).

3.3. Neutralizing effect of mAbs on the AahII binding on rat synaptosomes and on AahII toxicity in mice

The lethal effect of the scorpion venom is mainly due to the binding of AahII to voltage-dependent sodium channels. The capacity of mAbs to inhibit the biological effects of AahII was tested both in vitro and in vivo. The inhibition of the binding of ^{125}I-AahII to its site on rat brain synaptosomes was tested. All mAbs showed dose-dependent inhibition (Fig. 2). The efficiency of the inhibition was paralleled to the measured K_D of mAbs for the native toxin and clearly higher than the non-specific effect obtained with IgG from preimmune serum (Fig. 2). IC_{50} values (concentration of IgG giving half-binding inhibition of AahII) were 1 (23C6), 5 (37D3) and 8 μM (26D8).

The toxicity of AahII was measured in C57BL/6 mice by i.c.v. injection. The protective effect of mAbs was evaluated by preincubating each of a series of amounts of AahII with purified IgG from each mAb and injecting the mixture by the i.c.v. route to mice (six per AahII concentration). The controls were performed in the same way, mAb being replaced by IgG from mouse nonimmune serum at the same concentration (2.5 mg/ml). In the presence of mAb 23C6, 50% of the mice injected survived the injection of 2 LD_{50} whereas all the control mice died. The protective capacity of 23C6 mAb was about 200 LD_{50} by mg of purified IgG from ascitic fluid. No significant protection was observed with the other mAbs.

Table 1
Characteristics of anti-(Abu)8-AahII mAbs

mAbs	Isotype	p*I*	Titer (ELISA)[a] $\times10^{-9}$		K_D (ELISA)[b] $\times10^{-7}$	K_D (RIA)[c] $\times10^{-7}$
			(Abu)8-AahII	AahII	AahII	AahII
23C6	IgG1,κ	6.3	0.09	5	4	3.7
37D3	IgG1,κ	7.6	0.10	19	39	d
26D8	IgG1,κ	5.8	5.00	97	d	d

[a]Titer was the concentration of IgG giving half-binding to (Abu)8-AahII or AahII-coated plates.
[b]K_D from competitive ELISA were measured as described in Friguet et al. [15].
[c]K_D from competitive RIA was calculated as in Bahraoui et al. [6].
[d]No competition was observed at the highest concentration tested (5×10^{-6}).

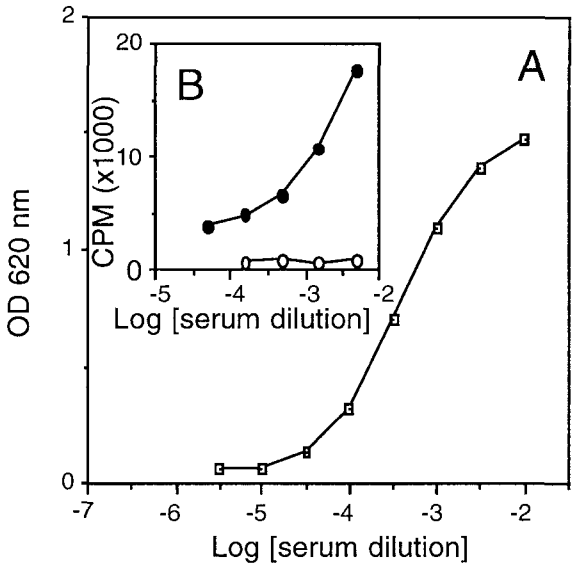

Fig. 1. Reactivity of polyclonal anti-$(Abu)_8$-AahII serum with AahII toxin. BALB/c mice were immunized with $(Abu)_8$-AahII. Serial dilutions of immune sera were tested on 1 μg/ml AahII-coated plates in ELISA (A) or against ^{125}I-AahII in RIA (B).

3.4. *Epitope mapping of mAbs using different sets of Aah II-derived synthetic peptides*

Two different sets of overlapping linear peptides were used for epitope mapping. They were 12–17 residues long. The cysteine residues were replaced by acetamidomethyl groups (Acm) in one set, and by α-aminobutyric acid (Abu) in the other. The mAbs 23C6 and 26D8 reacted strongly with peptides from the C-terminal part of the molecule (Fig. 3). MAb 23C6 recognized the C-terminal Acm (residues 49–64) or Abu (residues 52–64) peptides. MAb 26D8 recognized two Acm peptides (residues 45–59 and 49–64) and one Abu-peptide (residues 45–57). In order to confirm that the two sequences recognized by mAbs 23C6 and 26D8 were differents, an another synthetic peptide (sequence 50–59 [18]) was tested. It was not recognized by mAb 23C6 but gave a strong signal with mAb 26D8 (data not shown). MAb 37D3 reacted with Acm-peptides 6–22 and 23–37 but not with Abu-peptides. In addition, no mAbs (even at high IgG concentration) reacted with heptapeptides synthesized in the Pepscan format [11,19].

4. Discussion and conclusion

There are several possible strategies for eliciting mAbs active against highly toxic molecules. Low doses, chemical detoxification and synthetic peptides have been used. We have previously obtained a neutralizing mAb of high affinity using low-dose of native AahII [6]. Here, we tried to obtain additional mAbs by using a non-toxic immunogen, $(Abu)_8$-AahII. Our choice was validated by the successful mice protection experiments [8] using this analog which contains the entire sequence of AahII. In contrast, previous trials using short immunogenic peptides (residues 50–59 and 1–8), produced toxin-specific antibodies, but failed to protect mice against native toxin irrespective of how the immunogen was formulated ([20] and Chavez-Olortegui and Granier, unpublished). We obtained several mAbs by screening clones produced by cell fusion for reaction to the native toxin. They recognized AahII both in solid- (ELISA) or liquid-phase (RIA) assays but with lower affinity than that of mAb 4C1. The strongest reaction was obtained with mAb 23C6 which inhibited the binding of ^{125}I-AahII to rat brain synaptosomes (IC_{50} = 1 μM). The mAbs 37D3 and 26D8 also had a slight inhibitory effect (IC_{50} = 5 μM and 8 μM, respectively). In addition, preincubation of AahII toxin with 23C6 IgG caused a doubling of the LD_{50} in C57BL/6 mice.

We identified the antigenic sites of mAbs on toxin II using synthetic peptides from AahII sequence in ELISA. The mAbs 23C6 and 26D8 recognized the C-terminal part of AahII. Their sites overlapped extending from residues 49 to 64 and 45 to 64, respectively. The combination of the results obtained in ELISA with the two sets of overlapping peptides (acm- and abu-peptides) allowed to define minimal sequences recognized by mAbs as residues 52–64 (mAb 23C6) and 49–57 (mAb 26D8). The mAb 37D3 is unusual as it binds two non-overlapping Acm-peptides (residues 6–22 and 23–37) separated by two unreactive peptides, but does not recognize smaller sequences (Abu-peptides 8–19 and 23–34). All three mAbs failed to bind to smaller peptides (heptapeptides in the Pepscan format).

These antibodies, produced against a 64-residue synthetic analog devoid of disulphide bridges were selected on the basis of their cross-reaction with the intact toxin. They recognize peptides of 12–17 residues. This does not necessarily mean that all these residues are involved in the interaction between paratope and epitope. This size may be critical to mimic epitopes with a local secondary structure very similar to that found in the intact AahII toxin. Some residues, although not directly involved, may play a role in stabilizing the interaction and/or the relevant structure. All identified mAb sites contain sequences forming β-turns in the AahII X-ray model [21]: the fourth β-turn (residues 52–55) for both mAbs 26D8 and 23C6, first (residues 8–12) and second (residues 27–30) β-turns for mAb 37D3. In addition the defined mAb-binding sequences are the most accessible sites on the molecule [22,23].

The strong antigenicity of the C-terminus part of AahII was previously demonstrated using rabbit sera specific for toxins and synthetic peptides derived from the sequence of AahII [23] or obtained by the Pepscan method [11]. Polyclonal anti-

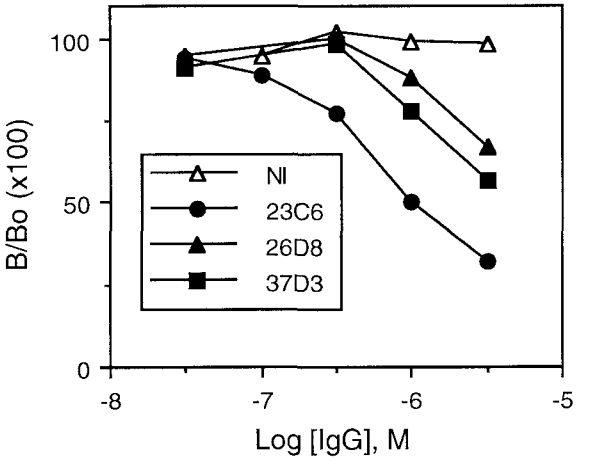

Fig. 2. AahII neutralizing capacity of anti-$(Abu)_8$-AahII mAbs. Binding of ^{125}I-AahII (10^{-10} M) to rat brain synaptosomes was inhibited by preincubation with various dilutions of the IgG mAbs. BALB/c preimmune serum was used as a control (NI). B and Bo was the binding measured in presence (B) or in absence (Bo) of IgG.

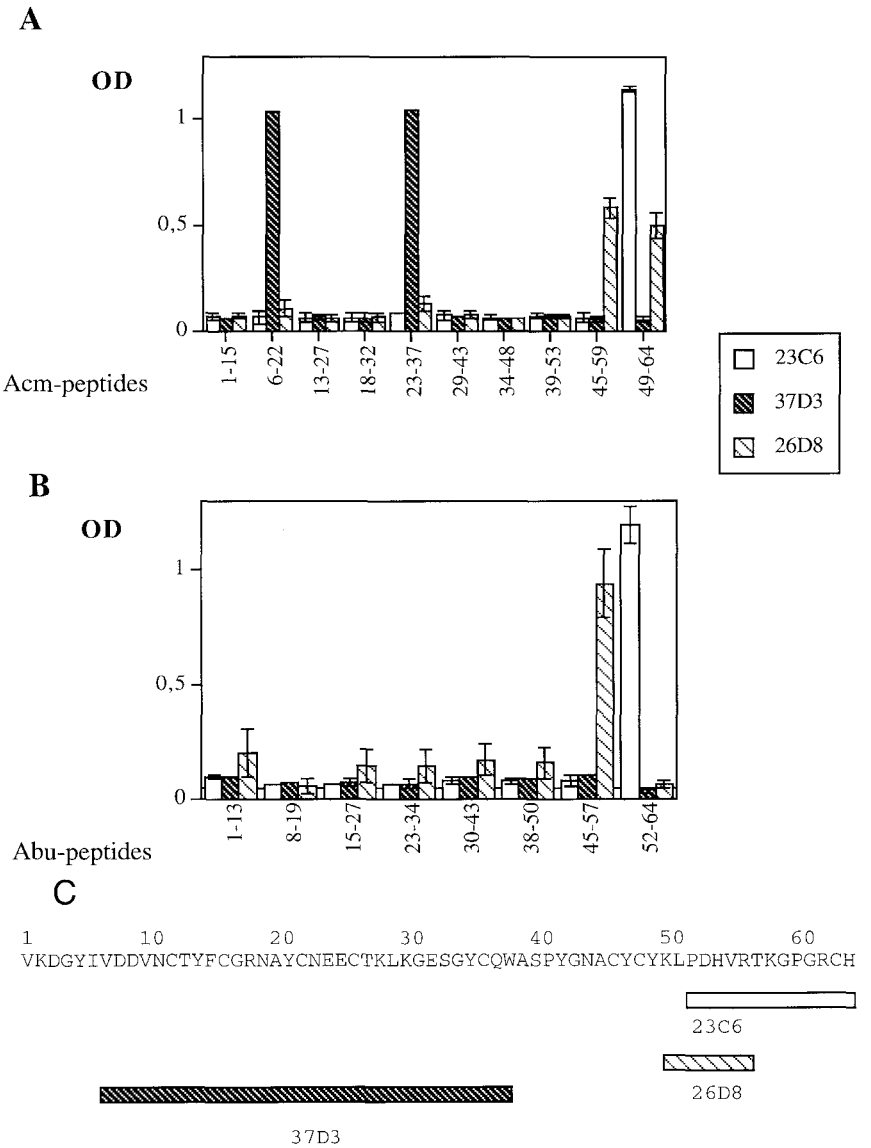

Fig. 3. Epitope analysis of mAbs. Antibodies were tested for binding to different synthetic peptides derived from the AahII sequence. (A) Binding pattern of mAbs to Acm-peptides or (B) binding pattern of mAbs to Abu-peptides as determined by ELISA. (C) Location of the binding sites of the mAbs on the AahII sequence as deduced from the ELISA data.

bodies could be induced by BSA-coupled peptide (50–59) in rabbits. They neutralized the lethal effect of the toxin [18] but we failed to obtain neutralizing antibodies in mice whatever was the presentation of the peptide [20]. Furthermore, the immunoreactivity of the neutralizing mAb 4C1 obtained by immunization with AahII was strongly altered when K^{58} or $H^{54/64}$ were modified [6]. The binding site of mAb 23C6 on AahII was a sequence containing the highly reactive K^{58} whose chemical modification abolishes binding of the toxin to its pharmacological site on Na^+-channel ([24] and Hassani et al. unpublished). The three mAbs induced by $(Abu)_8$-AahII were able to inhibit the binding of AahII to its receptor. It is unknown if this inhibition is due to steric hindrance of the pharmacological site or to direct interaction with this site. Complete description of antigen-antibody interaction involves both structural and functional analyses [25–27]. Functional immunoassays using synthetic peptides to define antigenic determinants on proteins have often been successfully used [28,29]. Our results with mAbs 23C6 and 26B8, together with previous work, suggest that there is a dominant antigenic domain on the AahII toxin. It consists of several overlapping functional epitopes.

This study shows that mAbs may be obtained using $(Abu)_8$-AahII as the immunogen. The diversity of these mAbs and the location of their binding sites may explain the strong protection of $(Abu)_8$-AahII-immunized mice against challenge by natural toxin [8]. If the entire accessible surface of a protein is a continuum of overlapping epitopes [30,31], a full-length analog may be more valuable than some carrier-coupled peptides as a non-toxic immunogen.

Acknowledgements: The authors are greatly indebted to M. Alvitre for animal care.

References

[1] Köhler, G. and Milstein, C. (1975) Nature 256, 495–497.
[2] Waldmann, T.A. (1991) Science 252, 1657–1662.

[3] Russell, S.J., Llewelyn, M.B. and Hawkins, R.E. (1992) Br. Med. J. 305, 1424–1429.
[4] Al Moudallal, Z., Briand, J.P. and Van Regenmortel, M.H.V. (1985) EMBO J. 4, 1231–1235.
[5] Goldberg, M.E. (1991) Trends Biochem. Sci. 16, 358–362.
[6] Bahraoui, E., Pichon, J., Muller, J.-M., Darbon, H., El Ayeb, M., Granier, C., Marvaldi, J. and Rochat, H. (1988) J. Immunol. 141, 214–220.
[7] Zamudio, F., Saavedra, R., Martin, B.M., Gurrola-Briones, G., Hérion, P. and Possani, L.D. (1992) Eur. J. Biochem. 204, 281–292.
[8] Zenouaki, I., Kharrat, R., Sabatier, J.-M., Devaux, C., Karoui, H., Van Rietschoten, J., El Ayeb, M. and Rochat, H. (1997) Vaccine 15, 187–194.
[9] Rochat, H., Rochat, C., Sampieri, F. and Miranda, F. (1972) Eur. J. Biochem. 28, 381–388.
[10] Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) Proc. Natl. Acad. Sci. USA 81, 3998–4002.
[11] Devaux, C., Juin, M., Mansuelle, P. and Granier, C. (1993) Mol. Immunol. 30, 1061–1068.
[12] Galfré, G. and Milstein, C. (1981) 73, 3–47
[13] Devaux, C., Defendini, M.-L., Alzari, P.M., Abergel, C., Granier, C. and Fontecilla-Camps, J.C. (1991) FEBS Lett. 286, 64–67.
[14] Rochat, H., Tessier, M., Miranda, F. and Lissitzky, S. (1977) Anal. Biochem. 85, 532–548.
[15] Friguet, B., Chaffotte, A.F., Djavadi-Ohaniance, L. and Goldberg, M.E. (1985) J. Immunol. Methods 77, 305–319.
[16] Gray, E.G. and Whittaker, V.P. (1962) J. Anat. (London) 96, 79–87.
[17] Jover, E., Martin-Moutot, N., Couraud, F. and Rochat, H. (1980) Biochemistry 19, 463–467.
[18] Bahraoui, E., Granier, C., Van Rietschoten, J., Rochat, H. and El Ayeb, M. (1986) J. Immunol. 136, 3371–3377.
[19] Devaux, C., Fourquet, P. and Granier, C. (1996) Eur. J. Biochem. 242, 727–735.
[20] Aït-Amara, D., Chavez-Olortegui, C., Romi, R., Mery, J., Brugidou, J., Albericio, F., Devaux, C. and Granier, C. (1992) Natural Toxins 1, 1–8.
[21] Fontecilla-Camps, J.C., Habersetzer-Rochat, C. and Rochat, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7443–7447.
[22] Novotny, J., Handschumacher, M., Haber, E., Bruccoleri, R.E., Carlson, W.E., Fanning, D.W., Smith, J.A. and Rose, G.D. (1986) Proc. Natl. Acad. Sci. USA 83, 226–230.
[23] Granier, C., Novotny, J., Fontecilla-Camps, J.-C., Fourquet, P., El Ayeb, M. and Bahraoui, E. (1989) Mol. Immunol. 26, 503–513.
[24] Darbon, H., Jover, E., Couraud, F. and Rochat, H. (1983) Int. J. Peptide Protein Res. 22, 179–186.
[25] Van Regenmortel, M.H.V. (1989) Immunol. Today 10, 266–272.
[26] Laver, W.G., Air, G.M., Webster, R.G. and Smith-Gill, S.J. (1990) Cell 61, 553–556.
[27] Benjamin, D.C., Berzofsky, J.A., East, I.J., Hannum, C., Sydney, J.L., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E. and Wilson, A.C. (1984) Annu. Rev. Immunol. 2, 67–101.
[28] Parry, N.R., Barnett, P.V., Ouldridge, E.J., Rowlands, D.J. and Brown, F. (1989) J. Gen. Virol. 70, 1493–1503.
[29] Gao, B. and Esnouf, M.P. (1996) J. Immunol. 157, 183–188.
[30] Van Regenmortel, M.H.V. (1986) Trends Biochem. Sci. 11, 36–39.
[31] Newman, M.A., Mainhart, C.R., Mallet, C.P., Lavoie, T.B. and Smith-Gill, S.J. (1992) J. Immunol. 149, 3260–3272.