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Production and characterization of monoclonal anti-idiotypic antibodies to muramylpeptide

T.Yu. Mareeva, O.V. Kotova, T.N. Golovina, V.A. Nesmeyanov*

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russian Federation

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Abstract Hybridomas producing monoclonal anti-idiotypic antibodies (anti-id MAbs) to *N*-acetylglucosaminyl-β1–4-*N*-acetylmuramyl-alanyl-D-isoglutamine (GMDP) were developed. Three clones of hybridomas demonstrated the properties characteristic for the Ab2β type of anti-id antibody: they bound to Fab-fragments of high-affinity MAb to GMDP; dose-dependent inhibition of this binding by GMDP was observed; immunization of mice with these MAbs resulted in production of GMDP-specific antibodies. When these antibodies were used to stain blots from SDS-PAGE of macrophage lysate, the same receptor proteins were specifically stained as upon staining with ^{125}I-labelled GMDP derivative.

Key words: Anti-idiotypic antibody; Monoclonal antibody; Muramyl peptide

1. Introduction

Muramyldipeptide (MDP) is the minimal structure of the bacterial cell wall capable of replacing mycobacteria in Freunds' complete adjuvant [1]. MDP and its analogues, containing the *N*-acetylglucosamine residue, demonstrate a variety of biological activities, in particular, adjuvant effect [2,3], induction of non-specific resistance to infections [4,5], and somnogenic activity [6]. The molecular basis of biological activity of muramyl peptides (MPs) is poorly understood, but the majority of data favors a receptor-mediated mechanism [7–10].

Macrophages are shown to be the main target of MPs [4]. The number of cell surface receptors for MPs is low, making their isolation and structural analysis difficult [7,8]. Like in studies of receptors of other biologically active ligands, monoclonal antibodies to GMDP-receptors could be of great aid, but their production by traditional procedures is hardly achieved.

Not long ago an alternative approach was developed based on the production of anti-idiotypic antibodies (anti-id) to corresponding ligands, capable of binding to ligand receptors. This approach is based on the Jerne theory of idiotypic network, claiming that the immune system consists of a set of interacting idiotypes and anti-idiotypes [11]. According to this theory for each exogeneous epitope an anti-id antibody exists mimicking the so-called internal image of this epitope by its hypervariable region. This antibody is designated as Ab2β [12].

Anti-id antibodies can be induced by immunization with anti-ligand antibodies, imitating the binding site of the receptor. Using this approach monoclonal and polyclonal antibodies to a number of receptors were produced [13–16]. The crucial step in obtaining anti-id MAbs capable of binding to the ligand receptor is identifying among hybridoma those clones producing antibodies of the Ab2β type to paratope-associated epitopes. In this study we developed murine hybridomas producing MAbs to the GMDP-binding site of mouse E6/1.2 MAb, and identified three antibodies mimicking the internal image of GMDP.

2. Materials and methods

2.1. Mice and reagents

Female Balb/c and F1 (CBA×C57BL/6) mice, 10–12 weeks old, were purchased from Stolbovaya breeding house (Moscow region).

GMDP, GMDP-Lys and GMDP-AL were synthesized by E. Makarov in the Laboratory of Peptide Chemistry, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, in accordance with earlier the developed procedure [2,24]. GMDP-OVA conjugate was synthesized as in [17].

Streptoavidin–horseradish peroxidase conjugate was a kind gift from Dr. V. Kovalenko, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry.

Cell culture reagents were purchased from Gibco (Scotland).

2.2. Production and purification of Fab-fragments of E6/1.2 MAb

Homogeneous E6/1.2 MAb was isolated from murine ascites by affinity chromatography on immobilized GMDP-Lys [17]. Papain hydrolysis of MAb was performed as in [19]. Briefly, 150 μl 10× PBS, 70 μl 0.1 M L-cysteine, 70 μl 0.02 M disodium EDTA and 40 μg papain in 70 μl PBS were added to 9 mg of affinity-purified E6/1.2 MAb in 1.5 ml PBS. The mixture was incubated for 2.5 h at 37°C with occasional shaking. The reaction was stopped by incubation for 30 min at room temperature with 70 μl 0.15 M iodoacetamide. Papain digest was dialyzed against 0.05 M Tris-HCl, pH 7.5, and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was applied to an FPLC Mono-Q 5/5 column, equilibrated with 0.05 M Tris-HCl, pH 7.5. Non-bound material was washed out with the same buffer and elution was carried out with a 0 to 1 M gradient of sodium chloride. Each fraction was analyzed by competition ELISA [17]. Peaks I and II (Fig. 1), containing the main part of GMDP-AL-binding activity, were dialyzed against PBS, pH 7.4. Each peak (30 μl) was analyzed by SDS-PAGE [20] on a 10% gel under reducing and non-reducing conditions.

Purified Fab-fragments were concentrated by ultrafiltration using PM 10 membrane and stored in PBS, containing 0.02% NaN_3, at 4°C.

2.3. Synthesis of Fab-MeBSA

Fab-fragments of E6/1.2 MAb (15 vg, 0.1 μmol) in 2 ml PBS were activated for 1 min at 18°C with 320 mg (2 mmol) EDC and 13.6 mg (0.1 mmol) 1-hydroxybenzotriazole, dissolved in 5 ml DMFA. Activated fragments were mixed with 6.7 mg (0.1 μmol) MeBSA in 5 ml PBS

*Corresponding author. Fax: (7) (095) 310 7007.

Abbreviations: MP, muramyl peptide; MDP, MurNAc-Ala-D-iGln; GMDP, GlcNAc-β1-4-MurNAc-Ala-D-iGln; L-GMDP, GlcNAc-β1-4-MurNAc-Ala-iGln; GMDP-Lys, GlcNAc-β1-4-MurNAc-Ala-D-iGln-Lys; PBS, 0.01 M sodium phosphate buffer, containing 0.15 M NaCl; PBST, PBS containing 0.1% Tween 20; BSA, bovine serum albumin; GMDP-OVA, GMDP conjugated to ovalbumin; GMDP-AL, GMDP conjugated to poly-Lys-poly-Ala polymer; MeBSA, methylated bovine serum albumin.

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and stirred overnight at 4°C. The reaction was stopped by dialysis against PBS, pH 7.4. The conjugate was stored in 20% glycerol at −20°C.

2.4. Immunization and hybridization

Balb/c mice were immunized by i.p. injections of 200 μg Fab-MeBSA conjugate. Two injections were made with a two-week interval in complete Freunds' adjuvant followed by three injections at one-week intervals in incomplete Freunds' adjuvant. Three weeks after the fifth immunization the mouse with the highest titer of anti-Fab antibodies was boosted during three consecutive days by i.p. injections with 100 μg Fab-MeBSA in PBS. Hybridization of splenocytes (5×10^7 cells) with SP2/0 myeloma cells (10^7 cells) was carried out by a standard procedure using PEG 6000 [25]. The suspension of hybrid cells in RPMI 1640, containing 20% FCS, was dispersed onto wells of four 96-well microtiter plates. The next day half of the culture medium was exchanged for HAT-medium. Twelve to fourteen days later supernatants from growing clones were screened for specific antibody production.

2.5. Binding ELISA

Supernatants from hybrid clones were screened for production of anti-E6/1.2 MAb by solid-phase ELISA using E6/1.2 Fab-fragments, biotinylated as in [22].

Supernatants were absorbed onto wells of 96-well microtiter plates at 4°C overnight. Remaining binding sites were blocked by 1 h incubation with 200 μl per well of 0.1% gelatin in PBS. After washing with PBST 100 μl of biotinylated E6/1.2 Fabs, diluted 1:2,000 with PBS, were introduced. The incubation was carried out for 1 h at room temperature. Plates were washed with PBST and incubated for 1 h at room temperature with 100 μl/well of 1:2,000 dilution of streptoavidin-HRP conjugate in PBS. After washing 100 μl of OPD solution (1 mg/ml) in 1% citrate buffer, pH 4.5, containing 0.05% H_2O_2, were added. The reaction was stopped 15–30 min later by addition of 100 μl/well of 1 M H_2SO_4. Optical density at 492 nm was measured.

2.6. Competition ELISA

GMDP-OVA (40 μg/ml) or GMDP-AL (10 μg/ml) in PBS were absorbed onto wells of microtiter plates for 1 h. Washing and blocking of remaining binding sites were performed as described in section 2.5. Fractions from the Mono-Q 5/5 column or serial twofold dilutions of anti-id MAbs (50 μl/well) in PBS and 50 μl of biotinylated E6/1.2 Fabs (1:4,000 dilution in PBS) were added. After 1 h incubation microtiter plates were washed and incubated for 1 h with 100 μl 1:2,000 dilution of streptavidin-HRP. The reaction was visualized as described in section 2.5. All incubations were carried out at room temperature.

Inhibition index (I, %) was calculated from:

$$I = \frac{A_0 - A_1}{A_0} \times 100$$

where A_0 and A_1 are optical densities of samples without or with inhibitor, respectively.

2.7. Purification and characterization of anti-id-MAbs

Hybridomas were grown in ascites of Balb/c mice. Mice were administered with 0.5 ml pristane and 14 days later inoculated with hybridoma cells (2×10^6 cells per mice).

MAbs were purified from ascites fluid by using affinity chromatography. E6/1.2 MAb Fab-fragments were immobilized on CNBr-activated Sepharose-4B according to instructions of the manufacturer. Elution was performed with 0.1 M glycine-HCl buffer pH 3.0. The purity of MAbs was characterized by SDS-PAGE in a 10% gel [20]. Isotypes and subtypes of MAbs were characterized by using commercial monospecific rabbit anti-mouse immunoglobulin antibodies, conjugated to HRP.

2.8. Induction of Ab3 by anti-id MAbs to GMDP

Groups of 5 female F1 (CBA×C57Bl/6) mice were administered i.p. 5 times with anti-id MAbs, 10 μg/mice in 0.2 ml PBS. The first two injections were made in complete Freunds adjuvant, the rest in PBS. A week after the fifth injection mice sera were screened for antibodies specific to GMDP by ELISA as described in [17]. GMDP-AL (20 μg/ml) was used as antigen. Serum from non-immune mice was used as a control.

2.9. Isolation of membrane fraction of WEHI-3 cells

WEHI-3 cells were grown in plastic flasks in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol.

Cells were dissociated from plastic by Versene solution (37°C, 20 min), washed with PBS and lysed by 0.002 M Tris-HCl, pH 7.5, containing 0.002 M $CaCl_2$ and 0.001 M phenylmethylsulfenyl fluoride. Intact cells and nuclei were sedimented by centrifugation (1,000 × g, 10 min). Membranes were pelleted by centrifugation at 105,000 × g for 60 min.

2.10. Immunostaining of WEHI-3 cell membrane proteins with anti-id MAb

Membranes (50 μg of protein) were dissolved in sample buffer for SDS-PAGE. Identical quantities of sample were introduced into several wells of the polyacrylamide gel. Electrophoresis was carried out according to Laemmli [20] on a 7.5% slab gel. Proteins were blotted onto a nitrocellulose membrane according to Burnett [21]. After incubating the membrane with 1 mg/ml BSA in PBS (4°C, 18 h) it was cut into strips along the sample lanes. Individual strips were incubated with: (a) 100 μg/ml solution of C5/F8 MAb in PBS; (b) 100 μg/ml solution of LNKB-1 MAb (IgG2b) to human IL 2.

The antibodies bound to membrane proteins were visualized with HRP-conjugated rabbit anti-mouse immunoglobulin antibodies (1:500 dilution, DAKO PATTS, Denmark) and the mixture of 3,3'-diaminobenzidine with 4-chloronaphtol [22]. The strip with molecular weight markers was stained with 0.5% Amido black in methanol/acetic acid/water (50:10:40).

3. Results and discussion

Not long ago we described the development of hybridoma clone E6/1.2, producing high affinity murine IgG1 (æ) MAb (K_a 2×10^9 M^{-1}) specific to GMDP [17]. Inhibition analysis revealed that E6/1.2 antibody besides GMDP could specifically bind GMDP-Lys and to a lesser extent L-GMDP. Interaction of E6/1.2 MAb with GMDP was slightly inhibited by disaccharide GlcNAc-MurNAc, but not by MDP, GlcNAc, MurNAc or dipeptide Ala-D-iGln. Thus, both sugar and peptide fragments of the molecule seemed to be crucial for GMDP binding, the most important being the intact disaccharide moiety and the Ala residue.

The aforementioned properties enabled us to use the E6/1.2

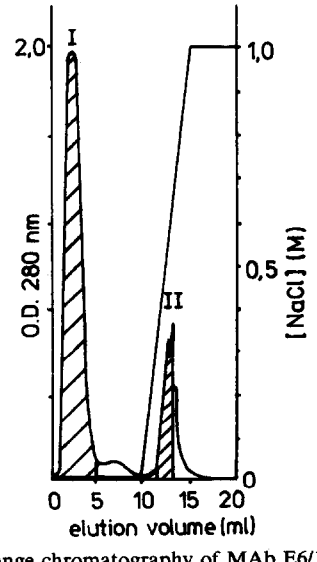

Fig. 1. Ion-exchange chromatography of MAb E6/1.2 Fab-fragments on FPLC Mono Q 5/5 column. Fractions positive in solid-phase ELISA are shaded.

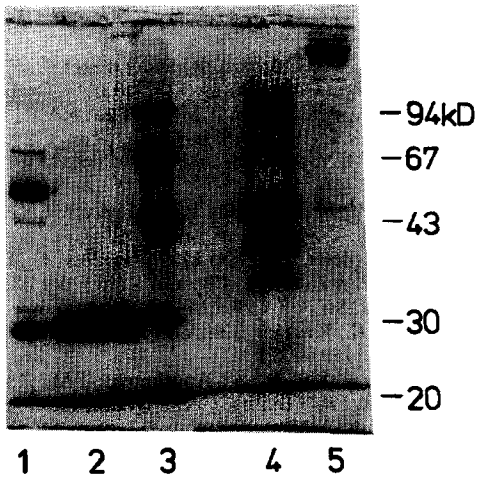

Fig. 2. SDS-PAGE of E6/1.2 Fab-fragments in 10% gel. Lanes 2 and 4, peak I; lanes 1 and 5, peak II under reducing or non-reducing conditions, respectively. Lane 3, protein standards.

antibody for the production of monoclonal anti-id antibodies to GMDP mimicking its internal image.

Fab-fragments of E6/1.2 MAb were used to mount an anti-id response in Balb/c mice. To obtain these fragments the E6/1.2 antibody was purified from ascites by affinity chromatography on immobilized GMDP-Lys [17] and subjected to papain hydrolysis [19]. Purification of Fab-fragments was achieved by DEAE-chromatography (Fig. 1). GMDP-binding activity eluted in two peaks. SDS-PAGE analysis (Fig. 2) revealed that Fab-fragments were eluted with flow-through fractions (peak I) whereas non-digested IgG eluted with NaCl gradient (peak II).

E6/1.2 Fab-fragments were conjugated to methylated BSA (Fab-MeBSA) in order to increase their immunogenicity. According to ELISA this conjugate retained the ability of E6/1.2 MAb to bind GMDP, confirming the preservation of native structure of GMDP-binding site on Fab-fragments.

Balb/c mouse was administered 5 times with Fab-MeBSA, and splenocytes were fused with SP2/0 myeloma. Positive hybridoma clones were selected 12–14 days later according to the ability of antibodies to interact with biotinylated E6/1.2 Fab-fragments in binding ELISA. In total 26 hybridoma clones were selected.

In order to identify clones producing MAbs to paratope-associated epitopes, the potency of hybridoma supernates to inhibit binding of biotinylated E6/1.2 Fab-fragments to GMDP-OVA was evaluated. According to competition ELISA seven positive clones were identified.

Hybridomas positive in both assays were cloned by the limiting dilution procedure. After recloning 8 clones, secreting MAbs to paratope-associated epitopes of E6/1.2 MAb, were selected. Isotypes and subtypes of anti-id MAbs were determined by using commercial antisera. For some of these, affinity to E6/1.2 MAb was determined by the procedure of Beatty et al. [26]. Properties of obtained MAbs are summarized in Table 1.

Among anti-id antibodies, binding of which to anti-ligand antibodies can be inhibited by ligand, few types are known to be present. Only one type, known as Ab2β, in fact mimics the internal image of the ligand. The most simple approach to identify Ab2β type MAb includes the study of specificity of antibody response to anti-id MAb, because administration of Ab2β has to result in the accumulation of ligand-specific antibodies (anti-anti-id antibodies, Ab3) [18]. In our case production of anti-GMDP antibodies had to be expected.

To induce the formation of Ab3 female F1 (CBA×C57Bl/6) mice (groups of 5 animals) were immunized 5 times with anti-id MAbs to GMDP. Serum from each mice was assayed for GMDP-specific antibodies by using direct and competition solid-phase ELISA [17]. GMDP-AL (20 μg/ml) was used as an antigen. In control samples, serum taken from non-immune mouse was substituted for serum from immune animals. Inhibition of Ab3 binding to GMDP-AL in the presence of free GMDP was evaluated as described above.

Though serum from any immune mouse to a certain extent bound to GMDP-AL (data not shown), specific dose-dependent inhibition was observed only for serum from mice immunized with MAbs C5/F3, C5/F8 and E1/B2 (Fig. 3). It should be noted that for these three anti-id MAbs specific, dose-dependent inhibition was observed with sera from all mice in the corresponding group.

Table 1
Properties of monoclonal anti-idiotypic antibodies to GMDP

Clone	Isotype, subtype	Binding constant to MAb E6/1.2 (M^{-1})	Induction of Ab3, specific to GMDP	Type
B4/B6	IgM	n.d.	–	γ
C5/F3	IgG2b	3.2×10^7	+	β
C5/F8	IgG2b	1.2×10^7	+	β
C10	IgG3	n.d.	–	γ
D3/G4	IgM	n.d.	–	γ
E1/B2	IgG1	5.5×10^7	+	β or γ
E6/F2	IgM	n.d.	–	γ
E9/C2	IgG1	2.7×10^7	–	γ

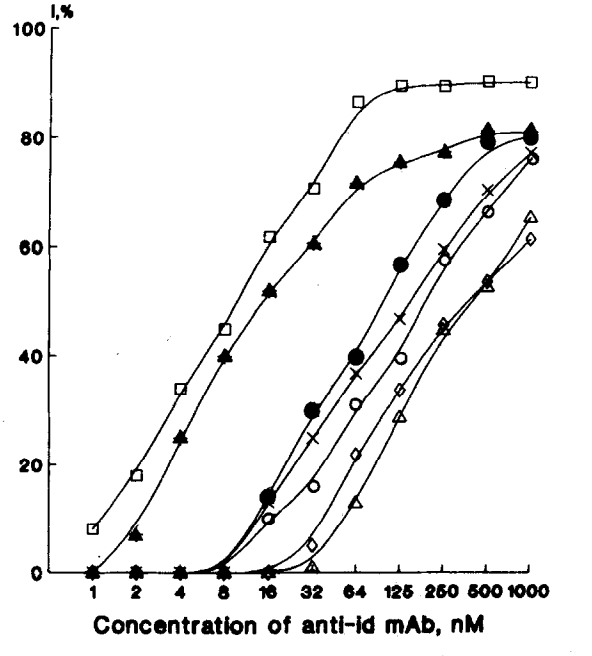

Fig. 3. Inhibition analysis of biotinylated E6/1.2 Fab-fragments binding to GMDP-OVA in the presence of anti-id MAbs: E1/B2 (●), C5/F8 (□), C10/F9 (△), E9/C2 (○), B4/B6 (×), E6/F2 (△) or D3/G4 (◇).

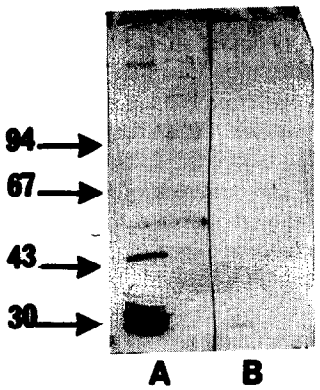

Fig. 4. Blot from SDS-PAGE of membranes from WEHI-3 cells. Staining in the absence (A) or presence (B) of GMDP with MAb C5/F8 followed by HRP-conjugated rabbit anti-mouse immunoglobulin antibodies and a mixture of 3,3′-diaminobenzidine with 4-chloronaphtol.

In order to investigate the possibility of using these antibodies in studying GMDP-receptors we compared the array of proteins of WEHI-3 cells capable of binding GMDP and MAb C5/F8. WEHI-3 cells were shown not so long ago to have intracellular GMDP-binding sites [30]. Cells were lysed by osmotic shock, and the membrane fraction was isolated and subjected to SDS-PAGE analysis. A blot from the gel was incubated with purified C5/F8 anti-id MAb. Subsequent incubation with HRP-conjugated anti-mouse immunoglobulin antibodies and peroxidase substrate revealed few proteins binding MAb C5/F8, with molecular weights 32–34, 45 and less than 30 kDa (Fig. 4A). Weak staining of 36 and 180 kDa bands was observed as well. The specificity of binding was confirmed by inhibition analysis: when the incubation with MAb was carried out in the presence of GMDP no binding occurred (Fig. 4B). No binding was observed also when irrelevant MAb of the same IgG subtype was substituted for C5/F8 MAb. It should be noted that proteins of similar molecular weight were identified as specific intracellular GMDP-receptors of murine peritoneal macrophages and WEHI-3 cells by affinity labelling technique (T.N. Golovina et al., submitted for publication).

The data obtained enable us to conclude that hybridomas C5/F3, C5/F8 and E1/B2 produce anti-id MAbs, classified as Ab2β, mimicking the internal image of GMDP. To our knowledge this is the first report describing the production of anti-idiotypic MAbs to muramyl peptide.

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