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Characterization of a Monoclonal Antibody Against Neopterin Using an Enzyme-Linked Immunosorbent Assay with Penicillinase as Label

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ABSTRACT

An active ester derivative of neopterin was prepared using 4-(*N*-maleimidomethyl) cyclohexan 4-carboxilic acid *N*-hydroxy succinimide ester (MCH-NHS), conjugated to bovine serum albumin (BSA) and injected for antibody production (for both monoclonal and polyclonal antibodies). High titer antibody producing spleen cells were removed and fused with myeloma cells of Sp2/0 origin. Neopterin was conjugated to the enzyme penicillinase by a one-step glutaraldehyde method, which was used as tracer. A novel enzyme immunoassay was developed using this conjugate to screen and characterize the monoclonal antibody (MAb) produced in these experiments. After limiting dilutions, it was found that antibody produced by one clone with a Ka value of 7.6 × 10–7 mol/L was specific for a number of structurally related molecules. This clone was found to be of IgG class and IgG_{2a} subclass. The standard curve was constructed with a sensitivity of 10 pg/well (100 pg/mL) covering up to 1 ng/mL.

INTRODUCTION

NEOPTERIN [D-erythro-6-(1,2,3,-trihydroxypropyl)pterin] is a low molecular weight compound derived biosynthetically from guanosine triphosphate (GTP). The key step in the biosynthesis of this molecule is the cleavage of GTP to 7,8-dihydroneopterin triphosphate by the enzyme GTP cyclohydrolase I (EC 3.5.4.16). The studies indicated that this activity of monocytes/macrophages was due to the induction of GTP-cyclohydrolase I, which was specifically stimulated with gama-interferon derived from activated T lymphocytes.[1–5] The function of neopterin is not completely known, however, determination of neopterin in body fluids is a powerful diagnostic and prognostic tool in a variety of diseases such as AIDS[6–8] infection and autoimmune disease[9–12] allograft rejection,[13] neoplastic diseases and leukemia.[14–18] The hazard of transmission of infectious pathogens by blood transfusion has been well known. It has been shown in a number of clinical and experimental studies that the determination of neopterin is a sensitive and specific method of detecting activated cell-mediated immune responses *in vivo,* which may ultimately be used as a large-scale blood screening method.[11,19] Procedures for neopterin measurement such as high-performance liquid chromatography (HPLC),[20,21] radioimmunoassay (RIA),[22] enzyme-linked immunoadsorbent assay (ELISA),[23,24] and fluroimmunoassay (FIA)[25] are reported. Immunochemical methods are found to be more rapid and easy to perform. In these assays, antibody plays a crucial rule because the specificity depends largely on the antibody quality.[26] Reports on polyclonal antibodies and MAbs against neopterin are available.[23,24,27] In this study we produced a specific MAb against neopterin and characterized it with a novel ELISA using penicillinase as label that was previously reported for a number of hapten molecules.[28–30]

MATERIALS AND METHODS

Reagents and media: RPMI 1640, tetrahydrofuran (THF), dichloroethan (DCE), fetal calf serum (FCS), penicillinase (B-lactamase, EC .3.5.2.6), polyethylene glycol (PEG) molecular

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Table 1. Clones Selected for Further Experiments After First Limiting Dilution

Clones	OD at 600 nm	OD at 450 mm		IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgM	IgA
		N-BSA	BSA						
N1D5	0.11	0.3	0.11	0.10	0.1	0.11	0.10	0.10	0.09
N1C2	0.85	0.9	0.12	0.08	0.47	0.08	0.10	0.11	0.08
N4E6	0.40	1.2	0.13	0.10	0.72	0.10	0.10	0.11	0.09
Cont +	0.20	1.5	—	0.12	0.09	0.10	0.09	0.10	0.10
Cont −	2.58	0.1	—	0.93	0.98	0.88	0.80	0.90	0.80

O.D., optical density; N-BSA, neopterin-conjugated BSA; Cont +, polyclonal antibody (1:500); and Cont −, Sp2/0 supernatant.

weight 8000 and 3700 (culture grade), penicillin V, streptomycin, hypoxanthine-aminopterin-thymidine (HAT), HT, neopterin, neopterin like compounds, bovine serum albumin (BSA), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant, tetramethylbenzidine (TMB), anti-mouse Fab-horseradish peroxidase(anti-M-Fab-HRP),4-(*N*-maleimidomethyl)- cyclohexane 4-carboxilic acid *N*-hydroxy succinimide ester (MCH-NHS), NaBH4 and glutaraldehyde (pure) were all obtained from Sigma Chemical Company (St. Louis, MO). ELISA plates (96 wells) and other plasticwares were obtained from Nunc (Denmark). Anti-mouse IgG was obtained from DAKO (Denmark). Myeloma cell line of Sp2/0 origin was obtained from Pasteur Institute (I.R. Iran). All other reagents used in this study were of analytical grade and obtained from standard sources.

Immunogen preparation and immunization

Immunogen was prepared following the method of Kitigawa et al.[31] with a minor modification briefly described as follows. Neopterin (2.5 mg, 50 mmol) was dissolved in 500/μL of bicarbonate buffer (50 mmol, pH = 10) MCH-NHS (3.4 mg, 50 mmol) was dissolved in THF. The above two solutions were mixed and stirred at 30°C for 2 h. Solvent was removed by nitrogen gas flushing and unreacted MCH-NHS was removed by extraction with DCE (3 mL × 3 times). The aqueous phase of this solution was used (solution 1). Ten milligrams of BSA was dissolved in 0.5 mL bicarbonate buffer (50 mmol, pH 10). Urea was added to the above solution such that and final volume for 6 molar solution is adjusted to 1 mL and EDTA, 0.1 molar was added. Ten miligrams of NaBH4 and 0.2 mL of normal butanol was subsequently added slowly and the resulting product was stirred at room temperature for 1 h. To stop the reaction, 0.5 mL of sodium monophosphate (0.1 mol) and 0.2 mL acetone was added (solution 2). The two solutions (1 and 2) were mixed, stirred at room temperature, and incubated at 4°C overnight. The product solution was then dialyzed against three changes of phosphate buffer (10 mmol, pH = 7.4) at 8 intervals, freeze-dried, and stored at 4°C until use. Another batch of immunogen was prepared following a one-step glutaraldehyde as explained elsewhere.[29] Ten BALB/c mice were immunized intraperitoneally primarily with 100 μg of immunogen in FCA and later with the same amount of immunogen prepared in IFA for injections each 17 days. Serum samples were prepared each month and high-titer antibody producing animals were sacrificed for hybridoma formation after three months of continuous immunization. Both immunogens were also injected for polyclonal antibody production and characterized as explained previously.[29]

Antibody detection

To detect antibody in serum or cell culture supernatant, neopterin molecule was conjugated to penicillinase using glutaraldehyde as a cross-linker.[29] To detect polyclonal antibody in mouse serum, purified anti IgG of normal mouse pre-

Table 2. Positive Clones After Second Limiting Dilutions

Clone	O.D.	*Class and Subclass					
		IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgM	IgA
1B3	0.31	0.09	0.3b	0.08	0.07	0.08	0.09
2B11	0.25	ND	ND	ND	ND	ND	ND
2E9	0.26	0.08	0.29	0.08	0.09	0.06	0.08
2F5	0.31	0.09	0.31	0.07	0.08	0.11	0.08
2B6	0.21	ND	ND	ND	ND	ND	ND
3B9	0.28	0.08	0.31	0.08	0.08	0.07	0.07
3C2	0.71	0.08	0.71	0.06	0.07	0.09	0.09
3D6	0.24	0.09	0.31	0.08	0.09	0.08	0.09
Con +	0.91	0.95	0.91	0.61	0.91	0.81	0.91
Con −	0.08	0.11	0.08	0.07	0.08	0.07	0.06

O.D., optical density at 450 nm; *Determined by isotyping kit, HRP as enzyme (450); and ND, not determined.

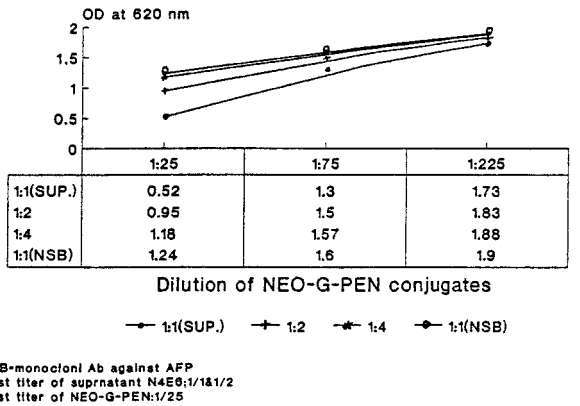

FIG. 1. Checker-board titration assay of anti-neopterin MAb (supernatant) using a neopterin-glutaraldehyde-penicillinase (Neo-G-PEN) as tracer and MAb against alfa-fetoprotein (AFP) as nonspecific binding index (NSB).

pared in rabbits was coated onto the wells of microtiter plate at 37°C overnight, washed, and blocked with a 0.5% solution of gelatin supplemented PBS (10 mM, pH = 7.2) for 30 min at 37°C, washed, and added with dilutions of polyclonal anti-neopterin antibodies (serum). In each experiment, normal mouse serum (NMS) with a dilution of 1:300 was used as nonspecific binding (NSB) index. The contents of the wells were incubated at 37°C for 2 h, washed, added with neopterin-penicillinase conjugate, and incubated at 37°C for 1 h. After incubation, wells were washed and added with 100 μL of 0.2 mol freshly prepared substrate solution penicillin V as explained previously.[28] The plate was incubated at 37°C for 1 h and added with 150 μL of starch iodin solution prepared as reported previously.[28] The reaction mixture was incubated at room temperature for 10 min and the color development was measured at 600 nm.

Hybridoma formation

High titer antibody producing animals immunized with immunogen prepared using MCH-NHS as cross-linker selected in the above way were sacrificed, spleens were removed, cells were prepared and fused with pretreated Sp2/0 myeloma cells in a 40% solution of PEG (3700). Hybridomas were cultured in a 20% FCS containing medium supplemented with HAT for 3 weeks. Positive clones were selected following the procedure explained below and subcultured in a 20% FCS-containing medium for another 2 weeks. Supernatant of the well containing monoclonal cell growth were characterized for titer, specificity, affinity, class, and subclass determination. Proper clones were selected, diluted, subcultured, and characterized for specificity, affinity, titer, class, and subclass identification. Finally selected antibody-producing clone (N4E6) was cultured in 50-mL flasks.

Monoclonal antibody detection and characterization

To screen antibody in supernatant of cell culture medium, we performed immunoassay in liquid phase. For this purpose, supernatant from each well were incubated at 37°C for 2 h with neopterin-penicillinase conjugate for specific binding and supernatant of Sp2/0 was used for NSB. At the end of incubation time, tubes were added with 200 μL of 1:30 dilution of second antibody (anti-mouse IgG antibody prepared in EIA buffer, containing 4% of PEG 8000) and incubated at 4°C for 20 min. The contents of the tubes were centrifuged, washed, wiped, added with 200 μL of substrate solution and the rest of experiments were performed as explained previously.[29] As a conformatory test, purified anti-mouse normal IgG antibodies were coated onto the wells of microtiter plate as explained previously,[30] added with supernatant of cell culture, incubated for 90 min, washed, and added with proper dilution of anti-M-Fab-HRP. After incubation for 90 min at 37°C, the plate was washed, treated with 100/μL of substrate TMB, and incubated for 10 min. The enzymatic reaction was terminated using a 50 μL of a 2 N sulfuric acid solution and the color development was finally measured at 450 nm. Suitable antibody-producing clones were selected in the above way and were titrated as follows. Different dilutions of supernatants were prepared and added to the tubes containing different dilutions of tracer. These were incubated for 90 min and the rest of the experiments were carried out as explained above. Proper dilutions of antibody and tracer, which were selected in the above manner, were used for standard curve preparation as follows. Standard curve was constructed in buffer using selected titer of supernatant of the best clone. For this purpose, in liquid phase EIA, different doses of neopterin standard preparation from 100 pg/mL up to 1 ng/mL were added to tubes containing a selected dilution of supernatant, incubated for 90 min, added with neopterin-penicillinase conjugate and incubated for 1 h. The rest of the experiments were followed as explained previously. For cross-reactivity study, neopterin-like compounds (100 pg/mL up to 100 ng/mL) were added in place of neopterin. Class and subclass determination was performed using Sigma (St. Louis, MO) isotyping test kit as advised by the manufacturer.

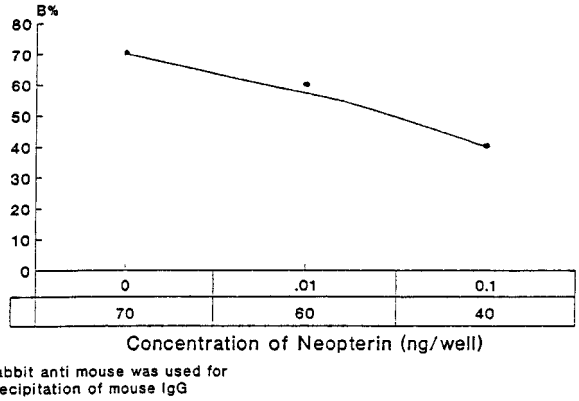

FIG. 2. A typical standard graph of neopterin measurement using MAb N4E6 (1:2 supernatant dilution) using Neo-G-PEN as tracer. The sensitivity is from 10 pg/well ranging up to 1 ng/well.

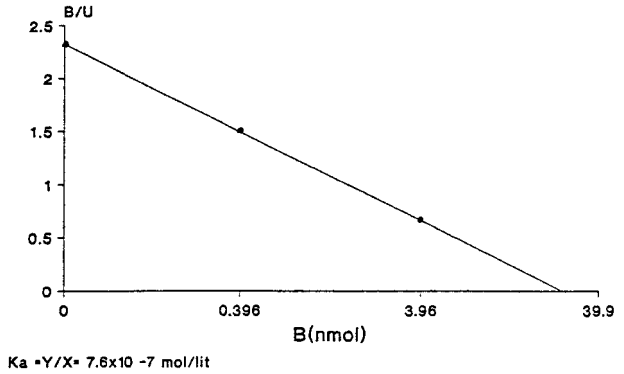

FIG. 3. Scatchard plot of anti-neopterin MAb using Neo-G-PEN as label.

RESULTS

Table 1 shows the results of optical densities (O.D.s) for three positive clones after first limiting dilution compared with the negative and positive control values. These were selected after the fusion and screening procedure. Note that O.D.s, when penicillinase was used as label, decreased while it increased when peroxidase was used as tracer. One of the antibodies (N4E6) showed better properties even after first limiting dilution, which was selected for further experiments. After further dilution of the above clone, several positive MAbs were detected, the results of which are shown in Table 2. However, because N4E6 showed suitability in term of viability of cells and other criteria such as specificity, this clone was selected for further experiments. The cells from the selected clone were cultured in 50-mL flasks, titrated, standard curve was prepared, and affinity was determined by Scatchard analysis (Figs. 1, 2, and 3). Table 3 presents our results for cross-reactivity study of the selected clone in terms of specificity toward structurally related molecules. These results showed much less cross-reaction with selected molecules for MAbs produced in this study. In the same table, a comparison between polyclonal and monoclonal antibodies is also given showing little difference between the two. The results of class, subclass, and affinity determination are also shown in Table 3.

DISCUSSION

The biological significance of neopterin measurement is the topic of many investigations in recent years.[6–19] Therefore, the quantitative measurement of this molecule has been tried by various methods.[20–21] Immunological methods are a prime choice for their rapid application and ease of performance.[22–25] In this study, we produced a highly specific monoclonal as well as polyclonal antibody for neopterin molecule conjugated to BSA via C2 amino group using MCH-NHS as cross-linker. This linker, because of having a long carbon chain mediated with a benzene ring in between, produced sufficient space between the hapten and carrier molecule. However our initial attempt to prepare an immunogen using a one step glutaraldehyde conjugation method although ending up with a good immunogen, producing a high-titer polyclonal antibody, was not suitable in immunoassay becuse there was a homology introduced (because enzyme tracer was also prepared in the same way). This effect was previously reported to influence the assay quality in case of other haptens used for immunogen and tracer preparations.[28] In our experiments, the tracer was prepared by conjugating neopterin to penicillinase via a glutaraldehyde cross-linker, although it was suggested that it was not possible to prepare neopterin-protein conjugates using glutaraldehyde as a cross-linker.[32] This successful attempt resulted into a heterologous combination of tracer and immunogen in cross-linking bridge type (chain heterology), which increased the detection limit of the assay and ease of antibody screening with more reliable results and less false-positive clones. Therefore, in all other experiments for MAb preparations, only a MCH-NHS derivative was used as immunogen. After the fusion, it was found that few positive wells were obtained which, when further diluted resulted in a number of monoclonals-producing antibodies. One of these clones, which was further studied and produced in larger amounts, showed high specificity and suitable affinity. The antibody obained here was of IgG class and $IgG2_a$ subclass. The results obtained in this study also showed that the specificity of polyclonal antibody was almost the same as that of MAbs. These types of conclusions have been previously reported by others.[22,27] Lack of cross-reactivity, especially with biopterin, which differs from neopterin only by a methyl moiety (instead of $-CH_2OH$ in neopterin), in monoclonal and polyclonal antibodies raised against neopterin may be because all immunogen preparations reported until now depended on C_2-NH_2 conjugation of carrier protein to hapten neopterin, which is far from the alkane side chain where the main difference between neopterin and its structurally related molecules exist. We also found no cross-reaction between the antibody and BSA molecule. This property was very important to avoid a false-positive clone selection in the process of large-scale screening. However, in conclusion it is to be noted that not much difference, at least from the specificity point of view, was found between monoclonal and polyclonal antibodies in the case of neopterin.

TABLE 3. CHARACTERIZATION OF SELECTED CLONE AND THEIR COMPARISON WITH POLYCLONAL ANTIBODY WITH RESPECT TO THEIR SPECIFICITY, AFFINITY, CLASS, AND SUBCLASS DETERMINATION

Monoclonal	*N4E6*	*Polyclonal**
Class	IgG	ND
Subclass	G_{2a}	ND
Affinity	7.6×10^{-7} M^{-1}	1×10^{-8} M^{-1}
Inhibitors	*Percent binding*	*Percent binding*
Neopterin	100	100
Biopterin	<0.05	<0.05
Pteroic acid	<0.2	<0.02
Pterin	<5	<5.0
Folic acid	<0.05	<0.05

ND, not detected; and *Prepared in rabbit.

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