PRODUCTION OF ANTI-HUMAN INTERFERON GAMMA MONOCLONAL ANTIBODIES

Cemalettin AYBAY, M.D., Ph.D.

Gazi University, Faculty of Medicine, Section of Immunology, Immunological Research and Application Center, Ankara
Gazi Medical Journal 2000; 11: 107-113

SUMMARY

Purpose: *Monoclonal antibody (mAb) allows identification of the antigen (Ag) with a unique and chosen specificity. Human interferon gamma (h-IFNg) is one of the immunoregulatory cytokines and is quantitatively assessed in various samples by using anti-h-IFNg mAb-based enzyme immunoassays. However all anti-cytokine mAbs and their enzyme-linked immunoassay (ELISA) kits are imported at extremely expensive rates. Thus, in this study, it was planned to produce hybridoma clones secreting unlimited amounts of mAbs specific for h-IFNg and to use these mAbs for the establishment of cytokine ELISA in the future.* ***Methods:*** *BALB/c mice were immunized with human IFNg. Fusion between splenocytes of the immunized mice and non-secreting FO myeloma cells was performed by conventional polyethylene glycol method. Anti-h-IFNg secreting hybridomas were screened with a lab-made ELISA. Cloning was made by single cell pick-up technique.* ***Results:*** *Among the specific hybridoma containing wells, six were selected and cloned (CAy-IFNg15, CAy-IFNg38, CAy-IFNg65, CAy-IFNg99C, CAy-IFNg111, CAy-IFNg124C), and the remainder were frozen in liquid nitrogen. All of the cloned CAy-IFNg15, CAy-IFNg38, CAy-IFNg65, CAy-IFNg99C, CAy-IFNg111, CAy-IFNg124C hybridomas secreted anti-h-IFNg mAbs in IgG1 isotype. Monoclonal antibody production capacities of the hybridoma clones were found to be higher than 10mg/ml per 1x106 cells/ml for 24h. As the by-products, several hybridomas secreted mAb specific for human albumin were also identified and frozen in liquid nitrogen in pre-cloning stage.* ***Conclusion:*** *This report represents the first study on production of mAbs against the cytokine IFNg in this country, similar to interleukin-2 mAb secreting hybridomas, also generated in our laboratory. Anti-h-IFNg mAb secreting hybridomas will provide unlimited amounts of mAb to establish the ELISA system, always available without waiting for samples from abroad and much cheaper.*

Key Words: *Monoclonal Antibody Production, Hybridoma, Human Interferon Gamma, Anti-Human Interferon Gamma Monoclonal Antibody.*

INTRODUCTION

A monoclonal antibody (mAb) is directed against a single antigenic determinant, the epitope (1). Thus, it possesses a unique specificity and an extremely high selectivity for the epitope. Monoclonal antibodies have made a triumphal entry into immunological and immunohistological diagnostics. For example, mAb against tumor markers, such as the carcinoembryonic antigen which colorectal

tumors release into the blood stream, can be used to monitor tumor progression and the success of therapy (2-4). The ELISA kits available commercially for cytokines, enzymes, tumor markers, hormones, and membrane antigens are increasing in number almost daily. In addition, mAbs have also improved both the diagnosis and differential diagnosis of viral and bacterial diseases (5,6).

Monoclonal antibodies are also sometimes used in vivo as immunomodulators, and above all as immunosuppressants (7). Studies employing monoclonal antibodies against IFNg indicate that inflammatory agents, such as bacterial toxin, induce local production of IFNg, which acts as an up-regulator of the primary phase of the inflammatory response (8). Anti-interferon-g antibody was demonstrated to be capable of protecting mice against the generalized Shwartzman reaction (9).

Detection of a specific cytokine such as IFNg in any sample is usually performed with specific anti-cytokine mAb-based ELISA system. Researches concerning IFNg usually needed the assessment of IFNg levels. However such an experimental research either before or after a manipulation as exemplified above, needs to assess IFNg levels with an expensive cytokine ELISA kit that costs nearly thousand dollars. Thus, this study was planned to produce anti-human IFNg mAb in order to establish ELISA system for future use and manipulating IFNg responses in any experimental system using anti-IFNg mAbs.

In this report, various hybridoma clones secreting monoclonal antibodies specific for human IFNg are presented

MATERIALS AND METHODS

Two six weeks old female inbred BALB/c mice were immunized with human IFNg (h-IFNg) in complete Freund's adjuvant (Sigma Chem. Co., MO, USA) by the intraperitoneal route. Each mouse was injected with 50mg of ImmuneronTM (Recombinant human interferon gamma, 1mg Study No: NCI, Activity: 2.4x107 U, Lot No: 10M37 A, Biogen Research Corp., Cambridge, MA 02142). The first injection of h-IFNg was followed by a second (in incomplete Freund's adjuvant, Sigma Chem. Co., MO, USA) and a third (in 0.9% NaCl) dose at 14 day intervals. After three days, blood samples were taken from the tails of the mice to evaluate their immunization status and then splenocytes of two mice were isolated, pooled and then used for fusion. Polyethylene glycol (PEG, Wt 3000-3700, Sigma Chem. Co., MO, USA) fusion between 120x106 erythrocyte-lyzed-splenocytes and 25x106 FO myeloma cells was performed. Hypoxanthine-aminopterine-thymidine (HAT, Sigma Chem. Co., MO, USA) selection was performed as described earlier (10) with some modifications. Briefly, hybridoma suspension was plated out in six peritoneal macrophage-seeded 24-well plates (Greiner GMBH, Nürtingen, Germany) and then incubated for 24 h at 37°C in a humidified atmosphere of 5% CO2. After incubation, HAT medium was added to the hybridoma cells. Exchange of supernatants with fresh HAT medium was done every day for the first three days and then once more on the 7th day. Ten days after the fusion, supernatants were screened continuously for the presence of specific antibodies.

Screening for the presence of anti-h-IFNg mAb in the culture supernatants of hybrid cells was performed with lab-made ELISA. High binding capacity 96-well flat-bottomed microtitre plates (Greiner GMBH, Nürtingen, Germany) were coated with human IFNg. To each well 50 ml of ImmuneronTM at 2mg/ml in phosphate buffered saline was added (PBS) and then incubated overnight at 4°C. After washing, potential binding sites were blocked with 200ml of 0.3% gelatin (Merck). Culture supernatants or standards, 50ml per well, were incubated with h-IFNg-coated solid phase containing 100ml of PBS supplemented with 0.05% Tween 20 (PBS-T), human serum albumin (HSA, fatty acid and globulin free, Sigma, Chem. Co., MO, USA) at 10mg/ml in PBS, or Immuneron at 10mg/ml in PBS for 1 h at 37°C. After washing five times, 100ml of horse radish peroxidase (HRP)-labeled anti-mouse IgG conjugate was added to each well (either Fab- or Fc-specific, Sigma Chem. Co., MO, USA), diluted to 1/6,000 in PBS-T, and then incubated for 1 h at 37°C. After washing five times, 100ml of TMB-chromogen/H2O2-substrate was added to each well and then incubated for 5 or 15 minutes at room temperature, avoiding direct light. Reaction was

stopped by adding 50ml of 2M H2SO4 per well and then optical density (OD) was evaluated with an ELISA reader (LP400, Diagnostics Pasteur, France) using 450nm filter.

Cloning was performed under inspection with an inverted microscope in a biological safety cabinet by picking up single cells with a thin-pointed pasteur pipette (hand-made) and transferring to wells of 96-well flat bottomed microtitre plates (Greiner GMBH, Nürtingen, Germany). Single cells were cultured in a thymocyte-conditioned medium consisting of RPMI-1640 (Sigma Chem. Co., MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Seromed, Biochrom KG, Berlin, Germany) and 10% thymocyte supernatant.

The isotype of the monoclonal antibody produced by the hybridomas was determined using a commercially available isotyping kit (ISO-1, ImmunoType, mouse monoclonal antibody isotyping kit, Sigma Chem. Co., MO, USA).

Reaction capacity against HSA was assessed on a 96-well plate coated with HSA (each well was added 50 ml of HSA at 10mg/ml) as described above. A commercially available anti-hIFNg (anti-hIFN-g, neutralizing antibody, Purified mouse monoclonal IgG2A, Catalog number: MAB285, 500mg, Lot: KWO15061, R&D Systems, Minneapolis, MN 55413) was also used as standard at defined concentrations.

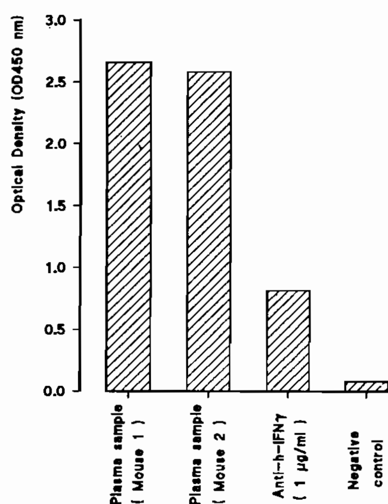

Fig. 1: Immune status of the mice. Plasma samples of hIFNg-immunized mice were diluted to 1/20 in PBS and then assessed for the presence of anti-human IFNg antibodies using ELISA. Reaction was stopped 5 minutes later after adding chromogen/substrate. Anti-human IFNg mAb at 1mg/ml and 1/20 diluted plasma sample of normal mouse were also included as the positive and negative controls respectively. Data were presented as the mean of duplicate wells.

Table 1 : OD450nm levels of supernatants of hybridoma containing wells on the hIFNg- or HSA-coated solid phases.

Well No	OD450nm of supernatants on the phase coated with	
	ImmuneronTM	Human Serum Albumin
2	0.912	0.204
15	1.411	0.107
33	2.339	1.849
38	1.817	0.093
39	1.414	0.166
51	1.864	1.398
52	1.851	1.386
55	2.167	0.084
57	1.990	1.690
65	1.448	0.091
73	1.200	0.148
97	1.682	1.512
99B	1.527	0.079
99C	0.870	0.082
107	2.130	2.122
109	2.167	1.879
110	1.921	1.489
111	1.699	0.369
113	1.544	1.447
114	0.796	0.444
116	1.593	1.291
120	0.835	0.550
124C	0.834	0.094
128	1.073	0.801
129	0.722	0.241
131	2.416	2.314
134B	1.003	0.085
140	0.800	0.233

RESULTS

Plasma samples from both mice contained high levels of anti-hIFNg when compared to a standard on the same ELISA system (Fig. 1). Thus, before the fusion it was observed that both mice were effectively immunized and seropositive for anti-human IFNg.

Out of 144 wells (six 24-well plates), 27 wells were found to be reactive against human IFNg and/or HSA (Table 1). Among them, some of the wells that were positive on hIFNg-coated solid phase but negative on HSA-coated solid

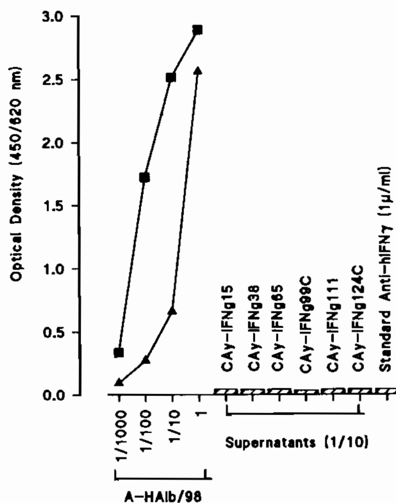

Fig. 2: Binding capacity of anti-HSA, standard anti-hIFNg, and mAbs secreted from various hybridomas to HSA-coated solid phase. Anti-HSA from A-HAlb/98 hybridomas (n) bound to HSA-coated solid phase and the binding reaction was competitively inhibited in the presence of HSA at 10mg/ml (s). Standard anti-hIFNg mAb and the mAbs from hybridomas did not bind to HSA-coated wells.

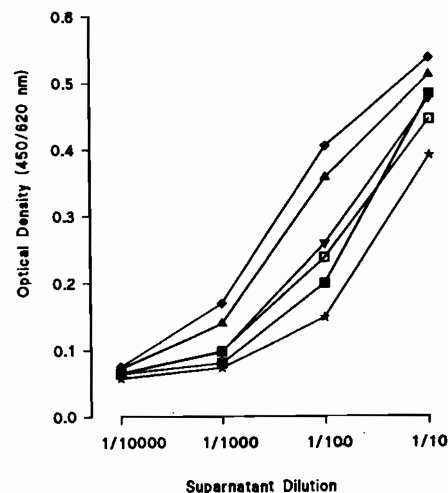

Fig. 3: Binding capacity of anti-mouse IgG, specific for Fc portion, to mAbs captured with hIFNg-coated solid phase. Supernatants of hybridomas at various dilutions were incubated with hIFNg-coated solid phase. HRP-labeled anti-mouse IgG (Fc-specific) conjugate bound to all of the mAbs secreted from CAy-IFNg15 (n), CAy-IFNg38 (s), CAy-IFNg65 (t), CAy-IFNg99C (u), CAy-IFNg111 (H), and CAy-IFNg124C (ÿ).

phase were selected for cloning procedure. Preclone well 15, 38, 65, 99C, 111, and 124C were arbitrarily chosen for cloning and the remainder were frozen in liquid nitrogen.

As shown in Figure 2, anti-HSA mAb containing supernatant of A-HAlb/98 hybridomas bound to HSA-coated solid phase in a concentration dependent manner and HSA itself competitively inhibited the binding reaction. However none of the supernatants from above-described hybridomas reacted with HSA-coated solid phase (Fig. 2). These results clearly demonstrated that hybridoma cells were cloned at a single cell level and did not contain any potential contaminant cells secreting anti-HSA

In order to determine isotypes of the mAb secreted from hybridomas, a different conjugate, specific for the Fc region of mouse IgG, was used in the first step. As shown in Figure 3, all mAbs reacted with Fc-specific conjugate in a titration dependent manner (Fig. 3). Thus, all hybridomas seemed to be secreting mAb in IgG isotype. Compatible with these data, it was confirmed that CAy-IFNg15, CAy-IFNg38, CAy-IFNg65, CAy-IFNg99C, CAy-IFNg111, and CAy-IFNg124C clones were found to be secreting mAb in IgG1 subtype (data not depicted).

When comparison was made against the curve obtained from the standard anti-hIFNg mAb, all of the above-mentioned hybridoma clones secreted mAb higher than 10mg per ml when cultured in 10% FCS-containing RPMI-1640 at a density of 1x106 cell/ml/well for 24 h (Fig. 4). These results demonstrated that all of the above-mentioned hybridomas were high-secretors.

All data presented in this study were representative. Thus, results were presented as the mean of duplicate wells and the standard deviations, which were less than 5% of the mean, were omitted. However, similar results were obtained many times in preliminary experiments.

DISCUSSION

Interferon-g (IFNg), also called immune or type II interferon, is a homodimeric glycoprotein containing two 21 to 24 kD subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains an identical 18kD polypeptide encoded by the same gene (11). IFNg has several important properties related to immunoregulations. Experimental researches relating to cytokines usually needed the use of

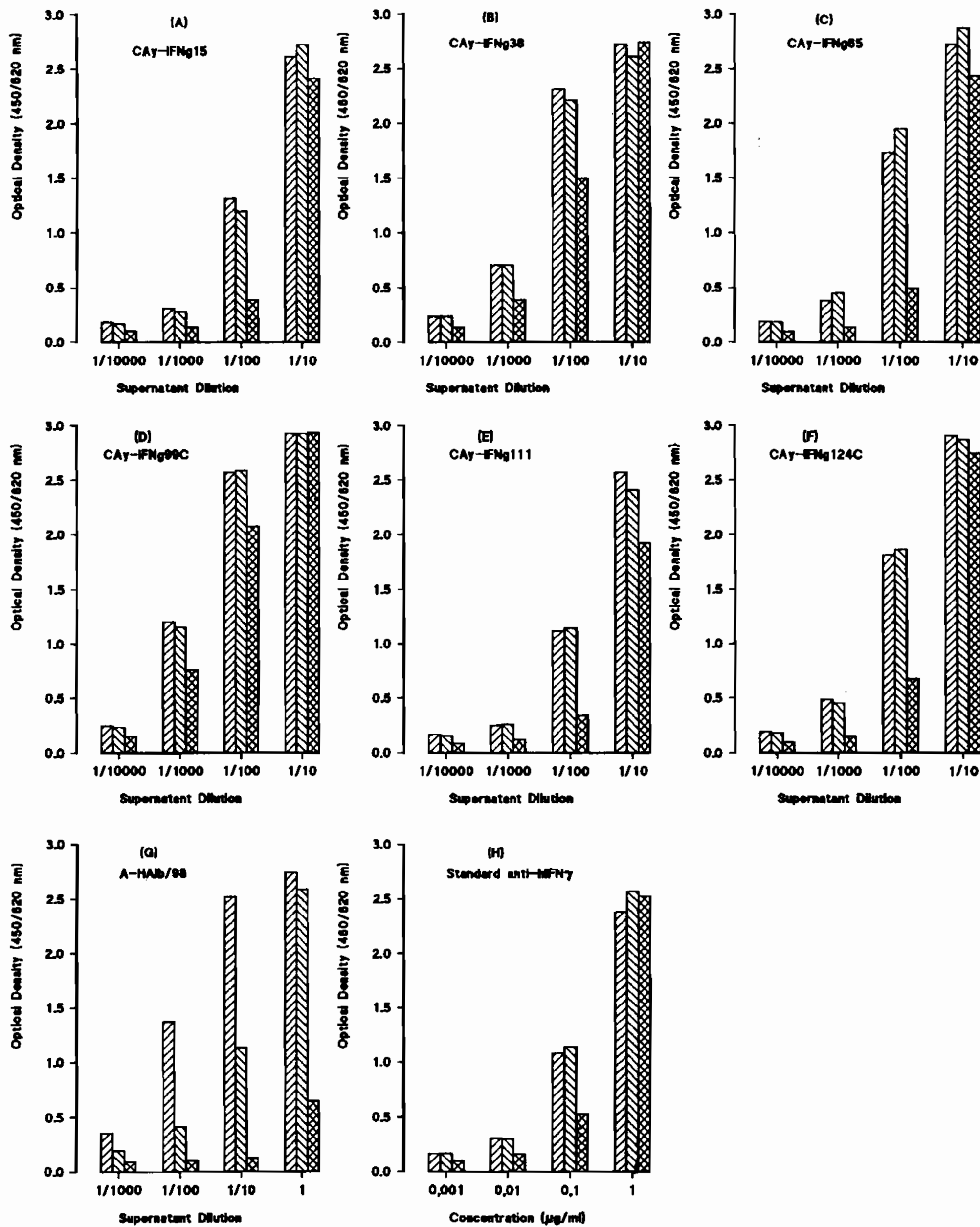

Fig. 4: Monoclonal antibody secreting capacities of hybridoma cells. 50ml of hybridoma culture supernatants and their corresponding ten fold dilution were added into hIFNg-coated wells either in the presence of 100ml of PBS-T (slashed bars), HSA at 10mg/ml (back slashed bars), or hIFNg at 10mg/ml (crossed bars). A-HAlb/98 culture supernatant bound to solid phase (G) because hIFNg, used for coating, also contained human albumin. Free albumin and hIFNg preparation, containing HSA, competitively inhibited binding of A-HAlb/98 culture supernatant especially at 1/10 or 1/100 dilution. As expected, free HSA did not inhibit binding of standard anti-hIFNg mAb (H) or any of the CAy-IFNg hybridoma culture supernatants (from A to F). However, free hIFNg at 10mg/ml inhibited the binding capacity of supernatants to some extent. When compared to the OD levels obtained with standard anti-hIFNg, all CAy-IFNg hybridomas produced mAb equal or higher than ^{3} 10mg/ml.

mAb-based ELISA systems. However, all of the mAb-based ELISA kits, including IFNg ELISA, are imported at high costs. Although some reports have recently been encountered in the local literature (10, 12-14), monoclonal antibody production technology has not yet been satisfactorily developed in our country.

In this study it was planned to produce mAb specific for human IFNg for multipurpose

usage. If the antigen of interest is highly immunogenic, almost any immunization scheme will stimulate the generation of many antibody forming cells (15). However many different immunization scheme have been proposed, and different protocols may work better for different immunogens (16-19). In addition, the fusion and the cloning procedures are rather cumbersome. Thus, the immune status of the mice was evaluated as the first step. As shown in Figure 1, plasma samples contained specific antibodies against hIFNg after immunization protocol. Thus, it seemed to be suitable for fusion procedure.

Six lines of mouse mAb against human interferon gamma were selected and cloned (Table 1). The reason for double positive reaction (against hIFNg and HSA-coated solid phase) obtained with the supernatants of some wells (Table 1) was that human IFNg preparation (ImmuneronTM), used for both immunization and coating, contained HSA to some extent. Thus, supernatants taken from wells containing anti-HSA mAb secreting hybridomas also reacted with Immuneron-coated wells. However, none of the selected CAy-IFNg15, CAy-IFNg38, CAy-IFNg65, CAy-IFNg99B, CAy-IFNg111, and CAy-IFNg124C hybridomas, which were propagated from the single-cell level, reacted with HSA (Fig. 2). For the next step, it was determined whether the mAbs were IgG isotypes. For this, a IgG specific conjugate was used. As shown in Figure 3, anti-IgG conjugate bound to all mAbs. Compatible with these data, all mAbs were identified as IgG1 subtype using ImmunoTypeTM kit. For the last step, the hybridomas were tested for their capacity to produce mAb. When compared to OD levels obtained with standard anti-hIFNg on the same system, all of the above-mentioned hybridomas produced mAb at concentrations higher than 10mg/ml. Thus, the production of mAb against human IFNg was achieved and all lines were regarded as high producer hybridomas.

Correspondence to : Cemalettin AYBAY, M.D., Ph.D.
Gazi Üniversitesi, Tıp Fakültesi
İmmünoloji Laboratuvarı
Beşevler
06500 ANKARA-TÜRKİYE
Phone: 312-214 10 00 / 6963
e-mail: aybayc@tr-net.net.tr

REFERENCES

1. Peters JH, Baron D. Properties and significance of monoclonal antibodies. In. Peters JH and Baumgarten H (eds): Monoclonal Antibodies. New York: Springer-Verlag; 1992. p.4-11.
2. Lamerz R. CEA determination in the follow-up of extracolorectal neoplasms.Int J Biol Markers 1992; 7: 171-178.
3. Forones NM, Tanaka M, Machado D. Increased carcinoembryonic antigen and absence of reccurence in monitoring colorectal cancer. Arq Gastroenterol 1998; 35: 100-103.
4. Foon KA, John WJ, Chakraborty M, Das R, Teitelbaum A, Garrison J, Kashala O, Chatterjee SK, Bhattacharya-Chatterjee M. Clinical and immune responses in resected colon cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen.J Clin Oncol 1999; 17: 2889-2895.
5. Palomäki P. Simultaneous use of poly- and monoclonal antibodies as enzyme tracers in a one-step enzyme immunoassy for the detection of hepatitis B surface antigen. J Immunol Methods 1991; 145: 55-63.
6. Feodorova VA, Devdariani ZL. Development, characterisation and diagnostic application of monoclonal antibodies against Yersinia pestis fibrinolysin and coagulase. J Med Microbiol 2000; 49: 261-269.
7. Beniaminovitz A, Itescu S, Lietz K, Donovan M, Burke EM, Groff BD, Edwards N, Mancini DM. Prevention of rejection in cardiac transplantation by blockade of the interleukin-2 receptor with a monoclonal antibody. N Engl J Med 2000; 342: 613-619.
8. Billiau A. Interferons and inflammation. J Interferon Res 1987; 7: 559-567
9. Billiau A, Heremans H, Vandekerckhove F, Dillen C. Anti-interferon-g antibody protects mice against the generalized Shwartzman reaction. Eur J Immunol 1987; 17: 1851-1854
10. Aybay C. Production of monoclonal IgM and IgG1 antibodies specific for hepatitis B virus surface antigen. Gazi Medical Journal 1999; 10: 11-15.
11 Abbas AK, Lichtman AH, Pober JS. Cellular and Molecular Immunology. 3rd Edition. Philadelphia: W.B. Saunders; 1997. p.269.
12. Aybay C, İmir T, Kayhan B. A competitive enzyme-linked immunosorbent assay (ELISA) for determination of human albumin in the nanogram range. Gazi Medical Journal 1999; 10: 63-67.

13. Yücel F, Bermek E, Çırakoğlu B. Production of monoclonal antibodies specific for Xanthomonas campestris pv. Phaseoli. Turkish Journal of Biology 1997; 21: 125-131.
14. Başalp A, Yorgancı Ü, Çırakoğlu B, Bermek E. Detection of tobacco mosaic virus by monoclonal antibody MAM-1F5. Turkish Journal of Biology 1997; 21: 397-403.
15. French D, Fischberg E, Buhl S, Scharff MD. The production of more useful monoclonal antibodies. Immunol Today 1986; 7: 344-346.
16. Larsson A, Nilsson BO. Immunization with nanogram quantities of nitrocellulose-bound antigen, electroblotted from sodium dodecyl sulphate-polyacrylamide gels. Scand J Immunol 1988; 27: 305-309.
17. Giraud A, Ataman-Onal Y, Battail N, Piga N, Brand D, Mandrand B, Verrier B. Generation of monoclonal antibodies to native human immunodeficiency virus type 1 envelope glycoprotein by immunization of mice with naked RNA. J Virol Methods 1999; 79: 75-84.
18. Krasemann S, Jurgens T, Bodemer W. Generation of monoclonal antibodies against prion proteins with an unconventional nucleic acid-based immunization strategy. J Biotechnol 1999; 73: 119-29.
19. Bashir I, Sikora K, Foster CS. Generation of a monoclonal antibody to P-glycoprotein peptides using tuberculin-PPD as a carrier. Virchows Arch 1998; 432: 279-87.