Appl Biochem Biotechnol (2010) 162:1249–1257
DOI 10.1007/s12010-009-8873-8

Generation of a Novel High-Affinity Monoclonal Antibody with Conformational Recognition Epitope on Human IgM

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Received: 6 September 2009 / Accepted: 16 November 2009 /
Published online: 18 February 2010

Abstract As IgM is the first isotype of antibody which appears in blood after initial exposure to a foreign antigen in the pattern of primary response, detection, and quantification of this molecule in blood seems invaluable. To approach these goals, generation, and characterization of a highly specific mAb (monoclonal antibody) against human IgM were investigated. Human IgM immunoglobulins were used to immunize Balb/c mice. Spleen cells taken from the immunized animals were fused with SP2/O myeloma cells using PEG (polyethylene glycol, MW 1450) as fusogen. The hybridomas were cultured in HAT containing medium and supernatants from the growing hybrids were screened by enzyme-linked immunosorbent assay (ELISA) using plates coated with pure human IgM and the positive wells were then cloned at limiting dilutions. The best clone designated as MAN-1, was injected intraperitoneally to some Pristane-injected mice. Anti-IgM mAb was purified from the animals' ascitic fluid by protein-G sepharose followed by DEAE-cellulose ion exchange chromatography. MAN-1 interacted with human IgM with a very high specificity and affinity. The purity of the sample was tested by SDS-PAGE and the affinity constant was measured $\left(K_a = 3.5 \times 10^9 M^{-1}\right)$. Immunoblotting and competitive ELISA were done and the results showed that the harvested antibody recognizes a conformational epitope on the μ chain of human IgM and there was no cross-reactivity with other subclasses of immunoglobulins. Furthermore, isotyping test was done and the results showed the subclass of the obtained mAb which was $IgG_1\kappa$.

Keywords Monoclonal antibody · IgM · Anti-human IgM antibody

Introduction

The human immunoglobulins are a group of structurally and functionally similar glycoproteins that confer humoral immunity. The immunoglobulin protein "backbone" consists of two

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identical "heavy" and two identical "light" chains. Five classes of immunoglobulins have been distinguished on the basis of non-cross-reacting antigenic determinants in regions of highly conserved amino acid sequences in the constant regions of their heavy chains [1].

Quantification of each immunoglobulin isotype in serum sample has allowed identification of a given pathology. For example, IgE rises in helminthic parasitic and allergic conditions like asthma [2], atopic dermatitis, and anaphylaxis [3]; IgG in identification of selective IgG subclass deficiencies and myeloma states [4]; and IgM in infection with *Toxoplasma gondii* during pregnancy [5], *Rubella* [6], CMV [7] and so on. IgM antibodies, a class of immunoglobulins with high molecular weight (900 KD), are the first antibodies which appear in response to an initial exposure to an antigen and release into the blood. This antibody is also the first immunoglobulin which is synthesized by the neonate. They account for 5% to 10% of the total serum immunoglobulins; with an average serum concentration of 1.5 mg/ml [8]. IgM is secreted by plasma cells as a pentamer in which five monomeric units are held together by disulfide bonds that link their carboxyl-terminal heavy-chain domains. In fact the ratio of IgM and IgG can indicate the various stages of a disease. Due to the fact that, in an early stage, there are more IgM antibodies in the serum, detection, and quantification of this isotype would be very promising for diagnosis of newly emerged diseases. Because of the homology in the sequence of immunoglobulin molecules, it is difficult to prepare the specific polyclonal antibodies against each of them. The development of hybridoma antibody technology by Kohler and Milstein [9] has allowed production of monoclonal antibodies that bind to specific antigenic determinant. The ability to generate molecules capable of highly specific molecular recognition, and to apply this approach to virtually any molecular target, makes monoclonal antibody production a powerful technology. In spite of presence of different polyclonal and monoclonal antibodies reactive with human IgM in market, our primary ongoing studies on the detection of IgM antibodies using the accessible commercial antibodies showed that the polyclonal ones are partially not applicable as it has been reported by others [10] (low specificity and sensitivity) and although the monoclonal ones were better they had the same problem as well. This problem prompted us to produce specific high-affinity anti-human IgM monoclonal antibodies which could be applicable in different medical diagnostic enzyme-linked immunosorbent assay (ELISA) systems.

Materials and Methods

Immunization and Selection of Donors

Three-month-old male Balb/c mice (provided by the animal house of Pasteur institute, Tehran) were injected subcutaneously with an emulsion of complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) containing 100 μg of human IgM (purchased from Sigma). One month later, the animals received 100 μg of the antigen in incomplete Freund's adjuvant by the same route. The third injection was done with a similar time gap. Then the titer of required antibody in the blood samples obtained from the mice venous tails was determined by ELISA. In order to maximize the quality and quantity of immune response the animal with the highest titer of required antibody received a booster injection intravenously containing 30 μg IgM in 50 μl phosphate-buffered saline (PBS). Three days later the selected mouse were euthanized and its spleen were cut out and separated cells were ready to be fused with non-secreting SP2/O cell line using 50% polyethylene glycol (MW 1450; purchased from Sigma).

All animal procedures were according to protocols approved by the institutional animal care and use committee.

Fusion

SP2/0 mouse myeloma cells were cultured regularly to reach the logarithmic growth phase. Then they were fused with spleen cells from the immunized mice by the procedure of Kennett (with minor modifications) [11]. The hybridoma medium was RPMI 1640 (GIBCO), supplemented with 20% fetal calf serum(GIBCO), 2 mM glutamine, penicillin at 100 U/ml, and streptomycin at 100 μg/ml. The culture plates were seeded with 3 $x10^3$ mouse peritoneal macrophages per well on the day before fusion, as suggested by Groth and Scheidegger [12].

Selection

The cells were then cultured in hypoxanthine-aminopterin-thymidine (HAT)-containing medium. In order to select monoclonal antibodies reactive with human IgM but not other isotypes of immunoglobulins, supernatant medium from microtiter cell culture plate wells containing macroscopically visible clones was tested for antibodies by using ELISA.

ELISA

Supernatants gathered from the wells with growing hybridomas were screened by ELISA using polystyrene microplates (Immunlon, Dynatech). The plates were coated with 1 μg/ml of human IgM in PBS (100 μl total volume) and incubated overnight at 25 °C after washing, they were saturated with 1% bovine serum albumin (BSA) in PBS (pH 7.4) for 1 h. From now on the plates were each time incubated for 1 h at 25 °C and washed five times with PBS containing 0.05% tween 20. Adding 100 μl of gathered supernatants, bound antibodies to immobilize antigen were detected with 100 ng/well of the anti-mouse IgG conjugated with horseradish proxidase (Abcam). The mAb was visualized by the addition of 100 μ1 tetramethylbenzidine (Sigma) substrate solution. The plates were read in an Anthos2020 ELISA-reader Apparatus at 450 nm.

Subcloning and Freezing

For subcloning, the limiting dilution method of Fazekas and Scheidegger was employed. Positive clones (detected by ELISA) from plates with cell growth in less than 30% of the wells were considered as true subclones and were picked and grown for further use. Hybridomas producing antibodies to human IgM were cloned four times in microtiter cell culture plates. In each step retaining the antibody producing capability were tested by a separate ELISA. For long-term storage, aliquots (0.5 ml) of harvested cells (10^6 per ml) in hybridoma medium containing 10% (vol/vol) dimethyl sulfoxide were frozen in a −80 °C freezer over night and then transferred to liquid nitrogen container 24 h later.

Ascitic Fluid Collection

A group of similar mice were prepared and 0.5 ml Pristane (2, 6, 10, 14 tetramethylpentadecane, Sigma) was injected intraperitoneally into each mouse. Ten days after priming with Pristane, 5×10^6 cells per 0.5 ml PBS, from the harvested clone, were injected into each

mouse .The mice were assessed daily for production of ascitic fluid. About 7 days after cell injection changes in the abdomen size of the mice were detected. About 10 days later, abdomens of the mice were enlarged enough and their skins were extended. Using 18 gage needles, their ascitic fluid was harvested and centrifuged at 12,000 rpm for 5 min and the related supernatants were collected for purification and further characterization.

Purification and Determination of Concentration and Purity

The antibodies were purified in a three-stage process that included precipitation into ammonium sulfate (50% saturation) and protein G affinity column chromatography and ion exchange chromatography on DEAE-cellulose as we have previously published [13]. Antibody concentration was measured by UV spectroscopy according to the following formula:

$$([\text{Antibody}]\ \text{mg/ml} = \text{OD read at } 280\ \text{nm}/\ 1.4)$$

Immunoblotting

We ran our protein samples including human IgM and two samples of IgG as positive and negative controls along with the protein marker on the SDS-PAGE by the method of Laemmli [14]. Proteins were transferred to nitrocellulose membrane using the procedure described by Towbin [15]. Transfer of the immunoglobulins from gel to nitrocellulose membrane was done at 150 V for 1 h. Then, nonspecific sites were blocked with 3% BSA solution. After three times of washing, the membrane was cut into strips and except one of the strips containing human IgG others were incubated for 2 h at 25 °C using our purified antibody. After washing, these strips were incubated for 1 h at room temperature with anti-mouse IgG conjugated to horseradish proxidase (HRP). The remaining strip was directly exposed to HRP-conjugated anti-human IgG (purchased from Abcam) as a positive control. Finally, after five times of washing, the strips were exposed for 10 min to 0.5 mg/ml of diaminobenzidine (Sigma) containing 0.05% hydrogen peroxide.

Competitive ELISA

This experiment was accomplished according to similar procedures of washing, incubation, and coating as described for direct ELISA. The binding ability of the harvested antibody to soluble antigen comparing to coated antigen was checked in this test. A serial dilution of soluble IgM was produced and was added to the IgM-coated plates. Then MAN-1 was added to the wells with a constant concentration. The visualization step was accomplished and a curve was drawn from the data gained.

Isotyping

The class and subclasses of the monoclonal antibody were determined by an enzyme immunoassay employing a mouse-hybridoma subtyping strip (ZYMED, US).

Affinity Measurement

The affinity was calculated using the method of Beaty [16]. Briefly different concentration of IgM was coated against different Man-1 concentrations and by using the formulas presented in this method $\left(K_{\text{aff}} = 1/2\left(2[\text{Ab}']_{\text{t}} - [\text{Ab}]_{\text{t}}\right)\right.$ affinity constant was determined.

Results

Fusion and Colony Selection

After fusing SP2/0 and spleen cells by PEG the supernatant of the cultured hybridomas in 96-well plates which had a visible cell colony were screened by ELISA to detect the presence of any anti-IgM antibody. Thirty-five positive colonies were detected. One of these which showed the highest OD value and did not have any cross-reactivity with other isotypes of immunoglobulins was selected for further study (Data not shown). Four rounds of limited dilution were performed and we obtained a colony in which all the cells were stable producers of a monoclonal antibody against IgM. We named this hybridoma MAN-1.

Purification of MAN-1 and Elucidation of its Isotype and Affinity

After injecting appropriate number of hybridoma cells to the peritoneal cavity, MAN-1 was purified from acsitic fluid using protein-G sepharose followed by DEAE-cellulose ion exchange chromatography and the purity was checked by SDS-PAGE in reducing and nonreducing conditions and the gel was silver stained to increase the detection sensitivity of available bonds (Fig. 1).

In order to clarify the isotype of MAN-1 the isotyping test was done and the results showed that the antibody harvested was IgG1 and its light chain was kappa.

The affinity constant (K_{aff}) of $3.5\times10^{9}M^{-1}$ was determined using the procedure of Beatty [16] (data not shown).

Assessment of MAN-1's Specificity to IgM

Direct ELISA

As it is shown in Fig. 2, there is a concentration-dependent high reactivity between MAN-1 and human IgM. Besides MAN-1 cannot react with other isotypes of immunoglobulins and it reacts specifically with IgM (Fig. 2).

Competitive ELISA

To address this question that weather MAN-1 has the capability to recognize its epitope while IgM is free in the solution, we did the competitive ELISA. The results presented here (Fig. 3) show the ability of MAN-1 to recognize and react with soluble IgM.

Immunoblotting

To further characterize MAN-1, the immunoblotting test was done involving human IgM and human IgG which the latter was used as a negative control. After accomplishing the visualization steps, as we expected there was no bond detected on the nitrocellulose paper for IgG that confirmed the ELISA results and did not show any reactivity between MAN-1 and this isotype of antibody; but surprisingly there was also no bond for IgM (Fig. 3); however, the results showed two clear bonds while visualizing human IgG directly by anti-human polyclonal IgG conjugated with HRP (Fig. 4b), which pointed to the successful transfer of immunoglobulins to nitrocellulose paper.

Fig. 1 Gel electrophoresis of MAN-1 in reducing (*A*) and nonreducing (*C*) conditions. 2-mercaptoethanol was added to the sample buffer in the latter condition. Silver staining was done. Marker (*B*) size is 60, 45, 25, and 14 KD. It is clear that our sample is very pure as no additional bond is detected

Discussion

IgM is the first immunoglobulin which appears in response to an initial exposure to an antigen and can be detected 7–15 days after infection, followed by a progressive decline and disappearance within several months. Presence of a high level of these antibodies in blood can be an indication of an acute infection.

From another point of view quantization of each immunoglobulin isotype in serum sample is valuable in identifying a given pathology. For instance in a disease called dengue fever (DF) which is an acute infectious disease caused by the dengue virus [17], the detection of virus-specific immunoglobulin M antibody in serum samples is used for diagnosis of infection.

Besides, detection of specific IgM in neonatal sera is also of major value in distinguishing between a congenital infection and passively acquired maternal antibody [18].

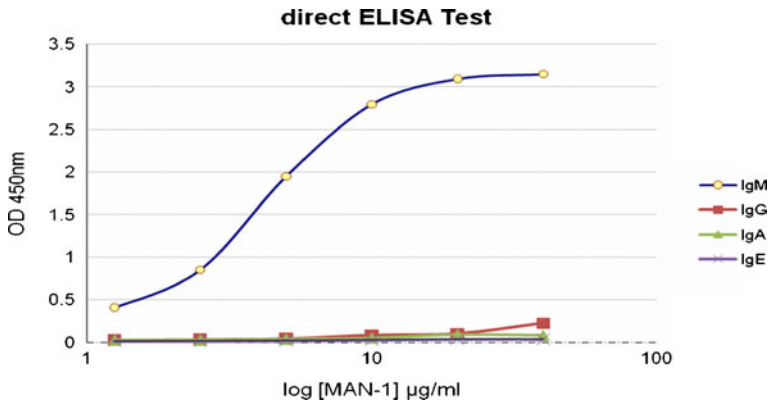

Fig. 2 The wells of a 96-microtiter plate were coated with 1 µg/ml of IgM, IgG, IgA, and IgE and a serially diluted MAN-1 solution in diluting buffer was added to each well. As it is shown no cross-reactivity was detected. On the other hand a concentration-dependent high reactivity exists between IgM and MAN-1

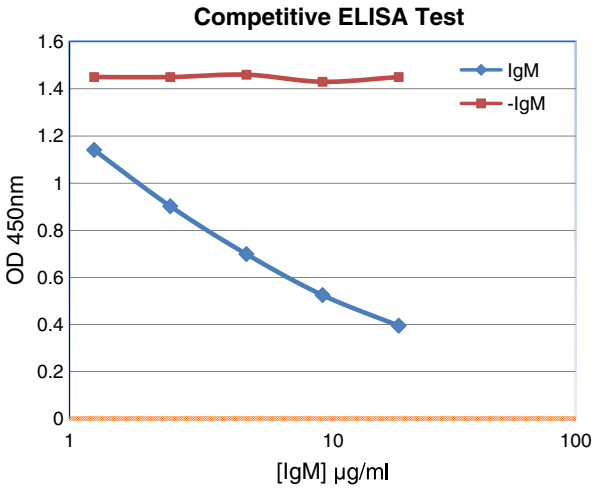

Fig. 3 In this experiment a specific concentration of IgM (1 µg/ml) was coated in microtiter plates and then a mixture of MAN-1 (with a definite concentration) and IgM with varying concentration were added into each well. In another experiment with the same specifications IgM was omitted as a control. The results clearly show the ability of MAN-1 to recognize its antigen (epitope) while in solution

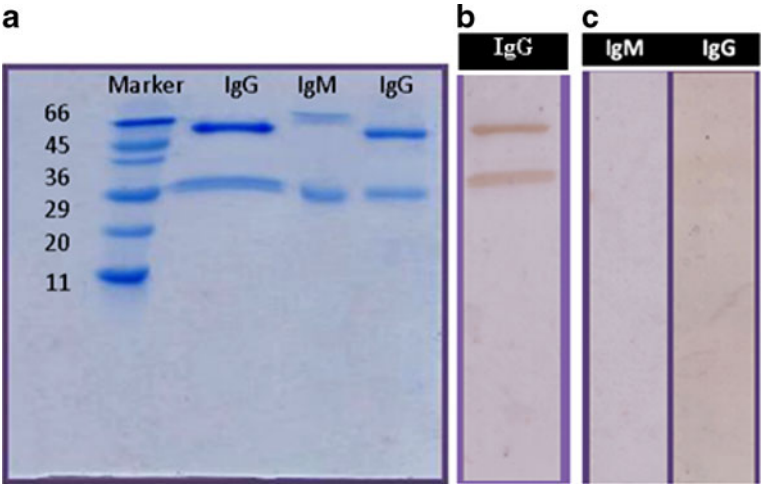

Fig. 4 **a** As it is shown in this figure which is a SDS-PAGE analysis (12.5% polyacrylamide gel) the first, second, third, and the fourth wells from the left have been loaded by protein marker, human IgG, human IgM, and human IgG, respectively (marker size 66, 45, 36, 29, 20, and 11 KD).the samples are in their reducing condition. **b** The strip corresponding to the second well was separated and immunoblotting test was done on it. This piece of nitrocellulose paper contains human IgG and was directly exposed to anti-human polyclonal IgG conjugated to HRP as a positive control. The two bonds clearly show the successful transfer of the proteins from gel to paper. **c** Immunoblotting test on the third and fourth wells. Human IgG is used as a negative control and human IgM is the antigen of MAN-1. As it is shown no bond was detected for IgG neither was detected for IgM light or heavy chain

As a result measuring the amount of IgM in blood and evaluating its concentration in a period of time and comparing it with the normal range could be a valuable indication of newly emerged infections and also different stages of diseases.

Although different anti-human IgM monoclonal and polyclonal antibodies have been developed [5, 19] and some of them are commercially available, we felt the need to produce a new one. In fact our first ELISA results on the detection of human IgM antibodies (the project has not been accomplished yet) confirmed the inapplicability of polyclonal and low sensitivity or specificity of accessible monoclonal antibodies we tested. Interestingly, different groups have encountered similar problems [20]. For instance a comparative analysis of six commercially available diagnostic kits which detect IgM antibodies against *T. gondii* has been fulfilled and as it is concluded two of the tested systems did not have the adequate sensitivity or specificity [21]. In addition, as far as we know the affinities of the anti-IgM monoclonal antibodies commercially available differ from 2×10^5 to 5.34×10^8 [19] and this will affect optimizing the measurement procedure. Therefore, we started raising this antibody in Balb/c mice. ELISA results on the harvested antibodies showed a high OD value which evidenced the existence of a high reactivity between our monoclonal antibody, MAN-1, and human IgM both in coated and soluble form. However, no bond emerged for reduced IgM on nitrocellulose paper. So we came to this conclusion that MAN-1 may recognize an epitope which will be distorted while the heavy and light subunits are separated from each other. In another words, we are dealing with a conformational epitope here. Besides MAN-1 cannot recognize other isotype of immunoglobulins and this guide us toward the location of this epitope which should be somewhere on the μ chain. Isotyping test showed that the harvested antibody was $IgG_1\kappa$. Another advantage of MAN-1 is its high affinity $\left(K_a = 3.5 \times 10^9 M^{-1}\right)$ which rendered it useful for immunologic tests.

In this study we produced a highly specific monoclonal antibody with a high affinity and specificity against human IgM for subsequent application in the detection of primary immune response to infectious agents.

Acknowledgments This work was financially supported by the Monoclonal Antibodies Research Centre, Simaye Iran street Tehran, Iran and all rights are reserved to this company.

References

1. Abbas, A. K., Lichtman, A. H., & Pober, J. S. (1994). *Cellular and molecular immunology* (2nd ed.). Philadelphia: W.B. Saunders.
2. Borish, L. (1999). Genetics of allergy and asthma. *Annals of Allergy, Asthma & Immunology, 82*, 413.
3. Gould, H. J., et al. (2003). The biology of IgE and the basis of allergic disease. *Annual Review of Immunology, 21*, 579.
4. Hanson, L. A., Bjorkander, J., & Robbins, J. B. (1986). IgG subclass deficiencies. *Vox Sanguinis, 51* (suppl 2), 50–56.
5. Balfour, A. H., Harford, J. P., & Margaret, G. (1987). Use of monoclonal antibodies in an ELISA to detect IgM class antibodies specific for *Toxoplasma gondii*. *Journal of Clinical Pathology, 40*, 853–857.
6. Morgan-Capner, P., Rodeck, C. H., Nicolaides, K. H., & Cradock-Watson, J. E. (1985). Prenatal detection of rubella-specific IgM in fetal sera. *Prenatal Diagnosis, 5*, 21–26.
7. Ficicioglu, C. & Tasdemir, S. (1996). Prevalence of cytomegalovirus IgG and IgM antibodies in the sera of postpartum women and cord sera of newborn babies and their correlation with socioeconomic status. *Medical Principles and Practice, 5*, 12–18.

8. Kindt, T. J., Goldsby, R. A., & Osborne, B. A. (2007). *Kuby immunology* (6th ed.). England: W.H. Freeman.
9. Kohler, G. & Milstein, C. (1975). continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*, 495–497.
10. Pouletty, P., Pinon, J. M., Garcia-Gonzalez, M., Desmonts, G., Thulliez, P., Thoannes, H., et al. (1984). An anti-human immunoglobulin M monoclonal antibody for detection of antibodies to Toxoplasma gondii. *European Journal of Clinical Microbiology, 3*(6), 510–5.
11. Kennett, R. H. (1981). Hybridomas: a new dimension in biological analysis. *In Vitro, 17*, 1036–1050.
12. de Fazekas, S. T., Groth, S., & Scheidegger, D. (1980). Production of monoclonal antibodies: strategy and tactics. *Immuno Methods, 35*, 1–21.
13. Mirshahi, M., Shamsipour, F., Mirshahi, T., Khajeh, K., & Naderi-Manesh, H. (2006). *Immunology Letters, 106*(1), 57–62.
14. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bactrriophage T4. *Nature, 227*, 680–685.
15. Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science, 76*, 4350–435.
16. Beatty, J. D., Beatty, B. G., & Vlahos, W. G. (1987). Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. *Journal of Immunological Methods, 100*, 173–179.
17. Balmaseda, A., Guzman, M. G., Hammond, S., Robleto, G., Flores, C., Tellez, Y., et al. (2003). Diagnosis of dengue virus infection by detection of specific Immunoglobulin M (IgM) and IgA antibodies in serum and saliva. *Clinical and Diagnostic Laboratory Immunology, 10*, 317–322.
18. Desmonts, G., Forestier, F., Thulliez, P. H., Daffos, F., Capella-Pavlovsky, M., & Chartier, M. (1985). Prenatal diagnosis of congenital toxoplasmosis. *Lancet, i*, 500–504.
19. Rudich, S. M., Winchester, R., Patricia, A., & Mongini, K. A. (1985). *Journal of Experimental Medicine, 162*, 1236–1255.
20. Liesenfeld, O., Press, C., Montoya, J. G., Gill, R., Isaac-Renton, J. L., Hedman, K., et al. (1997). False-positive results in immunoglobulin M (IgM) toxoplasma antibody tests and importance of confirmatory testing: the Platelia Toxo IgM test. *Journal of Clinical Microbiology, 35*(1), 174–8.
21. Verhofstede, C., Renterghem, L. V., & Plum, J. (1989). Comparison of six commercial enzyme linked immunosorbent assays for detecting IgM antibodies against Toxoplasma gondii. *Journal of Clinical Pathology, 42*(12), 1285–1290.
22. Daffos, F., Forestier, F., Capella-Pavlovsky, M., Thulliez, P., Aufrant, C., Valenti, D., et al. (1988). Prenatal management of 746 pregnancies at riskfor congenital toxoplasmosis. *New England Journal of Medicine, 318*, 271–275 (PMID: 3336419).
23. Zerbini, M., Musiani, M., Gentilomi, G., & Placa, M. (1986). Detection of specific immunoglobulin M antibodies to cytomegalovirus by using monoclonal antibody to immunoglobulin M in an indirect immunofluorescence assay. *Journal of Clinical Microbiology, 24*, 166–168 (PMID: 3013930).