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GENERATION OF HYBRIDOMAS PRODUCING HUMAN MONOCLONAL ANTIBODIES AGAINST HUMAN CYTOMEGALOVIRUS

Yoh-ichi Matsumoto, Tohru Sugano, Chikako Miyamoto and Yasuhiko Masuho*

Teijin Institute for Biomedical Research
Asahigaoka 4-3-2, Hino-city, Tokyo 191, Japan

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In vitro stimulation of human lymphocytes were studied in connection with cell fusion. When splenic lymphocytes were stimulated with human cytomegalovirus (CMV), they produced IgG but not IgM antibody against CMV. The stimulation with 50 ng/ml of CMV antigen induced the maximum antibody response, and higher concentrations of CMV antigen decreased antibody response and increased nonspecific IgG production. Human splenic lymphocytes were stimulated for 6 days with CMV antigen (50 ng/ml) and/or B-cell growth factor (BCGF), and then fused with mouse myeloma cells. Stimulation with a combination of antigen and BCGF were able to generate CMV-specific hybridomas synergistically. Two of these hybridomas were cloned by limiting dilution. The human monoclonal antibodies produced by them, C1 and C23, bound to CMV but not to other herpesviruses. C23 neutralized virus infectivity but C1 did not at all. This method for generation of hybridomas producing human monoclonal antibodies against a predefined antigen may be applicable to a variety of viral antigens.

Human cytomegalovirus (CMV), one of herpesviruses, causes life-threatening infections in immunocompromised hosts. Attempts at treatment with currently available antiviral agents have not been successful (1-3). Human serum immunoglobulin with a high titer to CMV prevented severe CMV infection in bone marrow transplant recipients (4,5). However, it is very difficult to prepare a sufficient amount of high-titer serum immunoglobulin for wide clinical use. If one could establish hybridomas producing human anti-CMV antibodies, cell culture of the hybridomas would be able to provide a large amount of human immunoglobulin with a high titer.

There have been many attempts at establishing cell lines producing human monoclonal antibodies (MAbs), but to date these attempts have met with only limited success (6-8). The largest obstacle is the difficulty of

*To whom all correspondence should be addressed.

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obtaining B lymphocytes highly immunized with a pertinent antigen. In vivo immunization in humans is restricted to special conditions. Recently, we showed that in vitro stimulation of human lymphocytes with an antigen and pokeweed mitogen (PWM) provided hybridomas producing human MAbs against the antigen (9). PWM increases the number of B-cell blasts which preferentially fuse with myeloma cells and enhances the generation of hybridomas (10). However, the nonspecific mitogenic effect of PWM increases not only antigen-specific hybridomas but also nonspecific hybridomas.

Current studies on B-cell differentiation have shown that BCGF induces proliferation of B cells that are already activated with an antigen (11-13). Stimulation of lymphocytes with an antigen and BCGF was thus expected to increase the antigen-specific hybridomas but not nonspecific ones. In this study, we examined in vitro stimulation of human lymphocytes with an antigen plus BCGF and generated hybridomas producing human MAbs against CMV.

MATERIALS AND METHODS

Cytomegalovirus (CMV). CMV strains were kindly provided by Y. Minamishima, Miyazaki Medical College. They were propagated and titrated in human embryonic lung (HEL) cells. Monolayers of HEL cells were grown in Eagle minimum essential medium containing 10% fetal calf serum (FCS), 40 μg/ml gentamicin and 2 mM glutamine. CMV antigen for in vitro stimulation was harvested by centrifuging the culture fluid of CMV-infected cells at 70,000 × g for 2 hr. CMV antigen for enzyme-linked immunosorbent assay (ELISA) was prepared by sonication of the CMV-infected cell pellet. These antigen preparations were irradiated with ultraviolet rays to inactivate infectious viruses.

B-cell growth factor (BCGF). BCGF was purchased from Cellular Products Inc., Buffalo, NY. The manufacturer prepared BCGF from the spent medium of human T cells that had been stimulated with phytohemagglutinin. BCGF was separated from the phytohemagglutinin, gamma interferon and T-cell growth factor by partial purification including DEAE Sepharose column chromatography.

In vitro stimulation of lymphocytes. A human spleen was obtained by splenectomy from a patient with idiopathic thrombocytopenic purpura. The splenic cell suspension was stored in a liquid N_2 tank. Before use, the frozen cells were thawed and splenic lymphocytes were separated with Ficoll-Paque solution (Pharmacia Fine Chemicals, Uppsala, Sweden). The splenic lymphocytes were suspended at the cell density of 1.5×10^6 cells/ml in RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 80 μg/ml gentamicin and 0.25 μg/ml amphotericin B. To determine in vitro antibody responses, 150 μl aliquots of this lymphocyte suspension were mixed with 10 μl of CMV antigen solution in a U-bottomed 96-well plate (Coster 3799, Cambridge, MA, USA), and cultured in a CO_2 incubator at

37°C for 6 days. After washing the cells twice with antigen-free culture medium, they were further cultured for 2 days in 150 µl of antigen-free medium. The culture fluids were then harvested and assayed for IgG and IgM antibodies against CMV and total IgG and IgM concentrations by enzyme-linked immunosorbent assay (ELISA). To generate hybridomas, 1-ml aliquots of lymphocyte suspension (1.5×10^6 cells/ml) were mixed with 0.1 ml of CMV antigen solution (500 ng protein/ml) and/or 0.1 ml of BCGF solution in each well of a flat-bottomed 24-well plate (Coster 3424), and cultured at 37°C for 6 days before cell fusion.

Cell fusion. Mouse myeloma cell line P3×63AgU1 (14) was used as the fusion partner. The stimulated lymphocytes which numbered 4.5×10^6 cells at the beginning of stimulation were fused with 5×10^6 myeloma cells as was described previously (9). The fused cells were seeded in one 96-well microculture plate. After 4-week culture, wells with macroscopic hybridoma colonies were counted and antibody activity in each well was measured by ELISA.

Enzyme-linked immunosorbent assay (ELISA). Human antibodies were measured by ELISA. To measure human IgG and IgM, microtiter plates were coated with 10 µg/ml of goat IgG anti-human IgG or IgM. For antibodies against CMV, microtiter plates were coated with 10 µg protein/ml of the CMV antigen. The assay procedures were the same as described previously (9).

Neutralization of virus infectivity by human MAbs. The neutralization activity of human MAbs was assayed basically as described by Reynolds et al. (15). Briefly, about 200 plaque-forming units of the AD169 strain were mixed with human MAbs and 5% (v/v) of guinea pig serum as a source of complement, and incubated at 37°C for 60 min. The residual infectious viruses were inoculated to monolayers of HEL cells. After 2-week culture, viral plaques were counted under a dissecting microscope. The neutralization activity of human MAbs was determined in duplicate and expressed as reduction of viral plaques.

RESULTS AND DISCUSSION

In vitro antibody response to CMV. The spleen from a CMV-seropositive donor was used as the lymphocyte source in this study. Before experiments on cell fusion, the in vitro antibody response was examined to optimize the conditions for in vitro stimulation. After splenic lymphocytes were cultured for 6 days with various concentrations of CMV, their productions of CMV-specific antibodies (Fig. 1A) and total immunoglobulin (Fig. 1B) were measured by ELISA. Although both IgG and IgM productions were observed, the CMV-specific antibody response was limited to the IgG class. The specific antibody response increased in a dose-dependent manner up to 50 ng/ml of CMV antigen, and decreased at the higher concentrations. On the other hand, total immunoglobulin production increased with increasing antigen concentrations within the range tested. The increase of total

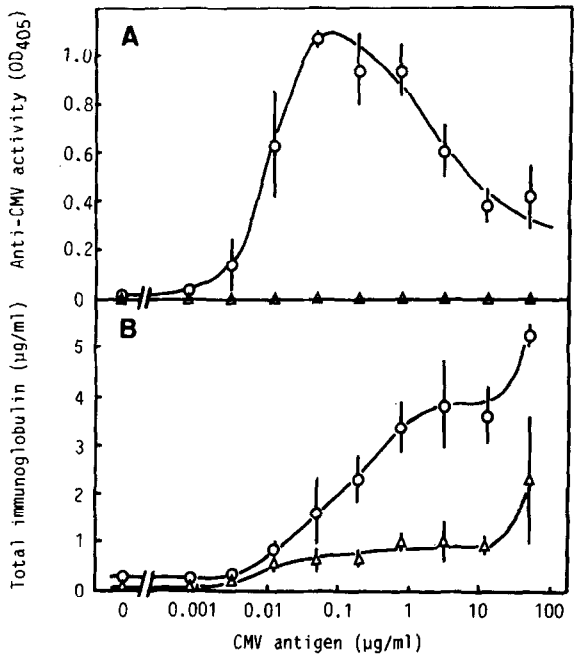

Figure 1. In vitro antibody response to CMV. Antibody response to CMV (A) and total immunoglobulin production (B) were measured by ELISA. IgG and IgM responses were indicated with circles and triangles, respectively.

immunoglobulin should be due to nonspecific effect of CMV which is known to be a potent polyclonal B-cell activator (16,17). Therefore, the optimum CMV concentration for antigen-specific stimulation is 50 ng/ml.

Generation of hybridomas producing human MAbs against CMV. Human splenic lymphocytes were stimulated in vitro with CMV alone, BCGF alone, CMV plus BCGF or CMV plus pokeweed mitogen (PWM), and then fused with mouse myeloma cells. Four weeks later, the generation of hybridomas and their antibody productions were compared among these stimulations (Table 1).

Table 1. Generation of hybridomas producing antibodies against CMV*

Stimulation	Growth	Total IgG	IgG anti-CMV	IgM anti-CMV
None	18	12	0	0
CMV	20	27	1	0
BCGF	81	93	6	0
CMV + BCGF	84	96	32	2
CMV + PWM	55	29	3	0

*This series of cell fusions was carried out at one time, using myeloma cells harvested from the same cell culture. Four weeks after cell fusion, wells with macroscopic hybridoma colonies were counted and the culture fluids were screened for total IgG, IgG anti-CMV and IgM anti-CMV by ELISA.

Preculture without either CMV or BCGF generated only 12 IgG-producing hybridomas, none of which was positive for anti-CMV antibody. Stimulation with CMV alone increased the number of IgG producers to 27, but only one of them was positive for anti-CMV antibody. BCGF alone greatly enhanced the generation of hybridomas, which included 6 CMV-specific ones. It has been shown that B lymphocytes which are previously activated with an antigen are capable of growing in response to BCGF (11-13). The increase in hybridomas by stimulation with BCGF alone suggests that some population within the splenic lymphocytes had already been activated in vivo with CMV and other unknown antigens. Among these in vitro stimulations, the combination of CMV and BCGF generated the greatest number of CMV-specific hybridomas. Of 96 wells, 32 were positive for anti-CMV IgG and 2 were positive for anti-CMV IgM. These results imply that in vitro stimulation of human lymphocytes with a mixture of CMV and BCGF synergistically enhances the generation of CMV-specific hybridomas. Furthermore, this combination generated more CMV-specific hybridomas than a combination of CMV and PWM.

The CMV-specific hybridomas thus generated were cloned by the limiting dilution method. After the cloning process, some hybridomas continued producing MAbs stably. Representative clones C1 and C23 have been producing MAbs for more than 6 months. The MAb concentrations in the culture fluids of these hybridomas have ranged between 1 and 10 μg/ml/day. The subclass of each of MAbs C1 and C23 was determined by immunodiffusion with sheep antibodies against each of human IgG1, IgG2, IgG3 and IgG4. The immunodiffusion analysis showed that both C1 and C23 were of the IgG1 isotype.

Binding specificities of human MAbs C1 and C23 in ELISA. As shown in Fig. 2, both C1 and C23 bound to all the tested CMV strains including laboratory strain AD169 and 6 clinical isolates, but neither MAb bound to a homogenate of uninfected host cells (HEL). In addition, neither C1 nor C23 bound to any other herpesviruses, i.e., herpes simplex virus (HSV) type 1 or type 2, varicella zoster virus (VZV) or Epstein-Barr virus (EBV). Since

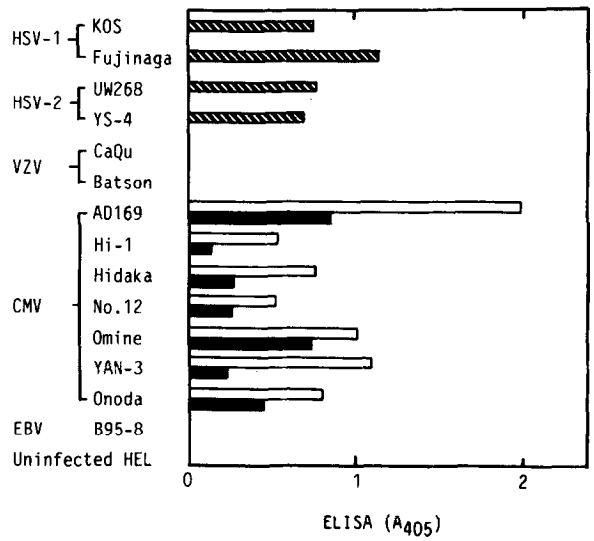

Figure 2. Binding specificities of human MAbs in ELISA. C1 (white bars), C23 (black bars) and H2 (shaded bars).

CMV-infected cells are known to have a large amount of Fc-binding substance (18,19), there remains the possibility that these MAbs bound to CMV through their Fc moieties. But MAb H2, which is also of the IgG1 isotype and specific for HSV, did not bind to any of the tested CMV strains in our assay system. Therefore, both C1 and C23 are specific for the CMV antigen conserved by various CMV strains.

Neutralization of virus infectivity by the human MAbs. It was determined by measuring the reduction of viral plaques (Table 2). While C1 did not reduce viral plaques at a concentration as high as 142 μg/ml, C23 reduced them by 50% at about 1 μg/ml. Baron and his co-workers (20,21) found antiviral substances other than antibodies in culture fluids of

Table 2. Neutralization of virus infectivity by human MAbs

Human MAb,	concentration	Reduction of viral plaques (%)
C1	142 μg/ml	0
	61	0
C23	3.4	80
	1.1	43
	0.37	12
*Purified C23	10	94
	1.0	22

*C23 in the culture fluid was purified by affinity chromatography on protein A Sepharose.

various tissue cells. The plaque reduction shown by the culture fluid of hybridoma C23 might be due to such antiviral substances. To examine this possibility, IgG was purified from the culture fluid by protein A affinity chromatography. The purified MAb C23 had the same level of neutralization activity as that of the crude preparation. Therefore, MAb C23 is clearly capable of neutralizing CMV infectivity.

In this study, we established hybridomas producing human MAbs against CMV by cell fusion between mouse myeloma cells and human lymphocytes which had been stimulated with CMV antigen and BCGF. This combination synergistically enhanced the generation of CMV-specific hybridomas. The synergism was greater than that of CMV and PWM. Most of them (32/34) produced IgG antibodies, suggesting that the in vitro stimulation induced the secondary immune response of lymphocytes which had already been sensitized with the antigen in vivo. Lymphocytes from seronegative donors did not provide antigen-specific hybridomas, even if they were stimulated in vitro with CMV and BCGF (data not shown). Therefore, it is also important to select a suitable lymphocyte source for efficient generation of antigen-specific hybridomas.

Xenogenic mouse × human hybridomas had been expected to be unstable in antibody production because of gene segregation (22). But our previous study showed that mouse × human hybridomas were more stable than human × human hybridomas (23). Actually, some hybridomas established in this study also have been producing anti-CMV MAbs in long-term culture. The cell culture of hybridomas provides a potentially unlimited amount of human MAbs which may be effective against CMV infections in humans. Studies on their antiviral activities are now in progress.

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