Human-Human Hybridomas Secreting IgM-like Immunoglobulin with α and μ Heavy Chains

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Human-human hybridomas were produced by fusion of a malignant 6-thioguanine resistant human B lymphoblastoid cell line and normal lymphocytes. The fusion efficiency with lymphocytes from peripheral blood was elevated by preculture of the lymphocytes in the presence of lipopolysaccharides. However, the preculture did not enhance the efficiency with lymphocytes from lung cancer tissue. The resulting hybridomas secreted mostly IgG and/or IgM. A small number of hybridomas secreted IgA. Two IgA secreting hybridomas, 9P-13-4 and 9L-27-9 also produced IgM-like immunoglobulin even after repeated cloning, indicating these hybridomas secrete IgA and IgM-like immunoglobulins simultaneously. The IgM-like immunoglobulin has a molecular weight of 900 K and contains 3 monomeric IgMs and 2 IgAs. This indicates that it is therefore possible to produce a IgM-like immunoglobulin binding to two different antigen determinants, by hybridoma technology.

Human monoclonal antibodies are highly promising for clinical uses.¹⁾ There are two methods to obtain human monoclonal antibodies. One is to transform human B lymphocytes with Epstein-Barr virus (EBV), and another is to make hybridomas by cell fusion of B lymphocytes with a malignant fusion partner cell line.²⁾ Though EBV transformation gives many clones of transformed B cell lines, production of immunoglobulin tends to be lost from the transformants during serial subcultivation.³⁾ For cell fusion to produce human hybridomas, a feasible fusion partner cell line originating from a human plasmacytoma had been desired.⁴⁾ We recently developed a new human lymphoblastoid cell line as a fusion partner.⁵⁾ HO323, the fusion partner, gave a very high incidence of hybridomas. HO323 is an IgM producer but not a secretor. In the process of making IgA secreting hybridomas by fusion with HO323 and human

lymphocytes, we observed not only IgA but also IgM-like molecules were simultaneously secreted by a single hybridoma line. We report here the production and characterization of the IgM-like immunoglobulin having both α - and μ -chains in the same molecule.

MATERIALS AND METHODS

Cells. The malignant fusion partner for the production of human-human hybridomas was a 6-thioguanine resistant lymphoblastoid cell line HO323, derived from WIL2-NS, that has been described elsewhere.⁵⁾ HO323 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and in a humidified atmosphere of 5% CO₂/95% air at 37°C. Lymphocyte cells to be fused with HO323 were obtained from lymph nodes of a patient with lung cancer, and from normal peripheral blood. Peripheral blood lymphocytes (PBL) were separated from reticulocytes by Ficoll-Paque discontinuous density gradient centrifugation. These lymphocytes were cultured at a cell density of 10⁶/ml in 10% FCS-RPMI 1640 medium containing either 5 μ g/ml

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lipopolysaccharide (LPS, *E. coli*: 0111:B4, Sigma) or 0.01% pokeweed mitogen (PWM, GIBCO) for 6 days before the cell fusion.⁶⁾

Cell fusion and screening of hybridomas. HO323 cells were fused with lymphocytes by the method of Köhler and Milstein⁷⁾ using 50% polyethylene glycol (PEG: M_w 4000, Sigma). In the fusion mixture, there were twice as many HO323 cells as lymphocytes. The cell mixture was plated in a 96 well microtiter plate (Falcon 3072) at the density of $3 \times 10^4 \sim 1.5 \times 10^5$ cells/well and cultured in a humidified atmosphere of 5% CO₂/7% O₂/88% N₂. Hybridoma cells were selected in 10% FCS-RPMI 1640 medium supplemented with 0.1 mM hypoxanthine/0.4 μ M aminopterin/16 μ M thymidine (HAT).

Enzyme-linked immunosorbent assay (ELISA) of immunoglobulins. Monomeric IgM and IgA consist of two light chains (L chains) and two heavy chains (H chains). Monomeric IgM has heavy chains of μ associated with light chains of κ or λ . IgA consist of heavy chains of α also associated with κ or λ (see Fig. 3A). Naturally occurring IgM is a macromolecule composed of five monomeric IgM's and a J chain peptide (Fig. 3C). Immunoglobulin in the culture supernatant of the hybridomas was measured with an enzyme-linked immuno sorbent assay (ELISA).⁸⁾ Microtiter wells (Nunc. Immunoplate II) were coated with a mixture of anti-human κ and λ light chain antibodies (TAGO) for 12 hr at 4°C. Then the wells were blocked with 1% bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS) to protect the immunoglobulin from attaching nonspecifically to the wells. Fifty microliters of culture supernatant of hybridomas were incubated for 1 hr at room temperature to combine immunoglobulin from the hybridomas with the anti-light chain antibodies immobilized on the wells. The wells were then washed with 0.05% Tween-PBS. Peroxidase-conjugated anti-heavy chain (α , γ , and μ for IgA, IgG, and IgM, respectively) antibodies (TAGO) were bound to the trapped immunoglobulin for 1 hr. After washing, enzyme activity was measured by the addition of substrate (0.3 mg/ml 2,2'-azino-di-(3-ethyl benzthiazolin sulfonic acid), 0.03% H₂O₂ in 0.1 M citrate buffer). The enzyme reaction was stopped by adding 1.4% oxalic acid. Absorbancy of the reaction product was measured at 405 nm by an ETY-96 ELISA Reader (Oriental Instruments).

To identify the species of L chain of immunoglobulin produced by the hybridoma, the immunoglobulin was added to microtiter wells coated with anti- κ - or λ -chain antibody. The immunoglobulin trapped by the anti-light chain class specific antibody was then linked with peroxidase conjugated anti-heavy chain antibody.

The heavy chains of the immunoglobulin from the hybridomas were classified by the same method as for the light chains, except that the immunoglobulin was trapped on well coated with anti-heavy chain class (α or μ)-specific antibody (Cappel) instead of anti-light chain class-specific

antibody.

Cloning of hybridomas. Hybridomas secreting IgA were cloned twice by limiting dilution. A human fibroblast cell line, Flow 11000 was used as feeder layer cells to assist propagation of the single hybridoma cell in a microtiter well.

Gel filtration of immunoglobulins secreted by hybridomas. Cloned hybridoma cells were cultured in serum-free HB103 medium (Hana Biologicals). Immunoglobulin fraction was precipitated from the culture supernatant by addition of 60% ammonium sulfate. Immunoglobulin then solubilized in PBS was chromatographed on FPLC (Pharmacia) with a Sephacryl S-300 column.

RESULTS

Fusion efficiency of HO323

Hybridoma colonies were microscopically detectable 2~3 weeks after the cell fusion, and completed after 6 weeks. Table I lists the incidence of hybridoma formation using lymphocytes from peripheral blood and a lung cancer tissue variously pretreated with mitogens. Several hybridomas were generally obtained among 10⁶ lymphocytes. Lymphocytes from lung cancer gave the best incidence to form 6 hybridomas/10⁶ lymphocytes (Table I, column 4). PBL produced the most hybridomas if previously sensitized with LPS for 6 days. Lymphocytes derived from lymph nodes of lung cancer tissue revealed the highest fusion efficiency without any pretreatment.

Detection of IgM in the culture supernatant of hybridomas secreting IgA

Immunoglobulin in culture supernatants of hybridomas grown in microtiter wells were classified (Table I). Most of the immunoglobulins secreted by hybridomas of lymphocytes from lung cancer patients were IgG and IgM. Some of the hybridomas derived from lymph nodes of cancer tissue secreted IgA. No hybridoma secreting IgA was obtained by the fusion with PBL. As shown in Table II, most of the IgA-secreting hybridomas derived from lung cancer tissue secreted not only IgA but IgM. There were some hybridomas exclusively secreting IgA in this case, however the level of the immunoglobulin concentration was very

TABLE I. FUSION FREQUENCIES OF HO323 CELLS

Tissue source Exp.	Lymphocytes fused (No. $\times 10^{-7}$)	Wells containing hybridomas (No. per 10^6 lymphocytes fused)	Hybrid wells secreting Ig		
			IgA	IgG	IgM
PBL ^a	4	1.3	18 (1.38)	0	0
	5 ^c	1.2	10 (0.83)	0	0
	6 ^c	1.2	17 (1.42)	0	2
	8 ^d	0.9	10 (1.11)	0	0
	10 ^d	0.9	27 (3.00)	0	0
Lymph node ^b	7	2.0	119 (5.95)	13	68
	9P ^c	2.0	63 (3.15)	4	24
	9L ^d	2.0	91 (4.55)	6	50
	11P ^c	2.0	27 (1.35)	0	10
	11L ^d	2.0	62 (3.10)	4	26

^a Peripheral blood lymphocytes.^b Lymph node from a lung cancer patient.^c Lymphocytes were cultured with 0.01% pokeweed mitogen for 3 days (5 and 9P) or 6 days (6 and 11P) before fusion.^d Lymphocytes were cultured with 5 μ g/ml of lipopolysaccharide for 3 days (8 and 9L) or 6 days (10 and 11L) before fusion.

TABLE II. IgA SECRETING HYBRIDOMAS ALSO SECRETE IgM

Exp.	IgA ⁺ IgM ⁺	IgA ⁺ IgM ⁻
7	11	2
9P	3	1
9L	6	0
11L	3	1
Total	23 (85.2%)	4 (14.8%)

TABLE III. CLONING OF HYBRIDOMA 9P-13 AND 9L-27 SECRETING BOTH IgA AND IgM

Cells	Cells seeded per well ^a	Wells with cell growth	IgA ⁺ IgM ⁺	IgA ⁺ IgM ⁻
9P-13	0.3	1	0	0
	1	3	3	0
	2	7	4	0
	5	11	5	0
9L-27	0.3	3	1	0
	1	5	2	0
	2	10	2	0
	5	10	4	0

^a Hybridomas were cloned on microtiter plates using Flow 11000 cells as a feeder layer. At each cell density, cells were plated in each of 24 wells.

small. Almost 30% of IgG-secreting hybridomas also produced IgM. Seventy percent of these hybridomas secreted only IgG in sufficient amounts to measure.

Cloning of IgA secretor

Two hybridoma lines, 9P-13 and 9L-27, which produced both IgA and IgM in their culture supernatants were further cloned to separate IgA and IgM secretors on a feeder layer using the fibroblast line Flow 11000. The cells were inoculated in each of 24 wells at the density of 0.3~5.0/well. Wells containing growing cells obeyed the Poisson distribution, suggesting successful cloning. All immunoglobulin-producing clones still secreted both IgA and IgM (Table III). The same cloning

procedure was repeated to separate IgA secretors from IgM secretors. The resulting hybridoma clones secreting immunoglobulin still produced both IgA and IgM. Cloning on soft agar also failed to obtain any clone secreting IgA but not IgM (data not shown). These results indicate that every IgA-secreting hybridoma clone made by HO323 and lymphocytes of any origin simultaneously secrete IgM. Two clones, 9P-13-4 and 9L-27-9 were thus obtained from 9P-13 and 9L-23 hybridomas, and secreted both IgA and IgM.

Fig. 1. Gel Filtration of Immunoglobulins Secreted by Hybridomas 9P-13-4 (A) and 9L-27-9 (B).

Culture supernatants of hybridomas were precipitated by salting out with ammonium sulfate. Precipitates were dissolved with PBS and fractionated on Sephacryl S-300 column (1.2×56 cm). Each fraction (0.6 ml) was collected and the concentration of IgA and IgM was measured by ELISA. Serum IgA and IgM were eluted fractions 51~55 and 39~43, respectively.

Gel filtration analysis of IgA and IgM secreted by cloned hybridomas 9P-13-4 and 9L-27-9

Immunoglobulins in serum-free culture supernatants of 9P-13-4 and 9L-27-9 clones were separated by Sephacryl S-300 gel chromatography. Immunoglobulin in fractionated eluates was detected by the reaction with either α - or μ -chain specific antibody (goat) conjugated with peroxidase (Fig. 1). Immunoglobulins from 9P-13-4 and 9L-27-9 hybridomas gave the same elution patterns. There were two immunoglobulins (I and II) in the chromatogram. Immunoglobulin I contained heavy chains of α but not μ type, and were the same size as IgA (160 k), suggesting IgA. Immunoglobulin II contained almost comparable amounts of α - and μ -chains, and was the same size as IgM (900 k).

These results suggest that immunoglobulin II is a pentameric IgM molecule having both α - and μ -chains as the heavy chain components. It was, however, still unknown whether immunoglobulin II was a mixture of two separate molecules of 900 k; one a typical IgM of which all heavy chains were μ type, and another might be a pentameric IgA of which the heavy chains were α , although such a large IgA had not been known. Immunoglobulin I and II secreted by hybridomas 9P-13-4 and 9L-27-9 were compared for their binding activities for anti- α - and anti- μ -chain specific antibodies coated on the microtiter wells. Binding of these immunoglobulins for anti-heavy chain was detected using peroxidase conjugated anti- α - and μ -chain antibodies (Fig. 2). The enzyme reaction was positive for immunoglobulin I only when the reaction mixture contained anti- α -chain antibody and peroxidase conjugated anti- α -chain (closed circles in Fig. 2B, D). This indicates that immunoglobulin I is IgA, since IgA having α -chains is exclusively trapped by anti- α -chain antibody on the wells and thus trapped IgA binds peroxidase conjugated anti- α -chain antibody but not peroxidase conjugated anti- μ -chain antibody.

The reactivity of immunoglobulin II was more complicated as shown in Fig. 2A and was the case of that of 9P-13-4. Immunoglobulin II was trapped by both anti- α - and μ -chain antibodies on the solid phase. The immunoglobulin II then, bound both anti- α - and μ -antibodies conjugated with peroxidase. This indicates that the immunoglobulin II molecule contains both α - and μ -chains in the same molecule. On the immobilized anti- μ -chain (triangle), the molar ratio of α - and μ -chains in the immunoglobulin II bound to peroxidase conjugated anti- α -chain antibody ($\blacktriangle$) or for peroxidase conjugated anti- μ -chain antibody ($\triangle$) was not so discrepant. On the immobilized anti- α -chain antibody (circle), the molar ratio of α -chain in the immunoglobulin bound to the former ($\bullet$) was much less than the μ -chain bound to the latter ($\circ$). Immunoglobulin II from 9L-27-9 revealed almost the same

FIG. 2. Heavy Chain Class Identification of Immunoglobulins Secreted by 9P-13-4 (A and B) and 9L-27-9 (C and D).

Immunoglobulin II (A and C) and immunoglobulin I (B and D) from gel filtration were analyzed by ELISA using heavy chain class-specific antibodies. Samples were put on anti- α (● ○) or anti- μ (▲ △) immobilized ELISA plates and peroxidase-labeled anti- α (● ▲) or anti- μ (○ △) were used to identify the heavy chain classes adsorbed on the plate.

profile of reaction (Fig. 2C). The molar ratio of α -chains/ μ -chains in immunoglobulin II from 9P-13-4 and 9L-27-9 cells were deduced to be 1.9/3.1 and 1.6/3.4, respectively. Light chains of immunoglobulin I and II were κ and λ (data not shown).

DISCUSSION

To obtain antigen specific human monoclonal antibodies, there are three main inconveniences. First, it is not necessarily easy to make monoclonal antibodies reactive to any desired antigens, since sensitization by antigens is sometimes not applicable for humans as it is operable for animals. Second, human hybridomas tend to be unstable and cease immunoglobulin production due to the selective loss of chromosomes of B-lymphocytes. Third, few human monoclonal antibodies highly specific to any antigen have been obtained so far. The first problem will be solved to some extent by applying a technique for *in*

vitro sensitization of human B-lymphocytes by antigen. Development of such a system to make human hybridomas in higher efficiency will not only facilitate obtaining hybridomas secreting antibody against rare antigens but also enable selecting a hybridoma clone stably secreting one highly specific monoclonal antibody among other hybridomas. The HO323 cell line is a new malignant fusion partner with human lymphocytes for the production of human-human hybridomas in high efficiency. This cell line should be useful for making monoclonal antibodies of human type. Many hybridomas made by fusion of HO323 secrete IgG or IgM. Though the IgA concentration is second highest following that of IgG among immunoglobulins in plasma or culture medium of lymphocytes stimulated by mitogen (PWM, LPS), the incidence of IgA-secreting hybridomas is only 5% (Table I). Such low incidence may be relevant to the fact that the α -chain coding gene (C_α gene) is the farthest downstream on the gene of a heavy chains of

FIG. 3. Proposed Structure of Immunoglobulin II.

Structure of IgA (A), immunoglobulin II (B) and IgM (C).

immunoglobulins.⁹⁾

Most of the IgA-secreting hybridomas so far obtained secrete IgM simultaneously in spite of repeated clonings. This suggests that single clones of the hybridoma secrete both IgM and IgA (Table III). Though HO323 is a non-secretor of immunoglobulin, this cell line synthesizes a small amount of μ -chain inside the cell.⁵⁾ Whether the μ -chains secreted by 9P-13-4 and 9L-27-9 originate from the of HO323 should be clarified by analysing the difference of the variable (V) regions of α - and μ -chains of these cells. We deduce, however, that the μ -chains are derived from HO323, since preliminary experiments found that IgM-secreting hybridomas have been obtained by fusing ouabain resistant HO323 cells with IgA-secreting EBV transformed lymphocytes which are not considered to cause any genomic class switching of immunoglobulins. It may not be assumed that α - and μ -chains having the same V regions are simultaneously synthesized from the gene for heavy chains.

Size of the IgM-like immunoglobulins secreted by 9P-13-4 and 9L-27-9 was estimated chromatographically to correspond to that of IgM. These results indicate that the IgM-like immunoglobulin is a pentamer of monomeric IgM's of which heavy chains are composed of both α and μ . Five monomeric IgM moieties and a J chain produced in the Golgi apparatus

join to complete IgM in cytoplasm. Human-human hybridomas 9P-13-4 and 9L-27-9 have potentiality to make monomeric IgM and IgA which can also combine with J chains.¹⁰⁾ We already know that molecules of single α - and μ -chains joined each other is not made in these hybridomas (data not shown). Considering that the molar ratio of α - and μ -chains of the IgM-like immunoglobulin was fairly close to 2:3, the immunoglobulin is profiled as shown in Fig. 3B. In this IgM-like immunoglobulin molecule, two of the five monomeric IgM moieties are replaced by two IgA molecules. Therefore, this IgM-like immunoglobulin is symbolized by Ig(A₂M₃). The size of Ig(A₂M₃) still corresponds to that of IgM by gel-chromatography. The IgM-like immunoglobulin has two types of light chains, κ and λ . The κ -chains were originally made for IgM by HO323 cells,⁵⁾ and the λ chains were for IgA by B lymphocytes. However, we do not deny that IgM moieties are composed of the λ - μ combination and IgA moieties, κ - α in the IgM-like molecule, since there are both κ - and λ -chains in cytoplasm of the hybridomas. It is still ambiguous why and how this kind of IgM-like molecule having two types of heavy chains is processed in the hybridoma. In the IgM-like molecules produced by hybridoma 9P-13-4 and 9L-27-9, the antigen determinants reactive to the IgM and IgA moieties are not known.

This experiment has, however, developed a method to make a "multifunctional" IgM molecule of which the 5 monomeric immunoglobulin moieties are reactive to different antigenic determinants.

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