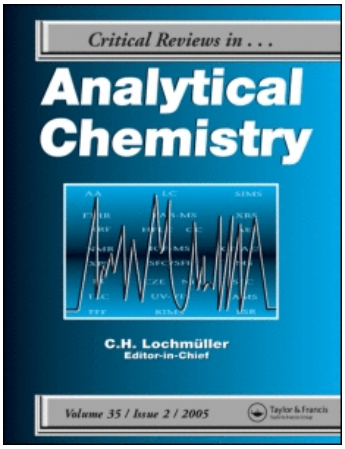

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Rapid Production of Chicken Egg Yolk Antibodies Against Multidrug Resistance-Associated Protein 1 (MRP-1)

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Resistance of cancer cells to a drug is usually accompanied by resistance to other drugs with different structures and cellular targets. This is called multidrug resistance (MDR). There have been many studies to develop anticancer agents and to identify new tumor markers. Multidrug resistance-associated protein 1 (MRP1), a 190 kDa protein found in a small-cell lung carcinoma line (H69AR), can confer cellular resistance to anthracyclines, vinca alkaloids, and epipodophyllotoxins and is considered to be primarily a multispecific organic anion transporter. A recent study showing the role of MRP1 in endobiotic excretion from cells has led to a suggestion that MRP1 also may have a physiological role in cells. Generation of antibodies in mammals is invasive. In addition, antibody production in mice requires sacrifice of the animals. Production of polyclonal antibodies against a mammalian protein in chicken and isolation of it from the egg yolk seems advantageous since the method is noninvasive, the animal does not need to be sacrificed, and the antibody titers in the eggs are higher than in mammals. Chickens are phylogenetically distant from mammals. Therefore, production of antibodies against a mammalian protein seems easier to produce in chickens.

Keywords egg yolk antibody (IgY), multidrug resistance-associated protein 1 (MRP1)

Unsuccessful chemotherapy of cancer patients often occurs as a result of drug resistance of the tumor to a limited number of chemotherapeutic agents. Resistance of cancer cells to a drug is usually accompanied by resistance to other drugs with different structures and cellular targets. This is called multidrug resistance (MDR). There have been many studies to develop anticancer agents and to identify new tumor markers, but the similarity between normal cells and tumor cells limits the number of anticancer agents and it is very hard to identify a specific new tumor marker.

At present, we know that there are a number of proteins which play a role in MDR. P-glycoprotein (P-gp) is the first one of these proteins found to be increased in a number of cancer cells. Currently it has been well demonstrated that a relationship between P-gp and MDR to chemotherapeutic agents exists in some cancers, and some clinical trials have been performed to overcome the P-gp–mediated MDR (1). However, the finding of some carcinoma cells exerting MDR but not expressing P-gp (2) directed the scientists to find other factors causing MDR. In 1992, Cole and coworkers cloned a gene (3) in a small-cell lung carcinoma line (H69AR), which was selected for adriamycin resistance from parental H69 cells (4, 5). This gene codes a protein with 1,531 amino acids. Molecular weight of the native protein is 171 kDa, and a mature protein seems to be 190 kDa (3, 6). This MDR-related protein is termed multidrug resistance-associated protein 1 (MRP1) (3). Similar to P-gp, MRP1 is an N-glycosylated integral membrane phosphoprotein that belongs to the ATP-binding casette (ABC) superfamily of transport proteins (3, 7). Although MRP1 and P-gp belong to the same family, they share less than 20% amino acid identity (3, 7). Most of their identity is in the nucleotide-binding domains, which are mostly conserved in the ABC superfamily. Computer-assisted hydropathy analyses suggest that MRP1 has a different secondary structure than other ABC members. The third membrane-spanning domain (MSD) in ABC superfamily is first found in MRP1. The first 5–6 transmembrane segment is mostly in the first 230 amino acids. The whole protein contains

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17–18 transmembrane segments (8–10). NH_2-proximal contains the first 11–12 segment and COOH-proximal contains the 6 transmembrane segment. However, P-gp and related transporters contain only two MSD which consist of 12 transmembrane segments divided into two halves (6 + 6). Another difference between MRP1 and P-gp is in the nucleotide-binding domains (NBD). The NBDs of P-gp are very similar and they do not have individual features (11). On the other hand, the NBDs of MRP1 are less similar and may have different features (12). Additionally, they reflect some differences in resistance to different drugs (13). The chromosomal locations of their genes are different. The MRP1 gene localizes in chromosome 16p13.1.

Since MRP1 is expressed in many human cancer cells, and the transfection of the MRP1 gene confers resistance to some anticancer agents in sensitive parental cells (13, 14), MRP1 is thought to be responsible for MDR (15–17). Molecular and immunohistochemical studies on human tumor samples and cell lines of breast cancer (18) and non-small-cell lung carcinoma cells indicated significant expression of MRP1 mRNA and protein (19). MRP1 can confer cellular resistance to anthracyclines, vinca alkaloids, and epipodophyllotoxins (2, 3, 13) such as vincristine, doxorubicin, etoposide, methodrexate, and epirubicin but is sensitive to drugs like paclitaxel, irinotecan, and cisplatine (13, 20–22). What determines the resistance phenotype is currently unknown.

MRP1 is located in the plasma membrane of cells (13). MRP1 is considered to be primarily a multispecific organic anion transporter (23). Although MRP1-overexpressing cells seem resistant to these drugs, it is not known how the substrates (drugs and most other endobiotic substrates) are transported by MRP1.

Leukotriene C_4 (LTC_4) is the first molecule that has been shown to be directly transported by MRP1 (24, 25). In these experiments transportation by MRP1 was inhibited by QCRL3 mAb, which recognizes a conformation-dependent sequence on the protein. MRP1-mediated, ATP-dependent vincristine transport was also shown, but only in the presence of reduced glutathione (GSH). GSH and vincristine accelerate their transport by MRP1 reciprocally, although they do not make a conjugate (24). This study also shows that GSH does not need to bind to all of the substrates of MRP1, but is necessary for efficient transport of some MRP1 substrates. The transport function of MRP1 depends on ATP. Discovering the role of MRP1 in LTC_4 transport indicates a physiological function of MRP1. MRP1 can transport compounds conjugated with GSH like aflatoxin B1, heavy metal oxyanions, prostaglandin A2 in an ATP-dependent manner (26–29). ATP is trapped in nucleotide-binding domain 2 (NBD2) (28), and NBD2 seems important for the transport function of MRP1 (28). A recent study showing the role of MRP1 in the transport of sulfated estrogens (endobiotics) is the first direct evidence that sulfated molecules can also be an MRP1 substrate (29). The demonstration of a tobacco-specific carcinogen NNAL-O-glucuronide excretion from cells has led to a suggestion that MRP1 also has a protective role in normal cells (30).

Many studies have been performed to demonstrate the relationship between MRP1 and multidrug resistance in different cancers. Recently, it has been shown that MRP1 is present in choroid plexus (31). Wijnholds and coworkers generated MRP1 knockout mice and directly demonstrated that MRP1 plays a role in transportation of drugs through the blood-brain barrier, so it can alter the concentration of drugs in cerebrospinal fluid (32). It should be noted that MRP1 knockout mice can live as wild-type mice. This demonstrates that MRP1 does not play a survival role in animal development. Thus, specific new MRP1 blockers can be developed to be used in cancer therapy regimens. Consequently, it might be of significant interest to clarify the role of MRP1 as a predictor of clinical response to chemotherapeutic agents.

Antibodies are very useful tools to detect and identify a protein, its structure, and its function. In this manner, different antibodies have been raised against MRP1 (13, 25, 33). These antiodies are used with limited proteolysis, site-direct mutagenesis, and epitope insertion studies to identify the exact topology of MRP1. To date, most of our knowledge about MRP1 membrane topology has been recovered with the use of antibodies. The protease hypersensitive region was detected with QCRL-1, NBDs of MRP1 were detected with mAbs (34, 35); OCRL-1 reacts with a linear epitope linker region of MRP1. The immunostaining assays indicate that this region is intracellular, as only the permeabilized cells can be detected with this antibody. Epitope mapping studies were performed with QCRL1, QCRL2, and QCRL3 monoclonal antibodies (33–35). OCRL-2, OCRL-3, and OCRL-4 recognize a conformation-dependent site of the protein, and QCRL-3 and QCRL-2 were shown to inhibit the transport function of MRP1 (6). Gao and coworkers demonstrated MSD1 and the first transmembrane segment of MRP1 to be important in LTC_4 transport using QCRL-1, MRPm6, and MRPr1 monoclonal antibodies (25). The N-glycosylation sites were also detected with these antibodies. Their study also demostrated the extracytosolic location of the N-terminal of MRP1, which is not a common feature of the ABC superfamily (36). Thus, generation of new antibodies which recognize different epitopes may help in understanding the exact features of the protein.

Generation of antibodies in mammals is invasive. In addition, antibody production in mice requires sacrifice of animals. General polyclonal antibody production protocols require blood collection and serum separation. Production of polyclonal antibodies against a mammalian protein in chicken and isolation of them from the egg yolk seems advantageous since the method is noninvasive, the animal does not need to be sacrificed, and the antibody titers in the eggs are higher than in mammals (37, 38). Additionally, chickens are phylogenetically distant from mammals (37–39). For this reason, production of antibodies against a mammalian protein seems easier to produce in chickens. Chickens have limited number of Ig genes than mammals

(40). Chicken antibodies (referred to as IgY) are quite different from mammalian IgG, for example, the heavy chain of chicken IgY is heavier than mammalian IgG (67–70 and 50 kDa, respectively). IgY does not bind to protein A or G (37). Egg contains as much Ig as serum (38, 41). So there is no need to take blood samples from chickens. Eggs contain approximately 200 mg Ig (38). Chicken housing is inexpensive and purification of antibodies from chicken eggs is fast and simple (42). The major problem in purification of IgY from eggs is the isolation of water-soluble proteins from lipoproteins and other fatty material (37, 43). Different methods have been suggested to overcome this problem (37, 39).

PREPARATION OF MRP1 FOR IMMUNIZATION

Because MRP1 is not commercially available, some methods to obtain MRP1 can be employed as described below. In one approach, cDNA of MRP1 can be fused with a protein such as glutathione S-transferase (GST) (13, 34, 44). Then, the fusion protein complex will be transfected to *Escherichia coli* and the MRP1-GST (or other protein) fusion complex is purified by affinity chromatography.

In another approach, membrane-enriched fractions of MRP1-rich cells (3) can be prepared. Long-term incubation of cells with chemotherapeutic drugs was found to cause more MRP1 expression than MRP1 gene transfected cells (3, 13).

PREPARATION OF MEMBRANE-ENRICHED FRACTIONS OF MRP1-EXPRESSING CELLS

Membrane-enriched fractions can be prepared according to Grant et al. (14). Briefly, cell pellet is resuspended in collection buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, and protease inhibitors) (Sigma-Aldrich Corp., St. Louis, MO, USA), then homogenized with a Potter-Elvehjem tissue homogenizer (Bellco Glass, Inc., NJ, USA) on ice approximately 80 cycles. The intact cells and nuclei are removed by centrifugation at 600 g at 4°C, and then the supernatant is centrifuged at 100,000 g at 4°C for 20 min to obtain the membrane-enriched fractions. The pellet is resuspended in resuspension buffer (10 mM Tris-HCl, pH 7.4 , 125 mM sucrose, and protease inhibitors). The membrane-enriched fraction is loaded on 5% stacking and 7% resolving SDS-PAGE. The band corresponding to MRP1 is excised from the gel and used for immunization (42).

CHICKEN IMMUNIZATION WITH MRP1

Nearly 24-week-old leghorn chickens are preferred for immunization (38, 45, 46). For the first immunization, approximately 100 μg of MRP1 protein is mixed with complete Freunds adjuvant (38, 41) to a final volume of 500 μL. Injections are made through the pectoral muscle and injections at different sites can give better results (47–49). Booster injections are made for 2- or 4-week intervals with the same amount of MRP1 in incomplete Freunds adjuvant (47–49). Eggs are collected after the second injection and stored at 4°C until antibody isolation process.

PURIFICATION OF IgY AGAINST MRP1

Many methods have been developed to isolate IgY from egg yolk (37, 39, 45, 49, 50). A simple method for purification of IgY against MRP1 is described here. Egg yolk is separated from egg white and washed with distilled water (46). Then egg yolk is placed on a funnel and its membrane is punctured, separated from its membrane, and yolk is poured into a graduate tube from the funnel. Nine volumes of water are added and the mixture is incubated for 6 h at 4°C. Then it is centrifuged at 10,000 g at 4°C and the supernatant containing polyclonal anti-MRP1 IgY is transferred into another tube. Nineteen percent sodium sulfate is added. Supernatant is removed and the precipitate is dissolved in phosphate-buffered saline (PBS), then dialyzed against PBS. For further purification of IgY, affinity chromatography is applied (42, 48, 49). The column is mixed with anti-chicken antibody, then the solution containing anti-MRP1 antibody is applied to the column and is eluted using acidic buffer (38, 42, 48). Antibody concentration is determined spectrophotometrically at 280 nm. Purified extracts of anti-MRP1 IgY are stored at 4°C in the presence of 0.1% NaN_3 as preservative.

Since chickens are phylogenetically distant from mammals, the possibility to gain specific anti-MRP1 polyclonal antibodies in chickens is high, and this methodology will increase our knowledge about the topology and funtions of MRP1 with the help of new chicken antibodies.

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