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Generation of Isotype Switch Variants from Hybridoma cells Producing anti-*Streptococcus pneumoniae* 6B Polysaccharide Antibody

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Hybridoma cells producing IgM anti-pneumococcal 6B polysaccharide antibodies were induced to switch to IgG-producing cells *in vitro* by treating with acridine orange. Treating 0.5 μg/ml of acridine orange for 24 hours generated maximal number of variant cells. The maximal isotype switch frequency was 3×10^{-5}, which is about 30-fold higher than the frequency of spontaneous switching. Resulting IgG-producing variants were enriched by sib selection and ELISA spot assay. Two IgG3-producing variant cells were finally cloned by limiting dilution. The variant cells produced similar amounts of antibodies as their parental cells did. The two switched antibodies had similar reactivity to pneumococcal 6B polysaccharide. When compared to their parental IgM antibodies, the switched IgG3 antibodies showed lower reactivity to 6B polysaccharide, which might be due to lower valency of IgG than that of IgM antibody. The antibodies will be useful as essential tools for comparative study of the role of heavy chain isotypes in protection against *Streptococcus pneumoniae*.

Key words: Isotype switching, anti-pneumococcal hybridoma cells

Streptococcus pneumoniae (pneumococcus) is a significant human pathogen which causes otitis media, sinusitis, pneumonia, septicaemia, and meningitis (6). The rapid spread of antibiotic resistant pneumococcus makes it necessary to develop efficient prophylactic measures to prevent pneumococcal diseases (1). Encapsulation is a prerequisite for virulence in pneumococcus, allowing the organism to evade phagocytic cells. However, in the presence of antibodies specific to capsular polysaccharide (PS), complement activation and phagocytosis can proceed (2). Currently available 23-valent vaccine is made of 23 capsular PSs of the 90 known serotypes of capsular PSs, but it is not immunogenic in young children, one of the groups at highest risk. To improve immunogenecity in young children, conjugate vaccines with PS linked to protein moiety are undergoing clinical trials (4, 8).

Human antibody reactivity to pneumococcal capsular polysaccharides is known to include several antibody isotypes, although several serotypes are shown to be dominated by IgG2 antibodies (5). And different types of vaccines might induce different isotype distribution. However, the functional differences among various isotypes of anti-pneumococcal antibodies are not well characterized because it is not easy to obtain antibodies to explore this. To better understand the function of each isotype in mediating protection from pneumococcal infections, it is necessary to have a panel of antibodies that have the exact same binding site associated with various isotypes.

Hybridoma cells are known to undergo isotype switching in vitro. But the low frequencies of spontaneous switching make it very difficult to isolate such a variant (10). The use of mutagens to increase switching frequency was reported by Spira *et al.* (12). They showed that acridine orange stimulated isotype switching of an IgG3 anti-*Cryptococcus neoformans* producing hybridoma cell to a panel of variants producing IgG1, IgG2b, IgG2a, IgE, and IgA. This approach could be applied to study functional differences among various isotypes of anti-pneumococcal PS antibodies. In this study, the effect of acridine orange on isotype switching of hybridoma cells producing anti-pneumococcal 6B PS antibodies were examined, and two isotype switched antibodies were characterized.

Materials and Methods

Assay of antibodies

Anti-pneumococcal 6B antibodies in culture supernatants were detected by enzyme-linked immunosorbent assay (ELISA). Microplate wells were coated with

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100 µl of pneumococcal 6B PS (10 µg/ml, American Type Culture Collection, Rockville, MD, USA) overnight in phosphate-buffered saline, pH 7.2 (PBS). The plates were washed with PBS containing 0.1% Tween 20 and incubated with blocking solution (PBS containing 1% bovine serum albumin and 0.05% Tween 20) for 30 min. Sample dilutions were added to wells and incubated for 2 h. Wells were washed four times followed by addition of goat anti-mouse polyvalent immunoglobulins-alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA). After incubation for 2 h, wells were washed, and p-nitrophenyl phosphate in diethanolamine buffer (915 mM diethanolamine, 0.24 mM $MgCl_2$, pH 9.8) was added. Absorbance was checked at 405 nm with a microplate reader. All the incubations were carried out at room temperature.

For quantification of mouse kappa chain antibodies, anti-mouse kappa monoclonal antibodies (5 µg/ml, Sigma) were used as capture antibody on the microplate well. And the mouse kappa chain antibodies bound to capture antibody were detected by anti-mouse kappa chain polyclonal antibodies-alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL, USA). Mouse IgG1/kappa monoclonal antibody (Southern Biotechnology Associates) was used for standardization of the assay.

Preparation of monoclonal antibody

5 µg of pneumococcal 6B PS suspended (1:1) in Freund's complete adjuvant was injected intraperitoneally into 5-week-old female BALB/c mice. After three weeks, the mice were injected with the same amount of 6B PS in Freund's incomplete adjuvant. And 3 weeks after 2nd immunization, 6B PS was injected intravenously into mice, and the spleen was removed for fusion after 3 days from the last injection. 2×10^7 Sp2/O-Ag14 myeloma cells were mixed with 10^8 spleen cells from immunized mouse. The cells were fused with 50% (vol/vol) polyethylene glycol 1450 (Sigma) in Dulbecco's modified eagle's medium (DMEM). After fusion, the cells were suspended in post-fusion medium containing 15% fetal bovine serum (HyClone, Logan, Utah, USA), 10% hybridoma cloning supplement (Boehringer Mannheim, Mannheim, Germany), 0.5% Nutridoma-SP (Boehringer Mannheim) and 0.6% lipopolysaccharide (Sigma) in DMEM. The cells were incubated at 37°C with 5% CO_2 for 24 h. Hybridoma cells were selected by incubating in hypoxanthine-aminopterin-thymidine medium (Sigma). Supernatants in hybridoma-growing wells were screened by ELISA for anti-pneumococcal 6B PS antibodies. Cloning was carried out by limiting dilution at a cell density of 5 cells per ml in HT medium. Cloned cell lines were cultured in DMEM containing 10% FBS. Culture supernatants were collected and used as antibody solution.

Isotyping of monoclonal antibody

The isotype of each monoclonal antibody was determined using a mouse-hybridoma subtyping kit (Boehringer Mannheim) and comfirmed by Ouchterlony double diffusion with isotype specific goat anti-mouse immunoglobulin (Sigma).

Mutagenesis

Isotype switching of hybridoma cell was induced by incubating cells with acridine orange (Sigma). The effect of acridine orange on viablity of cells was examined by incubating 10^6 cells per ml in 10% FBS/DMEM with various concentrations of acridine orange. After incubation for 24 h, the cells were washed three times with DMEM and diluted into soft agar (0.38% agarose in cloning medium: 15% FBS, 10% hybridoma cloning supplements, 0.5% Nutridoma-SP in DMEM). After incubation for 7 days, the number of viable clones was determined and compared with that of control sample which was not treated with acridine orange.

Concentration of acridine orange was optimized to increase the frequency of isotype switching. 10^6 hybridoma cells were suspended in 1 ml of 10% FBS/DMEM containing various amounts of acridine orange and incubated at 37°C for 24 h. After washing three times in DMEM, the cells were suspended in cloning medium and dispensed into microplate wells (1000 cells/well). At 50% cell confluency, some of the cells were taken and used in ELISA spot assay for determination of isotype switching frequency. The concentration of acridine orange which induced the maximal switching frequency was used to generate switch variants.

Isolation of isotype switch variants

The isolation of switch variant cells were carried out by sib selection (10) and ELISA spot assay (11). The cells in the well which showed highest number of isotype switch variants were diluted in cloning medium and about 1,000 cells were added to new wells. At about 50% cell confluency, some of the cells were examined for IgG-producing variants by ELISA spot assay. The well showing the highest switching was selected, diluted in cloning medium, and plated into new wells. By repeating this process, the isotype-switch variant were enriched, and finally the variants were cloned by limiting dilution.

ELISA spot assay

Isotype-switch variants producing IgG antibodies were detected by ELISA spot assay for IgG. Polysorb microtiter plate (Nunc, Rochester, NY, USA) was coated by incubating overnight at room temperature

with anti-mouse IgG (5 μg/ml. Sigma). After blocking and washing, 10^4 hybridoma cells were added to each well and the plates were incubated for 6 h in 37°C CO_2 incubator. Biotin-conjugated anti-mouse IgG (Serotec, England) and streptavidin-AP (Sigma) were sequentially added to the wells. Each step was carried out at room temperature for 2 h. Finally BCIP substrate (Sigma) was added to the wells, and the spots which were produced by IgG-producing variant cells were detected using dissecting microscope.

Reactivity of isotype-switched antibodies

Reactivity of parental and isotype-switched antibodies to pneumococcal 6B PS was tested by two types of ELISA. The first procedure was the same as the one used for the assay of antibodies in culture supernatant except that goat anti-mouse kappa-AP conjugate (Sigma) was used as developing antibody. The second procedure was inhibition ELISA. Microplate wells were coated with pneumococcal 6B PS (1 μg/ml, solid phase 6B) by incubating overnight at room temperature. After blocking, serially diluted 6B preparations (fluid phase 6B) and antibody solutions (final concentration, 2 μg/ml) were added to the wells, and the plates were incubated for 2 h. The antibodies bound to solid phase 6B were detected by sequential incubations with goat anti-mouse kappa-AP conjugate and NPP substrate. Each step was carried out at room temperature for 2 h.

Results and Discussion

Monoclonal antibodies to pneumococcal 6B PS

About twenty 6B binding clones were detected during the selection process of hybrodoma cells after fusion, and all of them produced IgM antibodies. The dominance of IgM monoclonal antibodies to pneumococcal 6B PS could be due to T cell-independent characteristic of 6B PS antigen. Finally, 2 cell lines were cloned, which produced IgM antibodies with kappa light chain (Fig. 1).

Mutagenesis and frequency of isotype switching

Although antibody producing cells switch isotype spontaneously, the frequency is very low and numerous number of samples need to be analyzed to isolate one isotype switch variant (10). To increase the frequency of isotype switching, hybridoma cells were treated with acridine orange. Since acridine orange is a potent mutagen, its effect on cell viablity was examined first. Acridine orange significantly affected the viability of hybridoma cells. 0.06 μg/ml of acridine orange started to affect cell viablility, and 2.0 μg/ml killed more than 90% of the cells in 24 h (Fig. 2).

To determine optimal concentration of acridine orange to induce isotype switching, IgM-producing cells were treated with various concentrations of acridine orange, the resulting IgG-producing variants were counted by ELISA spot assay. The frequency of isotype switching was greatly affected by the concentration of acridine orange (Fig. 2). Treating the cells with 0.5 μg/ml of acridine orange for 24 h yielded the highest number of variants (about 30 variants/microplate). About 10^6 cells were added to one microplate, and the maximal frequency of isotype switching of IgM-producing cells to IgG-producing cells by acridine orange was 3×10^{-5}, which is approximately 30 times higher than that of spontaneous switching (11). The abrupt decrease in variant frequency at concentrations above 0.5 μg/ml of acridine orange is probably due to the killing effect of acridine

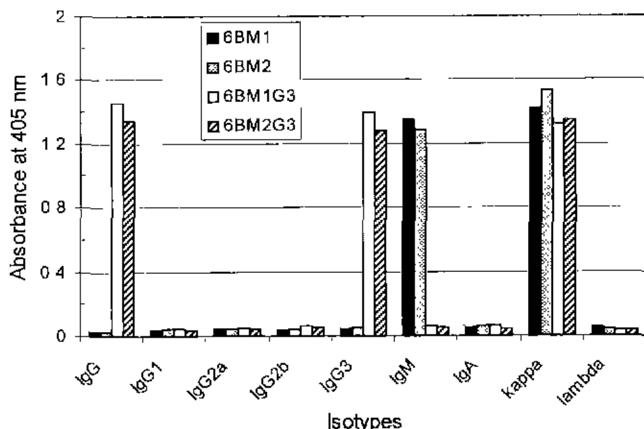

Fig. 1. Isotypes of monoclonal antibodies. The isotype of each monoclonal antibody was determined by ELISA with a mouse-hybridoma subtyping kit.

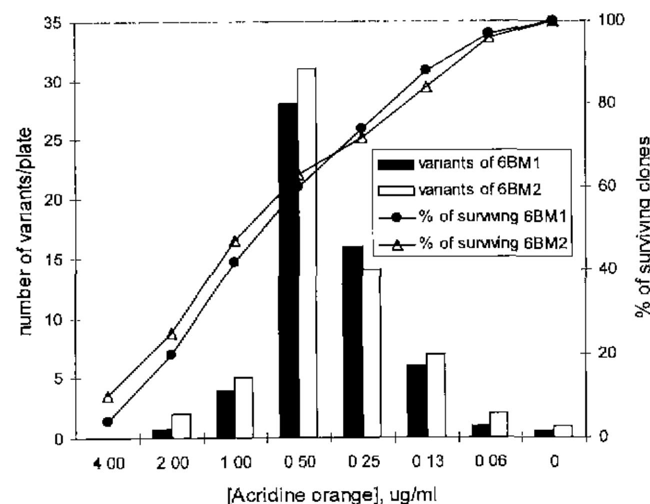

Fig. 2. Effects of acridine orange on cell viability (lines) and switching frequencies (bars).

orange.

It is not easy to isolate spontaneous switch variants from IgM-producing hybridomas because of the low frequency of spontaneous switching. This prompted the efforts to develop methods by which the frequency of switching could be increased. Immunoglobulin switching has been shown to be affected in vivo by a variety of interleukins. The production of gamma 1 and epsilon chain by mouse spleen cells, for example, is enhanced by interleukin 4 (IL-4) (9). IgM and IgA are enhanced by IL-5, and IgA is enhanced by transforming growth factor β(3, 13). However, attemps to increase the frequency of isotype switching of isolated cell lines using interleukins in vitro have been only partially successful. Due to the high variations in the concentration and exposure time of interleukins, only few cell lines have succeeded in switching at a 40-fold higher frequency (14). In addition to high variation at optimum conditions, the recombinant interleukins are too costly to be used routinely for isotype switching experiment. The approach to use mutagens for enhancing the frequency of isotype swiching was used for few hybridoma cells. In 1995, Paizi *et al.* (7) tested the effects of four mutagens (melphalan, mitomycin, ethyl methanesulfonate, and acridine orange) on the frequency of switching. Among the four mutagens, acridine orange enhanced isotype switching of hybridoma cells the most consistently. Our result showing approximately 30-fold increase in the frequency of isotype switching of two hybridoma cells producing anti-pneumococcal 6B PS antibodies be acrdine arange-confirms its usefulness in isotype switching of hybridoma cells.

Table 1. Characteristics of parent and isotype switched cell lines

Cell line	Isotype	Ig production (μg/ml)[1]	Reactivity to 6B[2]
6BM1	μ, κ	3.7	2.12
6BM2	μ, κ	1.3	1.68
6BM1G3	γ3, κ	2.4	0.39
6BM2G3	γ3, κ	2.1	0.34

[1] Cloned cells were cultured in DMEM containing 10% FBS at 100% confluency for one day and their supernatants were assayed for mouse kappa chain.
[2] absorbance at 405 nm in an ELISA where 2 μg/ml of antibody was applied, and substrate was developed for 30 minutes at room temperature

Characteristics of isotype switched antibodies

The antibody produced by the two isotype-switched variants had gamma 3 and kappa chains. And their antibody production capacities were not different from their parental cells (Table 1).

Reactivities of parental and isotype-switched antibodies to pneumococcal 6B PS were tested by two types of ELISA. To compare reactivities of antibodies, it is important to accurately determine the concentrations of antibodies tested. Since ELISA using antigen as capture molecule is greatly affected by the affinity between the antigen and antibody, an affinity-independent assay of antibody is necessary for accurate quantification. In addition, an ELISA procedure for mouse kappa chain was developed for accurate quantification of the antibodies because all the antibodies used in this experiment had kappa light chain.

When the two parental IgM anibodies were tested for their reactivity to 6B PS by ELISA, they showed similar reactivities to solid phase 6B PS (Fig. 3A). And the two isotype-switched IgG3 antibodies also showed similar reactivities to solid phase 6B PS. However, the IgM antibodies showed more than 6 times higher absorbance values than the IgG3 antibodies, which

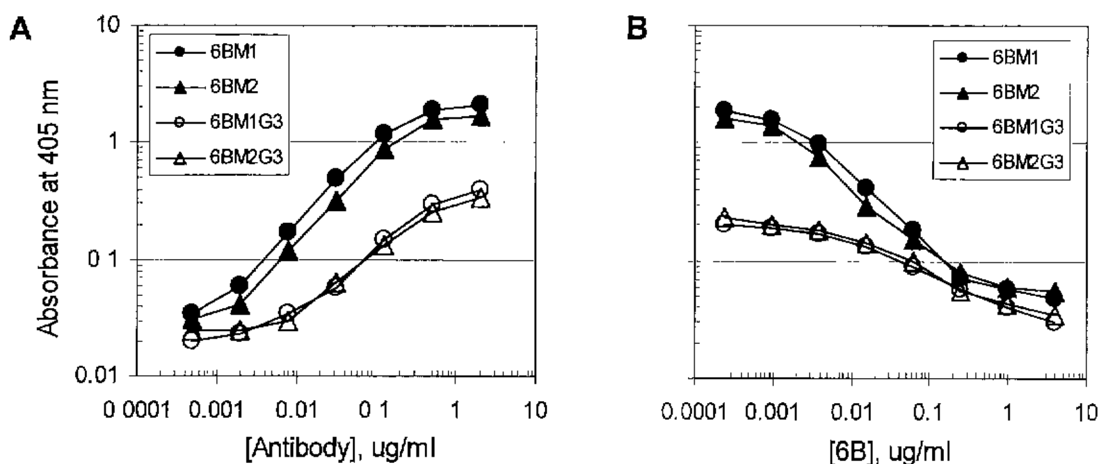

Fig. 3. Reactivity of each antibody to pneumococcal 6B polysacchride: binding to solid phase (absorbed to microplate wells) 6B polysaccharide in the absence of fluid phase 6B (A) or in the presence of fluid phase 6B (B).

means IgM antibodies have much higher reactivities to 6B PS than IgG3 antibodies. In the inhibition assay where fluid phase 6B inhibits the binding of antibodies to the solid phase 6B, 50% inhibition concentrations of fluid phase 6B for 6BM1 and 6BM2 antibodies were 9 ng/ml and 7 ng/ml, respectively (Fig. 3B). But the 50% inhibition concentrations of fluid phase 6B for the two isotype-switched IgG3 antibodies were 80 to 90 ng/ml, which are roughly 10 times higher than those for the IgM parental antibodies. Since the 50% inhibition concentration is relevant to the avidity of antibody to antigen in fluid phase, this result also indicates that the IgG3 antibodies have much lower avidity to 6B PS. This lower avidity of IgG3 antibodies than IgM antibodies is certainly due to the lower valency of IgG3 antibody.

IgM antibodies generally have low intrinsic affinity but due to their pentameric structure they could have high avidity. The change of heavy chain constant region of IgM to IgG antibodies will be accompanied by decrease in antibody valency, which will in turn decrease antibody avidity. This lower antibody avidity could restrict the usefulness of isotype-switched antibodies in some cases. According to a recent report (12), a hybridoma cell producing IgG3 antibodies against *Cryptococcus neoformans* was switched sequentially to IgG1, IgG2b, IgG2a, and IgE antibodies. The IgG3 antibodies prepared in this study could also be used for isotype switching to other IgG isotype or IgE antibodies. This could lead to the production of a panel of anti-pneumococcal 6B PS antibodies which are different in their heavy chain constant regions but are identical in variable region structures. Such antibodies will be valuable tools in the study of functional differences among antibody isotypes in the protective efficacy against *S. pneumoniae*.

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