Frequency and Distribution of *Pseudomonas aeruginosa* Serotypes 03, 06, 011 in Three Northwestern Ohio Hospitals as Determined by ELISA Using Specific Monoclonal Antibodies[1]

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ABSTRACT. Hybridoma producing monoclonal antibodies (mAbs), specific for three clinically significant *Pseudomonas aeruginosa* (serotypes 03, 06, and 011), were generated to investigate the prevalence of these serotypes in three Northwestern Ohio hospitals. Fusion products reacting with bacterial cells or membrane extracts were detected by enzyme-linked-immunosorbent assay (ELISA). Three mAbs designated: 72C, 11 H (IgM) and 11E (IgG2b), were selected. These mAbs reacted with approximately 40% of the clinical isolates of *P. aeruginosa* in each hospital. The incidence of serotype 011 varied in these hospitals, ranging from 13.2%-23.8%. Serotype 011 predominated in two of the three hospitals. The prevalence of serotype 06 was similar in all three hospitals (13.6-15%). In one of the hospitals (Hospital 2), the occurrence of serotype 06 was slightly higher (15%) than serotype 011 (13.2%). Serotype 03 occurred less frequently (1.5%) in one of the hospitals than in the other two (10-11%). None of the serotypes showed clear predilection toward any body site. The mAbs did not react with other strains of *P. aeruginosa*, nor with other gram-negative or gram-positive organisms. The results of Immunofluorescence and Western blot correlated well with ELISA. However, ELISA showed a higher sensitivity, indicating the usefulness of this technique for serotyping *P. aeruginosa*.

INTRODUCTION

The significance of *Pseudomonas aeruginosa* as an opportunistic pathogen has been recognized for many years (Bodey and others 1983, Olson and others 1984, Grundmann and others 1993). This organism is responsible for nosocomial infections among patients compromised by trauma, burns, respiratory illnesses, surgical wounds, cancers, and HIV (Singer and others 1977, McManus and others 1985, Kern and others 1990, Shepp and others 1994).

The increased prevalence of this multiple-antibiotic-resistant organism, in many hospitals is alarming. Despite the availability of some excellent anti-microbial agents against *P. aeruginosa*, the overall mortality and morbidity have not changed significantly. Thus, a reliable system for determining the source of infection is necessary to control the spread of this organism.

Various typing methods have been applied to differentiate isolates of *P. aeruginosa* and to determine the prevalence of the different strains. These methods include: biotyping, serotyping, bacteriophage typing, pyocin typing, and antimicrobial susceptibility testing (Bergen 1973, Pitt and Erdman 1977, Pitt 1981, Pitt and others 1986, Pitt 1988, Conroy and others 1983, Patzer and others 1986). In recent years, DNA typing and plasmid profiling have also been used (Poh and others 1988, Poh and Yeo 1993, Grundmann and others 1995, Lau and others 1995, Millesimo and others 1996).

Among the various differential characteristics, serotyping is used most frequently. This method is repro-ducible, easy to perform, and discriminatory (Webster and Ridgway 1988) The most complete serotyping system for *P. aeruginosa*, the International Antigenic Scheme (IATS), consists of 20 standard 0 serotypes (Liu and others 1983, Liu and Wang 1990).

Until recently, the slide agglutination technique using commercially available rabbit polyclonal antisera was employed for typing *P. aeruginosa*. Although this is a simple technique, its application has certain limitations. For instance, some strains are not typeable, and some are either autoagglutinable or polyagglutinable, therefore it can yield unreliable results (Pitt and others 1986). To enhance the efficiency of serotyping, monoclonal antibodies have been produced and applied for strain differentiation of *P. aeruginosa* (Lam, MacDonald and Lam 1987; Lam, MacDonald, Lam and others 1987). The application of these reagents has reduced the frequency of cross-reactivity and improved the overall efficiency of *P. aeruginosa* typing (Webster and Ridgeway 1988).

Among the twenty different serotypes of *P. aeruginosa*, only certain members have shown clinical significance; the prevalence of these strains varies from one region to another (Ullman and Schmülling 1980, Farmer and others 1982). Presently, there is no information available concerning the prevalence of *P. aeruginosa* strains in regional hospitals of Northwestern Ohio. In addition, the application of ELISA using specific monoclonal antibodies in epidemiological studies of *P. aeruginosa* has not been described adequately. In the present investigation, a panel of specific mAbs to three clinically significant strains of *P. aeruginosa* (serotypes 03,06 and 011) was produced to investigate their prevalence in three regional hospitals by ELISA. The reliability of ELISA was confirmed by IFA and Western Blotting.

MATERIALS AND METHODS

Microorganism

Samples of 17 original IATS of *P. aeruginosa* were obtained from the University of British Columbia (Courtesy of Dr. R.E.W. Hancock).

The 404 clinical isolates of *P. aeruginosa,* and other gram-negative and gram-positive microorganisms, were obtained from three major regional hospitals for use in this investigation. The sources of clinical isolates included; sputum, urine, wounds, feces, genital secretions, blood, body fluids, and so forth.

The microorganisms were first propagated on tryptic soy agar (TSA, Difco) to establish purity. Pure colonies of each microorganism were grown in tryptic soy broth (TSB) and aliquots were frozen at -80° C. The microorganisms were thawed and propagated on TSA before each application.

Preparation of Cell Membrane

The procedure of Yang (1982) was used for membrane preparation. *P. aeruginosa* (serotypes 03, 06 and 011) was propagated in TSB broth and incubated over night at 35° C. The cells were harvested by centrifugation, suspended in phosphate buffer saline (PBS, pH 7.2); and disrupted by sonication. The preparations were centrifuged at 10,000 X g for 10 min. Then the supernatant was collected and centrifuged at 40,000 X g for 90 min. The supernatant was discarded, the pellets were collected, resuspended in PBS and the protein concentration was determined and adjusted to about 5 mg/ml with the same buffer. Triton X-100, in 10% (w/v) solution, was added to a final concentration of 2:1 of the Triton and protein, respectively. After centrifugation at 40,000 X g for 1 h, the supernatant was collected and dialyzed against PBS. Protein concentration was adjusted to 2 mg/ ml and aliquots were frozen at -80° C for future use.

Immunization Procedure

The cell membrane preparation (2 mg protein/ml) was mixed with an equal volume of complete Freund adjuvant. BALB/c mice were immunized intramuscularly, (i.m.), with 0.2 ml of the above mixture. They were boosted at weekly intervals for eight weeks with the above preparation without the adjuvant. The last injection was administered intraperitoneally (i.p.) 48 h before the fusion experiment.

Fusion Experiment

A previously described procedure was used (Jamasbi and others 1992). BALB/c myeloma cells, SP2/0, were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2μM//ml L-glutamine, 100 μg/ml streptomycin, 100 μl/ml penicillin and 20% fetal bovine serum. The media and supplements were purchased from GIBCO Laboratories (Grand Island, NY). On the day of the experiment, an immunized mouse was sacrificed and its spleen was removed aseptically. A single-cell suspension was made, and 3×10^7 cells were fused with 5×10^7 myeloma cells. The fusion medium was 45% polyethylene glycol in DMEM. After fusion, the cells were washed and resuspended in DMEM containing 100 mM

hypoxanthine, 10 mM aminopterin, and 30 mM thymidine (HAT) medium. The cells were plated at 105 cells/ well in 96 well plates (Corning Inc., Corning, NY), and incubated at 37° C in an 8% CO_2 atmosphere with 100% humidity. At 10 to 14 days after fusion, supernatants of growing hybridoma cells were assayed for an antibody to *P. aeruginosa* using ELISA (see below).

Positive hybridomas were subcultured and tested against a battery of different bacterial species using ELISA. Three hybridomas producing specific antipseudomonas antibodies were subcultured and cloned by limiting dilution. The cloned hybridomas were expanded and supernatants were collected. The mAbs were precipitated from each hybridoma supernatant with 50% ammonium sulfate, reconstituted in 1/25 of the original volume with PBS, and dialyzed against PBS three times; the aliquots were then frozen and stored at -80° (Jamasbi and others 1992). This preparation (1:10-1:100 dilution) was used in all experiments. The subclass of each mAb was determined by ELISA using an immunoglobulin typing kit (Southern Biotechnology Associates, Inc., Birmingham, AL). The titer of mAbs was determined by preparing serial dilutions, and ELISA testing.

ELISA Procedure

A modification of a previously employed procedure was used (Jamasbi, and others 1992). For this purpose, 1 μg/well of bacterial protein (membrane preparation) in 50 μl of PBS was added to each well, fixed and allowed to dry. To perform ELISA, the plates were washed with PBS; 100 μl of undiluted supernatant, from hybridoma culture fluids, were added to each well and incubated at 37° C for 1 h. The wells were washed and incubated with 100 μl of diluted (1:400) ß-galactosidase-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, AL). After 1 h incubation at 37° C, the plates were washed, and 100 μl of 1 mg/ml substrate solution (p-nitrophenyl-ß-D-galactopyranoside) were added to each well, and allowed to develop at 37° C for 1 h. The optical density was measured at 410 nm using ELISA plate autoreader, MR-600 (Dynatech Laboratories, Inc.). When the whole bacterial cells were used as antigens, approximately 10^8 washed bacterial cells in PBS were fixed into each well and tested by ELISA.

Immunofluorescence Assay (IFA)

Bacterial smears were made, air-dried, heat fixed, washed and incubated with 100 μl of 1:10 dilution of each mAb in 1% BSA/PBS for 30 min at 37° C. The slides were washed and incubated with 100 μl of a 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, MO) for 30 min at 37° C. The slides were washed and examined by fluorescence microscopy. A limited number of direct smears provided by the hospitals were similarly tested.

Reactivity of mAbs With the Remaining Serotypes of *P. aeruginosa* and Other Microorganisms

To determine whether the mAbs reacted with the remaining serotypes of *P. aeruginosa* (IATS), bacterial

cells (different serotypes) were cultured in 96-well plates and tested by ELISA. The specificity of the mAbs was further tested by ELISA using other *Pseudomonas* species and several other gram-negative (*E. coli, E. aerogenes, Proteus* species) and gram-positive (*S. aureus, S. faecalis* and *Bacillus* cereus) organisms.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in 10% polyacrylamide gel (Jamasbi and others 1992). Approximately 20 µg solubilized protein/lane were applied. The gel was subjected to Western transfer (Towbin and others 1979). Following the transfer, the nitrocellulose membrane was blocked with 5% non-fat dried milk, washed twice in washing buffer (150 mM NaCI, 50 mM Tris HCI, pH 7.2 and 0.5% Tween-20), and incubated with the mAb (1:50 dilution) for 1 h at room temperature. The immunoblots were washed and stained with 1:500 dilution of secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG [Sigma]) and reacted with substrate (36 ml, PBS; 12 ml, 3 mg/ml in methanol of 4 chloro-1-naphthol and 20 µl of H_2O_2).

RESULTS

Determination of Binding Capacity and Specificity of Monoclonal Antibodies by *P. aeruginosa*

Three mAbs, designated 72-C (IgM), 11 E (IgG2b), and 11 H (IgM), were generated and found to react specifically with; serotypes 03, 06 and 011, respectively. The mAbs were tested against 404 clinical isolates of *P. aeruginosa* obtained from three Northwestern Ohio hospitals. Using ELISA, these mAbs reacted with 40.7%, 39.5% and 39.1% of the isolates received from each hospital (Table 1). The frequency of serotype 06 was not significantly different in the three participating hospitals (13.6-15%). However, the incidence of this serotype was slightly higher (15%) than serotype 011 (13.2%) in hospital #2. The occurrence of the serotype 011 was different in the three hospitals, ranging from 13.2% to 23.8% (Table 1), indicating that the incidence of this serotype differed in each of the clinical settings. The incidence of the serotype

03 (Table 1) was much lower (1.5%) in one of the hospitals than in the two others (10-11%).

The mAbs did not react with the remaining strains of IATS serotypes of *P. aeruginosa* or to any other pseudomonas species; nor did they react with any of the other gram-negative or gram-positive microorganisms (data not shown), indicating the high degree of specificity of the mAbs for the corresponding serotype of *P. aeruginosa*.

Site Distribution

To determine whether any of the three serotypes of *P. aeruginosa,* preferentially infect any specific organs, the frequency of the three serotypes in different anatomical sites were determined (Table 2). Again, serotype 011 showed higher frequency in all anatomical sites except the ears, where serotype 06 had a slightly higher occurrence.

Determination of Binding Capacity of Monoclonal Antibodies by IFA

To determine whether a similar degree of sensitivity and specificity observed by ELISA, can be achieved by IFA, direct smears or bacterial smears were stained by indirect IFA and studied by fluorescence microscopy (Counts and others 1988). A strong correlation between ELISA and IFA was obtained. Positive strains of *P. aeruginosa* showed strong fluorescence staining (Fig. 1), while negative strains remained negative (data not shown). The ELISA method displayed a much higher degree of sensitivity than the IFA technique. A 1:100 dilution of the mAbs resulted in an optimum ELISA reading; a dilution of 1:10 of the same mAbs was required to achieve an optimum IFA result.

Immunoblotting

Immunoblotting experiments were conducted to determine the specificity and pattern of reactivity of the mAbs with the soluble membrane antigen extracted from several positive strains (as determined by ELISA). The mAbs blotted the corresponding antigens specifically and produced ladder-like banding profiles on nitrocellulose membranes (Lam, MacDonald and Lam 1987;

TABLE 1

*Frequency of serotypes 03, 06 and 011 in each participating hospital as determined by ELISA using specific monoclonal antibodies.**

Participating hospitals	Total isolates	Monoclonal antibodies to:			% of Positive Stains
		Serotype 03 mAb-72C	Serotype 06 mAb-11 E	Serotype 011 mAb-11 H	
Hospital 1	221	22 (10.0)[†]	30 (13.6)[†]	38 (17.1)[†]	40.7
Hospital 2	53	6 (11.3)	8 (15.0)	7 (13.2)	39.5
Hospital 3	130	2 (1.5)	18 (13.8)	31 (23.8)	39.1

P. aeruginosa was isolated from different specimens, identified by an established microbiological procedure and serotyped by specific mAbs using ELISA.
[†]Numbers in parenthesis indicate percentage.

TABLE 2

*Frequency of serotypes 03, 06 and 011 in different
clinical specimens obtained from the three hospitals.**

Clinical sources	No. of isolates	Serotype		
		03	06	011
urine	135	10	14	20
respiratory secretions[†]	134	8	21	30
Wound	49	3	9	12
feces	28	4	2	5
ear exudate	15	1	6	4
blood	6	0	1	2
eye exudate	3	0	0	0
genital secretion	2	1	0	0
throat	3	0	0	0
pleural cavity	2	0	0	0
nasal sinus	1	0	0	0
gastric content	1	0	1	0
unidentified	25	3	1	3
Total	404	30	55	76

P. aeruginosa was isolated from different sources, identified by an established
microbiological procedure and serotyped by specific mAbs using ELISA.
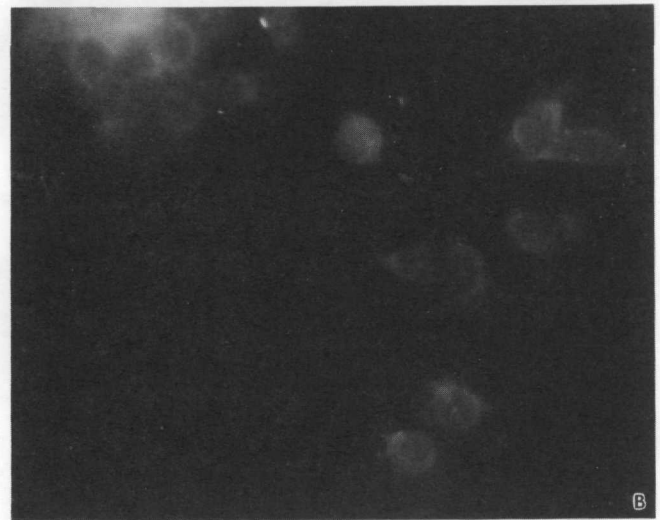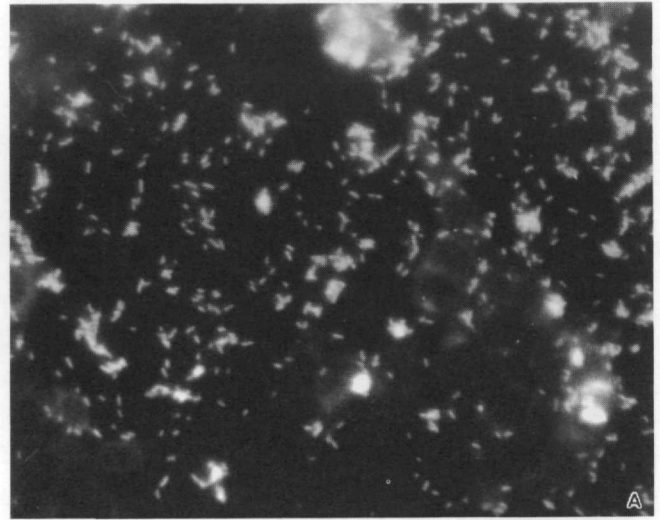[†]Sputum or tracheal washing.

FIGURE 1. Immunofluorescence assay: A direct smear of a *P. aeruginosa* strain was incubated with mAb-11 E (A), or with unrelated mAb (B) for 30 min at 37° C. The slides were washed and incubated with the secondary antibody (fluorescein isothiocyanate-conjugated rabbit antibody to mouse immunoglobulin) for 30 min. The slides were washed and examined by a fluorescence microscope (Olymus BH-2). Magnification 2,000X. Non-specific strain(s) did not stain (data not shown).

Lam, MacDonald, Lam and others 1987, Lam and others 1992). For the sake of brevity only one immunoblot is shown in Figure 2. Again, the results of the immunoblotting experiments correlated with the ELISA findings.

DISCUSSION

P. aeruginosa strains account for about 10-11% of all nosocomial infections (Bennett 1974, Jarvis and Martone 1992). In burn and cancer centers, it is the leading gram-negative isolate causing 30% of all infections (Cross 1985). Among the twenty different serotypes of *P. aeruginosa,* certain members appear to cause the majority of nosocomial infections. However, the prevalence of these stains varies from one location to another.

The prevalence of serotype 011 and its association with the outbreak of infection in different clinical settings have been reported (Yokota and Noguchi 1994, Hostacká and Majtán 1997). Englender and others (1990) found serotype 011 to be hospital-acquired and the most prevalent serotype, accounting for 23% of the isolates. Orsi and others (1994) recovered 73 environmental and clinical isolates from a single hospital and found that serotype 011 was most frequent in the environment, whereas serotypes 06, 012, 02, and 05 predominated among clinical isolates. Based on these findings, they concluded that environment was not an important source of *P. aeruginosa* infections in their hospital and that the transfer of the organisms was mainly from patient-to-patient. Poh and others (1988) reported that the dominant serotypes in their investigation, were 011 (38%) and 04 (12%).

Other investigators have found serotype 06 to be the most frequently detected serotype in their clinical settings. The high incidence of serotype 06 in pediatric patients was reported by Ullmann and Schmülling (1980). Young and Moody (1974) found the presence of serotype 06 in 36% of the cases. Pitt (1988) reported that this serogroup accounts for 21.6% of the strains identified in the United Kingdom. Patzer and others (1986) found that serotype 06 and 02 were the most frequently isolated strains. Bergan (1973) observed 0 serotypes 3 and 6 to be the most prevalent strains. Brokopp and others (1977) found 0 serotypes 1, 4, 6, and 11 to be the predominant

serogroup. Conroy and others (1983) found 44.2% of *P. aeruginosa* isolated from bacteremic patients belonged either to 01, 06, or 011. Richet and others (1989) found serotype 016 to be predominant in hematology and oncology patients. The above findings, and the results of the experiments reported here, indicate that the ecology of *P. aeruginosa* strains differs in different regions. However, it appears that serotypes 06 and 011 are the most frequently encountered strains in many clinical settings.

The incidence of serotype 03 was 4% and 6% in studies reported by Legakis and others (1982) and Ojeniyi and Høiby (1991), respectively. The high incidence of this serotype (10-11%) found in two of the hospitals in our study may suggest nosocomial spread of this serotype.

Of the 404 isolates recovered from the three participating hospitals, three sites of infection dominated. Specimens obtained from urine, respiratory secretions (sputum and tracheal washing), and wounds accounted for approximately 79% of the strains (Table 2). These are common infection sites associated with *P. aeruginosa*

infections (Sherertz and Sarubbi 1983). Although, in the present study, serotype 011 was the most frequently observed serotype in these organs, no correlation between the serotypes and site of infection was demonstrated. Other investigators (Webster and Ridgway 1988) have reached similar conclusions.

Other methods, such as antimicrobial susceptibility testing, bacteriocin typing, and phage typing, have been used to differentiate *P. aeruginosa* strains. Each of these typing methods has advantages and disadvantages, but they can be useful if applied with serotyping. The disadvantage of antibiotic susceptibility testing includes limited sensitivity for highly resistant organisms (such as *P. aeruginosa*). Bacteriocin typing lacks standardization and is not reproducible; the disadvantages of bacteriophage typing include lack of standardization, poor reproducibility, and limited availability of different bacteriophages (For review see Pfaller 1991).

In recent years newer techniques such as, plasmid profiling, and DNA typing have been used for strain differentiation. Plasmid pattern analysis provides a rapid characterization of bacterial isolates. However, this technique appears to lack sufficient sensitivity (Poh and Yeo 1993). Other DNA-based techniques, such as DNA probe (Grundmann and others 1993) or DNA finger printing (Grundmann and others 1995, Lau and others 1995), have been applied and found to be superior to phenotypic typings. It should be noted, however, that two different DNA typing systems may give conflicting results (Ojeniyi and Høiby 1991). The above descriptions indicate that a single typing method is insufficient for strain differentiation of *P. aeruginosa*. By combining several methods it might be possible to obtain a higher degree of discrimination between strains. Presently, we are conducting antibiotic susceptibility tests, biotyping, plasmid profiling and DNA typing on the positive serotypes to further substantiate the relatedness or variation among different strains in the same serogroup.

Regardless of limitation, our data indicate that serotyping by specific monoclonal antibodies, using ELISA, can provide valuable information as a primary screening method in determining the prevalence of different serotypes of *P. aeruginosa*, in various clinical settings.
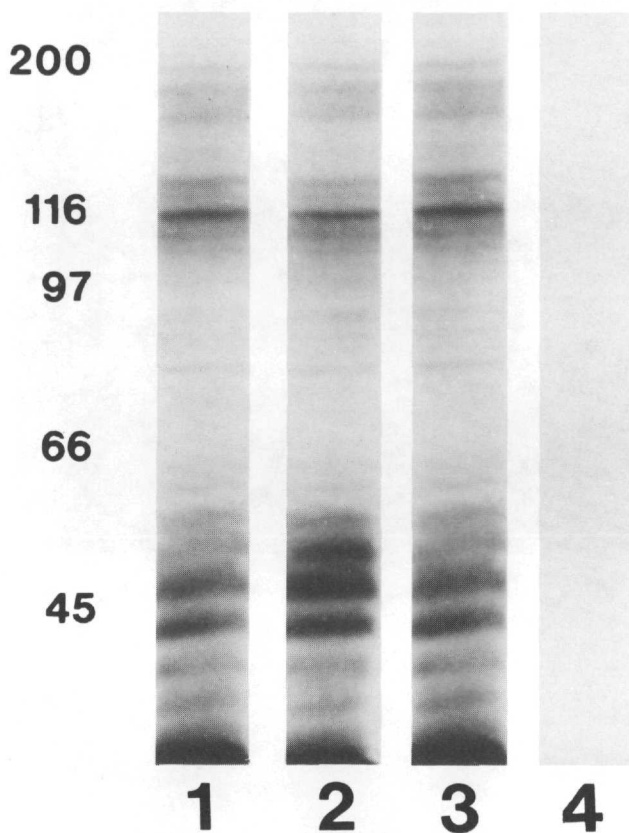

FIGURE 2. Western blot analysis: Solubilized membrane preparations (20 µg protein/lane of *P. aeruginosa* serotype 06 (lane 1) and two positive serotype strains from the clinical sources (as determined by ELISA; lane 2, 3, and one negative strain [lane 4]) were run on SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane. The membrane was washed, and reacted with mAb-11 E for 1 h at room temperature. The membrane was washed and further incubated with horseradish peroxidase-conjugated goat antibody to mouse immunoglobulin and detected by substrate solution (9 ml of 0.3% 4-chloro-1-naphthol in methanol, 27 ml PBS, pH 7.2 and 15 µl of 30% H_2O_2). Similar results were obtained under reducing or non-reducing conditions. Numbers on the left side indicate molecular weight standards.

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