Preparation of Polyclonal Antibody against Human MxA Protein and Its Specificity to Diversified Myxovirus Resistant Protein A[1]

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Objective To study the human myxovirus resistant protein A (MxA), a specifically induced peptide by interferon I, and to use its level as a diagnostic criterion for viral infections. **Methods** Anti-MxA antisera from immunized mice were prepared with the expressed MxA protein of pET32a-MxA in *E. coli* BL-21(DE3). To confirm the antiserum activity and specificity, the expression product of BL21, wild type MxA pEGFP-C1-wMxA and site-directed mutant MxA pEGFP-C1-mMxA(N589S) stably transfected 3T3 cells and induced A549 cells were detected by Western blot with the antisera using non-MxA transfected or non-IFN-β induced cells, intact A549, NIH 3T3 cells transfected with pEGFP-C1 and pET32a (+)-transformed BL-21 as controls. **Results** The antisera had specific positive immunoreactivity to the NIH3T3 cells transformed with pEGFP-C1-wMxA and pEGFP-C1-mMxA, INF-β induced A549 cells and BL21 proteins expressed with pET32a (+)-MxA. The hybridization signals from IFN-β induced A549 cells depended on the IFN-β inducing concentrations. Meanwhile, immunohistochemical assay showed that NIH 3T3 cells with pEGFP-C1-wMxA and pEGFP-C1-mMxA had > 98% of positive cells at 1:50 dilution of the serum and A549 cells induced by 20 ng/mL IFN-β for 48 h showed 95% positive cells. pEGFP-C1-transfected NIH 3T3 cells were all negative. **Conclusion** Anti-sera are highly specific to diversified MxAs. The antibody is detectable by Western blot, immunocytochemistry and immunofluorescence assay.

Key words: Myxovirus resistant protein A; Anti-sera; Specificity test

INTRODUCTION

Human myxovirus resistant protein A (MxA), an exclusively induced protein by interferon I, is a 76Kd GTP-banding protein[1-6]. Cytoplasmic MxA has a wide antiviral spectrum against different types of viruses, including bunyavirus, orthomxovirus, paramyxovirus, rhabdovirus, togavirus, picornavirus, and hepatitis B virus *in vivo* or *in vitro*[7-29]. These interferon-mediated effects are essential for the survival of higher vertebrates because they provide an early line of defense established within hours of viral infection before acquired immune responses are mounted[2, 30-33]. MxA may also become a potential agent against human influenza and avian influenza viruses[12, 16, 26, 28]. MxA is expressed at a fairly low level *in vivo* and *in vitro* due to its inductivity. Given that interferon I is up-regulated in some human individuals, a so-called anti-virus state, high MxA expression, occurs only in a few cells or in tissues such as PBMC. Since MxA exists in cytoplasm and is not secreted extracellularly, it is difficult to obtain purified MxA from human cells or tissues. Its expression *in vitro* is, therefore, an important means of clarifying its function using recombinant DNA techniques. Obtaining and utilization of a specific antibody against MxA are pivotal to detect this gene expression *in vitro*. In addition, MxA, a downstream protein to interferon I, may also be used both as an indicator of viruses and as a criterion for distinguishing infections with viruses as opposed to bacteria in diagnosis of infected diseases[33-38]. It is even more clinically significant to use anti-MxA antibody to detect MxA since the development of hepatitis B is somewhat associated with MxA expression level[19]. Eukaryotic expression vectors are not suitable for production of the protein due to their low lever of expression and difficulty in obtaining a sufficient amount of purified protein as antigen. In the present study, the prokaryotic expression vector

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pET32a (+)-MxA was used to produce MxA. The expressed product was dissolved and purified by SDS-PAGE. The purified protein was injected into mice to obtain antisera. The anti-sera were analyzed and evaluated using the stably transformed MxA NIH3T3 cells established in our laboratory and IFN-β induced human lung cancer cell line A549.

MATERIALS AND METHODS

Materials

Human lung cancer cell line A549（ATCC）was purchased from Cell Conservation Center (Shanghai, China). Wild type MxA (NIH-3T3-pEGFP-C1-wMxA), site-directed mutant MxA {NIH-3T3-pEGFP-C1-mMxA(N589S)} stably transformed NIH-3T3 cell strains, and non-MxA (NIH-3T3-pEGFP-C1) transformed NIH-3T3 line were all produced in our laboratory (The results will be published in another article). Prokaryotic expression vector pET-32a (+) and *E. coli* strain BL21 (DE3) were provided by Dr. Zhen-Min YE, Medical School of Suzhou University. Human IFN-β was from Cytolabs Company (USA). DMEM medium, Trypsin at a dilution of 1:250 (Japan), bovine serum (GIBCO Company), G418 (Merck Company), complete Freund' adjuvant (CFA), and incomplete Freund' adjuvant (IFA) were all purchased from Sino-American Biotechnology Company (Shanghai, China). Kunming mice for immunization were provided by Experimental Animal Center of our school. IgG goat anti-mouse IgG/ peroxidase, horseradish, HRP (Shanghai TIANGEN Company, China) and IgG goat anti-mouse IgG/FITC (Proteintech, USA), protein molecular weight markers were purchased from MBI Company and reagents for Western blotting were bought from Promega Company (USA).

Cell Culture and Collection

A549 cells were incubated in standard 50 mL cell culture flasks containing DMEM+10% inactivated bovine serum. A layer of fully grown cells was digested with 0.25% trypsin/0.01 mol/L PBS and sub-cultured for future utilization. Wild type MxA, site-directed mutant MxA and only expressing EGFP cells were raised in a culture medium with 1 mg/mL G418. A549 cell cultures grown to 60% confluence of a cell layer were chosen, medium liquids were removed and cell maintenance media were added with 2% bovine serum containing 0 ng/mL, 0.078 ng/mL, 0.3125 ng/mL, 1.25 ng/mL, 5 ng/mL, and 20 ng/mL IFN-β. Culture was continued for 48 h before the cells were collected. The collected cells were digested with 0.25% trypsin/0.01 mol/L PBS (pH 7.4), moved into 1.5 mL EP, centrifuged and washed 2 times with 0.01 mol/L iced PBS (pH 7.4) and precipitated cells were stored at -80 ℃ freezer for later use.

Methods

Construction and Expression of Prokaryotic Recombinant Vector for MxA Gene

A full length MxA gene was amplified from cDNA using human MxA-specific primers, cloned into pUC119 and sequenced. The sequenced MxA gene from recombinant plasmid-MxA was inserted into the prokaryotic expression vector pET32a (+) at the *Han*dIII and *Bam*HI sites and transformed into *E. coli* strain BL21. The open reading frame integrity of the recombinant plasmid was verified by DNA sequencing. *E. coli* strains containing a pET32a (+)-MxA plasmid were cultured in a 2YT medium to 0.3 OD_{600} and expression of the MxA was induced with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) at 28 ℃ for 4 h. After centrifugation at 4 000×g for 20 min, bacterial pellet was dissolved with 20×volume of 100 mmol/L PBS (pH 7.4), added with the same volume of loading buffer of 2 X SDS-PAGE, lysed by boiling for 3-5 min and analyzed by electrophoresis on 12% SDS-PAGE.

Purification of MxA Expression Product by SDS-PAGE and Preparation of Antiserum

Lysate was electrophoresed and separated by a 12% SDS-PAGE gel together with a protein molecular marker. After staining with Coomassie brilliant blue, the target protein bands of MxA were cut off, snipped and de-stained until the gels became clear. The snipped gels were collected in dialysis bags into which a correct amount of electrode buffer was added. The center of Western-blot transfer nip was set and electrophoresed at 100 mA for 3 h. The dialysis bag was dialyzed in 0.01 mol/L iced PBS (pH 7.4) for 24 h. The protein solution was transferred into a 1.5 mL EP and centrifuged at 10 000 r/min at 4 ℃ for 30 min. The supernatants were analyzed for protein concentration by Bradford assay (Bradford, 1976), and stored at -80 ℃ for future use. Several mice were injected intra-peritoneally with the purified MxA (0.01 mg/mouse) mixed with complete Freund's adjuvant in a 1:1 ratio. The mice were subsequently injected three more times with the protein mixed with incomplete Freund's adjuvant at two week intervals. Four days after the fourth injection, mouse blood was collected for testing antiserum with 1 mg/mL prokaryotic expression product as an antigen. The effect value of 1:8-16 in

immuno-precipitation reaction was considered an eligible antiserum, which was collected for use.

Western Blotting Analysis for the MxA Protein of Transformed Cell Stains

The procedure of Western blotting analysis[8] was summarized. In brief, 2×10^6 cells were lysed with 100 μL RIPA containing 150 mmol/L NaCl, 1% NP-40, 50 mmol/L Tris (pH 8.0), 50 μg/mL PMSF, and 1 μg/mL aporotinin, bathed in icy water for 30 min and centrifuged at 20 000 r/min at 4 ℃ for 30 min. After the protein concentration was measured with the Bradford method, supernatant samples were added with an equivalent volume of loading buffer, together with prokaryotic expressed MxA and a non vector bacterial lysate serving as controls, boiled for 3-5 min. Cell sample (50 μg), bacterial sample (10 μg) and pre-stained protein molecular weight marker (10 μg) were taken to electrophorese on 12% SDS-PAGE. The targeted protein bands were cut and transferred onto cellulose membranes using a transblot apparatus (BioRad) and then incubated for 5 h. The membranes were finally sealed with 5% BSA/0.01 mol/L PBS (pH 7.4) for 2 h at room temperature. Antiserum (1:100) and goat anti-mouse IgG/HRP (1:100) were incubated at room temperature for 2 h, washed four times every 10 min with 0.01 mol/L PBS (pH 7.4) following the instructions of DAB/H_2O_2 kit and photographed.

Analysis of Various Cell Strains by Immunocytochemistry and Immunofluorescence

The above mentioned cell strains were transferred onto the 6-cell culture plate with pre-deposited glass cover slips (14 mm×14 mm) and cultured until 30%-40% of a layer cell and then observed under a fluorescence microscope (OLYMPUS-IX71), counted and photographed at polarized light of 490 nm. For immunocytochemistry and immunofluorescence analysis, the cultured cells were collected, washed three times with 0.01 mol/L PBS (pH 7.4), fixed with 4% paraformaldehyde + 0.01 mol/L PBS (pH 7.4) at room temperature for 30 min, and vitrified with 0.2% Trpton-100/0.01 mol/L PBS (pH 7.4) for 30 min, sealed with % BSA /0.2% trypton-100/ 0.01 mol/L PBS (pH 7.4) at room temperature for 30 min. Twenty-five μL antiserum diluted at 1:100 and normal antiserum diluted at 1:100 as a control with goat anti-mouse IgG/HRP diluted at 1:100 were incubated at room temperature for 1 h, after washing four times every 10 min with 0.01 mol/L PBS (pH 7.4), displayed following the instructions of DAB/H_2O_2 kit, observed and photographed under a fluorescence microscope (OLYMPUS-IX71) at a polarized light of 490 nm. Meanwhile, A549cells transferred onto the 6-cell culture plate with glass cover slips (14 mm×14 mm) and cultured until 30%-40% of a layer cell. The medium liquid was removed, and a cell maintaining medium and 20 ng/mL IFN-β were added with 2% bovine serum, and cultured for 48 h. The cells were fixed and sealed, then 1:50 FITC conjugated goat anti-mouse IgG was incubated at room temperature for 1 h, washed, displayed, and photographed.

RESULTS

Identification of pET32a (+)-MxA by Double Restriction Enzymes

The products of pET32a (+)-MxA plasmid double digested by enzymes were 2 DNA fragment bands, 2 and 5.8 kb in length on 1% agarose gel, indicating that the MxA gene could be correctly linked on the pET32a (+) vector, while those of pET32a (+) plasmid digested with the same two enzymes showed only one band of 5.8 kb (Fig. 1A).

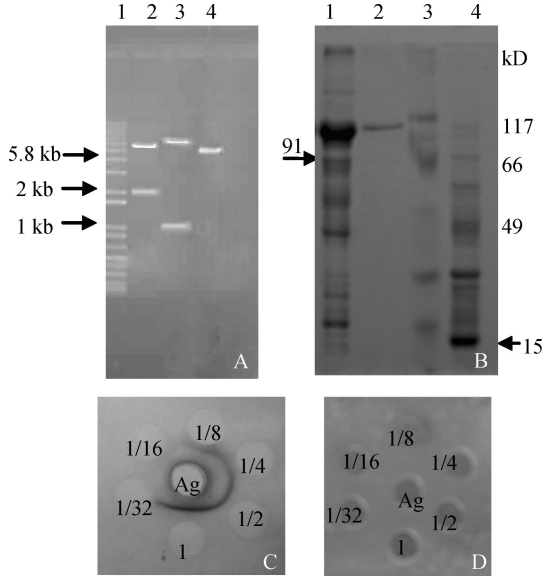

FIG. 1. Images of expression vector (A), crude and purified products of MxA gene expression (B), and immunodiffusion assay for mouse anti-sera.A: Identification of pET32a (+)-MxA. Lane 1: DNA Marker. Lanes 2-4: enzyme digestion of recombinant vector pET32a (+)-MxA at *Eco*R V/*Xba* I, *Kpn* I, and *Eco*R V/*Xba* I, respectively. B: Identification of crude and purified expression products of the MxA gene in *E.coli* on 12% SDS-PAGE. Lane 1: BL-12 with pET32a (+)-MxA; lane 2: purified expression protein; lane 3: pro-stain protein molecule weight marker; lane 4: BL-21 with pET32a (+). C: Immunodiffusion assay on ani-sera from mice immunized with MxA to rude MxA (Ag refers to rude MxA). D: Normal mouse sera as controls.

Identification of Expressed Proteins Purified by Gel Electrophoresis

The pET32a (+)-MxA and pET32a (+) individually transformed BL21 bacteria were induced by 0.2 μg/mL IPTG for 2, 4, and 6 h. Protein electrophoresis revealed a peak value for the target gene expression in the 4 h-induction. The images of protein bands of two bacterial strains on electrophoretic gel are given in Fig. 1B. The bacteria with pET32a (+)-MxA expressed a protein band (line 1) with a molecular mass of 90 kD, accounting for about 25% of the total proteins, with the expected molecular weight of 76 kD MxA+ 15 kD Trx, while the bacteria with pET32 a (+) expressed a 15 kD thioredoxin protein (line 4), suggesting that MxA could be successfully expressed in a form of fusion protein tagged with thioredoxin protein. Meanwhile, 15 mL of 300 μg/mL MxA was prepared by collecting, electrophoresing and electro-eluting 300 mL suspension of the bacterial pellets. The purified protein showed a single 90 kD protein band (line 4) on electrophoretic gel, indicating that the prepared protein with a purity of 90% can be used as an antigen to immunize mice for preparation of the antibody.

Identification of Antiserum Preparation by Double Immunodiffusion

Seven mice were immunized with MxA. Forty-five days after immunization, the fixed antigen to antibody (1:2) was tested by double immunodiffusion. Six mice were found to have an antiserum efficient value (1:8), only one mouse (No. 3) had an antiserum efficient value (1:16) and normal control serum showed no immuno-precipitation (Figs. 1C and 1D), indicating that usable anti-sera could be obtained for this immunoassay.

Western Blotting Analysis for Prokaryotic Expression Product and Various Cell Strains against Antiserum

Prokaryotic expression products showed a specific hybridization band at 90 kD and Western blotting revealed 100 kD hybridization bands of wild type MxA and site-directed mutant MxA transformed cells, while the non-MxA transformed *E.coli* containing pETa32 (+) and cells containing pEGFP-C1 showed no specific hybridization signal (Fig. 2A). Furthermore, all samples of A549 cells induced by different concentrations of IFN-β for 48 h had hybridization signals. The signal intensities showed in the IFN-β dose dependent manner, whereas non- IFN-β induced A549 cells showed no signal (Fig. 2B). When the antiserum was replaced with normal serum in Western blot showed no signal (images not shown). These results indicate that the anti-serum could not only specify the expression products of stable MxA-transformed cells, but could also recognize the natural form of induced MxA.

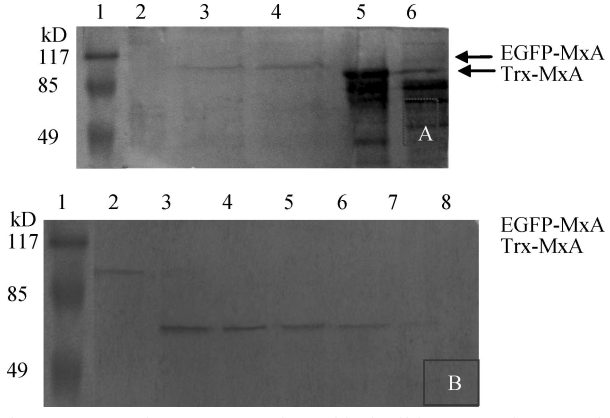

FIG. 2. Immunoblot showing mouse anti-sera to ectopic and inducible expressive proteins of the MxA gene by A: Immunoblot image of mouse anti-sera to ectopic MxA. Lane 1: Pro-stain protein marker; lane 2: NIH-3T3 transformed by pEGFP-C1 (control), lane 3: NIH-3T3 transformed by pEGFP-C1-wMxA; lane 4: NIH-3T3 transformed by pEGFP-C1-mMxA (589), lane 5: BL-21 pET32 (+)-MxA; lane 6: BL-21 pET32(+). B: Immunoblot image of mouse anti-sera to inducible MxA Lane 1: Pro-stain protein marker; lane 2: NIH-3T3 transformated by pEGFP-C1-wMxA; lanes 3-7: A549 cells induced with IFN-β at different concentration; lane 8: A549 cells (control).

Immunocytochemistry and Immunofluorescence Assay of Antisera against Various Cell Samples

Permanent MxA-transformed cells were analyzed with immune cell chemical techniques. The green fluorescence of cell strain pEGFP-C1 (control) was distributed both in nuclei and in cytoplasm, which occurred in a higher quantity and more uniformly in the former than in the latter. The fusion proteins of MxA-GFP were well localized in the form

of fluorescent particles in cytoplasm of wild type MxA-transformed cells, suggesting that the fused protein could be characterized with the natural MxA located in cytoplasm in the form of multiple polymers.

However, in site-directed mutant MxA transformed cells, the fused protein was also localized in cytoplasm but not in the form of fluorescent particles. Three cell strains had 98% positive cells by counting five vision fields randomly under a microscope (Fig. 3A1, B1, and C1). HRP analysis demonstrated that both wild and mutant MxA displayed color in cytoplasm, but the particles disappeared only in wild MxA, suggesting that inactivation of peroxidase originated from cells with H_2O_2 could destroy wild MxA polymers with 59% positive cells. No positive EGFP-transformed cells were observed (Fig. 3A2, B2, and C2). Controls with normal serum showed a negative response (Fig. 3A3, B3, and C3). Test of IFN-β induced A549 cells with FITC-indirectly labeled secondary antibody displayed a positive cytoplasm response with 95% positive cells. Partially enlarged images showed that MxA located in cytoplasm was in the polymer form (Fig. 4-1 and 4-2). Non-IFN-β induced A549 cells showed some weak positive responses, which might be due to the natural expression of MxA in cells (Fig. 4-3). Normal controls showed negative responses (Fig. 4-4), indicating that the antiserum could recognize the unreduced antigen.

DISCUSSION

The effect of vaccine on influenza virus has been decreased due to its antigenic drift. Scientists have shifted their attention to the studies on improving human and animal innate immunity or resistance to pathogens. Therefore, their immunity is associated with the innate immunity system. Type I interferon as an important member of the innate immunity system plays a key role in resistance to early invasion of viruses, especially the protective system of specific immunity is get not established. One of the functions of MxA, as a protein product of type I interferon, is to exert a direct anti-virus effect. However, because MxA is an internal cell protein, establishing a gene

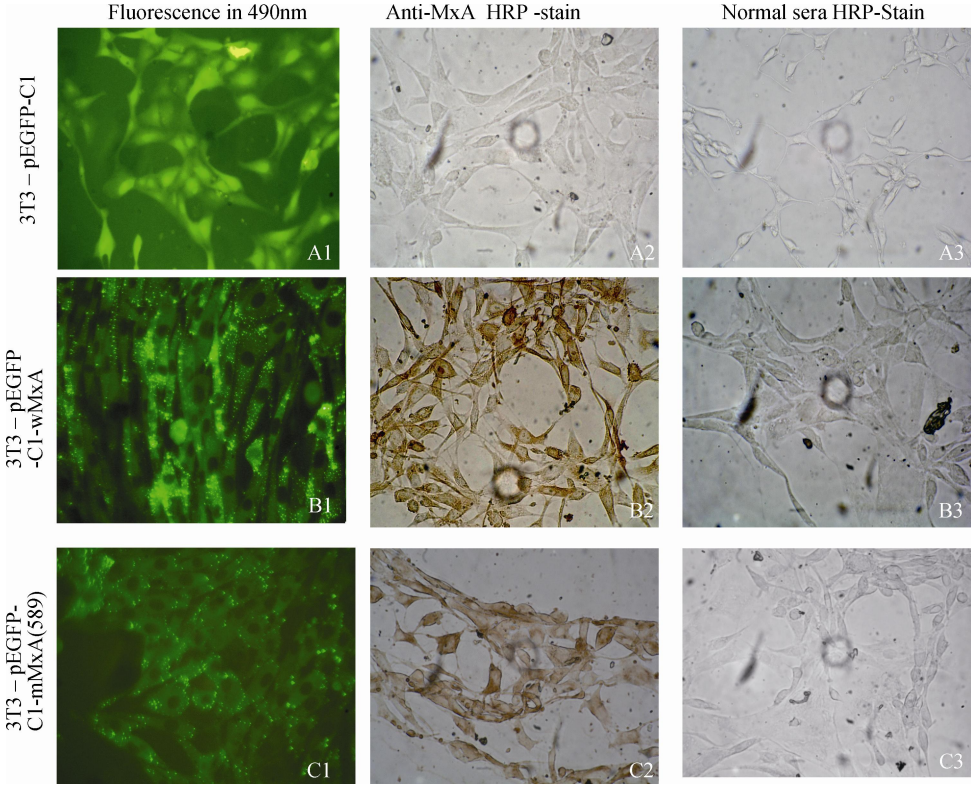

FIG. 3. Immunocytochemistry showing anti-MxA sera to ectopic expression of the MxA gene in fluorescence at 490 nm. A1-3: Stably transfected 3T3 cells y expressing enhanced green fluorescence protein (EGFP) with no positive signal (A2); B1-3 and C1-3: Stably transfected 3T3 cells expressing fusion proteins of EGFP-wild type (wt)/mutant type (mt) MxA (N589S) with ectopic expressing MxA proteins in NIH-3T3 recognized with anti-MxA sera (B2, C2) and no positive signals found in normal sera group (A3,B3,C3).

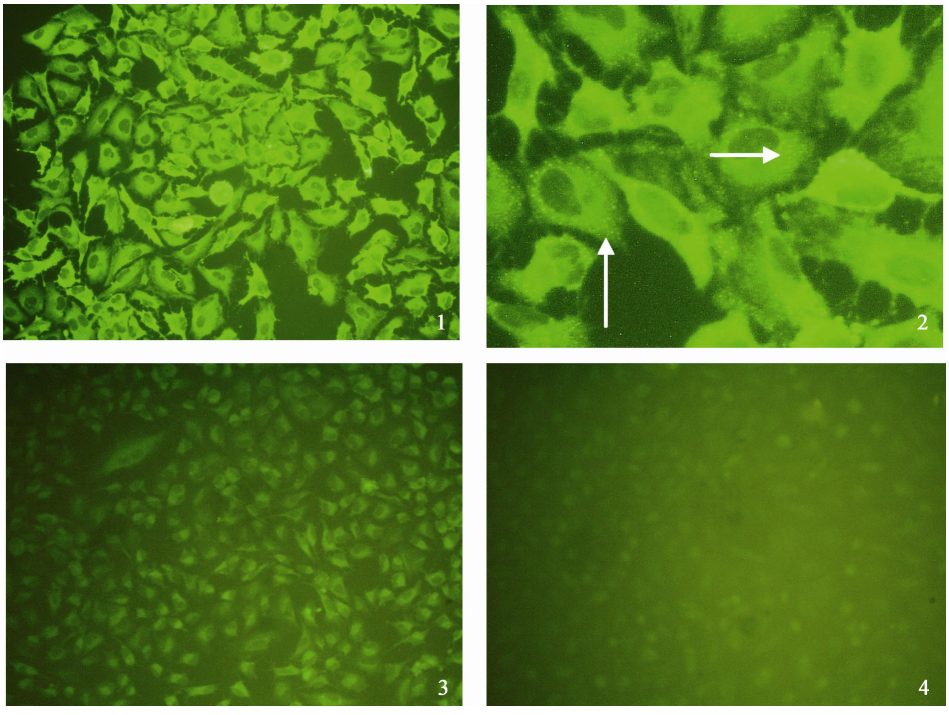

FIG. 4. Immunocytofluorescence showing anti-MxA sera to inducible expression products of the MxA gene. Images 1 and 2: A549 cells treated with 20 ng/mL IFNβ for 48 h + anti-MxA sera (1:50) in 200× and 500×, respectively; image 3: A549 cells + anti-MxA sera (1:50) in 100×; image 4: A549 cells treated with 20 ng/mL IFNβ for 48H + normal mouse serum (1:50). The results suggested that I-IFN inducible MxA in oligomer form is located in cytoplasm (arrow in Fig. 4-2).

stably transformed cell line or developing transgenic animals is an important way of studying the protein function. Chieux *et al*., Kanerva *et al*., Miura *et al*., and Schnorr *et al*.[15, 22, 25, 27-28] have investigated the MxA gene intensively by setting up the transformed cells. Arnheiter *et al*. and Jovan Pavloci *et al*.[9, 26] have shown that the resistance of transgenic mice to influenza virus and vesicular stomatitis virus has increased by 10 and 1 000 times, respectively, compared to thogoto virus.

No matter what transformed cells and transgenic animals are concerned, utilization of a specific antibody to detect the expression product is important. Although polyclonal or monoclonal antibodies have been used[8,15], they are not commercially available. In this experiment, polyclonal antibody against MxA was successfully used to detect various cell strains transformed with wild, mutant and IFN-β induced MxA.

MxA, which is stable and inducible in all types of cells and has a longer half life, may be used in clinical practice as criteria for distinguish viral and bacterial infections. Obtaining purified recombinant MxA, preparation of its antibody, establishment of MxA may provide a basis for early diagnosis of different bacterial and viral infections and clinical evaluation of I-IFN effect.

In this study, prokaryotic expressed products purified by SDS polyacrylamide gel electrophoresis were used as antigen to immunize mice for anti-sera to recognize the reductive linear antigen site. Western-blot analysis showed that the antibody could specify the reductive MxA antigen, while immunocytochemistry and immunofluorescence analysis revealed that the antibody could recognize the non-reductive and immobilized MxA antigen used, thus laying a foundation for future MxA studies.

A polyclonal antibody prepared using expressed MxA as an antigen to recognize eukaryotic expressed products cannot, sometimes, provide a strong stringency in antigen-antibody specificity. Although the two vectors are different in the expression system, they have the same open reading frame. The products show the same antigen site. In this study, Western-blot and immunofluorescence assay of the protein and cell samples from human lung cancer cell line A549 demonstrated that the anti-sera could fairly recognize the IFN-β induced MxA, suggesting that the prepared polyclonal antibody has a fair specificity. It is more interesting that the antibody prepared in our experiment can recognize three types of MxA, namely 76kD induced MxA, 91kD Trx-MxA, and

103kDa EGFP-MxA, thus providing a necessary condition for future research on MxA.

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