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Production and Characterization of Monoclonal Antibodies against Human Interferon-α

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Seven monoclonal antibodies were produced by fusing splenocytes from Balb/C mouse immunized with partially purified human interferon-α (HuIFN-α) with NSO plasmacytoma cells. They were identified as five IgG class (432.22: IgG2b/κ, 460.52: IgG2b/λ, 548.46: IgG2a/κ, 573.10: IgG2b/λ, 625.12: IgG2b/κ), one IgA class (460.50: IgA/κ) and one IgM class (465.27: IgM/κ), and all of them revealed highly sensitive to HuIFN-α. IgG class monoclonal antibodies have pIs ranged from 8.2 to 8.6. Ascites fluids produced from primed Balb/C mice and were purified through affinity column chromatography. The cytopathic effect (CPE) inhibition assay to examine neutralization of HuIFN-α by IgG class monoclonal antibodies, gave that MAbs 460.52, 548.46, 573.10 can neutralize HuIFN-α with varying degrees except 432.22. Therefore, it is deduced that these various monoclonal antibodies may recognize the distinct epitopes on HuIFN-α.

KEY WORDS: Human interferon-α, Monoclonal antibody, Neutralization

The interferons (IFNs) are a group of molecularly heterogeneous protein which have been classified into α (leucocyte), β (fibroblast), and γ (immune)(Stewart *et al.*, 1980). In the human genome, at least 14 functional genes code for human interferon-α (HuIFN-α), a family of small secretory proteins with its molecular weight ranging 16,200 to 26,000 (Henco *et al.*, 1985). HuIFN-α is known to affect a number of cell functions, including resistance to viral infection, inhibition of certain cell growth, alteration of cell sulface markers and immunomodulation of both cell-mediated and humoral responses like other IFN classes (Stewart, 1981). Nevertheless, as a group of proteins that mediate a variety of biological activities, it had been hampered by the scarcity of the material. Therefore simple and reliable methods for IFN research were required.

Monoclonal antibody produced from a single hybridoma clone resulting from the fusion of antigen specific splenocytes with plasmacytoma cells is extremely potent tool for characterization (Arnheiter *et al.*, 1983; Fish *et al.*, 1989; Adolf *et al.*, 1990) and quantitative analysis (Agui *et al.*, 1985; Meager *et al.*, 1986) as well as large-scale purification of a given immunogen (Secher and Berke, 1980; Tsukui *et al.*, 1986). Recently, monoclonal antibodies have facilitated characterization of various peptide domains within molecules by neutralizing or other affecting their properties. Staelin *et al.* (1981) produced 13 monoclonal antibodies and examined them for 6 various HuIFN-α subspecies. From the result, he suggested that there are at least three different epitopes in common on HuIFN-α. Arnheiter *et al.* (1983) showed that the carboxyl-terminus of HuIFN-α had no binding capacity to the receptor of cell membrane using specific monoclonal antibodies. In recent years, the functions of HuIFN-α

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domains were clarified by Fish *et al.* (1989). Neutralization experiments with a monocloal antibody directed against a conserved region from amino acid residues 113-149 indicated that this region of the HuIFN-α molecule may be important for antiviral activity. Monoclonal antibodies are also used to classify HuIFN-α subtypes. The IFN-ω1 is newly designated from IFN-α group, which shows approximately 50-60% homology in amino acid sequences with other HuIFN-α species but antigenically unrelated to HuIFN-α and -β as it is not neutralized by antisera or monoclonal antibodies to these two proteins (Adolf, 1990; Adolf *et al.*, 1990).

Although these studies have contributed to find out HuIFNs functions and structures, the HuIFN-α is so complex system that various monoclonal antibodies are required to study HuIFN-α subtypes. As the first step toward this end, we produced specific monoclonal antibodies against HuIFN-α.

Materials and Methods

Immunization

Three female Balb/C mice, 6 weeks old, were immunized with partially purified human interferon-α (HuIFN-α). Primary immunization was done by intraperitoneal injection with 20 μg of HuIFN-α emulsified with the same volume of complete Freund's adjuvant (Sigma). Secondary immunization was given at interval of 3 weeks by the same way with incomplete Freund's adjuvants (Sigma). Final intravenous booster was given 3 days prior to cell fusion with 30 μg of HuIFN-α to Balb/C mouse (Choi *et al.*, 1986). Test bleeds were run conveniently on small batches of serum prepared from eye of immunized mice. The first anti-serum was prepared after 2 weeks of primary immunization, second anti-serum after 1 week of secondary immunization and third anti-serum after 8 weeks of secodary immunization.

Hybridoma cell production

1×10^8 splenocytes from immunized mouse were fused with 2×10^7 NSO plasmacytoma cells using 50% polyethylene glycol (PEG 6000, Gibco) as described by Köhler and Milstein (1975). The cells were seeded into 96 well plates (Costar) at a density of 4×10^4 plasmacytoma cells per well in a volume of 0.2 ml of HAT medium, DMEM plus HAT (1×10^{-5} M of hypoxanthine, 4×10^{-8} M of aminopterine, 1.6×10^{-6} M of thymidine, Sigma) supplemented with 20% Fetal bovine serum (Gibco), 100 IU/ml of penicillin and 100 μg/ml of streptomycin. After 2 weeks culture supernatant from each well growing hybridoma clones was tested by a screening assay-ELISA and then the wells of positive were expanded in HT medium (HAT medium minus aminopterine). The positive hybridoma clones were subsequently cloned by limiting dilution method and rescreened for HuIFN-α.

Enzyme-linked immunosorbent assay

For screening assay, 100 μl of each culture supernatant (or diluted ascites fluid) was added into 96-well polystylene microtiter plate coated with 0.5 μg/ml of HuIFN-α per well. Non-specific binding sites were blocked with 1% BSA in phosphate buffered saline (PBS). Alkaline phosphatase conjugated with rabbit anti-mouse Igs (IgM + IgA + IgG, Cappel), was added and followed by ρ-nitrophenyl phosphate, 1 mg/ml in 10 mM diethanolamine (pH 9.5) containing 0.1 mM $MgCl_2$ solution. The colour change was read by absorbance at 410 nm on ELISA spectrometer (Dynatech Instrument Inc.).

Antibody class and subclass determination

It was carried out with mouse monoclonal sub-isotyping ELISA kit (Hyclone Lab, Inc.). Rabbit anti-mouse subtype immunoglobulins were added into microtiter plate coated with goat anti-mouse immunoglobulin. Then goat anti-rabbit IgG peroxidase conjugate was added and developed by diaminobenzidin containing 1% H_2O_2 in 50 mM PBS.

Affinity column chromatography

After inoculating 5×10^6 monoclonal hybridoma cells of IgG class intraperitoneally into primed Balb/C mice, ascite fluids were collected and purified by protein A-Sepharose CL-4B affinity chromatography (Pharmacia). Immunoglobulin was eluted with 0.2 M acetic acid (pH 3.0) containing 0.15 M NaCl and the pH was adjusted to

7.4 immediately with 1 M Tris-HCl. The eluate fraction was concentrated via ultrafiltration (XM 50 filter, Amicon) and stored at −20°C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

12% acrylamide gel electrophoresis was carried out according to the method described by Laemmli (1970).

Isoelectric focusing

It was conducted by non-equilibrium pH gradiant electrophoresis (NEPHGE). 5% acrylamide gel containing 2% ampholite (pH 3.5-10) was used for NEPHGE as described by O'Farrell *et al.* (1977).

Neutralization with cytopathic effect (CPE) inhibition assay

Kinetics of monoclonal antibodies to HuIFN-α neutralization was calculated according to Rubinstein *et al.* (1981) with minor modification. To determine the potency of monoclonal antibodies, a serial dilutions of them were incubated with a constant dose of HuIFN-α in comparison to controls in which regular RPMI-1640 containing 10% FBS was present for 1 hr at 37°C. 4×10^4 Madin-Darby bovine kidney (MDBK) cells were added as target into the plate and incubated for 2 hr at 37°C. And then 5,000 pfu (plaque-forming unit) of vesicular stomatitis virus (VSV) were challenged to MDBK cells. Finally the MDBK cells were fixed and stained with 0.1% crystal violet after incubation for 12 hr at 37°C. CPE inhibition was observed by microscope. The neutralizing unit was defined as the reciprocal of the highest dilution of monoclonal antibody that inhibited the antiviral activity of 10 interferon unit/ml.

Results and Discussion

Immune response from anti-sera of immunized mice

Partially purified HuIFN-α was used as an antigen described in materials and methods. But Staehelin *et al.* (1981) reported that the purity of interferon for immunization and screening of hybridoma was essential for obtaining many specific hybridomas. So highly purifed HuIFN-α was used in screening of hybridoma clones in the present experiments. In order to monitor development of immune response test bleeds were performed after respective immunization and antibody titration was measured by ELISA. As shown in Table 1, antibody titer of mouse #3 was higher than those of mouse #1 and #2 in the first test bleed at 2 weeks after primary immunization. The same profile revealed in the second test bleed at 1 week after secondary immunization. On the other hand, antibody titer of mouse #3 was rather lower than those of the others in the third test bleed at 8 weeks after secondary immunization. Because the level of circulating antibody is responsible for the booster efficiency by 'rapid clearance' of the same immunogen injected subsequently in the later immune response, it is necessary to extend until the level of circulating antibody drops down prior to the final booster. Considering this respect the mouse #3 was selected for the best responder and the final booster was carried out to mouse 3.

Cell-fusion and hybridoma production reactive with HuIFN-α

Splenocytes of immunized mouse were fused with NSO plasmacytoma cells, HPRT⁻ and non-

Table 1. Antibody titer of mouse anti-HuIFN-α sera by ELISA.

	Antibody titer		
	$1:10^2$	$1:10^3$	$1:10^4$
Control	0.105	0.069	0.051
Mouse #1			
1st anti-serum	0.887	0.758	0.624
2nd anti-serum	1.405	1.229	1.029
3rd anti-serum	1.071	0.807	0.724
Mouse #2			
1st anti-serum	0.960	0.756	0.650
2nd anti-serum	1.389	1.212	1.101
3rd anti-serum	0.955	0.754	0.658
Mouse #3			
1st anti-serum	1.272	1.064	0.876
2nd anti-serum	1.800	1.650	1.421
3rd anti-serum	0.695	0.430	0.300

Anti-sera are at dilution of $1:10^2$, $1:10^3$, $1:10^4$. Non-immunized mouse serum is used as negative control.

secretion of immunoglobulin. After HAT medium selection for 2 week 98 out of 512 culture wells showed hybridoma growth. When the culture supernatants of 98 growing wells were initially screened by ELISA using HuIFN-α, 15 wells gave positive result in the secretion of anti-HuIFN-α antibodies. These positive wells were expanded and rescreened. As a result of rescreening 8 hybridoma wells out of 15 positive clones turned into negative. This phenomenon may be due to chromosomal segregation or overgrowth of negative hybridoma cells mixed in the same well. The selected seven hybridoma wells showed stably positive and exhibited their antibody titer about 5 to 6 times as high as the others which were proved to be negative (Fig. 1). Seven monoclonal hybridomas were established by cloning and subcloning. To determine their isotypes, rabbit anti-mouse subclass specific immunoglobulins were used. As shown in Fig. 2, each monoclonal antibody revealed one heavy chain type except 573.10 exhibiting IgG2b and IgM. but it was proved single IgG2b by further cloning and isotyping test (data not shown). This was probably caused by mixed clones. The kinds of heavy chains were various like IgG2a, IgG2b, IgA, and IgM. And they had κ or λ light chain. In the NEPHGE analysis of IgG classes, their isoelectric points ranged approximately from 8.2 to 8.6 (Table 2). According to the isotype analysis shown in Table 2, it may be concluded that the seven HuIFN-α specific hybridoma clones are derived from seven different B-cell clones of the immunized mouse. Among them, 432.22 and 625.12 consist of the same heavy and light chains (IgG2b/κ), also 460.52 and 573.10 have the same IgG2b/λ. However they have distinct pIs 8.59 and 8.20 respectively in the former group and dissimilar neutralization capacity in the latter, which mean these MAbs are different from each other.

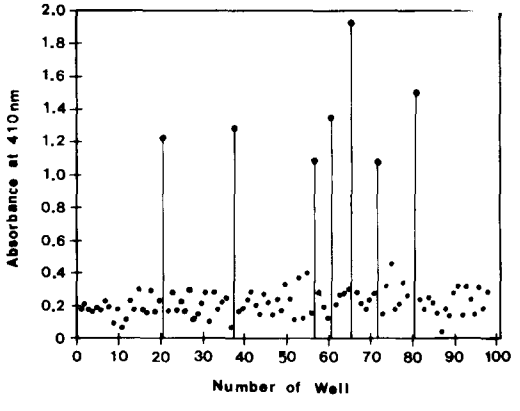

Fig. 1. Screening of positive wells for anti-HuIFN-α by ELISA. Among 512 wells, 98 wells were grown in HAT medium. Only 7 clones were positive for anti-HuIFN-α.

Preparation and purification of monoclonal antibodies

In order to prepare large amount of monoclonal antibodies, ascites fluids were collected from primed Balb/C mice. The monoclonal antibody titers of ascites fluids were 10^4 to 10^5 times as extremely high as those of culture supernatants

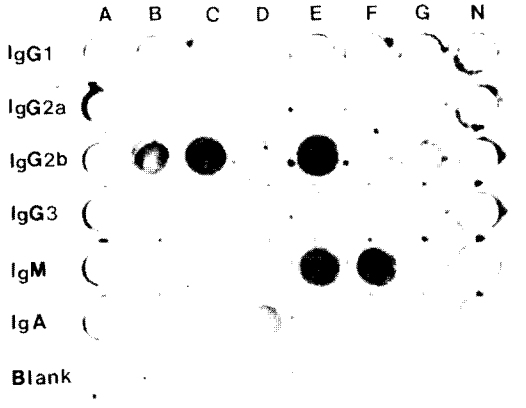

Fig. 2. Isotype determination of monoclonal antibodies. Subclass specific immunoglobulins are rabbit anti-mouse heavy chain; IgG1, IgG2a, IgG2b, IgG3, IgA, IgM. For the control, normal mouse serum was used. Lane A: 548.46, B: 432.22, C: 460.52, D: 460.50, E: 573.10, F: 465.27, G: 625.12, N: control.

Table 2. Isotypes of monoclonal antibodies and their pIs.

Hybridoma clone	Isotype	pI*
432.22	IgG2b/κ	8.59
460.50	IgA/κ	N.D**
460.52	IgG2b/λ	N.D
465.27	IgM/κ	N.D
548.46	IgG2a/κ	8.47
573.10	IgG2b/λ	8.59
625.12	IgG2b/κ	8.20

*Isoelectric point, **Not determined.

Table 3. Amount of antibody and antibody titer in ascites fluids.

Hybridoma clone	Amount of antibody in ascites fluid (mg/ml)	Antibody titer C.S* 1:1	A.F** 1:10^4	1:10^5
432.22	3-5	1.226	over	over
460.52	2	1.091	1.213	0.876
548.46	5-7	1.941	over	over
573.10	1	1.075	1.000	0.754
625.12	2	1.505	1.512	0.920
460.50	N.D***	1.283	N.D	N.D
465.27	N.D	1.352	N.D	N.D

Ascites fluids are at dilution of 1:10^4 and 1:10^5 and culture supernatants at dilution of 1:1.
*Culture supernatant, **Ascites fluid, ***Not determined.

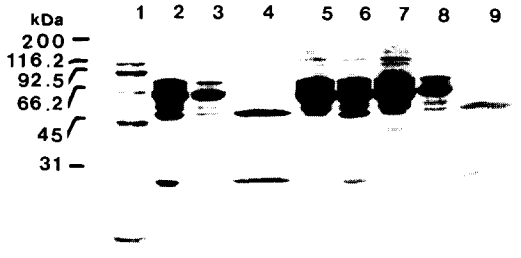

Fig. 3. Analysis of fractions from immunoglobulin G purification by SDS-PAGE. Lane #1: molecular weight standard, lane #2, #6: ascites fluid, lane #3, #5, #7. #8: flow-through (unbound protein), lane #4, #9: eluate (purified IgG).

(Table 3). Especially, MAbs 432.22 and 548.46 exceeded the O.D range in antibody titration and produced much large amount in ascites fluids. The seven monoclonal antibodies were not cross-reactive with HuIFN-β and -γ (data not shown). For the purification of IgG class monoclonal antibodies, protein A-Sepharose CL-4B affinity chromatography was conducted. The eluted IgG classes retained their own activity as usual (data not shown), and the purity was confirmed by SDS-PAGE. As shown in Fig. 3 the purified IgG was separated clearly into 50 kDa heavy chain and 25 kDa light chain.

Neutralization of HuIFN-α

Through CPE inhibition assay, 460.52, 548.46 and 573.10 monoclonal antibodies enabled

Table 4. Neutralization of HuIFN-α by monoclonal antibody.

Hybridoma clone	Isotype	Neutralization (N.U/ml)*	pI
432.22	IgG2b/κ	No	8.59
460.52	IgG2b/λ	1×10^3	N.D
548.46	IgG2a/κ	8×10^4	8.47
573.10	IgG2b/λ	2.5×10^3	8.59
625.12	IgG2b/κ	N.D**	8.20

*Neutralizing unit/ml, **Not determined.

HuIFN-α to be neutralized distinctly, but 432.22 monoclonal antibody did not. Among them, 548.46 has the strongest neutralization capacity to HuIFN-α (Table 4). This result implicates that the difference in neutralization capacity may result from the differential affinity for the same epitope or may reflect the different epitopes recognized by the respective antibody. These epitopes may be antiviral sites. But 432.22 may not recognize antiviral site of HuIFN-α. Staehelin *et al.* (1981) suggested that at least three different epitopes on HuIFN-α subtypes were recognized and defined by their 11 monoclonal antibodies using neutralization assay. But their isotypes were not various (8 out of 11 antibodies were IgG1/κ). Arnheiter *et al.* (1983) reported that monoclonal antibodies against the carboxyl terminus, 151-166, had no inhibition effect on biological activity of HuIFN-α. Meager and Berg (1986) found monoclonal antibody designated LO-22 with broad cross-reactivity to HuIFN-α subtypes distinguishes HuIFN-α_2 from HuIFN-αA, a subtype differing from HuIFN-α_2 by a single amino acid, lysine for arginine at position 23. Fish *et al.* (1989) showed that amino acid 113-149 region influences antiviral activity using neutralization experiments. Furthermore he reported that three functional domains of HuIFN-α are located in 10-35, 78-107, and 123-166. But these results do not include all the HuIFN-α subtypes thus do not elucidate the difference of their biological activities between subtypes completely. In order to analyze the function and structure of complex HuIFN-α system, the various analogs of HuIFN-α and specific monoclonal antibodies are required.

The seven monoclonal antibodies reported in the present study are to be valuable to examine

biological or biochemical activities and the structure of HuIFN α system for further research.

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인터페론 알파에 대한 단세포 군항체의 제조 및 특성

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부분 정제된 사람 인터페론 알파(사람 림프구 인터페론)로 면역시킨 Balb/C 마우스의 비장세포와 NSO 종양세포를 융합시킨 결과, 최종적으로 일곱 주의 단세포군 항체생산 융합세포주를 얻었다. 이들의 항체형을 조사한 바, IgG형 다섯주와 각각 하나의 IgA형, IgM형 세포주로 밝혀졌다: 432.22(IgG2b/κ), 460.52(IgG2b/λ), 548.46(IgG2a/κ), 573.10(IgG2b/λ), 625.12(IgGeb/κ), 460.50(IgA/κ), 465.27(IgM/κ). 이들 중 등전점이 8.2에서 8.6 사이의 값을 지닌 IgG형의 단세포군 융합세포를 Balb/C 쥐의 복강에 접종시켜 복수액을 얻은

후, 친화성 chromatography를 통해 IgG형 단세포군 항체를 정제하였고, 그 결과 복수액 1 ml당 평균 3 mg의 항체를 얻었다.

단세포군 항체가 인터페론의 항바이러스능을 중화시키는가의 여부를 세포병변 억제 분석 방법을 이용하여 조사한 결과, 460.52, 548.46, 573.10은 각기 다른 정도로 인터페론에 대한 중화능을 보였고, 432.22는 중화능이 없음을 확인하였다. 이러한 결과는 이들 다양한 단세포군 항체가 사람 인터페론 암과의 서로 다른 항원기를 인식하고 있을 가능성을 보여주는 것으로 사료된다.