Vol. 128, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1985 Pages 171-178

STUDY OF ANTIGENIC EPITOPES RECOGNIZED BY MONOCLONAL ANTIBODIES TO RECOMBINANT INTERFERON-GAMMA

Chi-Ming Liang, Suzanne Herren, Astrid Sand and Therese Jost

Laboratory of Immunology
Biogen SA
Geneva, Switzerland

Received February 28, 1985

Seven hybridomas (BG 1-7) which secreted monoclonal antibodies against recombinant interferon-gamma were produced. The ascites fluids containing four of the seven monoclonal antibodies (BG 1-4) neutralized the antiviral activity of both natural and recombinant interferon-gamma. Competition between labeled and unlabeled monoclonal antibodies for interferon-gamma in a solid phase immunoassay showed that BG 1 was competed by both BG 3 and BG 4 but not by BG 2; BG 2 was competed by BG 3 but not by BG 1 nor by BG 4. These results suggest that human interferon-gamma has at least two antigenic epitopes; one of the epitopes reacted with BG 1 & BG 4 while the other reacted with BG 2; BG 3 either binds to a region overlapping with the other two epitopes or reacts with both epitopes. The antigenic epitopes recognized by these four neutralizing monoclonal antibodies are likely at or closely related to the active sites of interferon-gamma.

Natural human interferon-gamma (IFN-gamma) is an important modulator of human immune responses (1-5). In vitro, it exhibits tumor inhibitory properties (6,7) and has a nonspecific antiviral effect on its target cells (8). Recently, recombinant DNA derived IFN-gamma has been produced and isolated from both bacteria and mammalian cells (9,10). The recombinant IFN-gamma, like natural IFN-gamma, is active in antiviral assay (9,10). The similarity in functional structures between recombinant and natural IFN-gamma, however, have not been well evaluated.

An important tool for monitoring the functional sites of biologically active molecules is the use of monoclonal antibodies (mAbs) (11,12). In this study, we report the production of mAbs to recombinant Escherichia coli-

Abbreviations used in this manuscript: ABTS, 2,2'-azino-di-(3'-ethyl benzothizaolin) sulfonic acid diammonium salt; ELISA, enzyme-linked immunosorbant assay; IFN-gamma, human interferon-gamma; rIFN-gamma, recombinant Escherichia coli derived human interferon-gamma; mAbs, monoclonal antibodies; PBS, phosphate buffered saline, pH 7.2.

0006-291X/85 $1.50

derived human IFN-gamma (rIFN-gamma) and the characterization of their binding epitopes on both rIFN-gamma and natural IFN-gamma.

METHODS

A. Materials:

Mengo virus and Hep 2 human larynx carcinoma cells were gifts of Dr. C. Weissmann (University of Zurich, Switzerland). Natural human IFN-gamma was a gift from Bioferon (Laupheim, W. Germany), rIFN-gamma (>95% purity) was prepared by the Department of Protein Chemistry, Biogen SA, Geneva. Peroxidase-labeled affinity purified goat anti-mouse immunoglobulins were from Kirkegaard & Perry Laboratories, MD; 2,2'-azino-di-(3'-ethyl- benzothiazolin) sulfoic acid diammonium salt (ABTS) and horseradish peroxidase (type VI) were from Sigma. Other sources are indicated in methods.

B. Procedures:

Immunization, fusion and cloning: Six female BALB/c mice (female, 10 weeks old) were injected intraperitoneally with rIFN-gamma (10 µg/mouse) emulsified in Freund's complete adjuvant. The mice were boosted twice with the same amount of rIFN-gamma at 21 day intervals. The mice with sera of high neutralization titer were injected intravenously with rIFN-gamma (10 µg in 0.5 ml phosphate-buffered saline) 4 days and 2 days before fusion. Spleen cells (1×10^8) from the rIFN-gamma immunized mouse were fused with 2×10^7 P3X63.Ag 8 variant 653 cells by using 50% (v/v) polyethylene glycol 3350 (J.T. Baker Chemical Co. NJ) as fusing agent. The procedure of Galfre and Milstein (13) was used. Hybridomas that secreted anti-rIFN-gamma antibodies were cloned and recloned by the limiting dilution method.

Preparation and purification of ascites fluids: The mice were injected intraperitoneally with 0.5 ml of pristan (Aldrich Chemical Co. Wis.) and 10 - 14 days later inoculated with 1×10^6 hybridoma cells/mouse. The ascites fluids which usually occurred 14 - 21 days after inoculation of the cells, were collected at 2-3 day intervals until the the animal was sacrified. The ascites fluids were purified either by precipitation with 40% ammonium sulfate or by protein A-Sepharose (Pharmacia, Sweden) chromatography according to the manufacturer's recommendation.

Preparation of enzyme labeled antibodies: Some of the purified antibodies were conjugated with horseradish peroxidase by the methods of Nakane and Kawaoi (14).

Neutralization of rIFN-gamma activity and antiviral assay: Fifteen microliters of hybridoma supernatant or ascites fluid at various dilutions were mixed with 45 µl of either natural IFN-gamma or rIFN-gamma (3.7 - 100 U/ml) under sterile condition and incubated at 37° C for 1 hr. The solution was tested in an antiviral assay (15). The criterion for positive antibody in the sample was defined as the ability of the sample to block the antiviral activity of IFN-gamma. Titer was defined as the reciprocal of the highest dilution of antiserum or ascites fluids which decreased the activity of IFN-gamma from 10 U/ml to 1 U/ml. A laboratory reference preparation of rIFN-gamma pre-calibrated against the NIH standard (Gg 23-901-530), was used to standardize and check all assays.

Sandwich Enzyme-linked immunosorbant assay (sandwich ELISA): An Immunoplate (Nunc, Denmark) was coated with anti-rIFN-gamma antibodies (10 µg/ml, 50 µl/well) at room temperature for 16-20 hr and then saturated with gelatin (10 mg/ml, 200 µl/well). After washing with phsophate buffered saline, pH 7.2 (PBS), a standard or unknown concentration of rIFN-gamma was added and incubation was carried out at room temperature for 1 hr. The unbound rIFN-gamma was washed off and peroxidase labeled anti-rIFN-gamma antibodies were added. After incubation at 4° C for 1/2-1 hr, the plate was

thoroughly washed with PBS containing 0.5% Tween 20. Fifty microliter of peroxidase substrate solution (containing 0.1% ABTS, 0.03% hydrogen peroxide in 0.1 M of sodium citrate and 0.2 M sodium phosphate buffer solution pH 4.0) was subsequently added to each well. Thirty minutes later, the optical density of the reaction mixture was measured at 630 nm in a Microelisa Auto Reader (Dynatech).

RESULTS

More than four hundred hybridomas were generated in the first fusion. The fused cells were screened by enzyme-linked immunosorbent assay (ELISA) and 3 hybridomas (BG 5-7) which secreted antibodies against rIFN-gamma were identified. None of these antibodies, however, neutralized the antiviral activity of rIFN-gamma. In subsequent fusion experiments, the generated hybridomas were screened by inhibition of rIFN-gamma antiviral activity. Four stable hybridomas (BG 1-4) were identified and these were cloned and recloned by limiting dilution. ELISA using rabbit antibodies specific for various subclasses of mouse immunoglobulins showed that BG 1, BG 2 and BG 4 were IgG_1 while BG 3 was IgG_{2a}(Table 1). The ascites fluids containing these four mABs

TABLE 1: Effects and Ig classes of anti-rIFN gamma mAbs

mAbs	Ig class	ELISA rIFN-gamma	neutralizing titers[a] against rIFN-alpha	rIFN-beta	rIFN-gamma	natural IFN-gamma
BG 1	IgG_1	+	<2	< 2	$3x10^5$	$3x10^4$
BG 2	IgG_1	+	<2	< 2	$2x10^5$	$2x10^4$
BG 3	IgG_{2a}	+	<2	< 2	$1x10^4$	$1x10^3$
BG 4	IgG_1	+	<2	< 2	$3x10^5$	$3x10^4$
BG 5	IgG_1	+	<2	< 2	< 2	<2
negative control (P3X63Ag8 653 myeloma)	-	-	<2	< 2	< 2	<2

a. Titer was defined as the reciprocal of the highest dilution of ascites fluids which decreased the antiviral activity of IFN from 10 U/ml to 1 U/ml.

neutralized the antiviral activity of rIFN-gamma with a titer of 300,000, 200,000, 10,000, and 300,000 respectively. The mAbs did not neutralize the activity of either rIFN-alpha or rIFN-beta, indicating that they bind selectively to rIFN-gamma.

To evaluate the binding between the mAbs and rIFN-gamma, one of the useful approach is to label the mAbs and then study their binding epitopes. Although ^{125}I labeling is easy and convenient, ^{125}I labeled proteins have a short shelf-life and are health hazard. We have thus used enzyme labeling; a preliminary experiment showed that horseradish peroxidase labeled mAb BG 1 recognized rIFN-gamma in ELISA. Biological assay demonstrated that the enzyme labeled BG 1 maintained 80% of its inhibitory effect on the antiviral activity of rIFN-gamma, indicating that peroxidase labeling does not abolish the binding ability of mAbs. All the other mAbs were thus labeled with horseradish peroxidase. The enzyme labeled BG 2,3 & 4, like BG 1 were active in their reactions with rIFN-gamma.

An effective means of distinguishing antigenic epitopes recognized by mAbs is to coat the plate with antigen and then study the competition between labeled and unlabeled mAbs (17). Since rIFN-gamma was denatured during the coating process (unpublished results), we modified the method by using sandwich ELISA to avoid the direct coating of the plate with rIFN-gamma. The plate was coated with an unlabeled anti-rIFN-gamma mAb and then rIFN-gamma was added. The bound rIFN-gamma was subsequently detected by each labeled mAb in the presence or absence of unlabeled mAbs. Our results (Table 2) showed that BG 1 was competed by BG 3 & BG 4 but not by BG 2; BG 2 was competed by BG 3 but not by BG 1 nor by BG 4. These results indicate that BG 1 & BG 4 bind to the same epitope while BG 2 binds to another, BG 3 either binds to a region overlapping with the other two binding sites or it binds to both sites respectively.

The mAbs also neutralized the antiviral activity of natural IFN-gamma, albeit to a lesser degree (Table 1). The binding of BG 1 to natural IFN-gamma was competed by BG 3 and BG 4 but not by BG 2 ; while the binding of BG 2 was

TABLE 2: Competition of unlabeled mAbs with labeled mAbs for bindings to rIFN-gamma[a] in Sandwich ELISA

unlabeled mAbs[b] (0.5 mg/ml)	labeled mAbs		
	BG 1	BG 2	BG 4
BG 1	0.046 [c]	0.634	0.005
BG 2	0.405	0.068	0.286
BG 3	0.115	0.242	0.071
BG 4	0.097	0.635	0.015
BG 5[d] (negative control)	0.426[e]	0.705[e]	0.345[e]

a. rIFN-gamma (1 µg/ml) was used in this assay.
b. Concentration as low as 6 µg/ml of unlabeled BG 1 blocked the binding of labeled BG 1 to rIFN-gamma. 0.5 mg/ml of unlabeled mAb, therefore, is more than sufficient.
c. The values shown are optical density (630 nm) in sandwich ELISA
d. BG 5, unlike BG 1-4 was a non-neutralizing mAb. It was used as a negative control in this competition experiment.
e. The diffence in these values was due to the fact that different labeled mAbs were used.

competed by BG 3 but not by BG 1 nor by BG 4, (Table 3) indicating that natural IFN-gamma, like rIFN-gamma, has at least two distinct antigenic epitopes for these neutralizing mAbs.

DISCUSSION

Monoclonal antibodies to natural IFN-gamma have been described by several investigators (18-23). mAbs made against rIFN-gamma, however, have not been reported. In this paper, we fused myelomas with the splenocytes of rIFN-gamma immunized mice and found seven hybridomas secreting mAbs (BG 1-7) to rIFN-gamma. Some of these mAbs (BG 5-7) only recognized the rIFN-gamma while the others (BG 1-4) not only bound to rIFN-gamma but also neutralized the antiviral activities of rIFN-gamma (Table 1).

Besides reacting with rIFN-gamma, BG 1-4 mAbs also neutralized the antiviral activities of natural IFN-gamma (Table 1), indicating that the

TABLE 3: Competition of unlabeled mAb with labeled mAbs for binding to natural IFN-gamma[a] in sandwich ELISA[b]

unlabeled mAbs (0.5 mg/ml)	labeled mAbs	
	BG 1	BG 2
BG 1	0.051	0.395
BG 2	0.179	0.005
BG 3	0.021	0.100
BG 4	0.042	0.360
BG 5 (negative control)	0.126	0.350

a. partially purified natural IFN-gamma (1 µg/ml; 50 µl/well) was used in the assay.

b. For details see Methods and legends of Table 2.

functional structure of rIFN-gamma is similar to that of natural IFN-gamma. However, since natural IFN-gamma is glycosylated while rIFN-gamma from Escherichia coli is not (23), minor differences in natural and recombinant IFN-gamma might be expected. This was demonstrated by the finding that our anti-rIFN-gamma mAbs were more selective for rIFN-gamma (Table 1), while the anti-natural IFN-gamma mAbs were reported to be more selective for natural IFN-gamma (20-22). Recently, Le et al. (24) described a mAb GIF-1 which recognized natural IFN-gamma but not rIFN-gamma. They proposed that natural and rIFN-gamma are not antigenically identical. In clinical trials of rIFN-gamma, however, we found little, if any, anti-IFN-gamma antibodies in 20 patients after 8 weeks of rIFN-gamma treatment (25), indicating that even if rIFN-gamma is antigenically different from natural IFN-gamma, it may not be a potent antigen to the humans.

Competition studies indicate that IFN-gamma has at least two binding epitopes for the neutralizing mAbs; BG 1 and BG 4 binds to one epitope while

BG 2 binds to the other. BG 3 not only competed with BG 1 & BG 4 but also with BG 2 (Table 2 & 3), we suggest that BG 3 recognized a region overlapping with the other sites or it binds to both sites independently. Since the binding of the mAbs on these antigenic epitopes blocked the activity of IFN-gamma, the antigenic epitopes are likely to be at or closely related to the active sites of IFN-gamma. It will be interesting to study whether IFN-gamma has several types of active sites corresponding to the antigenic epitopes or only one type of active site whose structure and function are affected by the binding of neutralizing mAbs to the antigenic epitopes of IFN-gamma.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. M. Hirschi for her assistance in bioassay, and to Dr. B. Otto for providing rIFN-gamma. We would like to thank Drs. J. Davies and R. Flavell for helpful comment. The technical assistance of C. Bouvier, M. Dumont, D. Williams and L. Bernasconi is appreciated.

REFERENCES

1. Epstein, L. (1981) in Interferon (Gresser, I., ed.) pp 13-14, Academic Press, New York.
2. Bloom, B.R. (1980) Nature 284, 593-595.
3. Steeg, P.S., Johnson, H.M., and Oppenheim, J.J. (1982) J. Immunol. 129, 2402-2406.
4. Basham, T.Y., and Merigan, T.C. (1983) J. Immunol. 130, 1492-1494.
5. Rubin, B.Y., and Gupta, S. L. (1980) Proc. Natl. Acad. Sci. USA 77, 5928-5932.
6. Glasgow, L.A., Crane, J.L., Jern, E.R., and Youngner, J.S. (1978) Cancer Treat. Rep. 62, 1881-1888.
7. Ito, M. (1984) J. Interferon Res. 4, 603-608.
8. Isaacs, A., and Lindenmann, J. (1957) Proc. R. Soc. Lond. B Biol. Sci. 147, 258-267.
9. Devos, R., Cheroutre, J., Taya, Y., Degrave, W., van Heuverswijn, H., and Fiers, W. (1982) Nucleic Acids Res. 10, 2487-2501.
10. Najarian, R., Simonsen, C.C., Derynck, R., Sherwood, P.J., Wallace, D.M., Berger, S.L., Levinson, A.D., and Goeddel, D.V. (1982) Nature 295, 503-508.
11. Kohler, G., and Milstein, C. (1975) Nature 256, 495-497.
12. Campbell, A.M. (1984) in Monoclonal antibody technology (Burdon, R.H., and van Knippenberg, P.H. eds.) pp 1-30 Elsevier Sci. Publishers, The Netherlands.
13. Galfre, G., and Milstein, C. (1981) Meth. Enzym. 73, 1-46.
14. Nakane, P.K., and Kawaoi, A. (1974) J. Histochem. Cytochem. 22, 1084-1090.
15. Weissmann, C. (1980) in Interferon (Gresser, I. ed.) Vol. 3, pp 101-134, Academic Press, London.
16. Youngner, J.S. (1977) Tex. Rep. Biol. Med. 35, 17-22.
17. Campbell, A.M. (1984) in Monoclonal antibody technology (Brudon, R.H., and van Knippenberg, P.H. eds.) pp 186-207 Elsevier Sci. Publishers, The Netherlands.

18. Chang, T.W., McKinney, S., Liu, V., Kung, P.C., Vilcek, J., and Le, J. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5219-5222.
19. Hochkeppel, H.K., and de Ley M. (1982) Nature 296, 258-259.
20. Novick, D., Eshhar, Z., Fischer, D.G., Friedlander, J., and rubinstein, M. (1983) EMBO J. 2, 1527-1530.
21. Rubin, B.Y., Bartal, A.H., Anderson, S.L., Millet, S.K., Hirshaut, Y, and Felt, C. (1983) J. Immunol. 130, 1019-1020.
22. Meager, A., Parti, S., Barwick, S., Spragg, J., and O'hagan, K. (1984) J. Interferon Res. 4, 619-625.
23. Kelker, H.C., Yip, Y.K., Anderson, P., and Vilcek, J. (1983) J. Biol. Chem. 258, 8010-8013.
24. Le, J., Rubin, B.Y., Keker, H.C., Feit, C., Nagler, C., and Vilcek, J. (1984) J. Immunol. 132, 1300-1304.
25. Liang, C-M., Herren, S., Fountoulakis, M., Otto, B., Hirschi, M., Gutzwiller, E., and Gerlis, L., Clin. Immunol. Immunopathol. (in press).