Distinct Functional Domains on the Recombinant Human Immune Interferon Molecule

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ABSTRACT

Two monoclonal antibodies directed against distinct epitopes of recombinant human immune interferon (rIFN-γ) were used to investigate the relationship between the molecular organization of IFN-γ and its various biological activities on cultured human melanoma cells. Both monoclonal antibodies inhibited the increase in the expression of cell surface human lymphocyte antigens Class I and II antigens and the antiproliferative and antiviral actions of rIFN-γ. On the other hand neither monoclonal antibody affected the binding of rIFN-γ to melanoma cells and its ability to reduce the expression of a high molecular weight-melanoma associated antigen. These data indicate that the functional domains of IFN-γ responsible for antiviral activity, increased human lymphocyte antigen expression and antiproliferative effects on human melanoma cells may be distinct from that (those) involved in reduced expression of the high molecular weight-melanoma associated antigen and in IFN-γ binding to cell receptors.

INTRODUCTION

Among the three major types of interferon, IFN-γ has been reported to have the strongest biological action on human tumor cells. It has marked antiviral and antiproliferative effects (1, 2), enhances the expression and shedding of cell surface HLA Class I and II antigens (3–5), and causes a reduction in the expression of the HMW-MAA (6). The mechanisms by which IFN-γ exerts its various biological activities on the target tumor cells are not clear and a definite relationship between the molecular organization of IFN-γ and its biological effects remains to be demonstrated. We have previously shown (7) that at the cellular level rIFN-γ modulates the expression and shedding of HLA Class II antigens through mechanisms different from those responsible for the antiproliferative action, modulation of the cell surface expression of melanoma-associated antigens, and of HLA Class I antigens. In this report by using two monoclonal antibodies directed against distinct antigenic determinants of human rIFN-γ, we show that at the molecular level several biological actions of IFN-γ on tumor cells may be mediated by distinct functional domains.

MATERIALS AND METHODS

Cell Line. Cultured human melanoma Colo-38 cells were grown at 37°C in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, 2.0 mm L-glutamine, and gentamicin sulfate (10 μg/ml). The cell line is Mycoplasma free.

Interferon. rIFN-γ, a generous gift from Dr. C. G. Sevastopoulos (Genentech Inc., San Francisco, CA), was produced in Escherichia coli and purified to homogeneity (8). It had a titer of approximately 10^7 antiviral units/mg. It was stored at 4°C in sterile ampuls. Dilutions were prepared in complete Dulbecco’s minimal essential medium at 1 × 10^3 units/ml prior to use. rIFN-γ was labeled with 125I to a specific activity of 5000 cpm/ng using the iodogen method (9).

Monoclonal Antibodies and Conventional Antisera. Anti-HLA Class I MoAb W6/32, the anti-β2-microglobulin MoAb NAM-1, the anti-HLA Class II MoAb Q2/80, and the anti-HMW-MAA MoAbs 225.28S and 902.5 were prepared and characterized utilizing a methodology described elsewhere (10–12). The anti-rIFN-γ MoAbs B133.1 and B133.3 were prepared by immunization of BALB/c female mice with 4 weekly s.c. injections of 10 μg of rIFN-γ and fusion of splenocytes with mouse myeloma P3X63-Ag8.653 cells 3 days after an i.v. injection of 20 μg rIFN-γ. Hybridoma culture supernatants were screened for their ability to block the HLA Class II enhancing effect of rIFN-γ on melanoma cells.

MoAbs B133.1 and B133.3 react with natural and rIFN-γ in a dose-dependent manner and display no reactivity with recombinant IFN-α and -β. Preliminary studies have shown that MoAbs B133.1 and B133.3 recognize determinants different from those recognized by MoAbs B1 and B3 described by Chang et al. (13). Ascitic fluids from mice immunized with the hybrid cell lines B133.1 and B133.3 were shown to contain mouse IgG1, 11.3 and 9.7 mg/ml respectively, as determined by a double-antibody immunosassay (14). The two monoclonal antibodies were used as ascitic fluid in the tests to analyze the functional activity of IFN-γ.

Affinity-purified rabbit anti-mouse IgG antibodies (heavy and light chain specific) were purchased from Cappel Laboratories (West Chester, PA).

Antibodies were labeled with 125I using either the iodogen (8) or chloramine-T method (15).

Serological Assays. All assays were performed in flexible microtiter plates. The indirect binding assay was carried out by sequential incubation of target melanoma cells with monoclonal antibodies and 125I-labeled rabbit anti-mouse IgG antibodies as described by Williams (16). Direct binding assay for 125I-labeled rIFN-γ to target melanoma cells was performed as described (7).

Immunoprecipitation. 125I-labeled human natural and rIFN-γ were incubated for 60 min with MoAbs B133.1 and B133.3 or the irrelevant anti-HMW-MAA MoAb 225.28S preimmobilized on rabbit anti-mouse IgG antibody Sepharose. Following six washings with phosphate-buffered saline (0.12 M NaCl, 0.003 M K2HPO4, 0.01 M NaH2PO4, pH 7.2–0.5% Triton X-100), packed beads were resuspended in the SDS-PAGE sample buffer and heated to 100°C for 10 min. Beads were removed by centrifugation, and the clear supernatant was loaded on a 12.5% polyacrylamide gel.

SDS-PAGE. SDS-PAGE was performed according to the method of Laemmli (17). Gels were fixed in 20% acetic acid, dried, and autoradiographed as described by Laskey and Mills (18).

Assay for Antiviral Activity of IFN-γ. Antiviral activity of IFN-γ was tested by inhibition of the cytopathic effect of vesicular stomatitis virus on human fibroblast strain Detroit 539, derived from a subject with trisomy 21. The IFN-γ concentration inducing 50% protection of the cytopathic effect on Detroit 539 cells corresponds approximately to 1 unit of NIH IFN-γ standard Gg-23-901-530.

RESULTS AND DISCUSSION

The anti-rIFN-γ MoAbs B133.1 and 133.3 bind to two distinct determinants on the rIFN-γ molecule, as determined by binding competition experiments in a solid phase radioimmunoassay.
The Colo-38 human melanoma cell line was then investigated. Anti-rIFN-7 was first incubated with cold MoAb B133.1 (•) or MoAb B133.3 (○) and B133.3 on rIFN-y. Microtiter plates were coated with rIFN-7 by incubating 50 μg/well of a rIFN-7 solution (50 μg/ml 0.1 M NaHCO₃, pH 9.4) for 18 h at 4°C. rIFN-7 was first incubated with cold MoAb B133.1 (○) or MoAb B133.3 (□) and then tested for its ability to bind 125I-labeled MoAb B133.1 or MoAb B133.3.

The neutralizing activity of MoAb B133.3 ascites is one-ninth of that expected on the basis of its antigen-binding capacity to rIFN-7 (specific activity, 10⁷ units/mg), assuming that all bound IFN-7 is neutralized, and it is two orders of magnitude higher than that of MoAb B133.1 ascites, although the two ascites preparations have similar antibody content and that all bound IFN-7 is neutralized, and it is two orders of magnitude higher than that of MoAb B133.1 ascites, although the two ascites preparations have similar antibody content and antigen-binding capacity.

The effect of MoAbs B133.1 and B133.3, at the final dilution of 10⁻², on the antigen modulating activities of rIFN-7 on the Colo-38 human melanoma cell line was then investigated. Anti-HLA Class I MoAb W6/32, anti-β₂-microglobulin MoAb NAMB-1, anti-HLA Class II MoAb Q2/80, and anti-HMW-MAA MoAbs 225.28S and 902.5 were used in an indirect immunoperoxidase assay (15) to monitor IFN-γ-induced changes in the expression of the corresponding antigens. rIFN-γ (600 units/ml; i.e., the minimum amount required to induce the maximum effect) enhanced the expression of HLA Class I and class II antigens on Colo-38 cells; this effect was completely inhibited by pretreatment of the IFN-γ preparation with a large excess (approximately 500 times the amount of rIFN-7 on a molar basis) of MoAb B133.3, but only partially blocked by pretreatment with MoAb B133.1. Cell surface expression of the HMW-MAA was reduced upon IFN-γ treatment of the target Colo-38 cells, and this decrease was unaffected by either of the two anti-IFN-γ monoclonal antibodies (Fig. 3).

In experiments to determine the effect of anti-IFN-γ monoclonal antibodies on the anti-proliferative activity of rIFN-γ, Colo-38 cells were cultured in the presence of rIFN-γ (600 units/ml) or the same amount of rIFN-γ preincubated with 10⁻² dilutions of MoAbs B133.1 and B133.3 or ascitic fluid obtained from mice immunized with NS1 myeloma cells. As shown in Fig. 4, the presence in the culture of IFN-γ preincubated with control NS1 ascitic fluid caused a 30% reduction in cellular proliferation; this effect was completely inhibited by MoAb B133.3, but never completely inhibited (85% inhibition) by MoAb B133.1. The viability of cell cultures was more than 95% before and after rIFN-γ treatment of the melanoma cells. All the investigated effects induced by rIFN-γ and natural IFN-γ preparations on melanoma cells were completely abrogated when the IFN-γ preparations were absorbed on Sepharose 4B beads to which MoAbs B133.1 and B133.3 were coupled (6) (results not shown).

To investigate the effect of anti-IFN-γ antibodies on the specific binding of 125I-labeled IFN-γ to cell receptors, target melanoma cells were first treated with sodium azide to block the internalization of the IFN-γ receptor complex (20) and then

![Fig. 1. Mapping of antigenic determinants recognized by MoAb B133.1 and B133.3 on rIFN-γ](image_url)

![Fig. 2. Immunochemical analysis of the reactivity of MoAb B133.1 and B133.3 with natural and recombinant IFN-γ. A, SDS-PAGE analysis of natural (lanes 1–3) and recombinant (lanes 4–6) human IFN-γ immunoprecipitated with the irrelevant anti-HMW-MAA MoAb 225.28S (lanes 1 and 4), with MoAb B133.1 (lanes 2 and 5), and with MoAb B133.3 (lanes 3 and 6). The molecular weight marker proteins were phosphorylase b (M, 92,000), bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 30,000), trypsin inhibitor (M, 18,000), and lysozyme (M, 13,000). b, SDS-PAGE analysis of 125I-labeled rIFN-γ. A 5-μg sample of rIFN-γ was electrophoresed on SDS-PAGE, the gel was fixed in 20% acetic acid, and washed extensively in phosphate buffered saline. The gel was sequentially incubated for 30 min with 0.1 M sodium phosphate buffer pH 7.5, containing 2.0 mCi Na125I and chloramine-T (10 μg/ml). The gel was washed for 24 h in 4 changes of 10% acetic acid, 20% methanol, dried, and autoradiographed.)
ascites fluid. The latter was supplemented with mouse IgG (10 mg/ml). Cells activity of rIFN-7 on cultured melanoma cells Colo-38. Melanoma Colo-38 cells (2.5 x 10^6/ml) were grown in complete Dulbecco’s minimal essential medium at 37°C for 48 h in the presence of sodium azide for 16 h with 6 ng of ^125I-labeled rIFN-7 preincubated for 16 h with NSI and MoAbs B133.1 or B133.3 ascites fluid (final dilution, 10^{-2}). Cells were centrifuged and washed six times with phosphate buffered saline-1% bovine serum albumin. The plate was dried, individual wells were cut, and cell-bound radioactivity was measured in a gamma counter. Radioactivity bound to target melanoma cells preincubated with unlabeled rIFN-7 was used as background radioactivity. Data are mean ± SD (bars) of 10 replicate wells in each experiment.

whether rIFN-7 was pretreated or not with either monoclonal antibody at a final dilution of 10^{-3}. The same conclusion was reached when cells were incubated with ^125I-labeled rIFN-7 at half-saturating level or at the final concentration of 3 ng/2 x 10^5 cells, when rIFN-7 was labeled with ^32P (21) and when the incubations were performed at 20°C.

The differential ability of MoAbs B133.1 and B133.3 to inhibit various biological activities of IFN-7 suggests that the human IFN-7 molecule may contain separate domains responsible for (a) binding to cellular receptors and reduced expression of the HMW-MAA and (b) antiviral activity, cell growth inhibition, and enhancement of HLA antigen expression, respectively. A similar possibility has also been suggested for certain biological activities of mouse IFN-7 by Celada et al. (22), who showed that one of their anti-IFN-7 monoclonal antibodies blocks IFN-7 binding to macrophages and inhibits IFN-7-induced macrophage activation, whereas the other one inhibits the antiviral activity of IFN-7 but does not affect its binding and its effect on macrophage activation. MoAb B133.3 very efficiently inhibits antiviral activity of IFN-7, although its maximal inhibitory activity is only about one-ninth of its antigen-binding activity. Chromatographic analysis of ^125I-labeled rIFN-7 under nonreducing and nonnaturating conditions, however, indicates that only 15% of the rIFN-7 molecules are monomeric, whereas the remaining are aggregated in trimers or larger complexes. Thus, under conditions of maximal precipitation, MoAb B133.3 recognizes only a fraction of the available antigenic determinants. Therefore, one might suggest that each interaction of MoAb B133.3 with IFN-7 leads to the inactivation of the IFN-7 molecule and that the determinant recognized by MoAb B133.3 is on or near the domain responsible for the antiviral and HLA antigen-enhancing effects. MoAb B133.1 recognizes an antigenic determinant distinct from that recognized by MoAb B133.3 and has a weaker ability to inhibit IFN-7 functions, suggesting that it reacts with a determinant less closely associated than that defined by MoAb B133.3 with the domain of IFN-7 responsible for its antiviral activity.

On the other hand, the differential affinity of the two anti-IFN-7 MoAbs is not likely to be a major factor in the different degree of neutralization of IFN-7, since both of them are effective in precipitating IFN-7 in various non-ionic detergents, a property not normally shown by low affinity monoclonal antibodies. The mechanism(s) by which the two antibodies inhibit some of the IFN-7 functions is not totally clear. The antibodies may impose or restrict certain conformational
changes of the IFN-γ molecule as suggested by Rubin et al. (23) or they may partially or completely block the internalization of IFN-γ that occurs after its binding to the cell membrane.

The antiviral activity of IFN-γ and its other functions, involving derepression of specific genes, might require internalization of IFN-γ (20) and an available molecular domain for its interaction with a second, possibly intracellular, receptor. On the other hand, the reduced expression of HMW-MAA and possibly, as suggested by the data in the murine system (22), macrophage activation might depend more directly on interaction of IFN-γ with the cell membrane; shedding and activation might then be triggered either directly by the binding of a IFN-γ domain to a specific receptor, or instead, by a secondary interaction following binding of a third functional domain.

ACKNOWLEDGMENTS

The authors wish to thank Drs. S. Pestka and J. Langer (Roche Institute of Molecular Biology, Nutley, NJ) for the gift of 32P-labeled IFN-γ and Edwina L. Jones and Vicky L. Temponi for the excellent secretarial assistance.

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