Immunochemical Mapping of α-2 Interferon

Nicholas B. Lydon, Catherine Favre, Sylvie Bove, Odile Neyret, Sylvie Benureau, Alan M. Levine, Gail F. Seelig, Tatanahalli L. Nagabhushan, and Paul P. Trotta*

Laboratoire de Recherches Immunologiques, UNICET, Dardilly, France, and Schering-Plough Corporation, Bloomfield, New Jersey 07003

Received October 15, 1984; Revised Manuscript Received February 27, 1985

ABSTRACT: A panel of five monoclonal antibodies, designated U1–U5, produced by murine hybridoma clones has been raised to recombinant interferon (IFN) α-2, and one monoclonal antibody, designated U6, has been raised to a mixture of cyanogen bromide fragments of IFN α-2. These antibodies have been characterized with respect to (1) neutralization of IFN antiviral and antiproliferative activities, (2) binding to four cloned IFN α subtypes (α-1, α-2, α-4, and α-7) that are naturally occurring and to two novel products of recombinant DNA technology (δ-4 α-1 and δ-4 α-2/α-1 hybrid), and (3) binding to three cyanogen bromide fragments of IFN α-2. Four of the six monoclonal antibodies inhibited IFN antiviral activity. In conjunction with the previously reported monoclonal antibodies III/21 [Arnheiter, H., Thomas, R. M., Leist, T., Fountoulakis, M., & Gutte, B. (1981) *Nature (London) 294*, 278–280] and NK-2 [Secher, D. S., & Burke, D. C. (1980) *Nature (London) 285*, 446–450], eight unique epitopes have been described. Analysis of cross-reactivity patterns with IFN α fragments and subtypes indicated that monoclonal antibodies U1 and NK-2, which neutralized both antiviral and antiproliferative activities, and U2, which was nonneutralizing in these assays, were directed to distinct epitopes located in a polypeptide consisting of the amino-terminal 15 amino acid residues linked to residues 60–110 by a disulfide bond. The epitope recognized by U1 was determined to reside, at least in part, between residues 5 and 15. Competitive binding studies indicated that neutralizing monoclonal antibody U3, which did not bind to any of the cyanogen bromide fragments, was directed to an epitope partially overlapping that of NK-2. Epitopes to which neutralizing monoclonal antibodies U3, U4, and U5 and nonneutralizing antibody U6 were directed were readily distinguished by cross-reactivity with IFN α subtypes. The nonneutralizing monoclonal antibody U6 was determined to be directed to an epitope between residues 22 and 58. The fact that δ-4 α-1 and the δ-4 α-2/α-1 hybrid were active in an antiviral assay indicated a lack of direct functional significance for the first four amino-terminal amino acid residues and the Cys_1–Cys_{98} disulfide bond. However, reduction with 2-mercaptoethanol of IFN α-2 altered the integrity of four of the eight epitopes. These data support a critical role for disulfide linkages in maintaining the native conformation of IFN α-2 and provide a potential basis for predicting the location of functionally important domains.

The IFNs[1] represent a multigene family that expresses a broad range of biological properties, including potent antiviral, antiproliferative, and immunomodulatory activities (Baron & Dianzani, 1977; Stewart, 1979; Nagata et al., 1980, 1981; Goeddel et al., 1981). The three major species of IFN are designated α (or leukocyte), β (or fibroblast), and γ (or immune) IFN. Within the group of human α interferons there are at least 13 distinct gene products, which express ca. 75% or greater sequence homology (Nagata et al., 1980, 1981; Goeddel et al., 1981; Weissmann et al., 1982; Zoon & Wetzel, 1983). All cloned human leukocyte IFNs share in common four highly conserved cysteine residues that have been demonstrated in one α subtype to exist as two disulfide linkages between Cys_1 and Cys_{98} and between Cys_{29} and Cys_{138}, respectively (Goeddel et al., 1981; Weissmann et al., 1982; Wetzel, 1981; Wetzel et al., 1981).

The cloning of a human IFN α gene into *Escherichia coli* by recombinant DNA technology has permitted the purification of gram quantities of a single subtype, IFN α-2 (Streuli et al., 1980; Nagabhushan et al., 1984), which has made feasible a detailed analysis of molecular structure–function relations. To date, however, identification of the domains of α IFN that are most critical for the expression of biological activity has been incomplete and controversial. For example, employing a chemically modified derivative of human IFN α-A, which differs from IFN α-2 at only one amino acid position (Streuli et al., 1980; Goeddel et al., 1981), Wetzel and colleagues (Wetzel et al., 1982, 1983; Morehead et al., 1984) have reported chemical modification studies implying that the integrity of the Cys_{29}–Cys_{138} disulfide bond is essential for maintaining the active three-dimensional conformation of the molecule. In contrast, Ackerman et al. (1984) have employed proteolytic digestion to generate a biologically active fragment of IFN α-2 containing only the amino-terminal residues 1–110 and therefore lacking the critical Cys_{29}–Cys_{138} linkage. This result suggests that a significant degree of active conformation may exist even in the absence of this disulfide bond. Thus, more data on the structure–function relations in α IFN are required, preferably from alternative methodologies that do not involve chemical or enzymatic modification of the parental molecule. One approach is the genetic engineering of novel protein variants, as demonstrated by the construction of fully active truncated variants of α IFN lacking the 10–13

* Correspondence should be addressed to this author at the Schering-Plough Corp., Bloomfield, NJ 07003.

[1] Abbreviations: IFN, interferon; CNBr, cyanogen bromide; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; TBS, Tris-buffered saline; EMEM, Eagle's minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EMC virus, encephalomyocarditis virus; VSV, vesicular stomatitis virus; IRMA, immunoradiometric assay; IU, international unit(s).

0006-2960/85/0424-4131$01.50/0

carboxyl-terminal amino acid residues (Streuli et al., 1981; Franke et al., 1982; Wetzel et al., 1982; Chang et al., 1983). Alternatively, immunochemical mapping with monoclonal antibodies can provide a further analysis of the functional significance of various domains. For example, a monoclonal antibody has been employed to establish that a segment of the primary sequence between the 10th and 16th residues from the carboxyl terminal is exposed on the surface of IFN α-2 and is nonessential for biological activity (Arnheiter et al., 1981, 1983).

A panel of eight monoclonal antibodies directed to IFN α-2 has been characterized in the present study. The regions of the molecule to which these antibodies are directed have been defined by a novel approach that combines information from cross-reactivity with a panel of six IFN α subtypes and three CNBr fragments of IFN α-2. Monoclonal antibodies that neutralize antiviral and antiproliferative activities have located potential regions of functional significance, and a nonneutralizing antibody has identified a primary sequence in a domain distinct from the receptor binding region. The fact that two cDNA clones produce IFN α variants that lack the first four amino-terminal residues but yet express high levels of antiviral activity provides strong support for the nonessentiality of this sequence in both IFN α-1 and α-2. Since neither of these truncated variants contains Cys_1, the data also provide further confirmation of the conclusion (Morehead et al., 1984) that the Cys_1–Cys_{98} disulfide bond is not required for the antiviral activity of human leukocyte IFNs.

Experimental Procedures

Materials. Cloned human IFN α subtypes α-2, δ-4 α-2/α-1, α-4, and α-7 were purified from *E. coli* K-12 to constant maximal specific antiviral activity. Purified IFN α-1 and α-7 were obtained from Biogen SA. Purified IFN δ-4 α-1 was provided by Charles Weissmann (University of Zurich). Alkaline phosphatase conjugated rabbit anti-mouse immunoglobulin was purchased from TAGO (Burlingame, CA), and class-specific rabbit anti-mouse immunoglobulin was purchased from Miles Laboratories (Elkhart, IN). Horseradish peroxidase conjugated goat anti-mouse immunoglobulin and 2,2′-azinobis[3-ethylbenzothiazolinesulfonate] were purchased from Kirkgaard Perry (Gaithersburg, MD). Purified mouse myeloma protein was obtained from Zymed (Burlingame, CA). Monoclonal antibody NK-2 was purchased from Celltech Ltd. (Berkshire, England). The hybridoma clone producing monoclonal antibody III/21 was obtained from H. Arnheiter (Arnheiter et al., 1981). All other monoclonal antibodies were raised in BALB/c mice against either IFN α-2 or CNBr fragments of IFN α-2, as described below. For competitive ELISA, alkaline phosphatase was conjugated to purified monoclonal antibody with glutaraldehyde (Avrameas et al., 1978). Female BALB/c mice were obtained from Jackson Labs and supplied by IFFA Credo (L'Arbresle, France). Diethanolamine, isopropyl alcohol, ammonium sulfate, PBS [NaCl (8 g/L), KH_2PO_4 (0.2 g/L), $NaH_2PO_4 \cdot 12H_2O$ (2.29 g/L), and KCl (0.2 g/L)], and Tween 20 were purchased from Merck (Darmstadt, West Germany). Fetal calf serum (lot no. 742) was obtained from IBF (Villeneuve La Garenne, France), and RPMI-1640, EMEM, L-glutamine, penicillin, streptomycin, and complete Freund's adjuvant were supplied by Gibco (Grand Island, NY and Paisley, Scotland). Hypoxanthine, aminopterin, thymidine, 2-mercaptoethanol, Tris (free base), MTT, alkaline phosphatase (type VII), and isopropyl alcohol were purchased from Sigma Chemical Co. (St. Louis, MO). Protein standards were a product of Polysciences (Warrington, PA). CNBr and formic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile was purchased from Burdick & Jackson (Muskegon, MI), and trifluoroacetic acid was obtained from MCB Reagents (Gibbstown, NJ). Acrylamide, *N,N′*-methylenebis(acrylamide), and sodium dodecyl sulfate were supplied by Bio-Rad (St. Louis, MO). The Microsorb C_{18} column (10 × 0.46 cm) used for amino acid analysis was purchased from Rainin (Woburn, MA); brij-35, sodium decyl sulfate, and sodium heptanesulfonate were obtained from Eastman Kodak Co. (Rochester, NY); constant boiling HCl (sequenal grade) was supplied by Pierce Chemical Co. (Rockford, IL). All chemicals were of analytical grade. Immunolon II microtiter plates were purchased from Dynatech Labs (Richmond, VA); Falcon microtiter plates for antiviral activity were purchased from Becton Dickinson Labware (Oxnard, CA).

Plasmid Construction. Cloned α genes were obtained as previously described (Streuli et al., 1980). The vector employed for expression of IFN α-2 in *E. coli* was a pBR322-based plasmid under the control of the β-lactamase promoter (Nagabhushan & Leibowitz, 1984). The vector employed for the IFN δ-4 α-2/α-1 hybrid was a pBR322-based plasmid under the control of the *lac* promoter (M. Ryan and P. Leibowitz, personal communication). The plasmid containing the IFN α-4 gene was also pBR322 based and under the control of the β-lactamase promoter; it was obtained from Biogen SA.

Monoclonal Antibody Production and Purification. Female BALB/c mice were injected intraperitoneally 4 times at 15-day intervals with 10 μg of IFN in complete Freund's adjuvant. A final immunization was performed by the latter procedure in combination with intravenous administration of 10 μg of IFN dissolved in PBS. Spleens were removed aseptically after 4 days, and single-cell suspensions of splenocytes were fused with mouse myeloma cells in the presence of poly(ethylene glycol), essentially as described by Kohler & Milstein (1975). Hybridoma colonies were selected in hypoxanthine–aminopterin–thymidine medium and screened for specific antibody production to IFN α-2 by ELISA. Positive hybridomas were cloned by limiting dilution. Selected clones were injected intraperitoneally into pristane-primed BALB/c mice for production of ascites tumors. After 7–10 days the ascites fluid was drained and centrifuged at 100000*g* for 30 min. Immunoglobulin G (IgG) was precipitated at 4 °C from the supernatant with 50% ammonium sulfate and redissolved in 20 mM Tris hydrochloride–40 mM NaCl, pH 7.8. IgG was further purified by chromatography on DEAE-cellulose employing a 0–1 M NaCl gradient for elution. Antibodies were determined to be ca. 90% IgG by SDS–PAGE.

Enzyme-Linked Immunosorbent Assay (ELISA). Hybridoma supernatants or purified IgG was screened for specific binding to IFN α-2 by employing an indirect solid-phase ELISA. Two alternative methodologies were employed. Method A was employed for all binding assays except for determining the effect of reduction with 2-mercaptoethanol (Table IV), for which method B was employed.

Method A. Microtiter plates were coated overnight at 4 °C with 200 ng of IFN α subtype or CNBr fragment per well dissolved in 200 μL of 50 mM sodium carbonate, pH 9.6, containing 0.02% sodium azide. After a 1-h incubation at 37 °C with 10% fetal calf serum, plates were washed 4 times with PBS containing 0.05% Tween 20. An aliquot (200 μL) of hybridoma culture supernatant or purified IgG diluted in RPMI-1640 was added to the wells, followed by a 1.5-h incubation at room temperature. After four washes with PBS–Tween 20 as described above, 200 μL of affinity-purified

alkaline phosphatase conjugated rabbit anti-mouse IgG was added to each well. After a 1.5-h incubation at room temperature the wells were again washed with PBS–Tween 20 four times, followed by addition of the substrate *p*-nitrophenyl phosphate (5 mg/L) dissolved in diethanolamine hydrochloride (0.9 mM, pH 9.8) containing 1 mM magnesium chloride. The optical density of each well at 405 nm was measured at 15-min intervals with a micro-ELISA autoreader (Dynatech Labs). Titers for antibody binding are expressed as the reciprocal of the dilution required for 50% maximal binding.

Method B. Microtiter plates were coated with 200 ng of IFN α subtype or CNBr fragment per well for 1 h at room temperature in 50 μL of TBS. The plates were washed 5 times with 0.02% Tween 20 dissolved in TBS. The wells were incubated 16 h at 37 °C with 50 μL of either TBS or TBS containing 0.1 M 2-mercaptoethanol. After the plates were washed 5 times with TBS–0.02% Tween 20, they were coated with 1% bovine serum albumin for 1 h at room temperature, washed again 5 times with TBS–0.02% Tween 20, and incubated with the hybridoma supernatant or purified antibody for 1 h at room temperature. The plates were washed 5 times with TBS–0.02% Tween 20 and incubated with 50 μL of horseradish peroxidase conjugated goat anti-mouse immunoglobulin at room temperature for 1 h. The wells were washed 5 times with TBS–0.02% Tween 20. Color was developed by addition of hydrogen peroxide and 2,2′-azinobis[3-ethylbenzothiazolinesulfonate].

Competitive ELISA. Competition of purified monoclonal antibodies for IFN α-2 bound to the solid phase was determined with a direct ELISA. Serial dilutions of purified monoclonal antibody were made in the wells of Immunolon II microtiter plates with EMEM as diluent. The amount of antibody ranged from 0.1 to 10 μg per well. A total of 4 μg of a second alkaline phosphatase conjugated monoclonal antibody dissolved in RPMI-1640 was added to each well to a final volume of 200 μL. After a 3-h incubation at room temperature, the wells were washed and further processed as described above for the standard ELISA.

Immunoglobulin Subclass. Antibody subclass and isotype were determined by an indirect ELISA employing subclass-specific rabbit anti-mouse IgG. Briefly, specific rabbit anti-mouse isotype diluted 1:500 with RPMI-1640 was incubated in microtiter wells overnight at 4 °C. After a 2-h incubation at room temperature with RPMI-1640 containing 10% heat-inactivated fetal calf serum, wells were washed with PBS–Tween 20, as described above, and 200 μL of diluted hybridoma culture supernatant was added to each well, followed by a 2-h incubation at room temperature. Bound mouse monoclonal antibodies were detected by addition of alkaline phosphatase conjugated rabbit anti-mouse IgG, as described for the ELISA.

Antiviral Activity. A cytopathic effect–inhibition assay was performed in 96-well microtiter plates essentially as described by Rubenstein et al. (1981) with either of two combinations of target cell and infecting virus: human foreskin cells (FS-71) and EMC virus (ATCC-VR129) or Madin-Darby bovine kidney cells (ATCC-CCL22) and VSV (Indiana strain), respectively. Serial 2-fold dilutions were prepared with EMEM containing 10% fetal calf serum. Plates were stained with crystal violet and read by visual inspection to obtain an IFN titer corresponding to an assay end point of 50% protection against viral infectivity. International units were determined by comparison to natural leukocyte standard WHO6919.

Antiproliferative Assay. Growth inhibition was assayed in microtiter plates on Daudi cells (ATCC-CCL213), a lymphoblastoid line of Burkitt's lymphoma origin, essentially as described by Evinger & Pestka (1981). Cell number was determined colorimetrically by reduction of MTT according to the method of Mosmann (1983). Absorbance was measured with a micro-ELISA autoreader at 570 nm.

Amino Acid Analysis. IFN α-2 dissolved in water or purified CNBr fragments dissolved in an acetonitrile–trifluoroacetic acid mixture were evaporated, dissolved in 0.50 mL of constant boiling HCl, and sealed in vacuo. After a 24-h hydrolysis at 110 °C, excess HCl was removed by evaporation, and the protein or polypeptide hydrolysate was dissolved in water. Amino acid analysis was performed on a Microsorb C_{18} high-pressure liquid chromatography (HPLC) column, essentially as described by Radjai & Hatch (1980). Peaks were detected by fluorescence in an Aminco Fluoro Monitor.

Protein Determination. Protein concentration was determined either as described by Lowry (1951) or as described by Bradford (1976). In both cases bovine serum albumin was employed as standard.

Results

Thirty-nine and thirty-two hybridomas were found positive for IFN α-2 and CNBr fragments, respectively, and were cloned by limiting dilution. Monoclonal antibodies were selected that were representative of different patterns of cross-reactivity with IFN α subtypes. These clones were injected into pristane-primed mice for the large-scale production of ascites fluid, from which the immunoglobulin fraction was purified. Five monoclonal antibodies (U1–U5) raised to IFN α-2 as immunogen and one monoclonal antibody (U6) raised to a mixture of CNBr fragments were characterized with respect to isotype, effects on IFN antiviral and antiproliferative activity, and epitope identification. Two other monoclonal antibodies were also similarly studied: (a) a monoclonal antibody (designated III/21; Arnheiter et al., 1981) produced to a synthetic 56-residue carboxyl-terminal fragment of IFN α-1 and (b) a monoclonal antibody (designated NK-2; Secher & Burke, 1980) raised against human leukocyte interferon and selected for its capacity to neutralize antiviral activity. Antibodies U1–U6 as well as III/21 were determined to belong to the IgG_1/K isotope; the same classification for III/21 was obtained by Arnheiter et al. (1981). NK-2 was classified as IgG_{2a}/K.

The principal approach for identifying the domains of IFN α-2 to which these monoclonals were directed, as well as for testing whether the same or different epitopes were recognized by the panel of antibodies, was analysis of binding to three CNBr fragments and six highly purified human IFN α subtypes.

Purification and Characterization of CNBr Fragments of IFN α-2. The six polypeptide fragments that are predicted from CNBr cleavage of fully reduced IFN α-2 are listed in Table I with the corresponding molecular weight values predicted from the reported gene sequence (Streuli et al., 1980). Methionine has been replaced with homoserine, a conversion that occurs during CNBr cleavage of methionyl linkages. Since in the absence of prior reduction, disulfide bonds exist between Cys_1 and Cys_{98} and between Cys_{29} and Cys_{138}, respectively, CNBr cleavage of native IFN α-2 is predicted to produce two major fragments referred to as 1-4 and 3-5, in which fragments 1 and 4 (for the first fragment) and 3 and 5 (for the second fragment) are covalently linked by disulfide bonds. Fragments 2 and 6 would be expected to be produced from both reduced and oxidized IFN α-2.

CNBr fragments were isolated and purified by HPLC employing a C_{18} μ-Bondapak column and an acetonitrile gradient, as described in Figure 1A. The column void volume was

Table I: Expected Fragments of IFN α-2 Produced by CNBr Cleavage[a]

fragment no.	amino acid composition from	through	M_r
1	Cys (1)	Hse (16)	1785
2	Leu (17)	Hse (21)	545
3	Arg (22)	Hse (59)	4522
4	Ile (60)	Hse (111)	5899
5	Lys (112)	Hse (148)	4477
6	Arg (149)	Glu (165)	1952

[a] These fragments are predicted on the assumption of complete reduction of disulfide bonds 1–98 and 29–138. Numbers in parentheses represent the corresponding position in the parent IFN α-2. The molecular weight values are calculated from the amino acid composition determined from the gene sequence (Streuli et al., 1980).

found to contain CNBr and formic acid; no protein could be detected in this fraction by the Bradford (1976) protein assay. Several peaks eluted by the acetonitrile gradient were detected by postcolumn derivatization with *o*-phthalaldehyde and by monitoring the relative fluorescence emitted at 440 nm. A number of minor components were typically observed as shoulders on each major peak. Their appearance is consistent with the various side reactions that are known to occur during the CNBr cleavage reaction, as, e.g., deamidation of glutamine and asparagine residues or oxidation of sulfur-containing amino acids. Initial identification of eluted peaks was performed by amino acid analysis of pooled fractions. Comparison of the observed and the theoretical amino acid compositions identified the positions of CNBr fragments 1-4, 3-5, 6, and 2, which eluted on HPLC with mean retention times of 72.0, 62.5, 42.0, and 22.0 min, respectively (Figure 1A). A peak corresponding to undigested IFN α-2 was observed at 75 min. The major peaks corresponding to CNBr fragments 1-4, 3-5, and 6 were rechromatographed on HPLC under the same conditions employed in Figure 1A. As shown in Figure 1B, greater than 90% purity was observed for each of these fragments. Purified fragments were found to be soluble in water and were generally stored lyophilized at −80 °C (in vacuo). Characterization of CNBr fragment 2 was not attempted since it was obtained in low yield and was not employed for epitope identification.

For further identification, fragments 1-4 and 3-5 were reduced with 10% 2-mercaptoethanol (15 min at 100 °C) and chromatographed on HPLC in the presence of 1% 2-mercaptoethanol in the eluting solvent; the remainder of the conditions for chromatography were identical with those described in Figure 1. For the reduced CNBr fragment 3-5 two new peaks in approximately equal amount were observed with retention times 1.2 min later and 4.1 min earlier, respectively, than that of the unreduced fragment. This result provided further evidence for the existence of a disulfide linkage between two polypeptide chains. The reduced CNBr fragment 1-4 eluted principally as a single peak 8 min later than the unreduced fragment. The latter result is consistent with the fact that CNBr fragment 1 contains no lysine residues (Streuli et al., 1980) and would therefore be predicted to react poorly with *o*-phthalaldehyde.

Fragments 1-4, 3-5, and 6 were tested for antiviral activity by employing a cytopathic effect–inhibition assay with EMC virus and human foreskin fibroblast cells as target. No activity was observed for fragments 3-5 and 6 even at a molar concentration ca. 100-fold greater than that required to demonstrate antiviral activity for IFN α-2. A very low level of activity associated with fragment 1-4 at less than 0.001% of the parental α-2 on a molar basis is most probably due to a minor contamination with native IFN α-2 since this fragment elutes adjacent to undigested IFN α-2 on HPLC (Figure 1A).

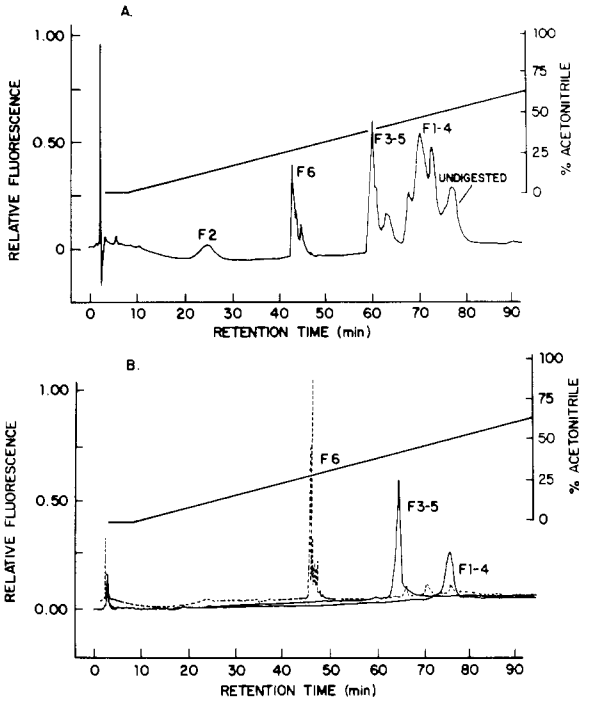

FIGURE 1: Reverse-phase HPLC of CNBr digest of IFN α-2. (A) Relative fluorescence as a function of retention time for the total digest. IFN α-2 (1.0 mg/mL) in 70% formic acid was reacted with freshly prepared CNBr (15 mg/mL) at room temperature for 20 h in a sealed glass tube. A 20-μL aliquot of the digest was applied to a 4 × 250 mm Waters μBondapak C_{18} column equilibrated with 0.01 M trifluoroacetic acid at 30 °C. After a 5-min isocrat with 0.01 M trifluoroacetic acid, peptides were eluted in a linear gradient between 0% and 62.7% acetonitrile containing 0.01 M trifluoroacetic acid for a total period of 85 min at a flow rate of 2.4 mL/min at 30 °C. The column effluent was mixed with *o*-phthalaldehyde dissolved in 0.2 M sodium borate, pH 9.75, containing 0.09% brij-35 and 0.2% 2-mercaptoethanol. Peaks were detected by fluorescence emission at 440 nm employing an Aminco Fluoro Monitor. F2, F6, F3-5, and F1-4 refer to the CNBr fragments defined in Table I. "Undigested" indicates residual unreacted IFN α-2. (B) Rechromatography of the three major CNBr fragments. Fractions corresponding to F6, F3-5, and F1-4 were pooled from seven consecutive chromatographies, lyophilized, dissolved in water, and rechromatographed under the same conditions as described above. A dashed line is employed for the profile obtained for fragment 6.

This conclusion is suggested by the fact that monoclonal antibody U3 (described more fully below), which does not bind to this fragment but does bind to native IFN α-2, was able to inhibit completely the antiviral activity found in the 1-4 preparation. Wetzel et al. (1982) also report that CNBr digestion results in an inactive mixture of fragments.

A mixture of the purified CNBr fragments 1-4, 3-5, and 6 was tested for its ability to competitively inhibit the antiviral activity of IFN α-2 by employing the same cytopathic effect–inhibition assay. Concentrations of the fragment mixture ranging from 0.012 to 124 nM, representing a ca. 3000-fold molar excess over the concentration of IFN α-2, resulted in no significant reduction in antiviral activity when human fibroblast FS-71 cells were incubated simultaneously with the CNBr fragments and IFN α-2. In other experiments, human fibroblast FS-71 cells were first preincubated with the individual purified CNBr fragments ranging in concentration from 15 to 25 nM for 4 h at 37 °C, followed by a 4-h incubation with IFN α-2 at 37 °C. Antiviral activity in this case was also found to be unaffected by the addition of any of the CNBr fragments.

Purification and Characterization of IFN α Subtypes. Six distinct IFN α subtypes were employed for epitope identifi-

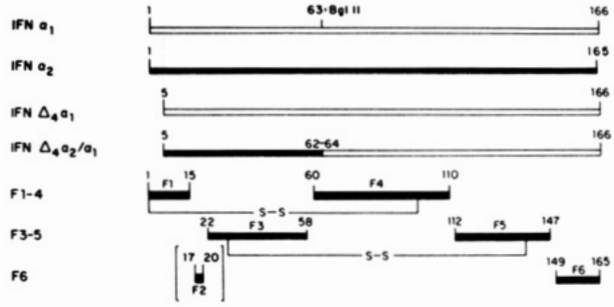

FIGURE 2: Schematic linear diagram of IFN α subtypes and CNBr fragments of IFN α-2. Filled horizontal bars indicate amino acid sequences in IFN α-2, whereas open horizontal bars refer to amino acid sequences in IFN α-1. Numbers above the linear representations indicate amino acid position. *Bgl*II is the restriction endonuclease employed in the preparation of the δ-4 α-2/α-1 hybrid. F1-4, F3-5, F6, and F2 represent CNBr fragments of IFN α-2, as defined in Table I. F2 is placed in brackets since it was not employed for epitope identification.

Table II: Specific Antiviral Activities of IFN α Subtypes[a]

IFN subtype	IU/mg	IFN subtype	IU/mg
α-1	7.1×10^6	α-2	1.7×10^8
δ-4 α-1	2.8×10^6	α-4	1.0×10^8
δ-4 α-2/α-1	1.0×10^8	α-7	2.0×10^7

[a] Activity was determined by the cytopathic effect–inhibition assay employing EMC virus and human foreskin cells, as described under Experimental Procedures. Data represent the mean of at least nine assays.

cation. Four of these (α-1, α-2, α-4, and α-7) are naturally occurring gene products whose gene sequence has been previously described (Streuli et al., 1980; Mantei et al., 1980; Nagata et al., 1980, 1981). The remaining two subtypes, δ-4 α-1 and the δ-4 α-2/α-1 hybrid, are novel products of recombinant DNA technology in which the first four residues from the amino terminal are deleted. Both of these molecules lack Cys_1 and, hence, are expected to contain only one disulfide linkage, Cys_{29}–Cys_{138}. Neither molecule contains the Cys_1–Cys_{98} disulfide linkage occurring in native IFN α-2. The δ-4 α-2/α-1 hybrid, which was produced by joining at a common *Bgl*II restriction site, consists of residues 5–62 of IFN α-2 and residues 64–166 of IFN α-1. The parental IFN α-2 and IFN α-1, the two novel δ-4 α IFNs, and the predicted IFN α-2 CNBr fragments are compared in a schematic linear diagram in Figure 2.

IFN α subtypes were purified from extracts of *E. coli* to constant maximal specific antiviral activity, essentially as described by Weissmann (1981). Chromatographies were performed on Matrex gel blue–Sepharose, Sephadex G-100, phenyl-Sepharose, and DEAE-Sepharose CL-6B. Elution from both Matrex gel blue and DEAE columns was achieved with a linear gradient of 0–1 M sodium chloride. For purification of the δ-4 α-2/α-1 hybrid, phenyl-Sepharose was replaced with a copper chelate column, from which the hybrid was eluted by lowering the pH to 3.0. The specific antiviral activities of the purified IFNs are summarized in Table II. It is notable that, despite a similar degree of purity for each IFN (see below), the specific antiviral activities span a range of approximately 2 orders of magnitude, from 2.8×10^6 IU/mg for δ-4 α-1 to 1.7×10^8 IU/mg for α-2. Variation in specific antiviral activity among IFN α subtypes has previously been reported (Streuli et al., 1981; Pestka et al., 1984; Seelig et al., 1984). The data further indicate that the two truncated IFNs that lack the first four amino-terminal residues express specific antiviral activity that is comparable to that

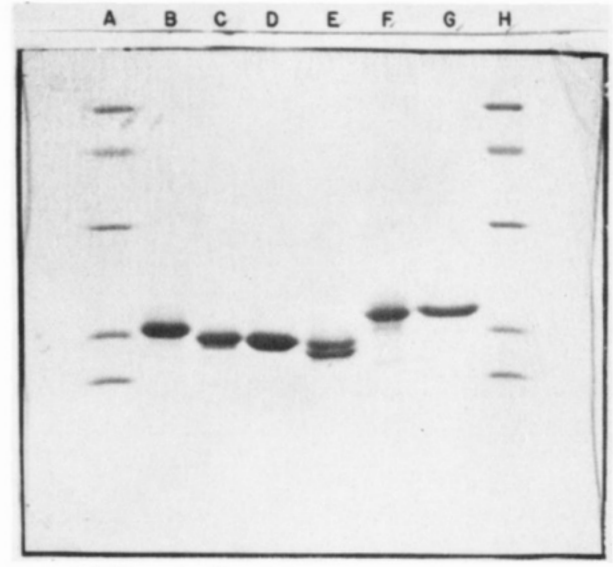

FIGURE 3: Polyacrylamide gel electrophoresis of purified recombinant IFN α subtypes. Prior to loading, 0.01 mg of each IFN α subtype was boiled for 2 min in 1% 2-mercaptoethanol, 1% SDS, and 10% glycerol dissolved in 0.06 M Tris hydrochloride, pH 6.8, containing 0.001% bromphenol blue. Discontinuous SDS–polyacrylamide gel electrophoresis was run according to Laemmli (1970). Lanes A and H, standard proteins, i.e., lysozyme, β-lactoglobulin, carbonic anhydrase, ovalbumin, and bovine serum albumin; lane B, IFN α-1; lane C, IFN δ-4 α-1; lane D, IFN α-2; lane E, IFN δ-4 α-2/α-1 hybrid; lane F, IFN α-4; lane G, IFN α-7.

of the parental molecule from which the amino terminus was derived (i.e., δ-4 α-1 compared to α-1, and δ-4 α-2/α-1 compared to α-2).

The IFN α subtypes were characterized for purity by SDS–PAGE. As shown in Figure 3, IFN α-1, δ-4 α-1, α-2, α-4, and α-7 each demonstrated one major species that represented 95% or greater of the total protein stained with Coomassie blue. No evidence for significant degradation or irreversible aggregation was obtained. Although approximately equal mixtures of two components were observed for the δ-4 α-2/α-1 hybrid, both of these expressed antiviral activity when eluted from the polyacrylamide gel with 0.1% SDS. The close migration of these components on SDS–PAGE further suggests that they are closely related and may be derived by posttranslational modification (e.g., proteolysis or deamidation). The exact cause of this apparent heterogeneity is at present unknown. IFN α-4 and α-7 displayed significantly slower migration than the other subtypes despite virtually identical molecular weight values predicted for the entire class of leukocyte IFNs (Nagata et al., 1980; Goeddel et al., 1981). It has been reported that a single amino acid residue difference can result in significant changes in migration on SDS–PAGE (Noel et al., 1979).

Characterization of the Monoclonal Antibodies. (1) Effects on IFN Antiproliferative and Antiviral Activities. The eight hybridoma supernatants were tested for their ability to inhibit the antiviral and antiproliferative activities of IFN α-2. Antiviral activity was measured in a cytopathic effect–inhibition assay, and the antiproliferative effect was tested on the Daudi cell line. As shown in Table III, complete inhibition of antiviral activity was observed for monoclonal antibodies U1, U3, U4, U5, and NK-2, compared to no effect on activity for antibodies U2, U6, and III/21. All antibodies that inhibited antiviral activity were also observed to inhibit antiproliferative activity completely, with the exception of U5, on which antiproliferative activity could not be evaluated due to an apparent cytotoxic effect on the Daudi cells. The neutralizing ability of the NK-2

Table III: Effect of a Panel of Monoclonal Antibodies on Antiviral and Antiproliferative Activities of IFN α-2[a]

monoclonal antibody	antiviral act.	antiproliferative act.	monoclonal antibody	antiviral act.	antiproliferative act.
U1	+	+	U5	+	ND[b]
U2	−	−	U6	−	ND[b]
U3	+	+	III/21	−	ND[b]
U4	+	+	NK-2	+	+

[a] Serial dilutions of hybridoma supernatants containing the indicated monoclonal antibodies dissolved in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin were incubated with varying amounts of IFN α-2 over the range of 1–100 IU in 96-well culture plates at 37 °C for 1 h. Antiviral activity was determined by employing VSV and Madin-Darby bovine kidney cells. Antiproliferative effects were evaluated with the Daudi cell line. Further details are described under Experimental Procedures. A plus sign (+) indicates 100% inhibition, and a minus sign (−) indicates no effect. [b] ND = not determined.

Table IV: Binding of a Panel of Monoclonal Antibodies to IFN α-2 and Its CNBr Fragments with and without Prior Reduction with 2-Mercaptoethanol[a]

monoclonal antibody	IFN α-2		fragment 1-4		fragment 3-5		fragment 6
	native	reduced	native	reduced	native	reduced	native
U1	+	−	+	−	−	−	−
U2	+	+	+	+	−	−	−
U3	+	−	−	−	−	ND[b]	−
U4	+	−	−	−	−	ND[b]	−
U5	+	+	−	−	−	ND[b]	−
U6	+	+	−	−	+	+	−
NK-2	+	−	+	−	−	−	−
III/21	+	+	−	−	−	−	+

[a] Antibody binding to IFN α-2 or CNBr fragments (see Table I) was determined by ELISA. ELISA method A was employed for all experiments except for testing the effect of reduction with 2-mercaptoethanol, in which method B was employed; further details are described under Experimental Procedures. For reduction with 2-mercaptoethanol, 200 ng of antigen was incubated in wells of microtiter plates at room temperature for 1 h. The plates were washed 5 times with TBS–0.02% Tween 20 and incubated with either TBS or TBS containing 0.1 M 2-mercaptoethanol. A plus sign (+) indicates binding, and a minus sign (−) indicates either no binding (i.e., no significant optical density increase compared to binding to carbonic anhydrase) or significantly diminished binding compared to that obtained with IFN α-2 or CNBr fragment. [b] ND = not determined.

Table V: Binding of a Panel of Monoclonal Antibodies to Purified Recombinant Human IFN α Subtypes[a]

monoclonal antibody[b]	% binding[c]					
	α-2	α-1	δ-4 α-1	δ-4 α-2/α-1	α-4	α-7
U1	100	−	−	73 ± 5[d]	−	−
U2	100	−	−	−	−	−
U3	100	−	−	−	−	12 ± 1.5
U4	100	56 ± 4	54 ± 3	37 ± 5	−	−
U5	100	67 ± 23	77 ± 23	43 ± 7	29 ± 5	−
U6	100	−	−	72 ± 14	−	−
III/21	100	88 ± 5	103 ± 6	74 ± 22	−	−
NK-2	100	−	−	−	−	10 ± 3

[a] Binding was determined by ELISA. Microtiter plates were coated overnight at 4 °C with 200 ng of IFN α subtype per well dissolved in 50 mM sodium carbonate, pH 9.6. A 200-μL aliquot of hybridoma supernatants (U1–U6) or purified antibodies (NK-2 and III/21 dissolved in RPMI-1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin) was added to each well, followed by a 1.5-h incubation at room temperature. After the wells were washed 4 times with 0.05% Tween 20 dissolved in PBS, 200 μL of affinity-purified alkaline phosphatase conjugated rabbit anti-mouse IgG was added to each well, followed by a 1.5-h incubation at room temperature. The wells were again washed 4 times with PBS–Tween 20, and the amount of bound antibody was quantified by hydrolysis of *p*-nitrophenyl phosphate, as described under Experimental Procedures. Data represent the mean of two determinations. A minus sign (−) indicates lack of binding, as measured by the failure to detect a measurable optical density increase at 405 nm compared to binding to carbonic anhydrase. [b] The immunogen for all monoclonal antibodies was IFN α-2, except for U6, which was raised against a mixture of CNBr fragments 1-4, 3-5, and 6, as described under Experimental Procedures. [c] Calculated from the ratio of the optical densities at 405 nm obtained for the various IFN α subtypes compared to IFN α-2. Binding to IFN α-2 was arbitrarily set at 100%. [d] ± values indicate the range of two determinations.

antibody and the lack of effect of III/21 are in agreement with previous reports of Secher & Burke (1980) and Arnheiter et al. (1983), respectively.

(*2*) *Epitope Localization.* (*a*) *Monoclonal Antibodies U1, U2, and NK-2.* These antibodies were grouped together since the epitopes that they recognized were located in CNBr fragment 1-4 (Table IV). However, they were distinguishable by their pattern of recognition of IFN α subtypes (Table V). Thus, U1, but not U2 and NK-2, recognized an antigenic determinant in the δ-4 α-2/α-1 hybrid. Since the hybrid contains only the amino-terminal 62 amino acid residues of IFN α-2, this epitope must contain amino acid residues 5–15 and/or residues 60–62, both of which are present in CNBr fragment 1-4 (Figure 2). As shown in Table V, U1 did not react with either IFN α-1 or IFN δ-4 α-1, both of which contain the same amino acid residues as α-2 at positions 60–62, namely, isoleucine at position 60 followed by two glutamine residues at positions 61 and 62, respectively. It should be noted that IFN α-2 contains 165 amino acid residues compared to 166 residues for IFN α-1 since it lacks the triplet coding for Asp_{44} (Streuli et al., 1980). These comparisons are based on insertion of an extra residue at position 44 in IFN α-2. Thus, the antigenic determinant recognized by neutralizing antibody U1 must reside, at least in part, between residues 5 and 15 on IFN α-2. It cannot be excluded, however, that a region of the sequence 60–110 that is similar in both IFN α-2 and IFN α-1 might also contribute to the epitope recognized by U1.

Although monoclonal antibodies U1, U2, and NK–2 bound to the same CNBr fragment, the epitopes they recognized were clearly distinguishable. U1 and NK-2 neutralized both antiviral and antiproliferative activities, but U2 did not (Table III). U2 was found not to inhibit these activities even at a 10^5-fold molar excess compared to IFN α-2. Purified preparations of U2 and NK-2 yielded similar titers for binding to IFN α-2 (8×10^{-4}/mg of protein and 4×10^{-4}/mg of protein, respectively), suggesting that a difference in affinity could not account for the failure of U2 to interfere with biological activity. U2 and NK-2 were also readily distinguished by the fact that NK-2 recognized IFN α-7 in an ELISA, compared to U2, which consistently was observed to show no binding to IFN α-7 (Table V). Finally, although both U1 and NK-2 neutralized biological activity, U1 interacted with the δ-4 α-2/α-1 hybrid, but NK-2 did not.

(*b*) *Monoclonal Antibodies U3, U4, and U5.* This class of antibodies did not react with any of the three CNBr fragments (Table IV) but did neutralize antiviral activity (Table III). However, patterns of cross-reactivity with IFN α subtypes indicated distinguishable patterns (Table V). Thus, monoclonal antibody U3 bound to IFN α-7 in contrast to U4, which recognized α-2, δ-4 α-1, and the δ-4 α-2/α-1 hybrid, but *not* α-7. In contrast, U5 displayed affinity for all IFN α subtypes except α-7. The epitope recognized by U5 was further distinguished from that to which U3 or U4 was directed since the former antibody bound to IFN α-4 whereas the latter two antibodies did not.

The uniqueness of these epitopes was further established by direct competition studies. U4 was unable to compete with alkaline phosphatase labeled U3 for immobilized IFN α-2 in an ELISA (data not shown). It is of interest that experiments employing the commercially available NK-2 IRMA kit (Celltech Ltd.) indicated that U3 was competitive with radiolabeled NK-2 for IFN α-2, while U2 and U4 did not compete. Although the epitopes recognized by U3 and NK-2 were clearly distinguishable by cross-reactivity with CNBr fragments (Table IV), their location was sufficiently close as to result in an apparent competition for the same site. Identification of precise domains of IFN α-2 to which U3, U4, and U5 were directed was not possible since none of these antibodies interacted with the CNBr fragments. The fact that U3 recognized IFN α-2 but not δ-4 α-2/α-1 suggests that the antigenic determinant resides between residues 63 and 165 or is composed of discontinuous sequences not present in the hybrid molecule. Since neither U4 nor U5 recognized IFN α-7, the epitopes to which they bind cannot reside completely in linear sequences that are identical in IFN α-2 and α-7.

(*c*) *Monoclonal Antibody U6.* None of the positive hybridomas raised against native IFN α-2 recognized either CNBr fragment 3-5 or CNBr fragment 6. In order to obtain antibodies that might recognize either of these fragments, mice were immunized with an equimolar mixture of all CNBr fragments (Figure 2), and hybridomas were selected for binding to both IFN α-2 and CNBr fragments in the ELISA. The majority of these hybridomas recognized either fragment 1-4 or fragment 3-5, although none were found to produce antibody that bound to fragment 6. Monoclonal antibody U6 represents one of the antibodies that recognized CNBr fragment 3-5. U6 was similar to U1 in that it recognized only the δ-4 α-2/α-1 hybrid in addition to IFN α-2, implying that the epitope must reside, at least in part, between residues 5 and 62 in IFN α-2. Since the amino acid sequence from residue 22 to residue 58 is present in both CNBr fragment 3-5 and the α-2 portion of the δ-4 α-2/α-1 hybrid, at least a portion of this antigenic determinant must be derived from amino acids contained in this sequence.

The epitope to which monoclonal antibody U6 is directed appeared to be located in a region distinct from the receptor binding region. U6 failed to neutralize the antiviral activity to IFN α-2 even when present at a 500 times higher concentration than that employed for the neutralizing antibody U1 (Table III). Binding studies in an ELISA indicated that both U1 and U6 demonstrated comparable affinities for the parental molecule. Thus, the failure of U6 to inhibit biological activity cannot be attributed to a low degree of binding.

(*d*) *Monoclonal Antibody III/21.* The immunogen for production of this antibody was the carboxyl-terminal 56-residue synthetic peptide of IFN α-1 (Arnheiter et al., 1981). This antibody has previously been demonstrated to (1) bind to a synthetic carboxyl-terminal 16-residue IFN α-1 peptide, (2) recognize recombinant IFN α-A bound to the surface of Madin-Darby bovine kidney cells, and (3) fail to neutralize antiviral activity (Arnheiter et al., 1983). In our study the lack of effect of monoclonal antibody III/21 on antiviral activity has been confirmed (Table III). It has been further demonstrated that this antibody binds to CNBr fragment 6, which consists of the 17-residue carboxyl terminal of IFN α-2 (Tables I and IV; Figure 2). These data represent additional information to that previously reported (Arnheiter et al., 1983) since the carboxyl-terminal 16 residues of IFN α-1 and α-2 differ at three positions. In other experiments it was demonstrated that CNBr fragment 6 inhibited binding of monoclonal III/21 to immobilized IFN α-2 in a competitive ELISA (data not shown). These results indicate that at least a portion of the 17-residue carboxyl terminal of IFN α-2 is exposed on the surface of the molecule and is not directly involved in receptor binding, in confirmation of the conclusions of Arnheiter et al. (1983).

Effect of Cleavage of Disulfide Bonds. The critical importance of one or both disulfide bonds in the expression of the biological activity of IFN α-2 was indicated by reports that reduction of α IFN at 37 °C leads to irreversible loss of antiviral activity (Fantes & O'Neill, 1964; Merigan et al., 1965; Wetzel et al., 1982, 1983; Pestka et al., 1983; Morehead et al., 1984), suggesting an important function in conformational stability. In order to examine the role of disulfide linkages in maintaining the integrity of the epitopes defined by the panel of eight monoclonal antibodies described here, IFN α-2 and the CNBr fragments 1-4 and 3-5 were immobilized on microtiter plates, followed by reduction with 100 mM 2-mercaptoethanol for 16 h at 37 °C. Immobilization was required since extensive and variable degrees of precipitation were observed if reduction with 2-mercaptoethanol was performed in solution. Appropriate controls were included in which the protein or polypeptide was incubated under the same conditions in the absence of 2-mercaptoethanol.

As shown in Table IV, the integrity of the epitopes on native IFN α-2 recognized by monoclonal antibodies U1, U3, U4, and NK-2 was destroyed or significantly diminished by reduction with 2-mercaptoethanol. In contrast, reduction appeared to have no effect on the binding of monoclonal antibodies U2, U5, U6, and III/21. Incubation of the immobilized antigens at 37 °C without 2-mercaptoethanol, or at 4 °C in the presence of 2-mercaptoethanol, did not appear to change the amount of antigen bound to the plate. Reduction of CNBr fragment 1-4 abolished binding of U1 and NK-2 but not that of U2, similar to the effect observed with IFN α-2. Reduction of CNBr fragment 3-5 failed to destroy the epitope recognized by monoclonal antibody U6, in confirmation of the results

Table VI: Summary of Epitope Identification for Monoclonal Antibodies Directed to IFN α-2

antibody	probable epitope[a]
U1	5–15[b]
U2	CNBr fragment 1-4
U3	unknown
U4	unknown
U5	unknown
U6	22–58[c]
III/21	149–165
NK-2	CNBr fragment 1-4

[a] Numbers represent amino acid residues from the amino terminal of IFN α-2 that constitute at least a part of the antigenic determinants. [b] Additional elements from residue 60 to residue 110 may also participate in this epitope on the basis of the binding of U1 to CNBr fragment 1-4 (Table IV). [c] Additional elements from residue 112 to residue 148 may also participate in this epitope on the basis of the binding of U6 to CNBr fragment 3-5 (Table IV). The CNBr fragment is defined in Table I.

obtained with IFN α-2. The fact that four of the eight epitopes recognized by this panel of antibodies were altered by reduction of the parental IFN α-2 at 37 °C implies significant conformational alterations as a result of disulfide bond cleavage. It cannot be excluded, however, that the apparent loss or retention of the integrity of the remaining four epitopes upon 2-mercaptoethanol treatment may be related to the fact that reduction was performed upon an immobilized protein.

A summary of the probable composition of the epitopes recognized by the monoclonal antibodies characterized in this study is shown in Table VI. It is yet to be determined whether additional amino acid residues from other regions of the molecule contribute to these epitopes as a consequence of the tertiary structure. Although precise identification of the epitopes for U3, U4, and U5 is unknown, the data support that each of the antigenic determinants is unique.

Discussion

The application of monoclonal antibodies for the mapping of functionally important regions of a protein is dependent on molecular probes that define various regions of the parent molecule. IFNs are ideal targets for elucidating structure–function relations with monoclonal antibodies since they represent a multigene family that contains conserved and nonconserved domains in both primary and secondary structure (Nagata et al., 1980, 1981; Sternberg & Cohen, 1982). With the use of recombinant DNA technology, novel hybrids and truncated molecules from the various subtypes can be constructed that represent powerful tools for identifying antigenic determinants. In this study, the naturally occurring leukocyte IFNs α-1, α-2, α-4, and α-7, in combination with two products of recombinant DNA technology, δ-4 α-1 and the δ-4 α-2/α-1 hybrid, have been employed for epitope identification on IFN α-2. We have also taken advantage of the fact that the location of the five methionine residues in the primary sequence of IFN α-2 results in the production of three CNBr fragments that retain epitopes present on the parental molecule. Monoclonal antibody cross-reactivity patterns with CNBr fragments of IFN α-2 have identified four classes of antibody: (1) antibodies binding to fragment 1-4 (U1, U2, and NK-2), (2) an antibody binding to fragment 3-5 (U6), (3) an antibody binding to fragment 6 (III/21), and (4) antibodies interacting with none of the CNBr fragments (U3, U4, and U5). No antibody was observed to react with more than one CNBr fragment, providing further evidence for the monospecific nature of these antibodies as well as for the purity of the preparations of CNBr fragments. All antibodies exhibited the ability to interact with the native IFN α-2 since the initial selection process was based on binding to IFN α-2 in a micro-ELISA. None of the antibodies that were raised to IFN α-2 as immunogen were able to recognize either CNBr fragment 3-5 or CNBr fragment 6. Thus, alternative approaches for mapping these regions were required. A mixture of CNBr fragments was employed as immunogen in an attempt to raise a monoclonal antibody that could react with both the native IFN α-2 and fragment 3-5 (i.e., monoclonal antibody U6). Similarly, the monoclonal antibody III/21 that recognizes carboxyl-terminal CNBr fragment 6 was originally raised to a synthetic 56-residue polypeptide derived from the carboxyl terminal of IFN α-1 (Arnheiter et al., 1981). These data imply that an effective panel of antibodies for the identification of determinants should be derived from both the parental molecule as well as from fragments that retain selected epitopes.

Two of the three antibodies binding to CNBr fragment 1-4 (U1 and NK-2) neutralized antiviral and antiproliferative activities of IFN α-2, of which U1 was shown to be directed to an epitope residing, at least in part, between residues 5 and 15. A comparison of the linear sequence of amino acids in IFN α-1 and α-2 between residues 5 and 15 indicates identity at all positions except residues 5, 10, and 11. Positions 5, 10, and 11 contain glutamine, glycine, and serine, respectively, in IFN α-2, compared to glutamate, aspartate, and asparagine in IFN α-1. U1, however, was observed to show no interaction with IFN α-1. Thus, it can be speculated that either one or both of the residues at positions 10 and 11 contribute to the epitope in IFN α-2 recognized by monoclonal antibody U1. Position 5 is a less likely candidate due to the structural similarity between glutamine and glutamate. A calculation of hydrophilicity as described by Hopp & Woods (1983) for residues in the amino terminal of IFN α-2 is consistent with this conclusion since it indicates a region of local hydrophilicity between residues 10 and 15. Thus, residues 10 and 11 are most probably oriented toward the surface of the molecule and are, therefore, potentially part of the antigenic determinant recognized by monoclonal antibody U1. These data suggest that the epitope recognized by U1 may reside in a smaller fragment of the amino-terminal 5–15 sequence. Regardless of the precise identification of this epitope, it is clear that it does not require the Cys_1–Cys_{98} disulfide linkage for its structural integrity since U1 binds to the δ-4 α-2/α-1 hybrid in which Cys_1 is absent. It is relevant with respect to the potential functional significance of amino-terminal residues 5–15 that Streuli et al. (1980) have demonstrated the production of biologically active IFN from a strain of *E. coli* containing an incomplete IFN gene that may be producing IFN α-2 lacking the first 15 amino-terminal residues. However, these data cannot be considered definitive since isolation and characterization of the truncated product have not been reported.

Amino acid sequences that may contribute to the epitope recognized by NK-2 include the first four amino acid residues from the amino terminus (on the basis of the lack of reactivity with the δ-4 α-2/α-1 hybrid) as well as residues 60–111 and 5–15 (on the basis of binding to CNBr fragment 1-4). Since the δ-4 α-2/α-1 hybrid displays both antiviral and antiproliferative activities, we conclude that the first four amino acid residues in IFN α-2 are not directly involved in biological activity. If the correct antigenic determinant for NK-2 does reside, at least in part, in the first four amino acid residues, inhibition of biological activity is probably a result of steric hindrance in preventing the binding of IFN α-2 to its specific cell surface receptor, which is the first step in the expression of the biological activity of IFN (Friedman, 1965; Besanscon

& Ankel, 1974; Aguet, 1980; Branca & Baglioni, 1981). Competitive binding studies indicate that this antigenic determinant must reside in proximity to that recognized by monoclonal antibody U3. An additional similarity between NK-2 and U3 was observed in cross-reactivity with IFN α subtypes, since both monoclonals were unique in recognizing only IFN α-2 and α-7.

Identification of antigenic determinants that do not result in neutralization of biological activity in the presence of antibody is of interest with respect to potential localization of domains distinct from the receptor binding site. Three such antigenic determinants were identified by monoclonal antibodies U6, U2, and III/21. The epitope to which U6 is directed was found to reside between residues 22 and 58 in IFN α-2 through a comparison of overlapping sequences present in CNBr fragment 3-5 and the amino-terminal sequence of the δ-4 α-2/α-1 hybrid. Although exact localization of the U2 epitope was not possible, examination of the CNBR fragment 1-4 indicates two nonconserved regions among human leukocyte interferons. The linear sequence of these regions extends from residue 75 through residue 90 and from residue 100 through residue 110, respectively. Since evidence to date indicates a common receptor for the various IFN α subtypes (Yonehara et al., 1983; Aguet et al., 1984; Seelig et al., 1984), lack of conservation of these residues among the leukocyte IFNs suggests a corresponding lack of functional significance. These sequences represent attractive potential candidates for recognition by monoclonal antibody U2. Identification of this antigenic determinant will depend on the preparation of smaller fragments that maintain the integrity of the epitope within the predicted regions. It is yet to be established that U2 and U6 remain bound to IFN α-2 after interaction with the cell surface receptor.

Morehead et al. (1984) have demonstrated that incubation of a disulfide-free thiosulfonate derivative of IFN α-A at 37 °C results in loss of binding in an ELISA that utilizes two monoclonal anti-IFN antibodies. Similarly, Pestka et al. (1983) have reported that reduction of the disulfide bonds of IFN α-A prevents detection in a radioimmunoassay also based on two monoclonal antibodies. These data are consistent with our observation that reduction of IFN α-2 with 2-mercaptoethanol at 37 °C for 14 h altered the integrity of four of the eight epitopes recognized by the panel of monoclonal antibodies. The implication is that one or both of the disulfide linkages are required for stabilization of IFN α-2 in its native conformation at 37 °C. However, these data do not imply that the two disulfide linkages are required per se for stabilization of the structural integrity of these epitopes under other conditions. For example, Morehead et al. (1984) have reported that several chemically modified derivatives of IFN α-A that lack the two disulfide bonds do maintain sufficient integrity for recognition by two monoclonal antibodies. Thus, the disulfide bonds appear to confer stability against extensive conformational alterations that occur as a result of incubation at 37 °C, which may result in aggregation with subsequent masking or destruction of epitopes. It will be important in future studies to establish the effect of alternative methods of disulfide bond cleavage on the conformational integrity of IFN α-2.

It is significant that the genetically engineered deletion of the four amino-terminal residues (Cys_1-Asp_2-Leu_3-Pro_4) from α-1 appeared to have a relatively insignificant effect on antiviral activity. Since 2-fold dilutions are employed in the cytopathic effect–inhibition assay, the somewhat lower activity noted for the truncated variant is not statistically significant. Similarly, deletion of the same four residues from IFN α-2 does not appear to result in a significant loss in antiviral activity since the δ-4 α-2/α-1 hybrid expresses specific antiviral activity that is among the highest for the purified IFN α subtypes, comparable to IFN α-2 (Table II). These data are consistent with the studies of Weck et al. (1981) demonstrating high antiviral activities for the IFN α-A/D (*Bgl*II) hybrid. The most straightforward interpretation is that none of these residues is directly essential for antiviral activity. Both of these truncated variants are also active as antiproliferative and immunoregulatory agents (Higgins & Platsoucas, 1984). In addition, since neither of these variants contains Cys_1, the data directly indicate that the Cys_1–Cys_{98} (Cys_{99} in IFN α-1) disulfide bond could not be required for stabilization of the biologically active conformation in either IFN α-1 or IFN α-2. Since human leukocyte IFNs as a class conserve the four cysteine residues (Streuli et al., 1981; Goeddel et al., 1981; Weissmann et al., 1982), it is reasonable to speculate that the nonessentiality of Cys_1–Cys_{98} may apply to each of the human α IFNs. Morehead et al. (1984) have made a similar conclusion on the basis of chemical modification data indicating that (1) an inactive thiosulfonate derivative of IFN α-A could regain its activity by regeneration of only the Cys_{29}–Cys_{138} linkage and (2) a carboxamidomethylated derivative of IFN α-A, which contained an intact Cys_{29}–Cys_{138} linkage, expressed full antiviral activity. The data presented here provide the additional information that the Cys_1–Cys_{98} linkage is probably required for maintenance of local conformational integrity, since 2-mercaptoethanol reduction of CNBr fragment 1-4 resulted in loss of binding of two monoclonal antibodies (Table IV).

Ackerman et al. (1984) have recently reported the preparation by proteolytic digestion of a biologically active amino-terminal 110 amino acid fragment of IFN α-2. This fragment, which retains ca. 1% of the specific antiviral activity of IFN α-2, apparently maintains sufficient native conformational integrity to express biological activity despite the absence of the Cys_{29}–Cys_{138} bond. These data are consistent with the fact that neutralizing antibodies U1 and NK-2 are both directed to CNBr fragment 1-4, which contains residues 1–16 linked to residues 60–111 by the Cys_1–Cys_{98} linkage. The observation that monoclonal antibody U3 was competitive with NK-2 for binding to native IFN α-2 indicates that it may also be directed to the amino terminal of the molecule. It is notable that experiments employing recombinant hybrid IFNs have indicated that the high specificity of IFN α-2 for human cells resides in the amino-proximal half of IFN α-2 (Streuli et al., 1981).

Several laboratories have raised monoclonal antibodies to both purified and relatively crude leukocyte IFNs (Secher & Burke, 1980; Morser et al., 1981; Staehelin et al., 1981; Allen et al., 1982; Laurent et al., 1982; Novick et al., 1982; Dreiding et al., 1983; Osterhoff et al., 1984). With the exception of the studies on the III/21 antibody (Arnheiter et al., 1983) and the recent report of a monoclonal antibody that may be directed to the receptor binding site (Dreiding et al., 1983), the majority of these antibodies have been applied to purification by immunosorbent chromatography or to the development of radioimmune assays. In contrast, in our study a panel of monoclonal antibodies has been employed to probe structure–function relations in IFN α-2. It is clear that the precise localization of the receptor binding region as well as other functionally important domains is difficult on the basis of the data presented here due to problems in interpretation arising from steric hindrance or conformational changes induced by

antibody binding. However, the future expansion of the number of monoclonal antibodies directed to IFN α-2 should allow further refinement in identification of epitopes critical for biological function.

ACKNOWLEDGMENTS

We thank Walter Protzman, Drs. Jerome Schwartz and Sheila Jacobs, Delores Surprenant, and Christine Risano for determination of the antiviral and antiproliferative activities and performance of the IRMA. We are grateful to Dr. Kathryn C. Zoon for helpful discussions, to Rosalinda Syto and Winifred W. Prosise for skillful technical assistance, and to Margarite Poepoe and Muriel Vatan for excellent assistance in the preparation of the manuscript.

REFERENCES

Ackerman, S. K., Zur Nedden, D., Heintzelman, M., Hunkapiller, M., & Zoon, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1045–1047.

Aguet, M. (1980) *Nature (London) 284*, 459–461.

Aguet, M., Grobke, M., & Dreiding, P. (1984) *Virology 132*, 211–216.

Allen, G., Fantes, K. H., Burke, D. C., & Morser, L. (1982) *J. Gen. Virol. 63*, 207–212.

Ankel, H., Chany, C., Galliot, B., Chevalier, M. J., & Robert, M. (1973) *Proc. Natl. Acad. Sci. U.SA. 70*, 2360–2363.

Arnheiter, H., Thomas, R. M., Leist, T., Fountoulakis, M., & Gutte, B. (1981) *Nature (London) 294*, 278–280.

Arnheiter, H., Ohno, M., Smith, M., Gutte, B., & Zoon, K. C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2539–2543.

Avrameas, S., Ternynek, T., & Guesdon, J. L. (1978) *Scand. J. Immunol. 8 (Suppl. 7)*, 7–23.

Baron, S., & Dianzani, F., Eds. (1977) *Tex. Rep. Biol. Med. 35*, 1–573.

Berman, B., & Vilcek, J. (1974) *Virology 57*, 378–386.

Besanscon, F. T., & Ankel, H. (1974) *Nature (London) 252*, 478–480.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Branca, A. J., & Baglioni, C. (1981) *Nature (London) 294*, 768–770.

Chang, N. T., Kung, H.-F., & Pestka, S. (1983) *Arch. Biochem. Biophys. 221*, 585–589.

Dreiding, P., Groebke, M., & Aguet, M. (1983) *Biol. Interferon Syst., Proc. Int. TNO Meet., 2nd, 1983*, 57.

Evinger, M., & Pestka, S. (1981) *Methods Enzymol. 79*, 362–368.

Fantes, K. H., & O'Neill, C. F. (1964) *Nature (London) 203*, 1048–1050.

Franke, A. E., Shepard, H. M., Houck, C. M., Leung, D. W., Goeddel, D. V., & Lawn, R. M. (1982) *DNA 1*, 223–230.

Friedman, R. M. (1965) *Science (Washington, D.C.) 156*, 1760–1761.

Goeddel, D. V., Leung, D. W., Dull, T. J., Gross, M., Lawn, R. M., McCandliss, R., Ullrich, A., Yelverton, E., & Gray, P. W. (1981) *Nature (London) 290*, 20–26.

Higgins, J. A., & Platsoucas, C. D. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1932.

Hopp, T. P., & Woods, K. R. (1983) *Mol. Immunol. 20*, 483–489.

Kohler, G., & Milstein, C. (1975) *Nature (London) 256*, 495–497.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Laurent, A.-G., Gruest, J., Krust, B., & Montagnier, L. (1982) *Hybridoma 1*, 313–322.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mantei, N., Schwartzstein, M., Streuli, M., Pantem, S., Nagata, S., & Weissmann, C. (1980) *Gene 10*, 1–10.

Merigan, T. C., Winget, C. A., & Dixon, C. B. (1965) *J. Mol. Biol. 13*, 679–691.

Morehead, H., Johnston, P., & Wetzel, R. (1984) *Biochemistry 23*, 2500–2507.

Morser, J., Meager, A., Burke, D. C., & Secher, D. S. (1981) *J. Gen. Virol. 53*, 257–265.

Mosmann, T. (1983) *J. Immunol. Methods 65*, 55–63.

Nagabhushan, T. L., & Leibowitz, P. J. (1985) in *Proceedings of the Second International Conference on Malignant Lymphoma* (Kisner, D., & Smyth, J., Eds.) pp 1–12, Nijhoff, Hingham, MA.

Nagabhushan, T. L., Surprenant, H., Le., H. V., Kosecki, R., Levine, A., Reichert, P., Sharma, B., Tsai, H., Trotta, P., Bausch, J., Foster, C., Gruber, S., Hoogerheide, J., & Mercorelli, S. (1984) *Interferon: Res., Clin. Appl., Regul. Consid., Proc. Int. Workshop, 1983*, 79–88.

Nagata, S., Mantei, N., & Weissmann, C. (1980) *Nature (London) 287*, 401–408.

Nagata, S., Brack, C., Henco, K., Schamick, A., & Weissmann, C. (1981) *J. Interferon Res. 1*, 333–336.

Noel, D., Nikaido, K., & Ames, G. F.-L. (1979) *Biochemistry 18*, 4159–4165.

Novick, D., Eshhar, Z., & Rubinstein, M. (1982) *J. Immunol. 129*, 2244–2247.

Osheroff, P. L., Tahara, S. M., & Chiang, T.-R. (1984) *Clin. Immunol. Immunopathol. 30*, 188–196.

Pestka, S., Kelder, B., Langer, J. A., & Staehelin, T. (1983) *Arch. Biochem. Biophys. 224*, 111–116.

Pestka, S., Kelder, B., Ortaldo, J., Herberman, R., Kempner, E., Moschera, J., & Langer, J. (1984) *Interferon: Res., Clin. Appl., Regul. Consid., Proc. Int. Workshop, 1983*, 59–77.

Radjai, M. K., & Hatch, R. T. (1980) *J. Chromatogr. 196*, 319–322.

Rubinstein, S., Familetti, P. C., & Pestka, S. (1981) *J. Virol. 37*, 755–758.

Secher, D. S., & Burke, D. C. (1980) *Nature (London) 285*, 446–450.

Seelig, G. F., Schwartz, J., Le, H., Smith, S., Nagabhushan, T. L., & Trotta, P. P. (1984) *Antiviral Res. 4*, 60.

Staehelin, T., Durrer, B., Schmidt, J., Takucs, B., Stocker, J., Miggino, V., Stahli, L., Rubinstein, M., Levy, W. O., Hershberg, R., & Pestka, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1848–1852.

Sternberg, M. J. E., & Cohen, F. E. (1982) *Int. J. Biol. Macromol. 4*, 137–144.

Stewart, W. E., II (1979) *The Interferon System*, Springer, New York.

Streuli, M., Nagata, S., & Weissmann, C. (1980) *Science (Washington, D.C.) 209*, 1343–1347.

Streuli, M., Hall, A., Ball, W., Stewart, W. E., II, Nagata, S., & Weissmann, C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2848–2852.

Weck, P. K., Apperson, S., Stebbing, N., Gray, P. W., Leung, D., Shepard, H. M., & Goeddel, D. V. (1981) *Nucleic Acids Res. 9*, 6153–6166.

Weissmann, C. (1981) *Interferon 3*, 101–134.

Weissmann, C., Nagata, S., Boll, W. Fountoulakis, M., Fujisawa, A., Fujisawa, J.-I., Haynes, J., Henco, K., Mantei, N., Ragg, H., Schein, C., Schmid, J., Shaw, G., Streuli, M., Taira, H., Todokoro, K., & Weidle, U. (1982) *UCLA Symp. Mol. Cell. Biol. 25*, 295–326.

Wetzel, R. (1981) *Nature (London) 289*, 606–607.

Wetzel, R., Perry, L. J., Estell, D. A., Lin, N., Levine, H. L., Slinker, B., Fields, F., Ross, M. J., & Shively, J. (1981) *J. Interferon Res. 1*, 381–390.
Wetzel, R., Levine, H. L., Estell, D. A., Shire, S., Finer-Moore, L., Stroud, R. M., & Bewley, T. A. (1982) *UCLA Symp. Mol. Cell. Biol. 25*, 365–376.
Wetzel, R., Johnston, P. D., & Czarniecki, C. (1983) in *Biology of the Interferon System* (Schellekens, H., et al., Eds.) pp 101–112, Elsevier, New York.
Yonehara, S., Yonehara-Takahashi, M., Ishu, A., & Nagata, S. (1983) *J. Biol. Chem. 258*, 9046–9049.
Zoon, K. C., & Wetzel, R. (1983) in *Handbook of Experimental Pharmacology* (Came, P. E., & Carter, W. A., Eds.) pp 79–100, Springer-Verlag, New York.

Macrophage Recognition of Immune Complexes: Development and Application of Novel Cell Surface Labeling Procedures†

Howard R. Petty* and William Dereski
Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202
Received January 24, 1985

ABSTRACT: A fluorescein- and lactoperoxidase-conjugated ferritin–anti-ferritin immune complex has been prepared for cell surface labeling experiments on immune recognition and effector function. Lactoperoxidase (LPO) has been covalently coupled to affinity-purified anti-ferritin antibodies with *p*-benzoquinone by a modified version of the method of Ternynck and Avrameas [Ternynck, T., & Avrameas, S. (1976) *Ann. Immunol. (Paris) 127C*, 197]. The conjugate is a heterodimer of M_r 230 000 with linkages to either or both of the heavy and light chains of the antibody, as judged by two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in the absence and presence of 2-mercaptoethanol. The conjugate retains antibody-binding activity as measured by a quantitative precipitin assay. When incorporated into immune complexes, the modified antibody also retains Fc receptor recognition ability as determined by erythrocyte–antibody rosette inhibition assays. Electron microscopy demonstrated that the antigen, ferritin, was monodisperse with complete apoprotein sheaths surrounding the core. Ferritin–anti-ferritin–LPO complexes were formed in 4-fold antigen excess. Complexes were verified by fluorescence and electron microscopy. Immune complexes were masked with "cold" iodine by use of the endogenous LPO activity. The complexes bound to cells at 4 °C as shown by electron microscopy and fluorescence video/intensification microscopy. The LPO delivered to the cell surface in this fashion can be utilized to iodinate the surface with ^{125}I. Under saturation conditions, the labeling with local LPO delivery followed by SDS–PAGE and autoradiography is identical with labeling with free LPO. Labeling has also been conducted under conditions of substrate deficit. In addition, bovine serum albumin has been employed to scavenge I^+, which may diffuse from the local LPO delivery sites. These conditions reveal that, during immune complex recognition, a local polymerization reaction occurs that can be at least partially reversed with 2-mercaptoethanol.

Macrophage cell surface receptors for the Fc domain of the immunoglobulin molecule can mediate immunologic recognition and trigger the internalization of particulate and soluble immune complexes. Studies on the nature of the Fc receptor typically employ erythrocyte–antibody rosetting or radiolabeled ligand binding measurements (Leslie & Alexander, 1980; Zuckerman & Douglas, 1979a,b). Recently, Mellman & Unkeless (1980) have employed a monoclonal anti-Fc receptor antibody to characterize this membrane glycoprotein. The receptor has been shown to function as a ligand-dependent ion channel (Young et al., 1983a,b). This change in membrane potential is likely associated with subsequent metabolic alterations. However, the biochemical events that accompany the triggering of endocytosis are not well characterized. In a recent study we have shown that membrane-impermeable sulfhydryl-blocking reagents inhibit endocytosis, but not binding, of immune complexes at nanomolar concentrations (Dereski & Petty, 1985). This suggests that a cell surface sulfhydryl group is associated with the triggering of endocytosis.

In this paper we describe the properties of model immune complexes that combine the advantages of morphological and biochemical analyses. These complexes (1) can be examined by fluorescence and electron microscopy and (2) provide an antibody-dependent method to deliver lactoperoxidase (LPO) to localized regions of immune recognition at the cell surface for simultaneous labeling with radioactive iodine. The results are relevant to understanding the transmembrane signaling required for endocytosis.

Materials and Methods

Cell Culture. The murine RAW264 macrophage cell line and the human U937 monocytic cell line were employed in these studies. Their origins and properties have been previously described (Raschke et al., 1978; Sundstrom & Nilsson, 1976; Petty et al., 1980a). The U937 cell line was obtained from the American Type Culture Collection (Bethesda, MD). The RAW264 cell line was grown as adherent cells. The U937

† This work was supported by NIH Grant AI-19075.

0006-2960/85/0424-4141$01.50/0