by picornaviruses that use the major group rhinovirus receptor [5]. Our studies suggest that a receptor-targeted approach to preventing coronavirus infection is worthy of further study. If the receptor for human coronaviruses is homologous to the glycoprotein receptor for murine coronavirus, oronasal treatment with a receptor-targeted ligand might also be a useful approach to prevention of human coronavirus infections.

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Neutralizing Antibodies to Interferon-α: Relative Frequency in Patients Treated with Different Interferon Preparations

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The frequencies of antibody development so far reported in patients treated with different interferons (IFNs) are not readily comparable because of differences in treatment regimens and assay methods. Thus the frequency of neutralizing antibody development was analyzed in a large sample of sera derived from a relatively homogeneous group of patients treated with different IFN-α preparations. The frequency of developing neutralizing antibody to IFN varied according to the IFN given. Particularly, the seroconversion frequency was significantly higher in patients treated with recombinant IFN-α2a (20.2%) than in patients treated with either recombinant IFN-α2b (6.9%) or IFN-αN1 (1.2%), a lymphoblastoid IFN-α. Furthermore, sera obtained from patients treated with either recombinant IFN neutralized both types of recombinant IFNs but failed to neutralize IFN-αN1.

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There are several reports of patients forming neutralizing antibodies while under treatment with interferon (IFN) preparations [1-7]. These antibodies may be clinically important, as shown by concomitant loss of beneficial effects of treatment [8-12]. Unfortunately, the data in these studies are heterogeneous in terms of the patients and diseases involved, the types and doses of IFN used, and the methods used to mea-

sure antibodies and calculate their titers. Thus, no definite conclusions can be drawn about the relative immunogenicity of the various commercially available IFN preparations. To allow for more quantitative conclusions, we tested serum samples from 296 patients under IFN treatment for chronic hepatitis B, non-A non-B (NANB) hepatitis, or delta hepatitis. This patient population was relatively homogeneous in terms of disease and also in the dose of IFN and schedule of treatment, but treatment involved recombinant (r)IFN-α2a, rIFN-α2b, or a lymphoblastoid IFN-α preparation (IFN-αNl).

These three commercial IFN preparations differ as follows: IFN-αNl is a natural mixture of IFN-α, produced by human lymphoblastoid cells after viral stimulation [13], whereas rIFN-α2a and rIFN-α2b are recombinant preparations obtained in *Escherichia coli* and represent only one subtype, IFN-α2. These latter preparations differ from each other and from the amino acid sequence of natural IFN-α2 by only one amino acid. IFN-α2a has a lysine molecule at position 23 instead of glycine in the natural sequence, which in IFN-α2b is substituted by an arginine.

At least three different assays are currently available for detection of antibodies to IFN: enzyme immunoassay (EIA), immunoradiometric assay (IRMA), and neutralization assay. In this study the neutralization assay was used because the same test may be used with all three types of IFN, while commercially available EIA or IRMA tests can only detect antibody to rIFN-α2; although significance of antibodies to IFN has not yet been clearly established, the neutralization assay is capable of detecting antibodies to an epitope(s) involved in biologic activity; and the neutralization assay is recommended by the World Health Organization for measuring circulating antibodies.

Materials and Methods

Patients. Sera were collected from patients treated in several clinical centers in Italy.

The 296 patients entering the study included 74 with hepatitis B (n = 39) or with NANB hepatitis (n = 35) treated intramuscularly (im) or subcutaneously (sc) with rIFN-α2a (dose range, 1.5–3 mega units [MU]/m^2 three times weekly); 144 with NANB hepatitis (n = 74) or with delta hepatitis (n = 70) treated im or sc with rIFN-α2b (3 MU/m^2 three times weekly); and 78 with hepatitis B treated im or sc with lymphoblastoid IFN-αNl (5 MU/m^2 three times weekly). The duration of therapy was similar for all patients, 6–12 months (mean, 8.1 for rIFN-α2a, 7.3 for rIFN-α2b, and 8.3 for IFN-αNl).

All trials adopted basically the same inclusion criteria as far as sex, age, diagnosis, and severity of disease.

Sampling from the clinical centers was randomized without prior knowledge of the type of IFN used or the therapeutic effects achieved. Samples were collected before initiation of therapy and at the end of therapy.

Detection of neutralizing antibodies to IFN-α. Antibody titers were determined by a neutralization test against 5 IU of rIFN-α2a, rIFN-α2b, and IFN-αNl, depending on the type of IFN administered in vivo. The sera were routinely inactivated at 56°C for 30 min before titration. A total of 60 μl of twofold serial dilutions of sample or control sera were incubated at 37°C with 60 μl of each type of IFN. After 1 h, 100 μl of individual mixtures were added to duplicate monolayers of human Wish cells in 96-well microtiter plates. After 18–24 h of culture, cells were challenged with Sindbis virus and incubated at 37°C for 24 h, as previously described [7, 14]. Controls included titrations of the IFN preparations used in respective assays and of a mixture of IFN and a known antibody to IFN-α. Antiviral activity and its neutralization were determined by observation of virus-induced cytopathic effect followed by hemagglutination assay [7, 14]. The titer was taken as the highest twofold dilution of serum that completely inhibited the antiviral activity of the added IFN [7, 14, 15]. Since the lowest serum dilution tested was 1:5, the limit of sensitivity of the assay described above is 5 neutralization units (NU)/ml, where 1 NU is defined as the amount of serum required to inhibit 5 IU/ml.

Serum samples were collected at least 24 h after IFN administration and were routinely assayed for and found free of endogenous or residual IFN activity.

To assay the specificity of the neutralizing antibodies, most positive sera were also tested against 5 IU/ml IFN-β or IFN-γ using the assay described above. In all cases they failed to show neutralizing activity against these heterologous IFNs.

Neutralizing activity was characterized as antibody mediated by adsorption to and subsequent elution from staphylococcal protein A-Sepharose CL-4B columns (Pharmacia, Uppsala, Sweden).

Only two patients had low-titered antibodies before IFN therapy (1:10 and 1:20, respectively) and were not included in the study.

Statistical methods. Seroconversion rates were compared by using the χ^2 test. Differences in geometric mean titers among study groups were analyzed by Student's t test.

Results

Table 1 summarizes our results. Of the 74 hepatitis patients treated with rIFN-α2a, 15 (20.2%) developed neutralizing antibodies, whereas neutralizing antibodies were detected in only 10 (6.9%) of 144 and 1 (1.2%) of 78 hepatitis patients treated with rIFN-α2b and IFN-αNl, respectively. Thus, the seroconversion frequency was significantly higher in patients treated with rIFN-α2a than in patients treated with either rIFN-α2b

Table 1. Incidence of neutralizing antibodies in patients treated with recombinant interferons (rIFN-α2a or rIFN-α2b) or lymphoblastoid interferon (IFN-αN1).

Treatment	Cumulative dose (MU/m^2, range)	No. positive/ no. tested (%)	Titer
rIFN-α2a	117–469	15/74 (20.2)	524 (10–20,480)
rIFN-α2b	234–469	10/144 (6.9)*	28 (10–160)
IFN-αN1	390–780	1/78 (1.2)*	NA

NOTE. MU = mega units. Positive refers to patients who developed neutralizing antibodies. Titers are geometric mean (range). NA = not applicable.

* $P < .01$, rIFN-α2a vs. rIFN-α2b treatment; $P < .001$, rIFN-α2a vs. rIFN-αN1 treatment.

($P < .01$) or IFN-αN1 ($P < .001$). In contrast, comparison of seroconversion rates between rIFN-α2b and IFN-αN1 treatment groups showed no significant difference.

Table 1 also shows that the titers of positive sera from patients treated with rIFN-α2a were significantly higher than those obtained from patients treated with rIFN-α2b ($P < .01$).

It should be pointed out that cumulative doses of IFN units received by patients in each group were not considerably different. In addition, the group of patients treated with IFN-αN1, who received the highest cumulative dose, showed the lowest rate of seroconversion. Thus, the differences in frequency and titers of antibodies that developed during therapy cannot be attributed to the amount of IFN given.

To determine whether there was any cross-reactivity between the different types of IFN, we compared neutralizing activity of all positive sera against IFN-α2a, IFN-α2b, and IFN-αN1 (table 2). Sera from patients treated with either of the two rIFN-α2 preparations neutralized equally well both IFNs but in most cases failed to neutralize IFN-αN1. This suggests that basically antibodies to one subtype of IFN are unable to neutralize all other subtypes of IFN-α present in IFN-αN1.

Table 2. Patients developing neutralizing antibodies: interferon (IFN) preparation used, cumulative dose, and cross-reactivity of their antibodies.

IFN-α type, patient no.	Dose (MU)	Time (months)	Neutralizing antibody titer vs		
			IFN-α2a	IFN-α2b	IFN-αN1
2a					
18	231	6	320	320	<10
23	231	6	2560	2560	<10
27	270	7	10	10	<10
30	270	7	640	320	ND
37	231	6	80	ND	<10
55	469	7	40	ND	<10
60	270	7	5120	5120	20
62	231	6	1280	1280	10
65	469	12	320	320	<10
70	231	6	640	1280	20
73	347	9	80	80	<10
76	469	12	5120	5120	<10
80	234	12	320	640	160
89	234	12	2560	5120	160
90	234	12	10,240	20,480	320
2b					
2	334	6	20	20	<10
45	334	6	40	80	<10
55	642	12	10	10	<10
67	334	6	80	160	10
69	372	7	10	20	<10
70	334	6	10	10	<10
74	372	7	20	20	<10
114	642	12	ND	80	<10
125	334	6	ND	40	<10
140	410	8	ND	10	<10
N1					
67	471	6	20,480	20,480	640

NOTE. ND = not done. MU = mega units.

Discussion

Many reports have shown that treatment with homologous natural and recombinant IFNs may cause the development of anti-IFN antibodies [1–7]. In several cases the appearance of such antibodies has been associated with the decreased clinical efficacy of IFN therapy [8–12]. The proportion of patients who developed antibodies during treatment varied from none to ~40% depending on the dosage, the diseases, and the assay method used. Therefore, comparison of the published accounts of the immunogenicity of different IFNs has not been readily possible. We addressed this problem by analyzing antibody development with identical methodology in a relatively homogeneous group of patients treated with three different IFN-α preparations.

Neutralizing antibodies were detected in 20% of patients treated with rIFN-α2a, a frequency significantly higher than in patients treated with either IFN-α2b or IFN-αN1 (~7% and 1%, respectively). Since the patients enrolled in the trials were chosen using basically the same criteria and since the dosing regimen and the route of administration and duration of therapy were comparable, the three groups of patients may be considered homogenous. Therefore, it is highly likely that the differences in frequency of neutralizing antibody development are correlated with the type of IFN used. Of course, the neutralization assay used in the study has the important limitation of not being capable of detecting nonneutralizing antibodies, which may also be clinically important, for instance by modifying pharmacokinetic properties of the IFN administered. Studies are in progress to address the presence of these antibodies in the same population of patients.

The results obtained also show that even high titers of neutralizing antibodies to rIFN-α2 do not substantially affect the biologic activity of IFN-αN1, a naturally occurring IFN-α mixture, indicating that this mixture can overcome the neutralizing activity of antibodies to one IFN-α subtype. This agrees with reports of the successful use of natural IFN mixtures in patients who relapsed after forming neutralizing antibodies while under treatment with an rIFN-α2 preparation [11, 12].

At this time it is difficult to explain the different immunogenicity of the types of IFNs used in this study. Several hypotheses have been considered. (1) It is possible that natural IFN, which is a mixture of at least 18 homologous subtypes, may be less immunogenic than rIFN since any subtype is actually present at a lower concentration than in an equal dose of rIFN, which contains only a single molecular species. (2) Since the rIFNs we used have different amino acid sequences, it is tempting to speculate that the tertiary structure and thus immunogenicity of the molecule is influenced by the specific amino acid substitutions that have been introduced into IFN-α2a and

-α2b compared with the natural IFN-α2 sequence. (3) Since the natural IFN mixture contains glycosylated IFN molecules [16], it is possible that the carbohydrates present on native IFN species may influence the antigenicity of the molecule by masking immunogenic sites.

In conclusion, our data confirm that IFN preparations can induce production of specific antibodies in humans and suggest that the different commercially available preparations differ significantly in this respect. Furthermore, our data strongly indicate that lymphoblastoid IFN-αN1 does not completely cross-react with rIFNs. Although we made no correlation between seroconversion and clinical outcome, our data support the view that IFN treatment of patients showing relapses due to the development of antibodies to rIFNs can be effectively continued with natural IFNs.

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