Incidence and Clinical Significance of Neutralizing Antibodies in Patients Receiving Recombinant Interferon Alfa–2a by Intramuscular Injection

LORETTA M. ITRI, MD,* MARILYN CAMPION,* ROBERT A. DENNIN, MA,* ALICIA V. PALLERONI, MS,* JORDAN U. GUTTERMAN, MD,† JEROME E. GROOPMAN, MD,‡ AND PATRICK W. TROWN, PHD*

More than 1600 patients with neoplastic disorders have received recombinant human interferon alfa–2a (Roferon®-A, Hoffmann-La Roche, Nutley, NJ) as part of ongoing or completed clinical trials. In this report, the efficacy of interferon alfa–2a therapy was compared with the incidence of antibodies to this interferon in 617 patients who received the drug by intramuscular administration. Antibody measurements were performed using a highly sensitive enzyme immunoassay, and an interferon antiviral neutralization bioassay. Partial or complete remission occurred in 28% (43 of 152) of the antibody-positive patients, and in 24% (112 of 465) of the antibody-negative patients ($P = 0.33$). The highest incidence of antibody formation occurred among patients with renal cell carcinoma and acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma (44% and 34%, respectively). Both the duration of treatment and length of survival were significantly longer for antibody-positive than for antibody-negative patients. No significant intergroup differences emerged for response rates or for time to onset or duration of therapeutic response. When results from the above assays were compared to those used for the detection of antibodies to recombinant interferon alfa–2b (Intron A®, Schering-Plough Inc., Kenilworth, NJ), the immunoradiometric assay method was determined to be seriously deficient for determination of antibody incidence. This decreased assay sensitivity may account for the reportedly lower incidence of antibodies to recombinant alfa–2b interferon.

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THE DEVELOPMENT of highly purified forms of human interferons (alpha, beta, and gamma) has made it possible to explore potential therapeutic indications for these proteins and to study the immune response during treatment. Since naturally occurring interferons have a high level of species specificity and antibodies are not normally made against "self" antigens, it was expected that interferons would not be immunogenic if used in homologous systems. Contrary to this hypothesis, in 1981 Vallbracht *et al.*[1] reported neutralizing IgG antibodies against natural human fibroblast interferon in a patient treated with that interferon.

Findings such as this prompted speculation that the efficacy of interferon therapy may be compromised by the body's ability to develop neutralizing antibodies to homologous interferons, whether produced by recombinant DNA technology or by cell culture. Recombinant DNA-derived interferon alpha has been shown to be an active antitumor agent in patients with acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma, malignant melanoma, B cell lymphomas, hairy cell leukemia, and cutaneous T cell lymphoma.[2–8] A 27% complete and partial remission rate has been reported for a group of patients with renal cell carcinoma treated with daily intramuscular (IM) injections of 20×10^6 U/m^2.[9–11] However, Quesada *et al.*[11] also reported the development of neutralizing antibodies in seven of 12 responding patients (58%), and nine of 29 nonresponding patients (31%) with metastatic renal cell carcinoma. Based on a comparison of the median duration of remission between antibody-positive and antibody-negative patients, this report further suggested that early relapse was associated with antibody development.

A recent report by Spiegel *et al.*[12] of 2017 patients receiving interferon alfa–2b (Intron A®, Schering-Plough Inc., Kenilworth, NJ) therapy by various routes of administration indicated a lower incidence of antibody formation. Using the immunoradiometric assay (IRMA)[13] to examine the sera of patients who received Intron A, these investigators detected neutralizing antibodies in only 2.4% of 537 patients receiving systemic therapy, and in less than 1% of the remaining 1480 patients (1326 intranasal, and 154 intralesional).

From *Hoffmann-La Roche Inc., Nutley, New Jersey, †M.D. Anderson Hospital and Tumor Institute, Houston, Texas, and ‡New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts.

Address for reprints: Loretta M. Itri, MD, Hoffmann-La Roche Inc., 340 Kingsland St., Nutley, NJ 07110.

Given the disparity in the reported incidence of antibody response to recombinant alfa-2 interferon therapy in different studies, we have evaluated the performance of antibody assays employed by various investigators.

The current study has attempted to resolve these issues by examining data on efficacy and antibody incidence from ongoing clinical trials, as well as the different methods used for antibody detection. Evaluation of the occurrence and possible pathogenic significance of circulating antibodies to interferon has been fundamental to our analysis of clinical trials, and very sensitive assays for antibodies to interferon have been developed and used to screen the sera of patients receiving interferon alfa-2a during all Phase I, II, and III studies.

Patients and Methods

Patients

Sixteen-hundred patients with different types of cancer received intramuscular injections of interferon alfa-2a (Roferon®-A, Hoffmann-La Roche, Nutley, NJ) for varying lengths of time. The inclusion and exclusion criteria for the clinical trials differed somewhat, depending on the indication being studied. In general, patients were eligible for entry if they were at least 18 years old, had histopathologically documented disease with a life expectancy greater than the duration of induction therapy (approximately three months), had a performance status of at least 60 on the Karnofsky scale, or at most two on the Eastern Cooperative Oncology Group scale, and required no palliative treatment during the study. Written informed consent was obtained from all patients.

Exclusion criteria were those common to oncologic clinical trials. Patients excluded from entry were pregnant and lactating women, fertile men and women not using effective contraception, patients who had undergone surgery within four weeks of study entry (unless they were fully recovered), and patients with a history of previous malignancy (excluding noninvasive cutaneous carcinoma). Other clinical criteria for exclusion were cardiac disease or any history thereof, central nervous system metastases or seizure disorders, or severe intercurrent infection. Impaired renal or hematologic functions (serum creatinine > 1.8 to 2.0 mg/dl; total bilirubin > 1.4 mg/dl; leukocyte count $\leq 3 \times 10^3/\mu l$; granulocytes $\leq 1 \times 10^3/\mu l$; platelets $\leq 10 \times 10^4/\mu l$, as well as serum calcium > 12 mg/dl) were the laboratory criteria for exclusion. Chemotherapy, radiotherapy, or immunotherapy received within four weeks of study entry for the primary diagnosis was also a criterion for exclusion.

Therapy

Induction therapy involved intramuscular injection and consisted of daily, three-times-weekly, or daily escalating dosing. The last schedule was developed in an attempt to abrogate some of the acute toxicity that occurs during the early stages of interferon therapy.

In general, doses ranged from 3×10^6 U/day, which is the current recommended dose for patients with hairy cell leukemia, to 136×10^6 U, which was confined primarily to early Phase I studies. The daily escalating dosage schedule required incremental doses given over 12 days beginning with 3×10^6 U, and increasing to 36×10^6 U. Therapy was continued at the highest tolerated dose for the remainder of treatment. Depending on individual patient tolerance, the duration of induction ranged from 8 to 16 weeks. Patients who responded (and, in some cases, patients whose disease remained stable during induction therapy) were allowed to continue treatment on a three-times-per-week maintenance schedule until the disease progressed, or the patient was removed from the study. Dose attenuation was determined on the basis of toxicity, criteria for which are described elsewhere.[14]

Assays for the Detection of Antibodies to Interferon Alfa-2a

Serum analyzed in the assays described below was routinely obtained from patients before the first injection of interferon alfa-2a and at the end of both the induction and maintenance intervals. Samples were obtained 48 hours or more after an injection in order to reduce assay interference from circulating blood levels of interferon alfa-2a. Patients were considered eligible for evaluation if they were negative for interferon antibody before therapy, and had at least one follow-up sample after receiving treatment.

Before testing in the interferon antiviral neutralization bioassay (ANB), all sera were prescreened by an enzyme immunoassay (EIA) that is sensitive to 5 ng specific anti-IFN-alfa-2a IgG/ml of serum. This EIA has been validated to detect all sera positive for neutralizing antibody.[15] The EIA also detects nonneutralizing antibodies and neutralizing antibodies present at titers below the detection limit of the ANB (see discussion following). If the serum was shown to be positive by the EIA, it was then tested in the ANB at the maximum sensitivity currently possible (100 INU/ml; interferon neutralizing units per milliliter). In order to address the methodologic questions alluded to earlier, we have assayed the antibody response of patients treated with interferon alfa-2a using the IRMA procedure described by Protzman *et al.*,[13] which is the assay employed by Spiegel *et al.*[12] for antibody evaluations in patients receiving interferon alfa-2b.

The Enzyme Immunoassay

The EIA used for the detection of specific antibodies to interferon alfa-2a is a solid-phase assay based on the "sandwich" principle (Fig. 1).

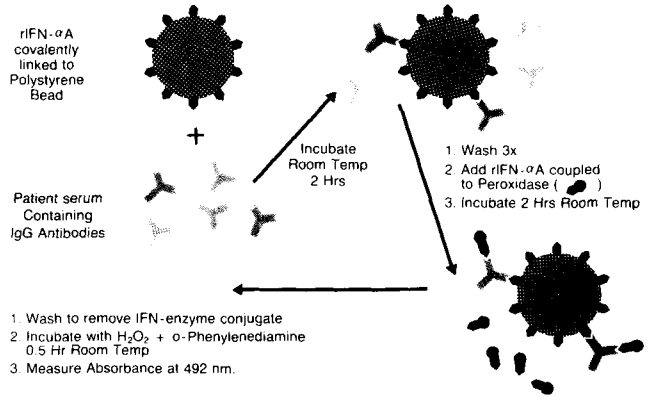

FIG. 1. A schematic representation of the enzyme immunoassay used for prescreening serum samples for the presence of antibodies Roferon®-A (recombinant interferon alfa-2a).

Serum samples (50 μl), diluted with phosphate buffered saline (PBS) were incubated for 2 hours at room temperature with interferon alfa-2a-coated beads (one bead per sample in a tube). Liquid was then removed and the beads were washed three times with 2 to 4 ml deionized water. Interferon alfa-2a-peroxidase solution was then added and incubated at room temperature for 2 hours in the dark. The beads were washed again three times with 2 to 4 ml deionized water and transferred to tubes containing the o-phenylenediamine/H_2O_2 solution. After 30 minutes at room temperature in the dark, the enzymatic reaction was stopped by adding 1 ml of 0.5 M sulfuric acid, and absorbance at 492 nm was determined. The color produced by the reaction was a function of the antibody concentration in the patients' serum. Using the conventional cut-off for semiquantitative EIAs, a serum sample was considered positive for antibody to interferon alfa-2a if its absorbance at 492 nm was greater than twice the mean absorbance obtained for the normal serum pool, which was used as a negative control.

Antiviral Neutralization Bioassay (ANB)

This method is described more fully elsewhere.[11,16] Briefly, serial twofold dilutions (lowest dilution 1:5) of the serum (100 μl) being tested were mixed with 10 μl of a 200 U/ml solution of interferon alfa-2a, (final concentration 20 U/ml) and incubated for 1 hour at 37°C. The mixtures were then transferred to preformed monolayers of Madin-Darby bovine kidney cells in 96-well microtiter trays. After incubation for 4 hours at 37°C, the contents of each well were removed, the monolayers were washed once with medium and then infected with vesicular stomatitis virus. The trays were incubated at 37°C for 16 to 24 hours. The cell monolayers were then stained with crystal violet and examined microscopically for viral cytopathic effect, after which the assay end point was determined (50% protection of the cell monolayer). The neutralization titer is the dilution of serum that reduces the effective concentration of interferon alfa-2a from approximately 20 U/ml to 1 U/ml. The antibody titer, expressed as interferon neutralizing units per milliliter (INU/ml), is equivalent to the number of units of interferon neutralized by 1 ml of the patient's serum, and is defined as the neutralization titer × 20 under the assay conditions described.

This neutralization bioassay was performed on those sera that were positive in a primary screen using the EIA. This was done because initial studies demonstrated that no EIA negative sera are ANB positive.[15]

Immunoradiometric Assay (IRMA)

This assay process has been described in detail by Protzman *et al.*[13] It has been used by the Schering-Plough Corporation (Kenilworth, NJ) to prescreen the sera of patients undergoing treatment with interferon alfa-2b for the presence of antibodies to that interferon. Only those sera found positive by this assay were further evaluated in an antiviral neutralization bioassay.[12,13]

The assay utilizes the Celltech (Celltech, Berkshire, United Kingdom) interferon immunoradiometric assay to quantitate the remaining interferon alfa-2b after incubation of the patient serum with a known amount of added interferon.[13]

In our studies, which used the procedure of Protzman *et al.*,[13] diluted serum samples (minimal dilution fivefold) were mixed with an equal volume (0.1 ml) of 20 U/ml interferon alfa-2a, and kept at room temperature for 30 minutes; ^{125}I-anti-interferon alpha monoclonal antibody was then added. After an additional 30 minutes, a polystyrene bead coated with sheep polyclonal antibody to interferon alpha was added, and incubation continued for 16 to 20 hours. The beads were washed, bound radioactivity was measured, and the amount of remaining interferon alfa-2a was determined from a standard curve. As described by Protzman *et al.*[13] a 50% or greater reduction in detectable interferon alfa-2a was considered positive. It has been claimed that this procedure can detect 50 neutralizing units/ml, where a neutralizing unit is defined as the amount of antibody required to neutralize one unit of interferon.

Comparison of Clinical Findings and Antibody Incidence

In order to assess the clinical importance of circulating antibody production, we compared both the time to, and the duration of, response as well as the length of survival between antibody-positive and antibody-negative patients.

TABLE 1. Proportion of Patients Developing Neutralizing Antibodies to Roferon®-A

Diagnosis	No. of patients	Antibody-positive patients	
		Number	(%)
Renal cell carcinoma	201	88	(44)
Kaposi's sarcoma	68	23	(34)
Lymphoma	66	13	(20)
Melanoma	149	21	(14)
Leukemia	82	3	(4)
Miscellaneous	87	12	(14)
Total	653	160	(25)

TABLE 2. Comparative Response Rates of Antibody-Negative and Antibody-Positive Patients

Classification of patients		No.	(%)
Patients evaluable for antibody		653	
Patients also evaluable for response		617	
Patients developing antibody		160	(25%)
Patients exhibiting CR or PR		155	(25%)
Evaluable patients developing antibody		152	
Antibody-positive patients responding	$P = 0.33$	43/152	(28%)
Antibody-negative patients responding		112/465	(24%)

CR: complete response; PR: partial response.

To do this we used a lifetable technique based on the log-rank, two-tailed test.

Results

Profile of the Patient Population

Of the 1600 patients who received interferon alfa–2a, 883 had adequate baseline serum samples available at the time of this analysis. Four of these patients were found to have preexisting antibody titers to alpha interferon before receiving Roferon®-A treatment. Six hundred fifty-three patients had adequate pre- and post-treatment sera available. Neutralizing antibody to interferon alfa–2a was detected in 160 of these patients (Table 1). Patients with renal cell carcinoma and AIDS-related Kaposi's sarcoma had the highest incidence of antibody formation.

The 653 patients were divided into comparative groups (antibody-positive *versus* antibody-negative) as shown in Table 2. Of these 653 patients, 160 (25%) developed neutralizing antibodies to interferon alfa–2a at some time during treatment. There were 617 patients evaluable for therapeutic response, and of these patients, 152 were positive for antibody. Of the 617 patients, 155 (25%) achieved either a complete or partial remission. Forty-three (28%) of the 152 antibody-positive patients achieved a partial or complete response. Of the 465 patients who did not develop antibody, 112 (24%) achieved a complete or partial remission. There was no significant difference (Fisher's Exact Test, $P = 0.33$) in the response rates of antibody-positive *versus* antibody-negative patients.

The Effects of Antibody Formation on the Outcome of Treatment

Differences in the median duration of therapy between the groups are shown in Table 3. Specifically, there was a statistically significant ($P < 0.01$) (Fig. 2) longer duration of treatment for antibody-positive patients overall, and for those with renal cell carcinoma and lymphoma. This finding indicates that patients who undergo longer treatment with interferon alfa–2a have a higher likelihood of developing antibodies.

The time required for a therapeutic response to occur and the duration of that response are shown in Figures 3 and 4, respectively. There was no significant difference between antibody-positive and antibody-negative patients for either parameter. Patients who developed antibodies to interferon alfa–2a survived significantly longer ($P < 0.01$) than those who did not (Fig. 5), although this is probably due to a longer duration of treatment.

Comparison of the EIA and IRMA Methodologies for Screening Patients' Sera for Antibodies

Spiegel *et al.*[12] have claimed that treatment of patients with interferon alfa–2b results in a low incidence of antibody production when measured by the IRMA method.

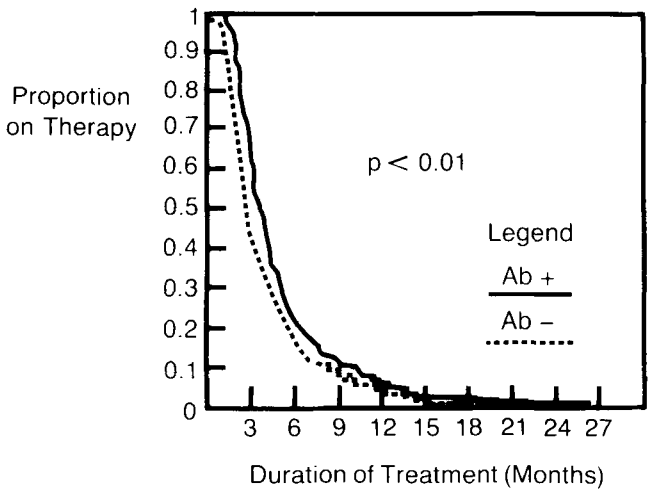

FIG. 2. Comparison of duration of treatment for antibody-positive (Ab+) and antibody-negative (Ab–) patients.

TABLE 3. The Median Duration of Therapy for Antibody-Positive and Antibody-Negative Patients

Type of malignancy	Months of therapy			
	Antibody-positive		Antibody-negative	
	Median	(Range)	Median	(Range)
Renal cell carcinoma*	3.0	(1.0–18.2)	2.0	(0.2–11.9)
Kaposi's sarcoma	5.2	(2.3–26.2)	4.3	(0.9–24.6)
Lymphoma*	5.4	(2.8–13.4)	2.7	(0.7–16.6)
Melanoma	2.9	(1.7–5.3)	2.1	(0.03–14.3)
Leukemia	10.0	(5.7–12.0)	5.8	(0.7–17.9)
Miscellaneous	2.8	(1.7–8.3)	1.7	(0.4–9.3)
Total*	3.4	(1.0–26.2)	2.4	(0.03–24.6)

* Significantly different ($P < 0.01$) by log-rank test from lifetable analysis.

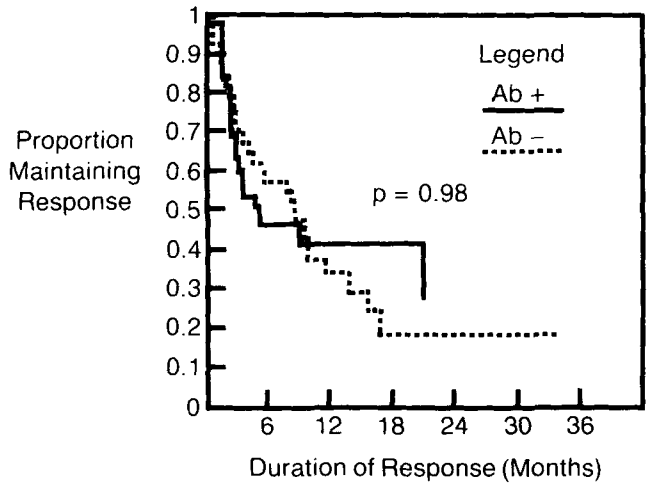

FIG. 4. The duration of therapeutic response for antibody-positive (Ab+) and antibody-negative (Ab–) patients.

This is in contrast to our data with interferon alfa-2a where, depending upon the duration of treatment and the underlying condition, approximately 25% of patients have detectable antibodies. It is possible that this difference may largely be due to differences in the relative sensitivities of the EIA and IRMA methods used to detect antibody in the patients' sera.

Since the IRMA is a commercially available radioimmunologic interferon assay kit (Celltech, Berkshire, United Kingdom), with procedural modifications,[13] we have been able to reevaluate the antibody response of patients treated with Roferon®-A in this assay. A total of 150 sera, 100 positive for interferon antibodies by the EIA and ANB, and 50 positive by the EIA but negative in the ANB, were tested. Of these sera, 98 of 150 (65%) were falsely interpreted as antibody-negative by the IRMA method. Forty-eight of 100 (48%) of the EIA-positive, ANB-positive samples were false negatives when measured using the IRMA technique; all 50 samples (100%) of the EIA-positive, ANB-negative sera were false negatives.

These findings reveal that the IRMA assay method employed by Spiegel *et al.* is much less sensitive than the EIA method for detecting interferon antibodies. Therefore, the reportedly low proportion of patients producing antibody to Intron A is probably due to an insensitive assay technique.

Discussion

Clinical Outcome

Antibodies to interferon were detected before treatment in four patients for whom adequate baseline serum samples were available. Of the 653 patients for whom adequate

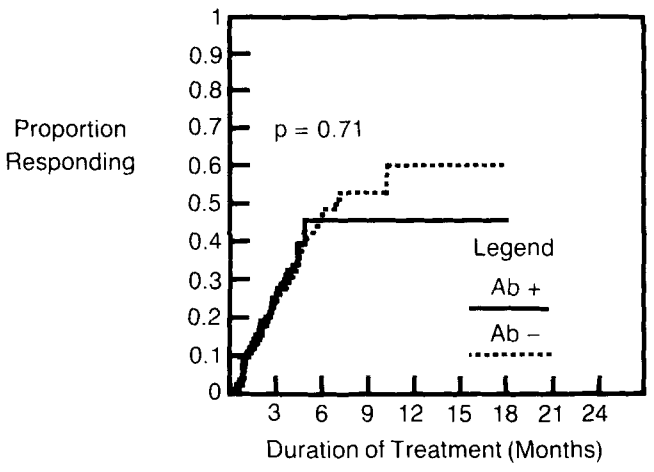

FIG. 3. The time required for a therapeutic response to occur for antibody-positive (Ab+), and antibody-negative (Ab–) patients.

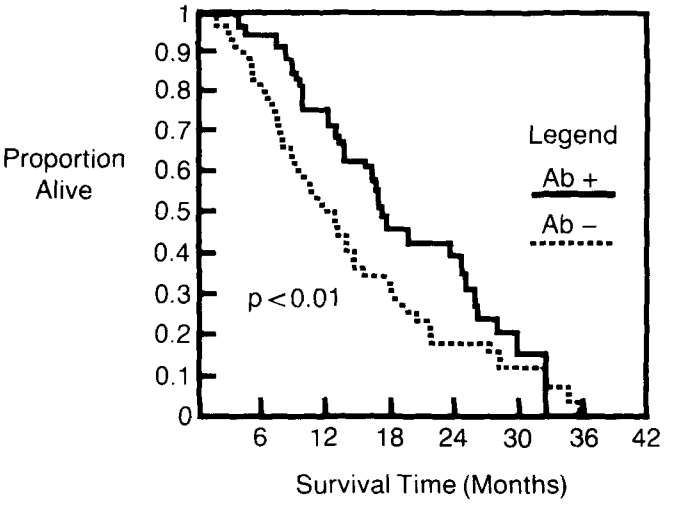

FIG. 5. Survival for antibody-positive (Ab+) and antibody-negative (Ab–) patients.

pre- and post-treatment samples were available,160 (25%) developed neutralizing antibodies to interferon alfa-2a during their course of treatment (Table 1). Six hundred-seventeen of the patients evaluated for antibody development were also suitable for analysis of therapeutic response by standard criteria. Of this group, 155 patients (25%) achieved either a partial or complete response (Table 2). Forty-three of the 152 (28%) antibody-positive patients, and 112 of the 465 (24%) antibody-negative patients responded.

When the course and outcome of treatment were analyzed by various criteria, several similarities and differences emerged between antibody-positive and antibody-negative patients. The duration of treatment was significantly longer ($P < 0.01$) for antibody-positive patients than for antibody-negative patients (Table 2 and Fig. 2). This suggests the probability that patients who receive treatment for prolonged periods of time have an increased likelihood of antibody formation.

The median survival rate for antibody-positive patients (17.0 months) was significantly longer ($P < 0.01$) than that for antibody-negative patients (11.4 months, Fig. 5). The most plausible explanation for this statistical difference is that those patients who are most likely to have a prolonged survival are those who respond to treatment. These patients will usually receive a more prolonged course of treatment, a circumstance that also increases the likelihood of antibody formation. However, it is important to note that it appears that antibody formation is not a disadvantage in terms of survival.

This is supported by the finding that the complete/partial response rates (Table 2) were similar for antibody-positive (28%), and antibody-negative patients (24%; $P = 0.33$). No significant difference was found between the two groups when the time required to achieve a therapeutic response (Fig. 3; $P = 0.71$), or the duration of response (Fig. 4; $P = 0.98$) were compared. These data do not support the publication of Quesada *et al.*[11] which implied that, for a small group of patients with renal cell carcinoma, the loss of therapeutic response was temporally related to the development of antibody. For the subpopulation of 201 patients with renal cell carcinoma in our study, there were no statistical differences in time to, or duration of, response between antibody-positive and antibody-negative patients.

Comparison of Assay Methods

Certain methodologic aspects of the IRMA technique used for the initial screening of all serum samples for the presence of antibodies appear to markedly underestimate the prevalence of antibody. Using the IRMA, patient sera were incubated with a murine monoclonal antibody, an immobilized sheep polyclonal antibody, and a known, added amount of interferon alfa-2b. There is potential for competition between the human, ovine, and murine antibodies for binding to the added interferon. If the affinities of the two heterologous antibodies are higher than those of the human antibodies, the latter will not be detected. Indeed, of the 60 samples for which results from both the IRMA and the neutralization bioassay are reported by Spiegel *et al.*[12] 13 (21.7%) were positive in the neutralization bioassay but negative by the IRMA. Nevertheless, these authors stated that only those sera that were positive by IRMA were further tested in the antiviral neutralization bioassay.

During the development of antibody assay methodologies we tested an assay configuration similar to the IRMA that used two murine monoclonal antibodies, and found that it yielded 20% false negative results (P. Trown and O. Bohoslawec, unpublished data). Given the importance of the phenomena under study, we feel that the IRMA method should not be considered sufficiently reliable. This concern has been amply validated by the similarly high false negative rate of antibody obtained when the procedure of Protzman *et al.*[13] was duplicated in our laboratories.

Conclusion

The development of specific antibodies consequent to any injected protein is to be expected, and monitoring for this phenomenon should be planned as part of both the early and ongoing investigational evaluation of any protein product. Since the configuration and sensitivity of the assays used to detect antibodies dramatically influence their apparent incidence, it is critical that the assays be carefully devised and validated. It is theoretically possible that high titers of circulating neutralizing antibody to interferon may be temporally related to a loss of therapeutic activity in isolated cases. Only careful and continued monitoring of this phenomenon in ongoing clinical investigations will provide answers to this intriguing question.

The relatively high incidence of antibody development that we have reported with Roferon®-A is probably due to the highly sensitive assay used to prescreen the patient sera, and it more realistically reflects the prevalance of this phenomenon when measured by this "state-of-the-art" technique. Rigorous statistical analysis in this study has revealed no deleterious effect on response rate, time to or duration of response, or survival among patients who develop neutralizing antibodies to interferon alfa-2a. It is also important to note that no clinical or laboratory adverse effects related to antibody development have been demonstrated in any patient receiving Roferon®-A or, to our knowledge, other recombinant alpha interferons.

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