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Biological and clinical significance of neutralizing and binding antibodies to interferon-alpha (IFN-α) during therapy for chronic hepatitis C

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SUMMARY

It is known that IFN therapy can induce the development of anti-IFN antibodies. In order to evaluate the biological and clinical significance of both neutralizing (NA) and non-neutralizing (binding) antibodies, 123 patients with chronic hepatitis C treated with recombinant IFN-α were examined. Among them, 15 were positive for NA and 24 for binding antibodies. The kinetics of NA appearance show that, in general, they develop early during the first 3 months of treatment. Moreover, NA seem to be clinically relevant, since they may be responsible for non-responsiveness to treatment in 53% of patients who develop them. The evaluation of the clinical significance of binding antibodies is more difficult. They appear significantly earlier in non-responders than in responders, but no differences were observed in the overall percentage of seroconversion between responders and non-responders. Thus, it is not possible at the moment to establish their possible role in inducing non-responsiveness.

Keywords antibodies to rIFN IFN therapy chronic hepatitis C

INTRODUCTION

IFN-α is generally recognized as the most important therapeutic agent in chronic hepatitis C. In fact, normalization of alanine aminotransferase (ALT) levels and improvement in chronic liver inflammation and necrosis are reported in approximately 50–60% of patients. However, 50% of these responder patients are known to relapse at the end of therapy [1,2].

Unfortunately, we are far from possessing a thorough understanding of the reason behind such a high non-responsiveness to therapy, and no reliable means of evaluating the prognostic factors of response are available as yet for identifying potential relapsing patients.

Among the several side-effects which have been reported during IFN therapy the most intriguing is probably the development of antibodies to IFN. This was originally described in 1981 by Vallbracht *et al.*, who observed the appearance of neutralizing anti-IFN antibodies (NA) during IFN treatment in a patient affected by nasopharyngeal carcinoma [3]. Since then, a percentage of patients developing NA, ranging from 0% to 56%, has been described in various reports [4,5]. This wide range is probably due to the patient selection, to the various schedules and dosages of treatment, to the methods used to detect antibodies, and finally to the type of IFN administered [4–6].

Although no definitive conclusion has been drawn, a role of these antibodies in diminishing the therapeutic activity of IFN-α has been postulated in neoplastic as well as infectious disease [7–12].

To gain new insight into the biological and clinical significance of antibodies to IFN, a retrospective investigation on patients affected by chronic hepatitis C, who developed anti-IFN antibodies during treatment, was planned.

To this purpose patients enrolled in two trials with rIFN-α2a and 2b, respectively, were studied. Specificially, rIFN-α2a-treated patients were chosen to evaluate the clinical role of NA, since this IFN has been reported to induce the development of NA in a number of patients [6]. In order to reduce the concomitant presence of both types of antibodies in the same subject, patients treated with rIFN-α2b, which is known to induce NA in a few cases [6], were chosen to study the role of binding antibodies.

The main goals of our study were: to analyse carefully the appearance kinetics of anti-IFN antibodies, and to evaluate further their clinical significance.

PATIENTS AND METHODS

Patients

Fifteen out of 63 patients (23·8%) treated with rIFN-α2a were

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positive for NA. Twenty-four out of 60 patients (40%) treated with rIFN-α2b were positive for binding antibodies; all these patients were found to be negative for NA.

None were hepatitis B surface antigen (HBsAg)-positive, anti-HIV positive, drug-addicted or homosexual.

Before starting therapy all the patients underwent liver biopsies to assess the histological degree of their chronic liver disease.

All patients were treated at the dosage of 3 MU intramuscularly three times weekly for 12 months, except for three patients who decided to stop treatment after 6 months. The patients were monitored monthly for anti-IFN antibodies.

Clinical response, evaluated at the end of therapy, was determined on the basis of the serum ALT values. Patients who showed a complete and persistent normalization of ALT were considered responders, whereas non-responders were defined on the basis of their irregular and/or abnormal ALT level.

Detection of neutralizing antibodies to IFN

Before starting therapy and monthly thereafter, patient sera were assayed for the presence of NA to IFN. Sera were collected after 36–48 h from the last IFN administration, to eliminate any residual IFN activity, and then stored at −20°C.

Antibody titres were determined by a neutralization test against 5 U of rIFN-α2a, rIFN-α2b, depending on the type of IFN administered *in vivo*. The sera were inactivated at 56°C for 30 min before titration. A total of 60 μl of two-fold serial dilutions of sample or control sera were incubated at 37°C with 60 μl of rIFN-α2a or -2b. After 1 h, 100 μl of the individual mixtures were added to duplicate monolayers of human Wish cells, in 96-well microtitre plates. After 18–24 h of culture, cells after an extensive washing were challenged with the Sindbis virus and incubated at 37°C for 24 h, as previously described [13,14]. Tests included titrations of the IFN preparations used in the respective assays and of a mixture of IFN and a known antibody to IFN-α. Antiviral activity and the neutralization were determined by evaluating the virus-induced cytopathic effect followed by a haemagglutination assay [13,14]. The titre was taken as the highest two-fold dilution of serum which completely inhibited the antiviral activity of the added IFN [13,15]. Since the lowest serum dilution tested was 1:5, the sensitivity limit 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 U/ml.

Detection of binding antibodies

Sera from a group of patients positive for binding antibodies were collected after 36–48 h from the last IFN administration, and stored at −20°C.

The binding antibodies to IFN were tested using a commercial EIA kit (Anawa Laboratories, Wangen, Switzerland), following the manufacturer's instructions. The test consists of a solid-phase enzyme immunoassay based on the sandwich system. Briefly, plastic beads sensitized with rIFN-α2b are incubated with patient serum samples; antibodies will be captured by the immobilized IFN. Then, IFN-α2 peroxidase conjugate is added and incubation continued. Captured antibodies bind to the conjugate by their remaining free combining site. After washing, enzyme activity on the beads is determined by incubation with the enzyme substrate. The optical density is read at the end of the reaction.

It should be observed that although the assay is specific for rIFN-α2b, the cross-reactivity between 2a and 2b is high, if not absolute [6,16].

Viral markers

The detection of anti-hepatitis C virus (HCV) was carried out by a second generation ELISA test (Ortho Diagnostic Systems, Raritan, NJ). The determination of the presence of HBsAg and anti-HIV antibodies was carried out by commercial kits from Abbott and Boehring.

HCV-RNA

RNA was extracted from serum by the acid guanidinium method [17], and transcribed into cDNA by a M-MuLV reverse transcriptase (Boehring Mannheim Biochemica, Mannheim, Germany) using an antisense primer synthesized from the highly conserved 5′-non-coding region of viral genoma (Genset, Paris, France). Then the cDNA was amplified by nested polymerase chain reaction (PCR). Twenty microlitres of the amplified products were analysed by agarose gel electrophoresis and stained with ethidium bromide [18].

Statistical analysis

Student's *t*-test was used to determine the 95% confidence interval (CI) for the appearance of binding antibodies. The geometric mean was performed to assess the titre mean of NA. χ^2 test was used to determine the 95% CI for the clinical role of anti-IFN antibodies. The one-tailed exact Fischer's test was used to statistically analyse the PCR data.

RESULTS

Neutralizing antibody-positive patients

The results of the monthly tests for NA are shown in Fig. 1.

Looking only at the NA-positive patients, it can be seen that 1/15 (6·6%) developed antibodies to IFN during the first month of treatment. Thereafter, this ratio increased to 12/15 (80%) after 3 months; all the patients were positive at month 8.

The kinetics of NA appearance was then analysed separately in responders and non-responders. The data indicate that the

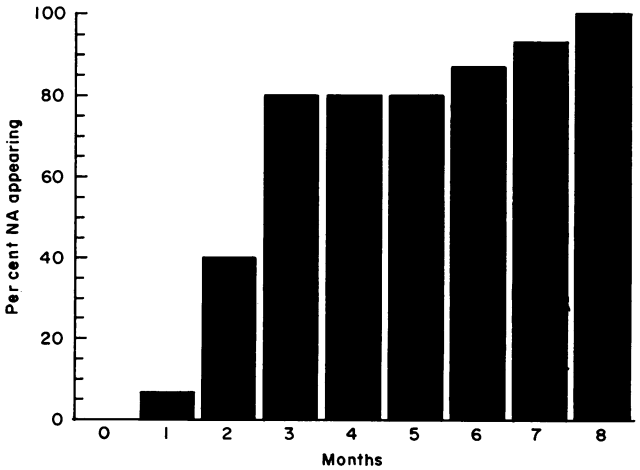

Fig. 1. Kinetics of cumulative percentage of patients who developed neutralizing antibodies (NA).

Table 1. Clinical response and kinetics of neutralizing antibody (NA) development in NA-positive patients

	No. of cases	%	Months of appearance
R	2	13	5·0 ± 4·2
NR	13	87	3·1 ± 1·7
Total	15	100	

R, Responsers; NR, non-responders.

appearance of NA is slightly but not significantly delayed in responders (3·1 ± 1·7 *versus* 5·0 ± 4·2 months) (Table 1). The antibody titre was also higher in patients who lost the clinical response than in non-responders (160 *versus* 63), although again this difference was not statistically significant. It should be mentioned that the lack of significance is likely to be due to the limited number of responder patients positive for NA, and perhaps, more importantly, to the high s.d. values (the data are referred to in fact as geometric mean).

It should be noted, however, that a correlation was observed between clinical response and the development of NA (Table 1). In fact, it can be seen that only 2/15 (13%) of servoconverted patients were responders, while 13/15 (87%) were non-responders. Importantly, this is consistent with the data obtained from the whole group of patients treated with rIFN-α2a. As already mentioned, the NA-positive patients represent the 23·8% of the 63 patients who were enrolled in the trial. Among the negative patients (76·2%), 36 out of 48 (75%) were responders and 12 out of 48 (25%) were non-responders. It can be suggested, therefore, that patients who had NA were more likely to be non-responders. Importantly, since the frequency of responders in the whole trial was 60·3% (38 out of 63) and the frequency of responders among the NA-positive patients was 13·3% (two out of 15), it is tempting to conclude that NA development could significantly ($P < 0·01$) affect therapeutic efficacy of IFN.

In the NA-positive patients the different pattern of response was examined to study the potential role of NA in influencing clinical response.

The different clinical response is described in detail for each patient in Fig. 2, where the trend of ALT and NA, examined monthly throughout IFN treatment, is reported. Table 2 shows that in eight out of 15 patients (53%), ALT underwent a

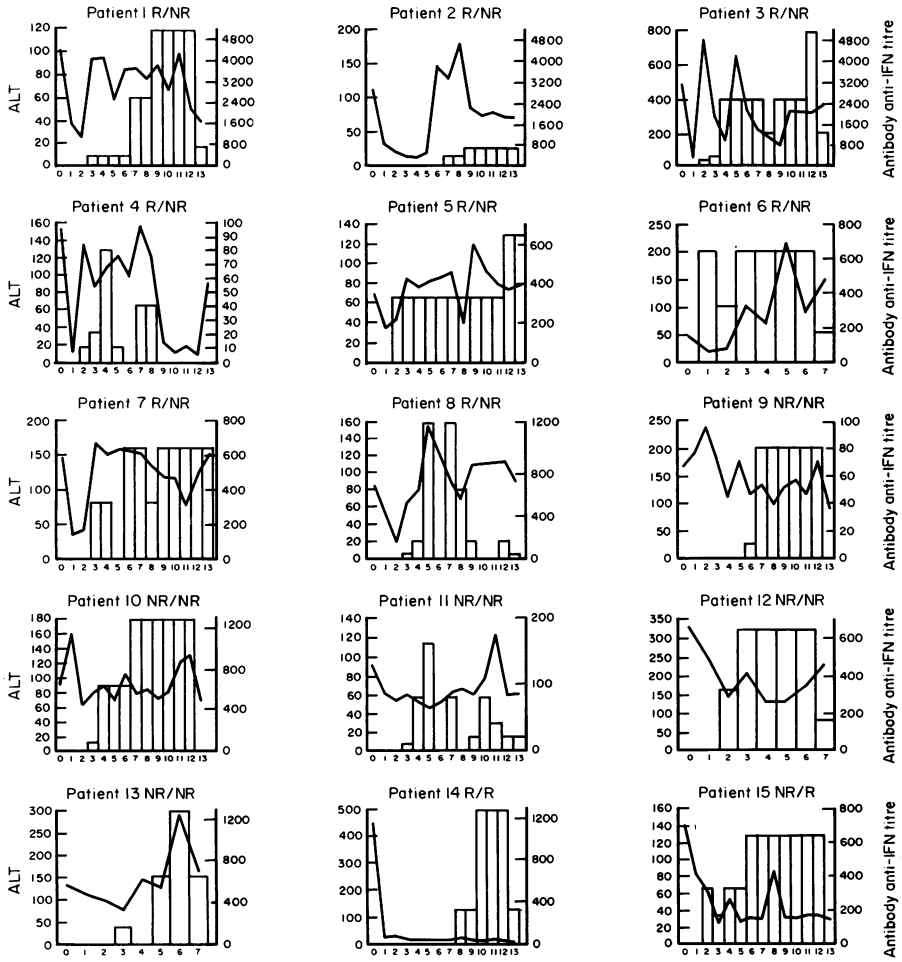

Fig. 2. Pattern of response (alanine aminotransferase (ALT) level) and neutralizing antibody (NA) development in 15 chronic HCV patients treated with rIFN-α2a.

Table 2. Correlation between the appearance of neutralizing antibodies (NA) and different clinical responses in NA-positive patients

Initial/final response	No. of cases	Month of response change	Month of NA appearance
R/NR	8	2·9 ± 1·3*	2.9 ± 1.7*
NR/NR	5	NA	3·4 ± 1·5
R/R	1	NA	8·0
NR/R	1	3·0	2·0

R, Responder; NR, non-responder; NA, not applicable.
* Index of correlation ($r < 90\%$).

dramatic decline during the first 2·9 ± 1·3 months of therapy, while the patients were still negative for anti-IFN antibodies. After 2·9 ± 1·7 months NA suddenly appeared. At this stage, ALT levels increased rapidly to fairly high values, which were maintained for the whole duration of treatment.

In the other five cases (33%), NA developed before any clinical response was evident. No relationship could be established between the appearance of antibodies and the clinical response, although it was not possible to exclude *a priori* any reciprocal influence. Of the remaining two patients, one showed complete response after only 1 month of therapy; this situation did not change when NA appeared during month 8 of treatment (Fig. 2, patient 14). In the second case, a complete response was obtained after 3 months of therapy, although NA had already appeared after 2 months (Fig. 2, patient 15).

The data in Table 2 strongly suggest that NA can significantly affect the clinical response only in those patients who lost a clinical response during therapy ($r > 90\%$).

In six out of eight of these patients (in the remaining two cases the sera were no longer available) we were able to evaluate the levels of serum HCV-RNA at the beginning of the therapy, before and after NA appearance. We found that each one had detectable viraemia at the start of IFN therapy, and apparently cleared the virus during the treatment. Interestingly, four of six patients did have viraemia concomitantly to the NA development ($P < 0·03$; data not shown).

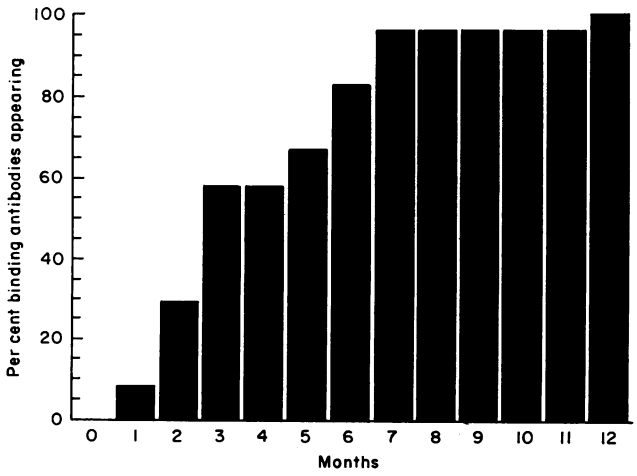

Fig. 3. Kinetics of cumulative percentage of patients who developed binding antibodies.

Table 3. Clinical responses and kinetics of developing binding antibodies to IFN in binding-positive patients

	No. of cases	%	Months of appearance
R	12	50	4·4 ± 3·0
NR	12	50	2·0 ± 1·7
Total	24	100	

R, Responder; NR non-responder

Binding antibody-positive patients

The results obtained for the kinetics of the appearance of binding antibodies in our group of patients, assayed monthly, are described in Fig. 3; they show that in two patients (8%) these antibodies were already present before starting the treatment. Moreover, a further 14 patients (totalling 67%) developed binding antibodies after 4 months, followed by four (totalling 83%) and three (totalling 96%), after 5 and 6 months of treatment, respectively. Finally, the remaining patient developed binding antibodies during the last month of therapy. Among the 24/60 (40%) patients who developed binding antibodies, 12/24 (50%) were responders and 12/24 (50%) were non-responders (Table 3). Therefore, binding antibodies do not seem to affect the clinical outcome of IFN therapy. In fact, in the remaining 36/60 (60%) patients, negative for binding antibodies, 21/36 (58%) were responders and 15/36 (42%) were non-responders. No statistically significant difference was observed between positive and negative patients in terms of clinical response ($P > 0·5$).

However, the kinetics of the appearance of binding antibodies was different in non-responders and responders. In fact, the former developed antibodies earlier (2·0 ± 1·7 months *versus* 4·4 ± 3·0 months; $P < 0·01$) (Table 3).

It should be mentioned that in a small number of cases (17%) these antibodies disappeared during therapy. After the antibodies appeared, they usually remained detectable for 1–3 months, disappeared during 1–3 months and, finally, reappeared for the remaining months of treatment (data not shown).

DISCUSSION

It is known that only about 50–60% of patients affected by chronic hepatitis C respond to IFN treatment. Approximately half of these relapses appear at the end of therapy [1,2]. The reason for the failure of IFN therapy in some HCV patients is not well understood, mainly due to our poor knowledge of both the natural history of HCV infection and the interaction between host immunity and IFN treatment. These problems hinder our understanding of the biological basis of non-responsiveness to antiviral treatment.

Among many possible explanations of this phenomenon, one is particularly worth mentioning, i.e. the development of antibodies to IFN.

It is known that anti-IFN antibodies can inhibit IFN action, thereby affecting the clinical response. This has in fact been observed in chronic myeloid and hairy cell leukaemia, mixed essential cryoglobulinaemia, and in chronic HBV hepatitis, in

which a significant correlation between the appearance of NA and the loss of clinical response has been reported [7–12]. As regards chronic hepatitis C, a first study indicating the clinical significance of NA has been reported [19]. Nevertheless, no definitive conclusions can be drawn about the clinical significance of this type of anti-IFN antibody. Furthermore, no data on the development of binding antibodies and the kinetics of the appearance of both binding and NA are available.

This study addresses these issues, trying to provide new insights into anti-IFN antibodies during therapy for chronic hepatitis C.

Our results show that in most of the patients who developed NA, this event occurred fairly early in the course of therapy, although in some patients seroconversion was detected only after several months of treatment. It appears that responders develop NA later than non-responders. However, this difference was not statistically significant, probably because of the low number of responder patients, one of whom developed antibodies at month 8 of therapy. It is therefore possible that the time of seroconversion may be a critical factor in determining the influence of NA on response. Indeed, all patients who showed an initial remarkable response and developed NA during the first 3–4 months of therapy became non-responders at the same time (Fig. 2, patients 1–8). In four out of six of these patients the clinical role of NA is also suggested by the increase of HCV-RNA serum levels.

Furthermore, the only patient who showed an evident improvement during the first 7 months of treatment and developed NA at month 8 remained a responder in spite of NA development. In this case (Fig. 2, patient 14) it is tempting to speculate that the previous 7 months were enough to induce complete remission. Similarly, the last patient (Fig. 2, patient 15), who showed a progressive and complete response during the first 3 months of therapy, developed NA during month 2. These two cases are consistent with the results of the clinical study previously published, reporting a sudden and clear response in responders after a few months of therapy [1]. Conversely, it was not possible to establish the role of these antibodies in patients (Fig. 2, patients 9–13) who showed only a partial response even before NA appearance.

It would be extremely interesting to establish whether there is a titre of NA above which loss of response is evident. Unfortunately our data do not allow one to reach any conclusion, probably because, as already stated, we have only a limited number of responder patients positive for NA.

Taken together, these findings suggest that development of NA to IFN can be associated to the therapeutic failure of IFN. However, since both a number of non-responder patients and some of the patients who relapsed after an initial response do not have serum NA, development of antibodies cannot entirely explain the high percentage of non-responders among chronic HCV patients. Indeed, several additional explanations do exist.

For instance, it is well known that 2′–5′ oligo-adenylate synthetase (an IFN-induced enzyme) plays a key role in the development of antiviral states. Low levels of this enzyme are probably unable to suppress virus replication successfully. Failure to produce this enzyme has been reported in non-responders [20]. It is likely that a deficit in intracellular IFN-mediated signalling could be associated with the lack of response.

Alternatively, sequestered sites of HCV replication, HCV variant resistant to the antiviral effect of IFN, or escape mechanisms developed during IFN therapy, could be taken into account to explain the non-responsiveness of some patients.

This study also examined for the first time the course of binding antibody development in patients affected with chronic C hepatitis and treated with rIFN-α.

Our data suggest that binding antibodies appear earlier than NA during IFN therapy. The percentage of patients developing binding antibodies seems to be lower after 3 months and higher after 6 months than that of patients developing NA. The reason for this irregular pattern is not known, but it may be consistent with the disappearance of binding antibodies during the therapy. It should be also mentioned that the comparison among NA and binding antibodies could be hindered by the different sensitivity of the assay used to detect them. It is interesting to observe that both NA and binding antibodies in most of the examined patients appeared during the first 6 months of therapy. Therefore, prolonging IFN treatment may not be a risk factor in the appearance of anti-IFN antibodies.

Although binding antibodies appeared significantly earlier in non-responders than in responders, they did not seem to influence clinical response. In fact, 50% of seroconverted patients were responders. However, these results may be only apparently contradictory, as: (i) it is not possible to exclude the existence of different types of these antibodies, which may be directed to different epitopes on the IFN molecule; (ii) it is also possible that in some patients, binding antibodies may change the pharmacokinetics of IFN; (iii) binding antibodies can fluctuate during therapy; and finally, (iv) it is not possible to rule out the possibility that a part of binding antibodies might, in some cases, neutralize IFN *in vivo*.

In conclusion, as indicated by previous reports [7–12,19], this study suggests that antibodies to IFN may be clinically important under certain conditions. Specifically, our data indicate that the appearance of NA can be responsible for the loss of clinical response in some HCV patients. As far as binding antibodies are concerned, further experiments are needed to establish the biological and clinical significance of this type of antibody.

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