

Comparative Study of Biosynthetic Human Growth Hormone Immunogenicity in Growth Hormone Deficient Children

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Abstract. The immunogenicities of six recombinant human growth hormone (rhGH) preparations, from KABI (A rhGH₁₉₁ and B rhGH₁₉₂), Eli Lilly (C), Nordisk (D), Sanofi (E) and Serono (F), used to treat 260 GH-deficient children, have been compared using a common specific and sensitive procedure for antibody determination. For this purpose we developed two immunoassays: a competitive liquid radioimmunoassay using ¹²⁵I-rhGH, and an immunometric solid enzymeimmunoassay in which the rhGHs were immobilized. Blood samples were collected from the GH-deficient children before treatment and after 3, 6, 9, 12, 18 and 24 months of therapy. Human GH antibodies were detected in children treated with 3 of the 6 rhGH preparations. Seven percent of the patients treated with hormone A, 14% with hormone B and 22% with hormone C formed antibodies against the respective rhGH. Differences in capacity and affinity of the hGH antibodies were observed between these anti-GH-positive groups. They could be divided into 2 groups according to their immunopotency. One group (7, 14 and 6% of the patients treated with hormones A, B and C, respectively) developed anti-hGH antibodies with very low binding capacities (30–100 fmol/ml). The other group (16% of the patients treated with hormone C) developed IgG-type antibodies to hGH with higher binding capacities (200–1,200 fmol/ml) and a measurable binding affinity ($K_a = 10^8 M^{-1}$). These hGH antibodies partially inhibited the binding of labeled GH to its specific liver membrane receptor. However, because of their low titer, they did not inhibit growth in the treated children. The antibody response profiles after 24–30 months of rhGH C therapy indicated a progressive increase in hGH antibody levels up to 6 or 9 months of treatment, followed by a decrease to undetectable levels at 30 months. These results show that currently available rhGH preparations have attained a great degree of purity and have a very low immunogenicity. Sensitive techniques, such as those used in the present study, may help to detect immunogenic differences between recombinant hormones and should be used to assess the quality of new preparations.

Introduction

Recombinant human growth hormone (rhGH) has proven to be equipotent to pituitary GH in GH-deficient children and produces no apparent clinically significant side effects [1–3]. The development of antibodies to GH has been reported in some patients treated with pituitary GH preparations [4] and with earlier preparations of

recombinant GH [1, 5, 6]. The concomitant presence of *Escherichia coli* polypeptide antibodies in the blood of rhGH-treated patients suggested that antigenicity may be related to the purity of the preparations [2]. However, more recent preparations display little antigenicity. The immunogenicity of commercially available rhGH is therefore a matter of considerable concern. The adjuvant effects of unrelated contaminants associated with hGH

by disulfide, ionic and/or hydrophobic links, as well as the changes in the intrinsic primary and secondary hGH structure may occur during the production and/or extraction of the hormone, and lead to potential immunogenicity.

The main concern with anti-GH antibodies is their ability to neutralize circulating GH and to inhibit its growth-promoting effect [4]. Such an effect has been reported in only a few patients presenting extremely high concentrations of antibodies with high binding capacities [7]. However, some children treated with GH seem to eventually develop GH antibodies with low binding capacities [8]. The long-term significance of any persistent circulating free and complexed GH antibodies remains unknown.

This study was performed to compare the immunogenic effect of preparations of rhGH presently available in France, to provide a measure of quality control and to evaluate short-term tolerance by a prospective multicenter study. Its purpose was to perform an independent comparison between several rhGH preparations. Therefore we developed highly sensitive enzymatic and radioimmunological assays to determine the capacity, specificity and affinity of plasma GH antibodies from treated patients. As the assay sensitivity limitations are due to nonspecific reactions and depend upon the type of assay used, GH antibody concentrations may vary from one report to another. A common and sensitive method was used to avoid differences between independent studies which are usually performed during clinical trials.

Materials and Methods

Recombinant Human Growth Hormones

Six commercially available hormones, distributed by the French Pituitary Agency, were assessed in this study. Four were hormones having the authentic GH sequence and synthesized by recombinant DNA techniques in *E. coli*. They were supplied by Kabi (Sweden) (indicated as GH-A in the Results), Eli Lilly (USA; GH-C), Nordisk (Denmark; GH-D), and Sanofi Elf Biorecherche (France; GH-E). A methionyl GH from Kabi (GH-B) and an authentic GH produced in a mammalian cell line by Serono (Italy; GH-F) were also investigated.

Patients

Between January 1987 and July 1989, a total of 260 boys and girls, aged from 2 years and 2 months to 18 years and 8 months, were treated with rhGH. Of these, 246 children were naive patients and 14 were transfer patients previously treated with extracted human pituitary GH. None of the transfer patients presented detectable circulating hGH antibodies on entering this study. All patients were given weekly rhGH doses of 0.4–0.6 U/kg, as a series of (generally) 6 daily subcutaneous injections.

Forty-three (16%) children received GH-A from Kabi and 29 (11%) GH-B from Kabi, 46 (18%) GH-C from Eli Lilly, 44 (17%) GH-D from Nordisk, 56 (21%) GH-E from Sanofi, and 42 (16%) GH-F from Serono. Blood samples for the measurement of antibody concentrations were collected before treatment and after 3, 6, 9, 12, 18 and 24 months of therapy. The interval between the last GH injection and blood sampling was at least 24 h. Blood was withdrawn into EDTA-treated plastic tubes and centrifuged at 3,000 rpm, the plasmas were transferred to polypropylene tubes, and stored at -20°C until assays.

Reagents

Radiolabeled ^{125}I -rhGH. Each rhGH was radioiodinated according to the radiolabeling method using 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Iodogen) by a modification of the Fraker and Speck [9] technique.

One microgram of Iodogen (Pierce, Netherlands) in 10 μl dichloromethane was introduced in a polypropylene microtube (Polylabo Paul Block, France) and the organic solvent evaporated off under N_2 . 20 μl 0.5 M phosphate buffer (pH 7.4), 5 μg rhGH in 5 μl 50 mM ammonium bicarbonate (pH 8.5) and 750 μCi ^{125}I (Amersham, UK) were added sequentially to the tube. After 5 min of incubation at room temperature, the reaction mixture was chromatographed by gel filtration on a 1.5×30 cm column of ACA 54 (IBF, France) and eluted with 0.1 M phosphate-buffered saline (PBS, pH 7.4), containing 5 g/l bovine serum albumin (BSA). After gel filtration all hormones gave a single major monomeric ^{125}I -hGH peak with specific activities of 90–120 $\mu\text{Ci}/\mu\text{g}$. There was a small amount of material migrating as dimeric and/or polymeric forms, up to 2.5% of the total radioactivity for rhGH C. The monomeric ^{125}I -rhGH was collected, aliquoted and stored at -80°C . Its immunoreactivity, calculated by incubating it with excess anti-hGH rabbit antiserum (L77-78), was between 95 and 100%.

Anti-hGH Rabbit Antiserum (L77-78). The affinity constant (K_a) of this antiserum, measured with extracted pituitary hGH and with the various rhGH preparations, was $1 \times 10^{10} \text{ M}^{-1}$ according to the method of Scatchard [10]. This antiserum (final dilution 1/10,000) bound the coated rhGH preparations with a maximum variation of 3% absorbance (mean value of 3 assays) in the enzyme-linked immunosorbent assay (ELISA).

Anti-hGH Human Serum (H-GER). The reference anti-hGH human serum (final dilution 1/200), bound 59% of 200 pg ^{125}I -extracted hGH, and 30–50% of 200 pg ^{125}I -rhGHs in the radioimmunoassay (RIA). The antiserum (final dilution 1/100) bound the various immobilized hormones (pituitary and recombinants) with a maximum variation of 8% absorbance (mean of 10 assays) in the solid phase ELISA.

Anti-Total Human Immunoglobulin Serum Used as Precipitant in the RIA. The precipitation curve obtained with this serum showed saturation under the assay conditions used. Consequently excess of this antiserum gave a maximal signal, even when the tested sample contained excess antibody. The nature of the anti-GH antibody was also determined by radioimmunoprecipitation with specific anti-human IgG globulins (Biosys, France) under the same conditions as used for the anti-human whole globulins.

Anti-Total Human Immunoglobulin Serum Conjugated to β -Galactosidase (Amersham, France). This affinity-purified immunoglobulin conjugated to β -galactosidase was used at a saturating concentration in the solid phase assay ELISA (1/100 final dilution).

Table 1. Frequency of detectable anti-GH antibodies in plasma from patients treated with six preparations of rhGH by competitive liquid phase RIA and noncompetitive solid phase ELISA

	rhGH type ¹					
	A	B	C	D	E	F
Patients	43	29	46	44	56	42
Patients with GH Ab	3	4	10	ND	ND	ND
Detected by RIA	3	4	10	ND	ND	ND
Detected by ELISA	1	3	5	ND	ND	ND

Results are expressed as number of positive anti-rhGH patients detected during 24 months of treatment.

¹ rhGH preparations were A (Kabi 191), B (Kabi 192), C (Eli Lilly), D (Nordisk), E (Sanofi) and F (Serono).

Procedures

GH Antibody Measurement by RIA. All determinations were carried out in duplicate. For each child, the origin of the iodinated rhGH preparation used for the measurement of antibodies was the same as the one used for treatment. We will call it 'homologous' rhGH. Twenty microliters of undiluted plasma were incubated with 100 μ l of 40,000 dpm ¹²⁵I-rhGH (corresponding to 0.2 ng or 0.014 pmol rhGHs) and 180 μ l PBS (0.1 M phosphate, pH 7.4, 0.15 M NaCl, containing 5 g/l BSA and 0.1 g/l Na azide; PBS-BSA), at 4 °C for 48 h. Then 40 μ l of undiluted anti-total human globulin (G, A, M) serum and 1 ml of 6% polyethyleneglycol were added to each tube and the mixture incubated at 0 °C for 45 min to precipitate the hormone-immunoglobulin complexes. The bound and free ¹²⁵I-rhGH were separated by centrifugation at 4,000 rpm for 20 min at 4 °C. The precipitated radioactivity, antibody-bound ¹²⁵I-rhGH, was determined by counting in a gamma spectrometer with 75% efficiency.

Each test included 2 negative controls (rabbit and human serum) and 2 positive controls (rabbit L77-78 and human H-GER antiserum) used at the same dilution as the unknown samples (20 μ l undiluted plasma per tube, 1/15 final dilution).

The detection limit of this assay, using the negative human serum (mean $\pm$ 2 SD) was 15–30 fmol GH-bound antibody/ml plasma. Antibody specificity was assessed in the presence of 100-fold excess unlabeled hGH (20 ng).

GH Antibody Measurement by ELISA. The wells of microtiter plates (Dynatech, France) were coated with 240 ng extracted hGH (NIDDK, Baltimore, Md., USA) or rhGH in 100 μ l 10 mM ammonium bicarbonate (pH 8.5)/well. The plates were sealed with Parafilm, covered and incubated at 37 °C for 18 h.

Hormone-coated plates were washed three times with mineral water containing 0.5 ml/l Tween 20 (washing solution) and 200 μ l of 20 ml/l Tween 20 in 0.1 M PBS (pH 7.4) was added to each well to coat residual binding sites (hydrophobic links) and reduce nonspecific binding. The covered plates were incubated for 1 h at room temperature and then washed 3 times with washing solution. Then 100 μ l diluted plasma (1/100 final dilution) in 0.1 M PBS (pH 7.4), 5 g/l BSA, 0.1 g/l Na azide and 2.5 ml/l Tween 20 (ELISA buffer) was added per well and the plates were incubated for 2 h at 37 °C. The plates were again washed 3 times and 100 μ l galactosidase-

linked anti-human immunoglobulins, diluted 1/100 in ELISA buffer, was added to each well and incubated for 2 h at 37 °C. The plates were washed and substrate (4 g/l orthonitrophenyl-galactopyranoside, ONPG) was added in a 100- μ l volume of 0.1 M PBS (pH 7.4) containing 2 mM Mg titriplex, 1 mM MgSO₄, 0.2 mM MnSO₄ plus 1 mM mercaptoethanol. Hydrolyzed ONPG was measured in an automatic ELISA reader at 405 nm (Multiskan, Flow Laboratory, France) within 1 h of starting incubation at room temperature.

All tests were performed in duplicate with specific rhGH and with extracted hGH. Each plate included 2 negative controls (human and rabbit serum) and 2 positive controls (human and rabbit L77-78 antiserum) at a final dilution of 1/100. The threshold of detection was established as 2 SD above the mean absorbance (A) obtained with the human plasma negative control. Results are expressed as percentages from the relationship: (test sample A – negative control A)/positive control A. Anti-GH binding was considered specific if at least 50% was displaced by 1 μ g hGH.

The binding for each patient plasma during the rhGH therapy was, in all tests, compared to the binding activity of his/her own plasma before treatment.

¹²⁵I-hGH Binding to Membrane Receptor. Cell membranes were isolated from pregnant rabbit liver by the method of Tsushima and Friesen [11]. One hundred microliters of membrane preparation (2 mg proteins/ml) were incubated with 100 μ l diluted plasma and 100 μ l ¹²⁵I-extracted hGH (80,000 dpm) plus 1.2 ml 50 mM Tris-HCl buffer (pH 7.4) containing 1 g/l BSA and 500 KIU/ml zymofren (Specia, France) for 3 h of incubation at room temperature. The reaction was stopped by adding 2 ml ice-cold Tris-BSA buffer. Membranes were sedimented by centrifugation at 4,000 rpm for 30 min at 4 °C and the ¹²⁵I-hGH in the pellet was counted in a gamma spectrometer. All tests were performed in triplicate. Nonspecific binding, determined in the presence of 500 ng unlabeled hormone, was about 5% of total activity. The percent specific binding was 14%.

Results

Anti-GH antibodies were found only in patients treated with the A, B and C hormones (table 1).

rhGH-D, -E and -F. Whatever assay was used, during the 18–24 months of therapy, no anti-GH antibodies were detected (ND) in the plasma of 44, 56 or 42 children treated with rhGH-D, -E and -F, respectively.

rhGH-A. During the 18–24 months of therapy, only 3 of 43 patients (7%) developed anti-rhGH antibodies detected by RIA. The binding capacity of these antibodies was very low, 30–100 fmol/ml plasma. The plasma of only 1 of the RIA-positive children was also positive in the immunometric ELISA using 'homologous' rhGH as well as extracted hGH.

rhGH-B. 4 of 29 patients (14%) had circulating low binding capacity anti-rGH antibodies (30–100 fmol/ml). In this group, some of the antibodies escaped detection by ELISA, as only 3 of 29 children gave positive results with both rhGH and extracted hGH.

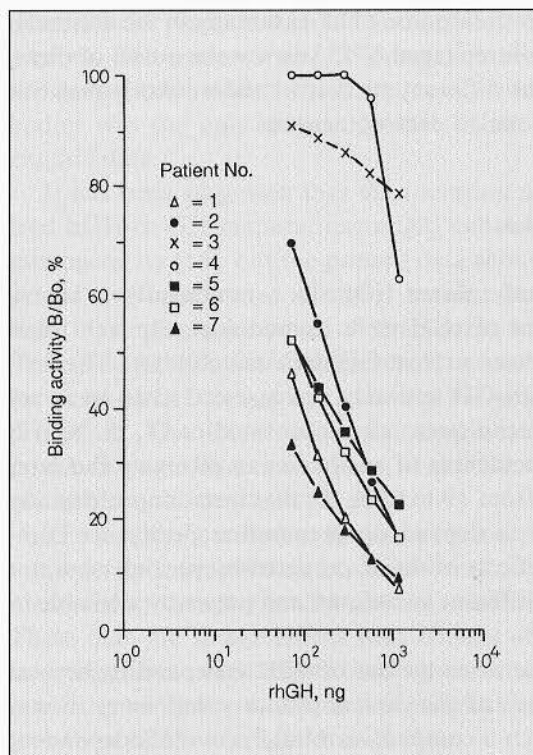


Fig. 1. Displacement curves for anti-GH plasma from patients treated with hormone C (see text). The anti-GH plasma were used at a dilution giving 30% binding with labeled hormone. Individual patients are identified by number.

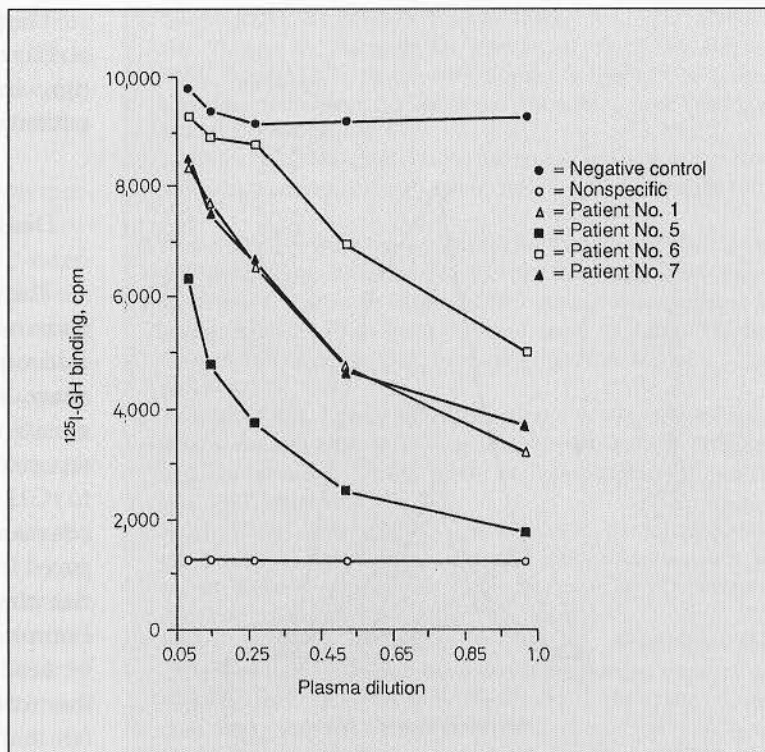


Fig. 2. Effects of 4 anti-rhGH-C plasmas and 1 negative control plasma on the specific binding of ^{125}I -hGH to its liver membrane receptor.

rhGH-C. Antibodies were found by RIA in 10 of 46 (22%) treated children. Three of the 10 patients had low capacity antibodies, 50–100 fmol/ml. The plasmas from the other 7 children contained specific antibodies with higher binding capacities, 260–1200 fmol anti-GH antibodies/ml plasma. Five of these 10 anti-rhGH-C-positive children had antibodies which also reacted with the immobilized GH in ELISA whether from recombinant or extractive hGH. Four of these 5 children with anti-GH-positive plasmas by ELISA belonged to the high binding-capacity plasma detected by RIA.

In 5 of the 7 patients on rhGH-C therapy with high binding capacity, ranging from 260 to 1,200 fmol/ml, the antibody association constant (K_a) used as an index of affinity, calculated by a Scatchard plot from the competition curves (fig. 1), revealed a major antibody population of $K_a = 10^8 \text{ M}^{-1}$ ($K_a = 5 \times 10^8 \text{ M}^{-1}$ for patients 1, 2, and 7; $K_a = 1.5 \times 10^8 \text{ M}^{-1}$ for patients 5 and 6). The other 2 patients (3 and 4) had antibodies with binding capacities of 300 fmol/ml and K_a inferior to 10^7 M^{-1} (fig. 1).

A specific hGH radioreceptor assay was applied to these plasma samples. The plasma from the 4 patients with the highest capacity and affinity for rhGH-C antibodies and 1 from a negative control subject were assayed for their ability to interfere with the binding of ^{125}I -hGH to its specific receptor. As shown in figure 2, all 4 plasmas inhibited ^{125}I -extracted hGH binding to the rabbit liver membranes. Half-maximal inhibition of binding was achieved with final plasma dilutions of approximately 1/15, 1/30, 1/40 and 1/120. In contrast, a negative control plasma did not inhibit ^{125}I -hGH binding at any dilution. The inhibition of ^{125}I -hGH-binding was correlated with the hGH-binding capacities estimated by RIA at 169, 281, 508, 1,231 fmol/ml, respectively.

Specific radioimmuno-precipitation indicated that the plasma of the 7 treated patients contained IgG-type anti-rhGH-C antibody. The profiles of plasma rhGH-C antibody concentrations of the 7 children with the highest capacity of GH binding over the 24–30 months of study are shown in figure 3. They contained no anti-GH

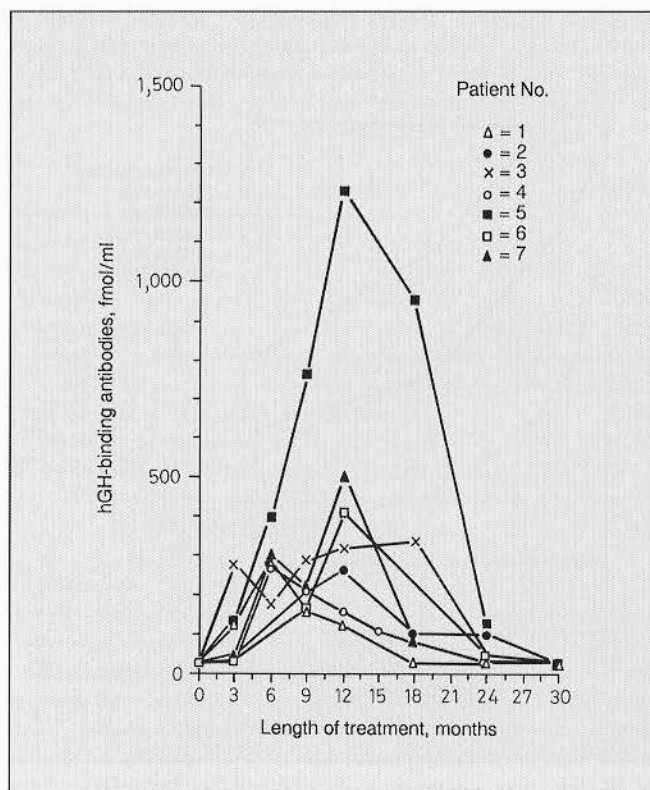


Fig. 3. Changes in the rhGH plasma antibody levels of 7 children during the first 30 months of treatment with biosynthetic hormone C.

antibodies before rhGH administration (< 27 fmol/ml). After 3 months of treatment, 2 of the 7 children had antibodies at the limit of RIA detection (33 fmol/ml) and the other 5 children had antibodies with GH-binding capacities of 50–136 fmol/l of plasma. At 6, 9 and 12 months all 7 children had plasma antibodies with capacities over 170 fmol/ml, the maximum values were 260–1,230 fmol/ml. The antibody concentrations decreased after 12 months of therapy, reaching concentrations near the detection limit at 24 months.

Antibodies were detectable late after 15 or 18 months of treatment in the plasmas of children treated with rhGH-A, and were still detectable at 24 and 30 months. Two of the children treated with rhGH-B had antibodies after 6 months of therapy and 2 others after 12 or 15 months. A GH-binding capacity of 30–100 fmol/ml was still measurable in all of them at the end of the study. In contrast, the low binding capacity antibodies found in 3 of the patients treated with rhGH-C, decreased after 6 or 9 months of treatment and were no longer detectable after 18 months.

The growth response to hGH therapy in the antibody-positive children (aged 6–13 years at the outset of therapy) was not different to that of the antibody-negative patients (detailed data not shown).

Discussion

This study shows that six commercially available preparations of rGH were immunologically well tolerated when used to treat GH-deficient children. The incidence of anti-GH antibody was assessed using two very sensitive techniques, as earlier studies [1, 5, 6, 12] reported incidences of antibodies to pituitary and later to rGH of from 10 to 66%. As that immunogenicity was considered to depend on preparation purity, we compared the effects of rhGH prepared by various laboratories using different techniques and presently available in France.

At a time when the use of hGH is expanding beyond the treatment of the clinical pituitary deficiency, it was felt that such a comparison would provide independent information on the tolerance of this synthetic hormone.

In the present study all the patients who generated antibodies presented significant titers after 3–9 months of rhGH therapy. Antibody levels decreased thereafter and in all cases disappeared during the 3rd year of treatment. The patients who developed anti-GH antibodies did not present any history of immune disease. They were sporadic cases and did not completely lack GH secretion, as do patients suffering from gene deletion [7, 13].

The number of anti-GH antibody-positive patients detected by RIA was higher than that detected by a non-competitive assay (ELISA). Although, in our case, the sensitivity of hGH ELISA was less than that of rhGH RIA, the very good relationship observed between both ELISA (one using rhGH and the other using extracted hGH) confirmed the high specificity of these assays toward GH. Three of the six rhGH preparations tested induced no antibody.

Moderate affinity antibodies ($K_a = 10^8 M^{-1}$), reaching binding capacities of 1,200 pmol/l, occurred with only one preparation. Similar findings have been reported in a recent study of the same rGH [8]. Low affinity antibodies ($K_a \leq 10^7 M^{-1}$) reaching very low binding capacities occurred with the other 2 preparations.

The difference to the various rhGH preparations in the immunological tolerance cannot be explained by our

data. All the preparations had similar chromatographic profiles. The presence of 2.5% non-monomeric GH in the preparation which most frequently induced antibodies was the only distinct feature of the chromatographic data.

It has been suggested that the inclusion of polymerized hGH in GH preparations might enhance their immunogenicity [14], but the present data should be interpreted with caution, as all rhGH batches had not been kept in similar conditions of time prior to the study (from 3 to 12 months). Furthermore it was also possible for some rhGH to aggregate during the radiochemical procedure. The monitoring of optical density elution profile from gel filtration column of rhGHs, in adequate quantity, could clearly cut it short.

The levels of antibodies found in several patients did not severely suppress their *in vitro* biological activity, as measured by the specific GH binding to liver membrane. These data are in agreement with clinical observations showing that a much higher binding capacity, above 50 nmol/l, is necessary to induce a significant inhibition of growth in GH-treated children [15].

In conclusion, there were slight differences in the capacities of the six rhGHs tested to induce anti-GH antibodies. These antibodies disappeared in all cases within 2 or 3 years. The titers induced were low and were only detected because of the highly sensitive techniques used. The significance of these antibodies in the long-term remains unknown. The results of this study should contribute to a better assessment of immunological tolerance and may be relevant to non-GH-deficient patients treated with rhGH.

Finally, it provides guidelines for improving those preparations which produce a significant frequency of immune reactions.

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