Incidence and Effects on Growth of Antibodies to Human Growth Hormone

SYLVIA R. CHALKLEY* and J. M. TANNER

From the Departments of Chemical Pathology and Growth and Development, Institute of Child Health and The Hospital for Sick Children, London

Chalkley, S. R., and Tanner, J. M. (1971). *Archives of Disease in Childhood*, 46, 160. **The incidence and effects on growth of antibodies to human growth hormone.** Antibodies to human growth hormone (HGH) have been measured by radioimmunoassay on one or more occasions in 98 children with short stature treated with HGH, and in 51 other children. Positive antibody binding reactions occurred in nearly a third of these 149 children, but the great majority of these showed an assay curve significantly non-parallel to the standard, denoting the presence of only non-specific antibodies. However reactions above 0.1% antibody binding capacity in relation to the M.R.C. standard anti-serum are mostly specific to HGH.

Specific reaction over 0.1% occurred in 19 sera taken from 10 children, all of whom were under treatment with HGH. Only 4 of these children showed a slowing down of growth associated with the development of antibodies, 2 children permanently and 2 only for a period of three months with spontaneous recovery of growth rate and drop of antibodies. The level of antibodies which appears to cause growth inhibition is between 0.5% and 1% binding capacity of the M.R.C. standard.

In all, 4 out of 42 children with isolated growth hormone deficiency have developed 'permanent' growth-retarding antibodies on HGH treatment. No children with other diagnoses have done so. Specific antibodies were not present in any of our patients before treatment.

All currently available growth hormone preparations are highly antigenic, and soon after treatment of children with human growth hormone (HGH) was begun patients were described whose initially increased rate of growth decreased abruptly as antibodies appeared in their blood (Prader *et al.*, 1964). The importance of monitoring for the formation of antibodies during treatment was soon realized, both to restrict waste of the exogenous hormone and to prevent a patient developing a high titre of antibodies which might react with the endogenous hormone thought to be produced in reduced quantities in some cases.

Since 1964 we have been following antibody levels every 3 or 6 months in a number of children treated with HGH, and we have correlated these with the children's rates of growth in height. We report two children whose growth response to HGH was permanently affected by antibodies and

a further two whose growth was only stopped temporarily.

Subjects

The main patients in this study were 98 children who had been treated with HGH for periods between 6 months and 7 years. Of these, 38 had hyposomatotropic short stature (or isolated growth hormone deficiency), 15 had craniopharyngiomas associated with failure to grow, 2 had pinealomas, and 1 tuberculous meningitis with short stature, 3 had panhypopituitarism of unknown cause, 16 were of short stature associated with low birthweight, 7 had genetic short stature, 6 had Turner's syndrome, 2 had steroid-induced short stature, 2 had glycogen storage disease, and there were 6 with unknown diagnoses, probably chondrodystrophies.

In addition, 51 children, including normals, asthmatics, and some with congenital anomalies, none of whom was treated with HGH, were studied each on one or two occasions.

HGH prepared by a modification of Raben's method by Hartree (1966) was used, which was distributed for trial by the Medical Research Council Sub-committee on Human Pituitary Hormones, to whom our thanks are due. The dose varied from 12 to 60 IU/week

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*Present address: Radioisotopes Division, Clinical Research Centre, Harrow, Middlesex.

given in saline in two intramuscular injections three or four days apart. The last injection before attending for antibody estimation was omitted, so that no child had had exogenous HGH during the 4 days before blood was taken for antibody determination. Most children received either 12 or 24 IU/week from one of 7 batches of hormone R6-11 and R13. The difference in dose did not result in any clear differences in height or skinfold response (Tanner and Whitehouse, 1967 a, b).

Method

Blood was obtained by finger-prick or occasionally vein puncture, left overnight at 4 °C, and the serum then removed and stored at -20 °C before being assayed.

Antiserum. Medical Research Council standard antibody 66/250 was used at the request of the M.R.C. and was kindly supplied by Dr. Bangham and Dr. Cotes, National Institute for Medical Research, Mill Hill. In a preliminary test, (M. Cotes, personal communication, 1966), 45 pg HGH was bound by 1.0 ml of this antiserum diluted 1:800,000. All results were expressed as antibody binding capacity (ABC), in terms of standard 66/250 so that valid comparisons could be made between different laboratories.

¹³¹I- and ¹²⁵I-labelled HGH. 5 µg HGH (Medical Research Council Standard A) was labelled according to the method of Greenwood, Hunter, and Glover (1963), with some modifications (Chalkley, 1969). The specific activity varied between 130 and 250 µCi/µg and the reagent blanks (i.e. the damaged hormone) were 5-10%. When the reagent blanks rose to 20% the hormone was discarded.

All sera were tested against at least two preparations of labelled hormone in order to minimize any non-specific effects due to the iodination damage.

Determination of antibodies. A radioimmunoassay using vertical electrophoresis in polyacrylamide gel was used (Chalkley, 1969), modified from that of Fitschen (1964), which was based on the original method described by Ornstein and Davis (1962).

In this method, labelled HGH reacts with antibody in an upper gel layer of large pore size for 60 min. Separation takes place in the lower gel layer of small pore size. After 60 min the antigen-antibody complex has migrated 0.5 to 1.0 cm from the origin and the unbound HGH 2.0 to 2.5 cm. The labelled HGH therefore moves through the antibody layer, so that there is a close proximity of reagents during the reaction period.

The standard antiserum, 66/250, was used at three dilutions: 1:6250, 1:125,000 and 1:250,000. The 1:6250 was prepared in bulk and frozen in 2.0 ml portions at -20 °C. One vial of this, when diluted for use, was sufficient for four assays.

Test sera were diluted 1:5 and 1:10 for preliminary assay. If the result was negative, the serum was re-assayed at these dilutions against a different preparation

of iodinated HGH. If the result was positive, the serum was re-assayed at higher dilutions, until a dilution was reached which gave a percentage binding equivalent to that of the reagent blank.

All standards and sera were done in duplicate in each assay, except for the blanks, of which there were four per assay. The electrophoresis apparatus held 34 tubes, so that these consisted of 4 blanks, 3 standards in duplicate, and 6 sera in duplicate at two dilutions.

The lower gel layer was polymerized in each tube, and then the upper gel layer was added and photopolymerized in two stages. 80 µl diluent (for blanks) or diluted antiserum or test serum were incorporated into the first stage, and 5 µl iodinated HGH (= 31 pg HGH) were incorporated in the second stage. Electrophoresis took two hours at 2.5 mA/tube at 37 °C. After this, the upper gel layer was discarded and the lower gel layer divided into 'bound', i.e. the antigen-antibody complex region, and 'free', containing the unbound HGH. These two portions were counted.

Calculation. The percentage HGH 'bound' by each serum sample was estimated by a technique which has for many years been established in bioassay (see Finney, 1952). A plot of the percentage bound against antiserum dilution gives the familiar sigmoid curve, with about 90% bound at very low dilutions of antiserum and about 20% bound at very high dilutions. On probit paper, then, the percentage bound plotted against log dilution gives a straight line.

The standard antiserum is assayed at three dilutions and a straight line fitted by least squares to the three resulting points. Similarly the test serum sample is assayed at three (in this work more usually two) dilutions and a straight line fitted. Two comparisons may then be made between the two lines. First they can be examined for parallelism (Youden, 1951) and second the difference in log dilution between them corresponding to any given percentage bound can be estimated. The percentage bound selected is 35% (Farr, 1958) because this is the midpoint of the probit curve, hence has the lowest error estimate. The final calculation is thus: Antibody Binding Capacity (ABC) as a percentage of standard antiserum capacity = 100 (log dilution at 35% bound of test serum - log dilution at 35% bound of standard antiserum). The actual calculations are simply carried out on an Olivetti desk computer.

The test for parallelism is of much importance, for non-parallelism between the assays indicates heterogeneity between the standard and the test serum and probably means non-specific antibodies are present. In Tables II to V assays which show non-parallelism significant at 5% are given one asterisk, and those at 1% a dagger. Note that we assume as null hypothesis that the assays are parallel, so that we must be failing to asterisk a number of assays which are really non-parallel (and hence non-specific, see below), rather than the other way around.

Growth. The children were measured every three months when awaiting and when on treatment, and every

six months after treatment finished. All measurements were done by the same measurer, R. H. Whitehouse, using the same equipment and techniques. Only heights are considered here: a full report is given in Tanner *et al.* (to be published). In the cases in which antibodies developed, we quote the three-monthly increments of height (converted to yearly rates, i.e. cm/yr) for comparison with antibody levels. In most circumstances we prefer only to give the averaged rate over a whole year, first because there are seasonal changes in growth rates affecting the comparison of successive 3-monthly periods; and second because the absolute error of measurement (a maximum of about 3 mm in skilled hands) is constant and hence constitutes a greater percentage of the 3-monthly than of the yearly increment. This limitation of 3-monthly rates should be borne in mind when considering Tables II to V. Where errors of measurement could have been very important, however, as in Cases 3 and 4, figures for sitting height, weight, skinfolds, and other dimensions have been carefully scrutinized.

Results

Specific and non-specific reactions. 28% of the serum samples from the children with short stature, and 31% of the samples from the 'control' children gave positive ABC reactions. However all samples taken from the short children before HGH treatment, and all samples from the control children, showed significant non-parallelism to the standard and must be presumed to represent non-specific antibodies. Many of the samples taken during HGH treatment also showed non-parallelism. Only 27 sera gave reactions which were possibly parallel and hence represent specific antibodies, and of these only 19 were also above 0.1% ABC. These 19 occurred in 10 individual children, all of whom had been treated with HGH.

It seems that there is a specific antibody to HGH down to about the level of 0.1% ABC, but not below. Out of 23 ABC values above 0.1%, only 4 were significantly non-parallel at the 5% level and a further 5 at the 10% level. Of the 25 specimens with ABC from 0.025% to 0.09%, 19 were significantly non-parallel at 5% and a further 2 at 10%. Of the 52 specimens with ABC from 0.005% to 0.025%, 50 were significantly non-parallel at 5% and a further 1 at 10%.

Two sera which gave high ABC levels were used to see whether the addition of unlabelled HGH to the assay system would inhibit binding of labelled HGH (Frasier and Smith, 1966). The sera were R.K. (Table II) 1.34% ABC, and P.T. (Table IV), 0.45% ABC. Serum R.K. was run at dilutions of 1:40 and 1:80, and serum P.T. at dilutions of 1:20 and 1:40. 1.208 ng unlabelled HGH per tube was added. The addition of unlabelled

HGH caused a decrease of between 1.75% and 8.25% in the binding of labelled HGH to the given antiserum.

HGH batches. Four different batches of HGH used in treatment have been compared for the number of reactions they produced (Table I). There seemed little difference, either in 'specific' or 'non-specific' reactions.

TABLE I
Comparison of Batches of HGH in Relation to type of Antibodies Produced

HGH batch no.	R8	R9	R10	R11
Total no. of samples*	9	22	74	40
% positive ABC 'non-specific'†	11	22.5	19	20
% positive ABC 'specific'‡	22	22.5	15	12
Total	33%	45%	34%	32%

*Each sample represents a serum specimen taken at the end of 3 months' treatment with the batch of HGH.

†With slope significantly non-parallel to standard slope.

‡Slope not significantly different from standard slope.

Antibodies and growth rate. Of the 10 children who showed possibly specific antibodies at above the level of 0.1% ABC on one or more occasions, only 4 appear to have had their growth

TABLE II
Patient R.K.; Male with Hyposomatotrophic Short Stature

Age (yr)	Approximate Duration of HGH Treatment (yr)		% Antibody Binding Capacity	Growth Rate (cm/yr) Over Preceding Period	
2·1	Off	0·75	Not done	5·2	
3·1	R6, 7	1·00	Not done		19·3
3·4	R8	1·25	Not done		18·1
3·6	R9	1·50	Not done		13·9
3·9	R9	1·75	Not done ¹		2·4
4·1	R9	2·00	Not done		2·4
4·5	Off	0·25	Not done	0·0	
4·6	Off	0·50	44·7	3·3	
5·0	Off	1·00	1·34	1·6	
5·2	Off	1·25	1·58	1·6	
5·7	Off	1·50	0·51	0·0	
6·0	Off	2·00	0·30	1·1	
6·2	Off	2·25	0·30	2·8	
6·5	Off	2·50	0·19	0·9	
7·0	Special ²	0·1	6·74		2·6
7·5		Off	0·5	1·0	
8·0	Off	1·0	0·10	0·2	
8·0	Wilhelmi ³		3·16		—
8·0	—	0·02	7·10		—
8·1	—	0·04	4·16		—
8·1	—	0·06	3·90		—

¹Haemagglutination antibodies demonstrated in high titre (Székely).

²3 injections 10 mg mixed Wilhelmi-Raben preparation.

³Single injection 1 mg Wilhelmi preparation.

Fig.—Growth in height (left) and height velocity (right) of R.K. (Table II). Rates over 3-monthly periods of growth are plotted as points joined by dots, yearly rates by solid lines. Antibody levels as indicated.

rate affected, 2 permanently and 2 only temporarily. The details of these are given in Tables II to V.

R.K. (Table II and Fig.) is the most striking of our cases, and the most unusual. He responded extremely well to Raben-type HGH for a full 18 months, and then, while still on batch R.9, suddenly stopped growing. At this time, high titres of antibodies were demonstrated by haemagglutination, kindly done by Dr. Széky of Zurich. HGH was stopped, but six months later he still had the highest level of antibodies we have ever seen, 44.7% ABC. This decreased, but was still 0.2% when a single injection of what was supposed to be the Wilhelmi-type growth hormone was given. In fact, owing to an error in ampouling, the injection contained the Raben as well as the Wilhelmi preparation; antibodies at once rose to 6.7%. They then decreased once more, but the growth rate has stayed very low and this patient's growth status is now not much better than it would have been without any treatment. Recently a single injection of 1 mg of a purified Wilhelmi type preparation was given and the antibody level followed weekly thereafter. At once it rose to 3% and then 7% before gradually declining.

The second patient, D.R. (Table III), developed

TABLE III

Patient D.R.; Male with Hyposomatotrophic Short Stature

Age (yr)	Approximate Duration of HGH Treatment (yr)	% Antibody Binding Capacity	Growth Rate (cm/yr) Over Preceding Period
10.4	Off 1.00	Not done	3.4
10.7	R5 0.50	0.45	10.0
11.0	R5 0.75	Not done	1.3
11.3	R5 1.00	25.1	0.0
12.2	Off 1.00	Not done	2.7
13.1	Off 2.00	Not done	4.2
14.0	Off 2.75	0.01*	3.0
14.6	Off 3.25	0.02†	3.5
15.1	Off 3.75	Nil	2.9

*Significantly non-parallel to standard slope at 5%.

†Significantly non-parallel to standard slope at 1%.

antibodies during the first six months on growth hormone, but they were insufficient in amount to stop his good response (10.0 cm/yr) during this time. During the second six months they rose much higher and his growth entirely ceased. The antibodies went down to insignificant levels after two years off the hormone and growth was resumed at the pre-HGH, hyposomatotrophic rate.

The third patient, P.T. (Table IV), is interesting

TABLE IV

Patient P.T.; Male with Hyposomatotrophic Short Stature

Age (yr)	Approximate Duration of HGH Treatment (yr)	% Antibody Binding Capacity	Growth Rate (cm/yr) Over Preceding Period
15.9	Off 1.00	Nil	3.6
16.1	R8 0.25	0.04	16.0
16.3	R8 0.50	0.45	13.7
16.6	R8 0.75	1.03*	4.0
16.8	R10 1.00	0.20	12.8
17.3	Off 0.50	0.03†	4.0
17.9	Off 1.0	0.01	3.8
18.1	R11 0.25	Nil	6.1
18.4	R11 0.50	0.57	8.6
18.6	R11 0.75	1.10	9.2
18.9	R11 1.00	1.80	6.8
19.1	R13 1.25	1.21	6.6
19.3	R13 1.50	0.49	3.4
19.6	R13 1.75	0.49	1.3

*Significantly non-parallel to standard slope at 5%.

†Significantly non-parallel to standard slope at 1%.

in that he showed a sudden decrease of growth rate associated with a rise of ABC from 0.45% to 1.02% (even though this assay was significantly non-parallel at 2.5%). There was no significant change of HGH dose and no change of batch except after the high titre. During the whole year of treatment, puberty was gradually occurring, but the change rate cannot be explained by this, nor by a measuring error, for it appeared in a number of body measurements. When he was restarted on HGH, his antibodies gradually rose again but apparently this time without affecting his growth rate, the response to growth hormone being about as much as can be expected at his degree of development. (When 18.1 years, his bone age was 15.6

TABLE V

Patient H.S.; Male with Hyposomatotrophic Short Stature

Age (yr)	Approximate Duration of HGH Treatment (yr)	% Antibody Binding Capacity	Growth Rate (cm/yr) Over Preceding Period
7.5	Off 0.5	Nil	5.4
8.1	Off 1.0	0.01	3.8
8.4	R10 0.25	0.03	9.0
8.7	R11 0.50		10.2
8.9	R11 0.75	0.54	2.6
9.2	R11 1.00	0.23	7.2
9.6	Off 0.50		2.4
10.1	Off 1.00	0.22	2.1
10.4	R13 0.25	0.04	7.6
10.7	R13 0.50	0.07	7.2
10.9	R13 0.75	0.02	8.8

and testes size 8/12, genitalia stage 4, pubic hair stage 4; at 19.3 years, bone age was 15.9, testes 15/20, genitalia 5, and pubic hair stage 5). Thus it seems that on one occasion a rise of 1% ABC stopped growth and on another it did not do so.

The fourth patient, H.S. (Table V), also showed a temporary 3-month period of growth deceleration associated with a high antibody titre, just like the third patient. The antibody value was 0.5% at the immediate ending of the slow-down (velocity of 2.6 cm/yr in Table V). Unfortunately we do not have the value at the beginning. As in the previous child, the antibodies dropped and growth resumed spontaneously. When treated a second time a good growth response occurred, with no untoward antibody rise to date.

Discussion

Incidence of growth-retarding antibody levels. Of our 38 hyposomatotropic children treated with HGH, 2 (patients R.K. and D.R.) have developed antibodies in a 'permanent' way, precluding further treatment with present preparations. The levels of these 'permanent' antibodies were high, all being well over 1% ABC. The threshold level of antibody which seems to affect growth is between 0.5% and 1% ABC. In addition, before beginning this study we have had 2 earlier hyposomatotropic patients (out of a total of 4) stop responding on development of antibodies shown by the haemagglutination technique. Thus the total incidence in our series is 9%. Prader *et al.* (1967) also using the Raben preparation reported 8 out of 18 cases, an incidence of 44%. Seip and Trygstad (1966) on the other hand, using the Gemzell preparation, had no cases of antibody growth-arrest among 12 patients. Kaplan *et al.* (1968) using the Wilhemi preparation reported no growth arrests in 53 hypopituitary patients of whom 16 were isolated GH deficiencies and 19 multiple trophic hormone deficiencies. They did remark however that in about a third of their patients low levels of antibody were detected which had no effect on growth response. Their percentage agrees exactly with ours for low-level non-specific antibody development. Parker, Mariz, and Daughaday (1964) had one growth-arrested patient out of 13 with Raben HGH. It seems possible that significant levels of antibody arise more frequently in cases of isolated GH deficiency than in others.

Two patients developed *transient* high antibody levels, though only of the order of 1%, for completely unknown reasons. Such an occurrence has not, so far as we know, been remarked before. It

seems to have little clinical significance; after a temporary slow-down of growth for a few months the child continues to respond to HGH. Development of antibodies, of course, is not the factor responsible for the gradual diminution in response shown by all children in the growth 'catch-up' situation.

Our first patient, R.K., is curious in developing antibodies only after 18 months of treatment. Previous investigators (Fraser and Smith, 1966; Prader *et al.*, 1964; Roth *et al.*, 1964) have reported that growth-inhibiting antibodies appear always within the first three months of treatment.

Since none of our patients gave evidence of the presence of specific antibodies before treatment, it appears that the short stature is not due to autoimmunity to HGH in any of our cases.

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Correspondence to Professor J. M. Tanner, Department of Growth and Development, Institute of Child Health, 30 Guilford Street, London W.C.1.