Development of antibodies during long-term therapy with corticotrophin in rheumatoid arthritis

I. Porcine ACTH

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Development of antibodies to various polypeptide hormones has been extensively reported. In the case of certain hormones, notably insulin and growth hormone, antibodies are associated with resistance to their therapeutic actions (Oakley and Cunliffe, 1968; Prader, Wagner, Széky, Illig, Touber, and Maingay, 1964).

Corticotrophin (ACTH), a straight-chain polypeptide of 39 amino acids, is a relatively poor antigen; nevertheless antibodies to it have been reported (Fleischer, Abe, Liddle, Orth, and Nicholson, 1967; Landon, Friedman, and Greenwood, 1967). Imura, Sparks, Grodsky, and Forsham (1965) have demonstrated in rabbits that the antibody-combining site on the ACTH molecule is towards the 'C' terminal end, away from the biologically active 'N' terminal sequence of the molecule (Fig. 1). Landon and others (1967) found that antisera to the complete peptide, which had developed in children who had received porcine ACTH, bound the labelled complete peptide more readily than the labelled 'N' terminal 1-24 fragment.

The subjects reported by Fleischer and others (1967) and by Landon and others (1967) who had developed antibodies to ACTH were mostly suffering from non-rheumatoid conditions. Production of autoantibodies is a feature of rheumatoid arthritis, and Meiselas, Zingale, Lee, Richman, and Siegel

(1961) have reported increased antibody production to *Brucella* vaccine in rheumatoid arthritics, together with alterations in other antibody systems. Shearn, Epstein, and Engleman (1963), however, were unable to confirm this. The consideration that a disordered immune response is characteristic of rheumatoid arthritis, and the fact that porcine ACTH is extensively used in its treatment, led us to investigate a series of rheumatoid arthritics receiving long-term therapy with porcine ACTH. We have attempted to ascertain the frequency of development of antibodies to the peptide, and to investigate the sites of combination between antibodies and antigen.

Materials and methods

A series of 33 subjects with rheumatoid arthritis, who had been treated with gelatin-linked porcine ACTH ('Acthargel', Armour) as their only form of ACTH therapy over a period ranging from 2 to 18 years (mean 7.5), was studied. The daily dose administered ranged from 6 to 30 units (mean 14). An equal number of subjects suffering from rheumatoid arthritis, but who had never received any form of hormone therapy, served as controls.

Blood was taken by venepuncture, and the serum was separated and stored at -20°C. until analysed.

The following peptide fragments of the ACTH molecule were used in the various experiments: synthetic α_1 -16, α_1 -1-24, α_1 -1-28, and α_1 . The nomenclature for corticotrophin used here is that of Li (1959) and throughout

FIG. 1 Human ACTH molecule, showing principal fragments used in this study.

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this paper, 1-16, 1-24, and 1-28 peptides refer to α_h^{1-16} , α_h^{1-24} , and α_h^{1-28} respectively, and 1-39 to α_p .

The peptides were iodinated with ^{125}I by the method of Greenwood, Hunter, and Glover (1963) and were homogeneous on electrophoresis.

The antigen-binding technique used for the detection of antibodies in serum was essentially that described by Landon and others (1967), labelled 1-39, 1-28, and 1-24 peptides being used for the incubation. All sera were incubated in 0.05 M. phosphate buffer (pH 7.4) at a dilution of 1:8, and mercaptoethanol to a final concentration of 0.2 per cent. with 400 K.I. units of Trasylol (Bayer) was incorporated in the buffer (Berson and Yalow, 1968). The separation of bound and free peptide was carried out using dextran-coated charcoal (Herbert, Lau, Gottlieb, and Bleicher, 1965). With each batch of test sera a batch of control sera of equal size was incubated in parallel.

Significant antibody titres were judged to be present when the degree of binding of the peptide by the test serum was more than three standard deviations above the mean non-specific binding found in control sera. The binding was shown to be reversible by acidification to pH 2 and by displacement of the bound labelled peptide with an excess quantity of the unlabelled peptide.

In four subjects, studies of displacement of labelled antigen were performed, using increasing concentrations of unlabelled 1-39, 1-24, and 1-16 peptides. The concentration of added peptide was then plotted against the percentage of the labelled antigen which remained bound, allowing comparison of binding efficiency for the three different peptides.

Two subjects with antibodies, and one without, were investigated for lymphocyte transformation *in vitro* with the same porcine preparation of ACTH used for therapy but without carrier gelatin. A purified lymphocyte suspension was prepared from peripheral blood (Maini, Bryceson, Wolstencroft, and Dumonde, 1969) and triplicate 3-ml. suspensions of $1 \times 10^6/\text{ml}$. lymphocytes were cultured with serial dilutions of ACTH in screw-capped Bijou vessels. Simultaneous cultures with dilutions

of phytohaemagglutinin (PHA) were also set up as an internal standard to confirm that the lymphocytes were reactive. A 2 μCi tritiated thymidine pulse was added for 18 hrs from the 126th to the 144th hour of culture, and after the cultures were harvested incorporation was assayed by liquid scintillation counting. The results were expressed as the ratio:

$$\frac{\text{mean uptake of tritiated thymidine in the present of ACTH or PHA(T)}}{\text{mean uptake of tritiated thymidine without antigen (C)}}$$

A T/C ratio of greater than 1.5 in this system (using antigens associated with known forms of hypersensitivity) indicates significant stimulation of thymidine incorporation as a measure of lymphocyte transformation.

Results

Ten out of 33 sera (*i.e.* from 30 per cent. of the subjects) bound labelled 1-39 ACTH. Three of the ten sera also bound labelled 1-28 ACTH and two of them bound labelled 1-24 ACTH. The percentages of 1-39 peptide bound were respectively as follows: 5, 10, 10, 12, 15, 17, 25, 60, 65, 70 (mean 28 per cent.).

Of the four subjects in whom displacement studies were carried out using increasing concentrations of 1-39, 1-24, and 1-16 peptides, two showed progressive displacement only with the 1-39 peptide; the 1-16 and 1-24 peptides failed to displace. The curves from one of the two antisera are shown in Fig. 2 (subject T.L.).

The other antisera tested with increasing amounts of unlabelled peptide showed binding with labelled 1-24 peptide. In both these instances progressive displacement was obtained with increasing amounts of unlabelled 1-39 and 1-24 peptides. Again, one such set of curves is shown in Fig. 3 (subject E.M.).

At the levels of ACTH used, lymphocyte transformation was not significantly increased when the

FIG. 2 Displacement curves—subject T.L., showing relative failure of 1-24 and 1-16 fragments to displace labelled 1-39 peptide.

FIG. 3 Displacement curves—subject E.M., showing relative failure of 1-16 fragment to displace labelled 1-39 peptide.

results with porcine ACTH were compared with phytohaemagglutinin (see Table).

There was no difference with respect to dose, duration of therapy, or frequency of administration between those who developed antibodies and those who did not.

Discussion

In this series consisting entirely of rheumatoid arthritics, one-third developed antibodies. This is a smaller proportion than described in previous series: eight out of nine by Fleischer and others (1967), thirteen out of nineteen by Landon and others (1967), five out of six by Norman and Sanders (1969), and 34 out of 66 by Ratcliffe, Pritchard, and El-Shaboury (1969). Clinical details are not available in all the series but, where reported, rheumatoid arthritis formed a small minority and asthmatics appeared to predominate.

It is uncertain whether the higher incidence of antibodies in the other series is related to the different pathologies in the groups investigated, or whether it is methodological. Our criteria for identifying antibodies were strictly defined in terms of a control series investigated under identical conditions; hence, although there may be variations in sensitivity between different laboratories, this should not influence a comparison of the incidence of antibodies, unless binding is being overestimated due to 'damage'.

The demonstration of apparent binding of a peptide by serum does not itself prove the presence of antibodies. Incubation of ACTH with serum leads to varying degrees of damage to the peptide, probably due to enzyme activity. A 1:8 dilution of serum together with preservative agents was employed during the incubation in this study in an effort to minimize such damage (Berson and Yalow, 1968). Damaged peptide may not be absorbed by dextran-coated charcoal and thus would give rise to over-estimates of binding. Antibody binding is reversible at pH2; hence the acidification step in the method helps to distinguish true from apparent binding. Additional evidence that binding is by specific antibodies is given by the antigen displacement studies. The excess peptide added competitively

displaces a proportion of the labelled peptide from antibody, thus reducing the percentage of radioactivity bound. These experiments do not exclude the possibility of binding by serum proteins other than immunoglobulins, but the comparison with the control sera makes this possibility unlikely.

A further factor in the lower incidence of antibodies in this group might be the long duration of therapy when compared with other series. Also, the carrier substance may act as an adjuvant to the antigenicity of the corticotrophin. Fleischer and others (1967) and Norman and Sanders (1969) both used in part a zinc-linked preparation, and it is possible that this enhances antigenicity (Ratcliff and others, 1969). In our study the ACTH preparation used employed gelatin as the vehicle.

The failure of eight of the ten antisera to bind 1-28 or 1-24 ACTH supports the view that the usual site of combination is towards the 'C' terminal sequence of the porcine ACTH molecule. In previous studies in humans, displacement curves with unlabelled antigen have not been reported. Arquilla, Ooms, and Mercola (1968) have shown that iodination, even to a relatively low specific activity, can alter the antigenic properties of peptides. Hence, displacement curves with unlabelled peptides produce better evidence of antigenic identity than direct combination with labelled peptide. We have been able to carry out this type of study on four antisera which were of sufficient titre. Two such sets of curves are shown (Figs 2 and 3).

In subject T.L. (Fig. 2), the antigenic site would appear to lie between amino acids 24 and 39, since unlabelled 1-24 and 1-16 fragments failed to displace the labelled peptide. In subject E.M. (Fig. 3), a combining site must rest somewhere between amino acids 16 and 24, since displacement was observed with both 1-39 and 1-24 peptides, but not with 1-16 fragments. Since parallelism between dose-response curves suggests identity of the immunologically active sites (Aubert and Felber, 1969), such displacement studies provide additional evidence that these antisera are directed particularly to the 'C' terminal sequence, although other binding sites do exist. Results of studies using direct combination with labelled antigen are thus confirmed.

The poor lymphocyte responsiveness to PHA

Table *T/C ratio: lymphocyte transformation (Dr. R. N. Maini)*

<i>Patient no.</i>	<i>p.ACTH (μg./ml.)</i>					<i>P.H.A. (μg./ml.)</i>			<i>ACTH antibodies</i>
	1.6	8	40	200	1000	1	10	100	
1	0.7	0.8	0.03	0.008	0.004	0.9	3.0	8.9	+
2	1.2	0.88	0.3	0.006	0.004	2.7	9.1	44	+
3	1.1	0.7	0.08	0.02	0.02	2.9	2.9	0.4	—

could be the result of high corticosteroid levels produced by the ACTH therapy. Further experiments with amounts of ACTH less than $1.6 \mu\text{g}$. may be necessary before definite conclusions about lymphocyte responsiveness in these patients can be reached as the ACTH appeared to be inhibitory in high doses.

This investigation suggests that adults with rheumatoid arthritis form antibodies to ACTH with the same specificity as those suffering from non-connective tissue disorders and experimental animals. Although a humoral immune response can be readily demonstrated, no evidence was obtained that cellular immunity developed as measured in three subjects by lymphocyte transformation.

Summary

The antibody response to long-acting natural porcine corticotrophin has been studied in 33 subjects with rheumatoid arthritis. Ten (30 per cent.) of the

subjects were found to have developed antibodies. The majority of antisera bound towards the 'C' terminal amino-acid sequence of the corticotrophin molecule, but other binding sites were shown to occur in some subjects.

In three of the subjects who developed antibodies, lymphocyte transformation studies failed to demonstrate cellular immunity.

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