

The Proteolysis of Immunoglobulin G with Long-Acting Thyroid-Stimulating Activity

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1. Proteolysis of immunoglobulin G with long-acting thyroid-stimulating activity with papain and pepsin shortened the duration of thyroid stimulation in mice prepared for the McKenzie (1958) bioassay. The time-course appeared to be related to molecular size. 2. The activity after proteolysis was recovered solely in the electrophoretically slow F_{ab} -fragment. 3. There was no immunological cross-reaction between pituitary thyroid-stimulating hormone and the active fragments of the long-acting thyroid stimulator despite their similar behaviour in the bioassay. 4. The implications of these findings are discussed.

Chemical and immunological studies on the LATS* detectable in the serum of some patients with thyrotoxicosis have shown it to be associated with IgG (Adams & Kennedy, 1962; McKenzie, 1962; Dorrington, 1964; Kriss, Pleshakov & Chien, 1964). Attempts to dissociate the thyroid-stimulating activity from IgG have been unsuccessful (McKenzie, 1962; Dorrington, 1964), suggesting that LATS activity may be an inherent property of specific IgG molecules.

Much information has been obtained relating the gross structure of IgG to serological activity, particularly in rabbit antibodies. Available evidence indicates that human IgG is essentially similar (Cohen & Porter, 1964). The four-polypeptide-chain structure proposed by Porter (1962) has been generally confirmed by subsequent work.

Analytical methods developed to study antibodies have been used in an attempt to determine the distribution of thyroid-stimulating activity in the peptide chains of LATS-IgG. Reduction of active IgG indicated that the heavy (γ) chains (Fleischman, Pain & Porter, 1962) carry the active portions of the molecule (Meek, Jones, Lewis & Vanderlaan, 1964; Dorrington, Munro & Carneiro, 1964). Separation of the peptide chains resulted in a variable loss of activity which probably accounts for discrepancies between the results from the two groups.

This paper describes results obtained with proteolytic enzymes used to degrade LATS-IgG

* Abbreviations: LATS, long-acting thyroid stimulator; the nomenclature used for the immunoglobulins and their subfractions is that recommended by the World Health Organisation (see Cohen, 1965).

in an attempt to locate the activity within the molecule.

METHODS

Bioassay of thyroid-stimulating activity. The method of McKenzie (1958) was used with minor modifications (Major & Munro, 1962). To control assay conditions separate groups of mice (White Swiss; University of Sheffield Breeding Unit) were injected with 0.15 M-NaCl buffered with phosphate to pH 7.4, two doses of International Standard thyroid-stimulating hormone and two doses of a standard for LATS (Dorrington & Munro, 1964). Groups of five mice were used for each material tested. The volume injected intravenously was 0.5 ml. Blood samples (0.1 ml.) were withdrawn immediately before and 3 hr. and 10 hr. after injection.

The significance of the responses in the test groups has been tested against the control animals injected with 0.15 M-NaCl by using Student's *t* test and $P < 0.05$.

Isolation of IgG. Immunochemically pure IgG was obtained from active and control sera by precipitation with $(NH_4)_2SO_4$ followed by chromatography on DEAE-cellulose (Levy & Sober, 1960) with 0.01 M-phosphate, pH 6.6. The isolated IgG accounted for 10–12% of the total serum protein with $S_{20,w}$ 6.5–6.6 s.

Papain digestion of IgG. This was performed according to Porter (1959). Chromatographic analysis of the papain digest was effected by gradient elution from DEAE-cellulose (Edelman, Heremans, Heremans & Kunkel, 1960) at pH 7.8. The starting buffer was 5 mM-phosphate and the limit concentration 0.5 M-phosphate.

Protein determinations. Protein concentrations were determined by absorption at 276 m μ in a 1 cm. cell of an automatic recording spectrophotometer [Optica (U.K.) Ltd.].

Peptic digestion of IgG. Digestion with pepsin was carried out by the method of Nisonoff, Wissler, Lipman & Woernley (1960). In some experiments L-cysteine hydrochloride was

added after digestion to a concentration of 0.01 M and the mixture incubated at 37° for 2 hr.

Production of antisera. Antisera have been produced in rabbits (New Zealand Whites, 2.5–3.5 kg.) against LATS-IgG, papain F_{ab}-fragment, papain F_c-fragment and human pituitary thyroid-stimulating hormone.

The antigens were dissolved in 0.15 M-NaCl (1.0–5.0 mg./ml.), emulsified with an equal volume of Freund's complete adjuvant (Difco Corp.) and 0.1 ml. was injected intradermally at five sites. The injections were repeated 10 days later and the first bleeding (30–50 ml.) was performed after 2 weeks.

Neutralization studies. In the immunological cross-reaction studies the mixed antigen and antiserum were incubated for 1 hr. at 37° and then stored overnight at 4°. In all instances the proportion of antiserum was one-third of the total volume of the mixture. Before injection into assay animals any precipitate was removed by centrifugation at 900g for 10 min.

Ultracentrifuge analysis. Sedimentation-velocity measurements were performed at 59 780 rev./min. in a Beckman-Spinco model E ultracentrifuge equipped with schlieren optics. Samples were studied at 5–10 mg./ml. in 0.01 M-phosphate–0.15 M-NaCl buffer, pH 7.4, at 18–21° in a double-sector cell. Calculated values of the sedimentation coefficients were corrected to the viscosity of water at 20° (*S*_{20,w}).

RESULTS

Digestion of LATS-IgG with papain resulted in a striking change in the time-course of response in the McKenzie (1958) assay. The characteristic long-acting response of LATS-IgG was greatly shortened to give a maximal response at 3 hr., resembling pituitary thyroid-stimulating hormone (Table 1). This change accompanied the characteristic reduction in sedimentation rate of the digested

IgG and separation of the antigenic determinants on immunoelectrophoresis (Edelman *et al.* 1960).

Chromatographic analysis of the papain digest on DEAE-cellulose separated the F_{ab}- and the F_c-fragments. With this system F_{ab} could be isolated free from F_c, as judged by immunoelectrophoresis. The short-acting thyroid-stimulating activity exhibited by the whole papain digest of LATS-IgG was localized entirely in F_{ab}; F_c was devoid of activity (Table 1).

After incubation of LATS-IgG with pepsin at pH 4.0 approximately two-thirds of the protein was recovered after dialysis. The sedimentation coefficient of the recovered protein was 4.9s and when assayed the response to this component was long-acting, as the original IgG (Table 1). However, when this fragment was incubated with cysteine the sedimentation rate dropped further to 3.5s and the assay response became short-acting, resembling the effect of papain (Table 1).

Since it was impossible to distinguish between the thyroid-stimulating activity of F_{ab} from LATS-IgG and pituitary thyroid-stimulating hormone from their bioassay responses, the relationship between the two was assessed by immunological cross-reaction studies (Table 2).

The long-acting response to LATS-IgG was completely annulled on incubation with anti-IgG, anti-F_{ab} and anti-F_c. It was not significantly affected by anti-(human thyroid-stimulating hormone) serum.

Incubation of the papain digest of LATS-IgG with anti-IgG and anti-F_{ab} abolished the short-acting response. However, anti-F_c and anti-

Table 1. *Effect of papain and pepsin on LATS-IgG as judged by the time-course of discharge of thyroidal ¹³¹I in mice*

Each value represents the mean response of five animals. The type of response is indicated either as long-acting (L.A.), i.e. 10 hr. response > 3 hr. response, or short-acting (S.A.), i.e. 3 hr. response > 10 hr. response.

Sample	Mean blood radioactivity (% of initial value ± s.e.m.)		Type of response
	At 3 hr.	At 10 hr.	
LATS-IgG (10.0 mg./ml.)	740 ± 101	1436 ± 163	L.A.
Papain digest (10.0 mg./ml.)	396 ± 37	345 ± 29	S.A.
F _{ab} -fragment (10.0 mg./ml.)	745 ± 51	541 ± 64	S.A.
F _c -fragment (10.0 mg./ml.)	97* ± 6	93* ± 5	—
LATS-IgG (10.0 mg./ml.)	436 ± 108	967 ± 145	L.A.
Peptic digest (10.0 mg./ml.)	548 ± 67	671 ± 79	L.A.
Peptic digest + 0.01 M-cysteine (10.0 mg./ml.)	402 ± 31	276 ± 37	S.A.
Standard thyroid-stimulating hormone (0.06 milliunit/ml.)	510 ± 54	200 ± 25	S.A.

* Not significantly different from aq. 0.15 M-NaCl controls.

Table 2. *Cross-reaction studies between LATS-IgG, F_{ab}-fragment and human thyroid-stimulating hormone*

Values are for mean blood radioactivity (% of initial value \pm s.e.m.) at 10 hr. for the first row and at 3 hr. for the second and third rows.

Antigen	Antiserum None	Anti-IgG	Anti-F _{ab}	Anti-F _c	Anti-(human thyroid-stimulating hormone)
LATS-IgG (1.0 mg/ml.)	1193 \pm 250	181 \pm 10*	148 \pm 16*	113 \pm 33*	1182 \pm 87
Papain digest LATS-IgG (1.0 mg./ml.)	419 \pm 59	136 \pm 13*	77 \pm 8*	359 \pm 29	465 \pm 32
Human pituitary thyroid-stimulating hormone (approx. 0.8 milliunit/ml.)	508 \pm 72	403 \pm 49	509 \pm 64	491 \pm 54	134 \pm 10*

* Not significantly different from controls (33% antiserum in aq. 0.15 M-NaCl).

(human thyroid-stimulating hormone) sera were ineffective under identical conditions.

The assay response to human pituitary thyroid-stimulating hormone was only neutralized when incubated with the homologous antiserum.

DISCUSSION

The effect of papain and peptic proteolysis on the bioassay response to LATS-IgG confirms and extends the previous findings of Meek *et al.* (1964). These workers were able to isolate pieces I and II, using the nomenclature for rabbit IgG (Porter, 1959), although only piece I was active at the concentrations tested. It was suggested that the short-acting activity may have been due to thyroid-stimulating hormone bound to IgG, although there is considerable evidence that the two thyroid stimulators are distinct (Adams, 1965; Dorrington & Munro, 1965). Kriss *et al.* (1964) reported that papain destroyed all the activity of LATS-IgG although their modified McKenzie assay is insensitive to thyroid-stimulating hormone and probably also to a short-acting papain digest.

The results of the chromatographic and sedimentation-velocity studies on LATS-IgG agree with previous studies on human IgG with papain (Edelman *et al.* 1960; Franklin, 1960; Hsiao & Putnam, 1961) and on rabbit IgG with pepsin (Nisonoff *et al.* 1960).

Proteolysis of IgG has been shown to result in a decrease in molecular size (Charlwood, 1959; Noelken, Nelson, Buckley & Tanford, 1965) and it is reasonable to suppose that the change in assay response to LATS-IgG was associated with this decrease. This is supported by the finding that the 4.9s fragment produced by pepsin (molecular size \sim 100 000; Nisonoff *et al.* 1960) has a similar type of response to IgG. On reduction, however, the time-course of response became short-acting and the sedimentation rate decreased to 3.5s.

Convincing evidence has been obtained that the thyroid-stimulating activity of LATS-IgG is associated exclusively with F_{ab}.

(1) Chromatographic analysis of the papain digest of LATS-IgG showed that only F_{ab} possessed activity. The isolated F_{ab}-fragments were immunochemically free of F_c.

(2) Biological activity in F_{ab} could only be neutralized by antisera containing antibodies to F_{ab} (i.e. anti-IgG and anti-F_{ab}). An antiserum to F_c was ineffective.

(3) Pepsin destroys F_c yet the activity remained and on reduction was associated with F'_{ab}. The F'_{ab}-fragments formed under these conditions appear to be similar to F_{ab} produced by papain (Mandy, Rivers & Nisonoff, 1961; Goodman & Gross, 1963).

The specific association of thyroid-stimulating activity with F_{ab} may be of significance, as the antibody-combining site of antigen-specific IgG is located on this fragment (Cohen & Porter, 1964). The disulphide bond-reduction studies of Meek *et al.* (1964) suggested that the A-chains were active when prepared from LATS-IgG. A combination of these two pieces of evidence implies that F_a (A-piece) carries the active sequence. However, more work needs to be done on isolated A- and B-chains in view of the discrepancies in the results obtained by Meek *et al.* (1964) and Dorrington *et al.* (1964).

The results presented in this paper provide circumstantial evidence that LATS activity is a property of peptide fragments within specific IgG molecules. On the basis of this evidence, however, it is not possible to decide whether LATS is an antibody.

An autoimmune aetiology for thyrotoxicosis has been suggested (Adams, 1965), involving the interaction of LATS-IgG with an unidentified thyroidal antigen. Various suggestions have been put forward as to the nature of this antigen. An intracellular thyroid-stimulating hormone binding

site has been implicated (Adams, 1965; Dorrington, 1964), possibly associated with the nucleus (Kriss *et al.* 1964) or some factor which normally inhibits thyroid metabolism (Dorrington, 1964; Kriss *et al.* 1964). However, in the last analysis proof of this hypothesis will depend on the characterization of a thyroidal antigen which initiates the synthesis of a specific IgG with LATS activity. The search for such an antigen is being actively pursued in several Laboratories.

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REFERENCES

- Adams, D. D. (1965). *Brit. med. J.* i, 1015.
- Adams, D. D. & Kennedy, T. H. (1962). *Proc. Univ. Otago med. Sch.* 40, 6.
- Charlwood, P. A. (1959). *Biochem. J.* 73, 126.
- Cohen, S. (1965). *Immunology*, 8, 1.
- Cohen, S. & Porter, R. R. (1964). *Advanc. Immunol.* 4, 287.
- Dorrington, K. J. (1964). Ph.D. Thesis: University of Sheffield.
- Dorrington, K. J. & Munro, D. S. (1964). *J. Endocrin.* 31, 21.
- Dorrington, K. J. & Munro, D. S. (1965). *Clin. Sci.* 28, 165.
- Dorrington, K. J., Munro, D. S. & Carneiro, L. (1964). *Lancet*, ii, 889.
- Edelman, G. M., Heremans, J. F., Heremans, M.-Th. & Kunkel, H. G. (1960). *J. exp. Med.* 112, 203.
- Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. 1, 174.
- Franklin, E. C. (1960). *J. clin. Invest.* 39, 1933.
- Goodman, J. W. & Gross, D. (1963). *J. Immunol.* 90, 865.
- Hsiao, S. & Putnam, F. W. (1961). *J. biol. Chem.* 236, 122.
- Kriss, J. P., Pleshakov, V. & Chien, J. R. (1964). *J. clin. Endocrin.* 24, 1005.
- Levy, H. B. & Sober, H. A. (1960). *Proc. Soc. exp. Biol., N. Y.*, 103, 250.
- McKenzie, J. M. (1958). *Endocrinology*, 63, 372.
- McKenzie, J. M. (1962). *J. biol. Chem.* 237, 3571.
- Major, P. W. & Munro, D. S. (1962). *Clin. Sci.* 23, 464.
- Mandy, W. J., Rivers, M. M. & Nisonoff, A. (1961). *J. biol. Chem.* 236, 3221.
- Meek, J. C., Jones, A. E., Lewis, U. J. & Vanderlaan, W. D. (1964). *Proc. nat. Acad. Sci., Wash.*, 52, 342.
- Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960). *Arch. Biochem. Biophys.* 89, 230.
- Noelken, M. E., Nelson, C. A., Buckley, C. E. & Tanford, C. (1965). *J. biol. Chem.* 240, 218.
- Porter, R. R. (1959). *Biochem. J.* 73, 119.
- Porter, R. R. (1962). In *Basic Problems of Neoplastic Disease*, p. 177. Ed. by Gellhorn, A. & Hirschberg, E. New York: Columbia University Press.