The Antigen-Binding Capacity of the Peptide Chains of Horse Antibodies

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(Received 6 January 1966)

The antigen-binding capacity of the peptide chains of horse anti-(diphtheria toxin) has been studied by using ^{125}I -labelled toxoid and electrophoresis of antibody-antigen mixtures on cellulose acetate. The heavy chains retained about 20% of the activity of the whole antibody and the light chains less than 5%. Recombination of specific heavy and light chains gave about 60% recovery of activity and recombination of specific heavy chains and non-specific light chains about 40% recovery. It is suggested that these results favour the heavy chain as the major site of the antigen-binding activity.

Papain digestion of rabbit antibodies showed that the antigen-binding sites were contained in the two fragments now known as Fab, and subsequent work has shown that these are formed of a light chain and the *N*-terminal half of a heavy chain. Repeated attempts have been made to decide whether the antigen-binding site is contained in the heavy or light chain of the molecule, or whether it is formed jointly by both chains (see Cohen & Porter, 1964). No decisive answer has yet been obtained, though the present majority opinion appears to favour the view that the site is formed jointly by both chains. Fleischman, Porter & Press (1963), using a somewhat unsatisfactory assay technique, gave evidence that appeared to show that the site was entirely in the heavy chain and, though it is now clear that these results were in part due to contamination of the heavy chain by undissociated antibody molecules, we now present further evidence that suggests again that the combining site is primarily located in the heavy chain.

MATERIALS AND METHODS

Horse antisera to crystalline diphtheria toxin, to purified tetanus toxoid and to lecithinase from *Clostridium welchii* and the corresponding antigens were given by the Wellcome Foundation Ltd., Beckenham, Kent. IgG and IgA(T) and their constituent peptide chains were prepared from normal horse serum and from horse antiserum, as described in the preceding paper (Weir & Porter, 1966). Diphtheria toxoid (given by the Wellcome Foundation Ltd.) was prepared by formaldehyde treatment of the crystalline toxin and low-molecular-weight contaminants were removed by passage through Sephadex G-100. Iodination of toxoid with ^{125}I Cl was carried out as described by McFarlane (1958). Non-protein-bound radioactivity was

removed by dialysis and the final preparation contained less than 5% of radioactivity soluble in 5% (w/v) trichloroacetic acid.

The method of assay of antigen-binding activity was based on that used by Berson, Yalow, Bauman, Rothschild & Newerly (1956) to assay insulin antibodies. In this method, antibody or fraction of antibody was mixed with ^{125}I -labelled toxoid in a buffer (13.4 g. of tris and 7.2 g. of EDTA/l. and conc. HCl to pH 7.4). The ^{125}I -labelled toxoid (about 20 μg . in 0.01 ml. of buffer) was mixed with antibody or fraction (75–250 μg . in 0.05 ml. of buffer) so that about 50% of the toxoid was bound to the antibody. The mixture was kept at 37° for 1 hr. and at 2–4° for 16 hr. Then 0.005 ml. of the reaction mixture was fractionated by electrophoresis on cellulose acetate strips (18 cm. $\times$ 2.5 cm.) in the cold room. Electrophoresis was for 4 hr. at 10 v/cm. and a current less than 1 mA/strip. The strips were soaked in 10% (w/v) trichloroacetic acid and, without washing, were transferred to an aqueous solution of 0.5% Coomassie Brilliant Blue for 10 min. The strips were then washed in deionized water and dried at 100° for 30 min. The strips were cut into 2 mm. sections and placed with 10 ml. of scintillation solution in vials for counting in a Nuclear-Chicago liquid-scintillation counter.

Isolated peptide chains from the Sephadex columns were concentrated by dialysis under pressure against the tris buffer, pH 6.5. When reassociated chains were studied they were combined in the 0.5N-acetic acid and again concentrated and neutralized as above.

Control solutions contained antibody that had been reduced with 0.1M-mercaptoethanol (and the thiol groups blocked with iodoacetamide) but not acidified. This treatment did not alter affinity for the toxoid, as judged by inhibition of precipitation, but it prevented precipitation with toxoid during incubation and hence was a more satisfactory standard. Blank solutions contained only toxoid in buffer and gave a measure of non-specific binding of toxoid to the cellulose acetate strip. A typical result is illustrated in Fig. 1. The control solutions were assumed to have 100% activity and all other results were expressed relative to this.

Fig. 1. Diagrammatic illustration of the method of estimating binding of ^{125}I -labelled diphtheria toxoid by antibody. Mixtures of diphtheria toxoid (dt) and anti-diphtheria toxin IgA(T) (A-dt) or anti-lecithinase IgA(T) (A-I) were fractionated by electrophoresis on cellulose acetate strips; the components were located by protein staining; the strips were sectioned and the radioactivity of the sections was estimated. Further details are given in the Materials and Methods section.

Preparations of heavy chain, light chain, reassociated chains and undissociated antibody molecules behaved very similarly on electrophoresis on the cellulose acetate strips and could not be distinguished under these experimental conditions.

Calculation of free and bound toxoid was made by estimating the counts/min. associated with the toxoid or antibody peak respectively. The values were corrected for the small amount of non-specifically bound toxoid found in the blank assay. Activities are expressed on a molar basis as a percentage of the binding capacity of the reduced and alkylated antibody.

RESULTS

As found in previous work, there is substantial loss of antigen-binding activity when the reduced alkylated anti-toxin is acidified before separation

Table 1. Antigen-binding activity of IgA(T) from anti-(diphtheria toxin) sera and of the constituent heavy and light polypeptide chains

Results are expressed as percentages of the mol. of antigen bound per mol. of IgA(T) or per 2 mol. of each peptide chain.

Protein	Relative activity		No. of assays
	Average	Range	
Reduced alkylated antitoxin	100		
Reduced alkylated acidified antitoxin	60	55-72	6
Heavy chain	25	15-37	5
Light chain	4	2-7	5
Heavy chain + light chain (equimolar)	65	59-80	4

of the peptide chains and no more satisfactory method of dissociation of the chains was found. There is some variation from experiment to experiment, but the average recovery relative to the activity of the reduced alkylated protein is 60%. Lowering the molarity of the acetic acid against which the reduced protein was dialysed from 0.5N to 0.1N did not decrease this loss of activity. The results in Table 1 show that there was a further substantial loss of activity when the peptide chains were separated and isolated. Such activity as survived was found almost completely in the heavy chains. This second loss of activity was, however, completely reversed when the two chains were remixed in equimolar proportions. The recovery of activity appeared to be proportional to the content of light chain added, up to the maximum reached in equimolar ratio, and could not be increased beyond this level by several-fold excess of light chains (Fig. 2). This suggests that about 25% of the heavy chains retain activity in the apparent absence of light chain, a further 35% are capable of reactivation by the addition of specific light chains, and some 40% have been irreversibly inactivated by the conditions used to dissociate the chains.

The specificity of these reactions was investigated by the use of non-specific IgA(T). As this component is barely detectable in serum from a non-immunized horse, it was prepared from the serum of horses immunized with tetanus toxoid or with *C. welchii* culture filtrates that contained lecithinase. Neither serum contained any detectable anti-(diphtheria toxin) activity, either by testing the whole antiserum by double-diffusion techniques or by diphtheria-toxoid-binding activity in the present system. Similarly, the isolated peptide chains were inactive. However, the addi-

tion of non-specific light chain to anti-(diphtheria toxin) heavy chain increased considerably their antigen-binding activity, though leaving it substantially below that of an equimolar mixture of specific heavy and light chains (Table 2). Again, adding excess of non-specific light chains either singly or mixed did not increase the activity significantly. The addition of light chains from IgG prepared from the serum of non-immunized horses caused a consistent slight increase in activity of the anti-(diphtheria toxin) heavy chain, but it was so small (about 5%) as to be within the error of the method and its significance is uncertain.

No competition between IgG light chains and specific IgA(T) or non-specific IgA(T) was observed. The addition of any combination of light chains neither decreased nor increased the activities measured when specific IgA(T) light chains were added alone.

DISCUSSION

If accepted at face value, these results suggest that in our preparations there were four classes of heavy chain: (1) able to bind antigen without the

Fig. 2. Effect on the antigen-binding activity of anti-(diphtheria toxin) IgA(T) heavy chain of adding different amounts of anti-(diphtheria toxin) IgA(T) light chain.

addition of light chains (about 20–25% of the total), (2) activated by the addition of specific or non-specific IgA(T) light chains (15–20%), (3) activated only by the addition of specific IgA(T) light chains (20%) and (4) incapable of activation under any conditions tried (about 40%).

Workers in several other Laboratories doubt the existence of class (1), i.e. free heavy chains retaining capacity to bind antigen (Edelman, Olins, Gally & Zinder, 1963; Franěk & Nezlin, 1963*a,b*; Franěk, Kotýnek, Šimek & Zikán, 1964; Roholt, Onoue & Pressman, 1964; Roholt, Radzinski & Pressman, 1965). The uncertainty arises partly from the different methods of assaying antigen-binding but, more important, the difficulty in deciding whether the heavy-chain preparation contains light chain or not. As discussed in the preceding paper (Weir & Porter, 1966), further material resembling light chains could be dissociated from our heavy-chain preparation by a solution of 1% sodium dodecyl sulphate, but the much higher carbohydrate content suggested that it consisted in part of split heavy chain and that no more than 1–2% of light chain by weight could be present. The quantitative effect of adding light chain (Fig. 2) suggests that a much heavier content of light chain would have been necessary to account for the activity observed. Thus, if the ratio of activity to the weight of added light chain is extrapolated to zero activity and it is assumed that this represents heavy chain free of light chain, it would imply that the heavy chain as isolated contained 10–15% of light chain by weight or 30–50% on a molar basis. It therefore seems likely that a fraction of the heavy chains retained affinity for the antigen in the absence of associated light chain. The increased activity on the addition of non-specific and specific light chain has been reported by several authors (Edelman *et al.* 1963; Franěk *et al.* 1964) and the differences with their results are primarily quantitative. Thus Franěk and collaborators, using bovine anti-dinitrophenyl antibody and measuring hapten by equilibrium dialysis at a constant concentration of

Table 2. *Antigen-binding activity of mixtures of specific and non-specific peptide chains of IgA(T)*

Results are expressed as percentages of the mol. of antigen bound per mol. of IgA(T) or per 2 mol. of each peptide chain.

	Individual values	Average
Reduced alkylated anti-(diphtheria toxin) IgA(T)	100	100
Anti-(diphtheria toxin) heavy chain	25, 18, 20	21
Anti-(diphtheria toxin) light chain	4, 2, 0	2
Anti-(diphtheria toxin) heavy chain + anti-(diphtheria toxin) light chain	59, 57, 49	55
Anti-(diphtheria toxin) heavy chain + anti-lecithinase light chain	38, 33, 31	34
Anti-(diphtheria toxin) heavy chain + anti-(tetanus toxin) light chain	35	35
Anti-lecithinase heavy chain + anti-(diphtheria toxin) light chain	4	4
Anti-(tetanus toxin) heavy chain + anti-(diphtheria toxin) light chain	2	2

hapten, found that neither heavy nor light chain alone bound any hapten. When specific heavy and light chains were mixed in a 2:1 weight ratio, 0.58 mol. of hapten was bound/2 mol. of each; 0.86 mol. of hapten was bound/2 mol. when specific heavy chain was mixed with a fourfold excess of non-specific light chain. No such effect has ever been observed in the present study.

It was possible that activity depended on the recombination of the heavy and light chain and that the loss of activity was due to aggregation of part of the heavy chain so that it could not recombine. Neutralized mixtures of specific and non-specific chain were therefore fractionated on Sephadex G-200 and recombination was estimated by the size of the peak in the position of the untreated IgA(T). In every case some aggregate and uncombined light chain are also present. It was found that non-specific IgG light chain recombined, as judged by these criteria, with anti-(diphtheria toxin) IgA(T) heavy chains as effectively as specific light chains, and yet the mixture had little more activity than heavy chain alone. Hence there was no correlation between the percentage of re-formed molecules of molecular weight 150 000 and the activity recovered.

Similarly, an attempt was made to fractionate isolated heavy chains at neutral pH on Sephadex G-200 to test whether the fractions behaving as dimers under these conditions differed in activity from those that were aggregated. Again there was no evidence of this and aggregates had similar activities to the dimers. Estimates were also made of the apparent contamination with light chain, as judged from re-running heavy-chain fractions on a column in 1% sodium dodecyl sulphate, and again no correlation was found.

It is not possible to draw any firm conclusion from these results, but they are consistent with the view that the antigen-binding site is located on the heavy chain but that the light chain plays a

semi-specific role in at least some of the molecules in assisting in the recovery of the native configuration after the dissociation in acid. It would follow that, if conditions for the separation of heavy and light chains could be found that caused little alteration of the configuration, much higher activities would be retained by the isolated heavy chains. Persistent attempts to achieve this have been unsuccessful, even in very dilute solutions such as Haber (1964) and Whitney & Tanford (1965) used to recover antibody activity after complete reduction in 6M-guanidine solutions.

We thank the U.S. National Science Foundation for financial support and the Wellcome Trust for the award of a Fellowship to R. C. W. We also thank the Wellcome Foundation Ltd. for generous gifts of toxoids and antitoxins.

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