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Cleavage of Bacterial Flagellin with Cyanogen Bromide

ANTIGENIC PROPERTIES OF THE PROTEIN FRAGMENTS

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1. Four polypeptide fragments, obtained by cyanogen bromide treatment of the protein flagellin from *Salmonella adelaide*, were tested for their antigenic activity by using them as inhibitors in three different assays: bacterial immobilization, haemagglutination of sensitized erythrocytes and quantitative micro precipitation. Immunodiffusion studies were also performed on the protein fragments. 2. Cleavage of the flagellin molecule in this way gave no detectable loss of antigenic determinants. Fragment A (mol.wt. 18000), the largest of the polypeptides, contained all the antigenic specificities present on flagellin that were recognized by the antisera used. In one test, fragment B (mol.wt. 12000) also contained antigenic activity to an extent not easily explainable by contamination with fragment A. Fragments C (mol.wt. 5500) and D (mol.wt. 4500) appeared to be antigenically inactive.

In the preceding paper (Parish & Ada, 1969*a*) some chemical and physical properties of four polypeptide fragments produced by treatment of *Salmonella adelaide* flagellin with cyanogen bromide were described. These fragments were arbitrarily called fragments A, B, C and D and were found to have molecular weights of about 18000, 12000, 5500 and 4500 respectively. In this paper we report the antigenicity of the four fragments as reflected by their capacity to inhibit the reaction between various pairs of flagellar antigens and antisera, and their immunogenicity as indicated by their ability to induce antibody production when injected with adjuvant into animals. Four types of flagellar antigens were used in three different inhibition systems. The techniques were bacterial immobilization, haemagglutination of sensitized erythrocytes and quantitative micro precipitation. In addition, immunodiffusion studies of the antigens and antisera were carried out.

MATERIALS AND METHODS

Antigens and antigen fragments. The preparation of flagellin and polymerized flagellin (polymer) from flagella of *S. adelaide*, and of the purified fragments, A, B and D, resulting from the treatment of flagellin with CNBr, was as described by Parish & Ada (1969*a*). Fragment C was not isolated free from fragments B or D. Fragments A and D were labelled with [^{125}I]iodide for assay in the micro precipitation and chromatographic techniques. The stock solutions of labelled proteins were kept at −20° in a 0·05M-tris–HCl buffer, pH 7·4, containing 3% (w/v) of bovine plasma albumin (B.P.A., fraction V; Armour and Co., Kankakee, Ill., U.S.A.).

Antisera to flagellin and to the fragments. Antisera were raised in adult New Zealand rabbits (average age about 6 months) or in Wistar rats (either sex, 10–12 weeks old) by initial injections of flagellin, the complete digest or the fragments (0·5mg.) emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) into the hind-foot pads. Then 1 month later, the rabbits were injected intravenously with 0·5mg. of the appropriate antigen in 0·9% NaCl; some rabbits were given a further intravenous injection of antigen later. All rabbits were bled 7–10 days after the final injection. The rats were injected once only with antigen (0·5mg.) in Freund's complete adjuvant and bled 13 weeks later.

Antigen–antibody reactions

Ouchterlony reactions. Double-diffusion analyses in two dimensions were carried out in 1·0% (w/v) agar containing 0·01% (w/v) NaN_3–0·12M-NaCl–0·02M-sodium phosphate buffer, pH 7·3. Undiluted serum was placed in the centre well and antigen solutions in the peripheral wells. Reaction was allowed to take place at room temperature for up to 3 days. The plates were washed free from soluble protein with 0·15M-NaCl and with water containing 1% (v/v) glycerol, dried and then stained with a solution of Amido Black (0·25%, w/v) in 7% (v/v) acetic acid.

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Immobilization and immobilization-inhibition techniques. Serum antibodies were regularly estimated by the immobilization technique as described by Ada, Nossal, Pye & Abbot (1964). Dilutions of sera were tested for their ability to immobilize a standard preparation of motile *Salmonella derby*, which has the same flagellar antigens (fg) as *S. adelaide*, but has different O antigens. In each series of analyses, two dilutions of a standard antiserum were included as controls. An inhibition technique was also used in which the ability of an antigen to neutralize the immobilizing activity of a standard dilution of antiserum was measured. In this technique, two- or three-fold serial dilutions of the preparation to be assayed (0·25 ml.) were mixed with a dilution of standard antiserum (0·25 ml.). After the mixtures had stood at room temperature for 30 min., serial twofold dilutions of each mixture were made (final volume of each, 0·5 ml.) and 0·25 ml. of the bacterial suspension was added. These mixtures were incubated for 30 min. at room temperature, and the degree of bacterial immobilization was estimated as described by Ada *et al.* (1964). The end point was read as the dilution of the substance being tested that decreased the titre of the antiserum by two dilutions (i.e. a 75% reduction).

Haemagglutination of tanned sensitized erythrocytes. Sheep erythrocytes were tanned and sensitized with polymer as described by Wistar, (1968). Antisera raised against flagellin or the fragments were titrated by the following procedure. Haemagglutination-inhibition tests were performed with five agglutinating doses of antiserum and serial twofold dilutions of the inhibitor, the mixture being incubated for 45 min. at room temperature. The cells were added and the end point was read as the minimum amount of inhibitor giving complete inhibition

Micro precipitation technique. The method was described in detail by Wistar (1968). Two diluents were used: BSA-dil, which was 3% (w/v) bovine serum albumin in 0·05 M-tris–HCl buffer, pH 7·4, containing 0·01% NaN_3, and S-dil, which was BSA-dil to which had been added 10% (v/v) of normal rabbit serum.

^{125}I-labelled antigen (100 μl. portions each containing about 10 ng. of flagellin, fragment A or fragment D) in BSA-dil was dispensed in tubes (6 mm. × 50 mm.). Serial dilutions of antiserum in S-dil (50 μl.) were then added. After mixing, the tubes were incubated for 3 hr. at 37° followed by 16 hr. at 4°, and were then centrifuged at 10000 *g* for 10 min. at 4°. Of the supernatant, 50 μl. was removed and the radioactivity counted in a Packard Autogamma counter. The percentage of the label precipitated in each tube was calculated and plotted as shown in Fig. 1(*a*).

For the inhibition assay a dilution of antiserum was chosen that precipitated the labelled antigen just less than maximally (i.e. in the region of slight antigen excess). A series of tubes were set up each containing 100 μl. of labelled antigen. To these were added serial dilutions (10 μl.) of the unlabelled inhibitor substance. After mixing, 50 μl. of antiserum was added and the tubes were handled as for the direct precipitin assay. Controls included 100 μl. of labelled antigen, 10 μl. of diluent and 50 μl. of antiserum (for maximal precipitation), or 100 μl. of labelled antigen plus 60 μl. of diluent (for zero precipitation). Various inhibitors were compared by determining the amount of material (in ng.) that gave 50% inhibition of maximal precipitation for a particular set of labelled antigen–antiserum mixtures. In Fig. 1(*b*), about 12 ng. of fragment A

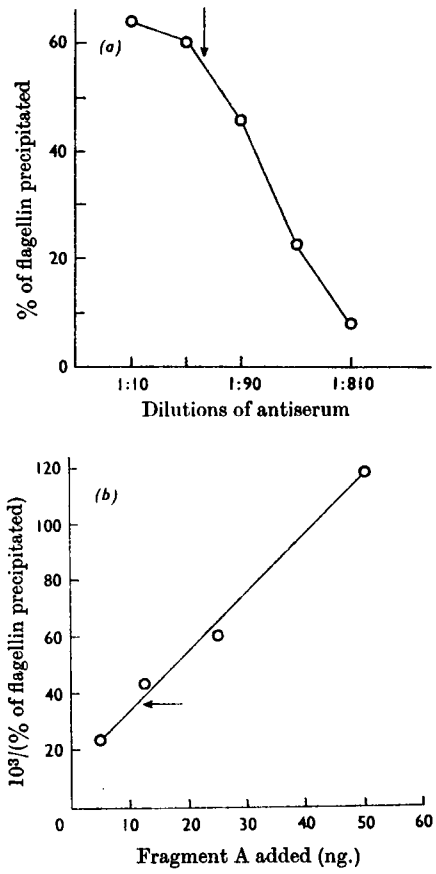

Fig. 1. Micro precipitation technique. (*a*) Precipitation of ^{125}I-labelled flagellin (8·3 ng.) by dilutions of rat anti-(fragment A). The arrow indicates the antiserum dilution used for the inhibition studies. (*b*) Inhibition of the precipitation of ^{125}I-labelled flagellin (8·3 ng.) with rat anti-(fragment A) (1:50) by fragment A. The arrow indicates 50% inhibition.

was needed to cause 50% inhibition of near-maximum precipitation of labelled fragment A. The reproducibility of the method was determined in an experiment in which twelve replicate assays were carried out with labelled fragment A, rabbit anti-(fragment A) antiserum and flagellin as inhibitor. The mean value for 50% inhibition (± S.D.) was 41 ± 10·7 ng. (range 30–55 ng.).

RESULTS

The activity of antisera raised against flagellin and its fragments and the ability of these substances to inhibit activity were tested by a variety of techniques.

Immobilization and haemagglutination techniques. The ability of antisera raised against flagellin or the fragments to immobilize organisms or to agglutinate

Table 1. *Ability of antisera to flagellin, the cyanogen bromide digest of flagellin or the isolated fragments to immobilize* S. derby *or to agglutinate tanned erythrocytes sensitized with polymer*

Antiserum	Species	Immobilization titre (dilution of antiserum)	Haemagglutination titre (dilution of antiserum)
Anti-flagellin	Rabbit	250000	160000
Anti-(CNBr digest) 1*		4800	8000
Anti-(CNBr digest) 2*		200	1280
Anti((fragment A)		< 100	800
Anti-(fragment B)		< 100	1600
Anti-(fragment A) 1†	Rat	14000	48000
Anti-(fragment A) 2†		8000	20000

* Two different antisera to the CNBr digest.
† Two different antisera to fragment A.

Table 2. *Ability of polymer, flagellin, the cyanogen bromide digest of flagellin and the isolated flagellin fragments to inhibit the activity of antisera to polymer, flagellin and fragment A as tested by the immobilization and haemagglutination tests*

N.D., Not determined.

			Quantity of inhibitor required for inhibition (ng.)						
Test	Antiserum	Species	Polymer	Flagellin	CNBr digest	Frag. A	Frag. B	Frag. B+C+D	Frag. D
Haemagglutination	Anti-polymer	Rabbit	25	25	N.D.	200	> 400	N.D.	> 450
	Anti-flagellin	Rabbit	12	12	N.D.	25	100	N.D.	> 450
	Anti-(fragment A)	Rat	6	< 3	N.D.	12	50	N.D.	> 450
Immobilization	Anti-flagellin	Rabbit	100	70	125	78	5000	15000	> 20000

sensitized erythrocytes was tested, the results being recorded in Table 1. With most sera, the two techniques gave similar answers, the haemagglutination titre being slightly higher than the immobilization titre. Three sera, however, had very poor immobilizing activity but exhibited haemagglutinating activity. Sera prepared in rabbits against the digest flagellin had low immobilizing or haemagglutinating activity, suggesting that the antibody produced was of a poor quality for these purposes or that the digest was poorly immunogenic in rabbits. Rats responded better to fragment A than did rabbits.

Despite the apparent low immunogenicity of the flagellin fragments in rabbits, fragment A retained very well the ability to inhibit the immobilizing and haemagglutinating activity of rabbit anti-flagellin serum (Table 2). This was also the situation with rat anti-(fragment A) serum, but not, at least in the haemagglutination test, with anti-polymer serum. In the latter case, fragment A was significantly less effective than flagellin. Fragment D was inactive in all cases, but fragment B showed activity in some tests. In the immobilization test with anti-flagellin serum, the low activity of fragment B could be explained by a 2% contamination with fragment A. In the haemagglutination test with the same antiserum, a contamination of up to 25% with fragment A would be needed to explain the relatively high activity of fragment B, and a similar result was found with rat anti-(fragment A) serum.

These tests showed, therefore, that fragment A was a poor immunogen in rabbits but somewhat better in rats. In most cases, the complete digest or fragment A was as efficient as flagellin itself in inhibiting the activity of antisera. Fragment D was inactive in all tests, but fragment B was active in some tests.

Immunodiffusion analyses. In these tests, the antigens used were flagellin, the complete cyanogen bromide digest of flagellin, fragments A, B and D and a mixture of fragments B, C and D. The rabbit antisera used were prepared against the polymer, flagellin, the complete cyanogen bromide digest of flagellin, and fragments A and B. However, the precipitating activity of antisera against fragment A was slight and that of antisera against fragment B negligible. A range of antigen concentrations was used, allowing in some cases a distinction to be made between intrinsic antigenic activity and contamination with variable amounts of a more antigenic component.

The results in Table 3 show that antisera to polymer, flagellin and the complete cyanogen

Table 3. *Immunodiffusion reactions between flagellar antigens and fragments and antisera raised against them*

+, Strong precipitin line; tr., faint precipitin line; −, no detectable precipitin line. Numbers represent the concentration or range of concentrations (μg./ml.) of antigen used in the reactions. N.D., Not determined.

Antiserum	Antigen ...	Activity					
		Flagellin	Total digest	Frag. A	Frag. B	Frag. B+C+D	Frag. D
Anti-polymer		+ 100	+ 100	+ 100	N.D.	− 100	N.D.
Anti-flagellin		+ 50–1500	+ 50–1500	+ 50–1000	−* 50–500	− 50–200	− 10–100
Anti-(total CNBr digest)		+ 250	+ 250	+ 250	− 250	− 200	− 100
Anti-(fragment A)		tr. 200	tr. 200	tr. 200	tr. 200	N.D.	N.D.

* With one serum tested, fragment B showed a weak line of precipitation at 200 μg./ml. antigen concentration.

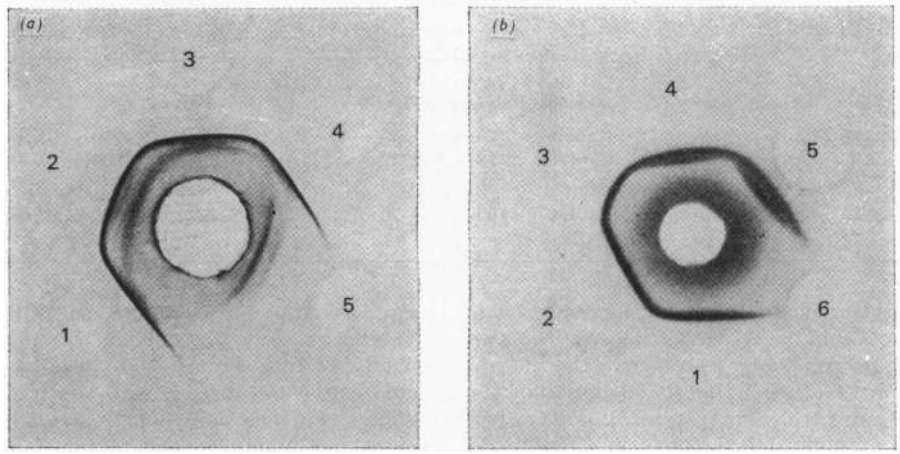

Fig. 2. Immunodiffusion reactions between flagellar antigens and various rabbit antisera. (*a*) The peripheral wells contained: 1, flagellin; 2, CNBr digest; 3, fragment A; 4, flagellin; 5, fragment B. The centre well contained sera from a rabbit injected five times with flagellin over a period of 8 months. Earlier bleeds from this rabbit did not show the line of precipitation with fragment B. The sample of fragment A used was subsequently found by amino acid analysis to be contaminated with fragment B. (*b*) The peripheral wells contained: 1, flagellin; 2, fragment A; 3, flagellin; 4, CNBr digest; 5, flagellin; 6, fragments B, C and D. The centre well contained sera from a rabbit after multiple injections with polymer. The same preparation of flagellin was used to fill wells 1 and 5.

bromide digest of flagellin showed strong precipitation when tested with flagellin; the complete cyanogen bromide digest and fragment A. In contrast, fragments B, C and D showed no precipitating activity when tested against these antisera with the exception of one anti-flagellin serum against which fragment B showed a weak line of precipitation. This precipitin line was close to the antiserum well, so the observed reaction was probably not due to a possible contamination with fragment A (Fig. 2*a*). Fig. 2(*b*) shows the reaction between flagellin, the complete cyanogen bromide digest or fragment A and anti-polymer serum. In both Fig. 2(*a*) and Fig. 2(*b*), lines of identity occur between flagellin, the complete cyanogen bromide digest and fragment A.

Inhibition reaction by the quantitative micro precipitin technique. Polymer, flagellin and fragments A, B and D of flagellin were tested for their ability to inhibit the precipitation of either labelled flagellin or labelled fragment A by antiserum against flagellin, the complete digest or polymer (all rabbit

Table 4. *Ability of polymer, flagellin and cyanogen bromide-digestion fragments of flagellin to inhibit the precipitation of flagellar antigens by antisera as tested by the micro precipitation-inhibition technique*

Results are expressed as the amounts of inhibitor (ng.) that gave 50% inhibition of precipitation of 8ng. of flagellin or 14ng. of fragment A by the various antisera.

Antiserum and species	Labelled antigen	Quantity of inhibitor required for inhibition (ng.) Flagellin	Polymer	Frag. A	Frag. B	Frag. D
Anti-flagellin (rabbit)	Flagellin	30	65	17	1800	>900
Anti-(total CNBr digest) (rabbit)		55	460	200	770	>900
Anti-(fragment A) (rat)		64	1480	14	2100	>900
Anti-flagellin (rabbit)	Fragment A	20	60	12	1500	>900
Anti-(total CNBr digest) (rabbit)		50	700	14	600	>900
Anti-polymer (rabbit)		56	110	30	2000	>900
Anti-(fragment A) (rat)		150	1200	30	1300	>900

antisera) or rat anti-(fragment A) serum, the results being shown in Table 4. For ease of comparison and for accuracy, 50% inhibition values are quoted. However, in all cases where 50% inhibition occurred, complete inhibition was obtained if sufficient inhibiting substance was added. One exception was the system labelled fragment A–rat anti-(fragment A) serum; complete inhibition with 10μg. of polymer was not observed, and higher concentrations could not conveniently be tested.

The main conclusions to be drawn from the results are as follows. (1) In all situations when they were tested as inhibitors, fragment A and flagellin behaved similarly, fragment B was less effective than fragment A and fragment D was ineffective. (2) With both labelled antigens and all antisera tested, polymer was less efficient as an inhibitor than was flagellin or fragment A. This was most noticeable when rat anti-(fragment A) serum was used. (2) Although generally inefficient as an inhibitor, fragment B approached the activity of polymer in this regard when tested in the presence of either antigen with anti-digest or anti-(fragment A) sera. This does not imply a similarity of antigenic determinants between polymer and fragment B.

DISCUSSION

Three types of antibody assay have been used to study the antigenic properties of the cyanogen bromide fragments of flagellin in relation to themselves and to flagellin and polymer. The first was the technique of bacterial immobilization, in which antibody reacted with the flagella particle, thereby immobilizing the *Salmonella* organisms. The second was the technique of haemagglutination, with erythrocytes sensitized with the polymeric form of flagellin, which differs from flagella because of the absence of the 'acid-insoluble residue'. Thirdly, the precipitin reaction was used either in a qualitative manner as simple immunodiffusion or as a quantitative micro precipitin reaction. The latter technique proved to be most useful, as either the direct or the inhibition reaction could be carried out with some accuracy. Before actual results are discussed, it is worth while to outline the interpretation of the possible types of results obtained with the micro precipitin-inhibition reaction. (1) If the labelled antigen was multivalent and the antiserum contained antibody to each antigenic specificity, complete inhibition with an inhibitor could not be obtained unless the inhibitor and antigen shared all antigenic determinants. (2) The quantity of inhibitor needed to get complete inhibition was a reflection of several factors, among which may be mentioned (*a*) the degree of contamination of the inhibitor preparation with another antigen and (*b*) the avidity of the antibodies for the inhibitor. (3) Partial as distinct from complete inhibition indicated incomplete sharing of antigenic determinants. This would yield a minimal estimate of the extent of sharing.

Several conclusions can be drawn from the studies reported. (1) Cleavage of flagellin with cyanogen bromide did not apparently destroy any of the antigenic specificities present on the native molecule. (2) By most tests fragment A contains all the specificities recognized by antiserum to flagellin or polymer. Both these conclusions can be drawn from immunodiffusion studies, which showed lines of identity between flagellin, fragment A and the whole cyanogen bromide digest with anti-flagellin antiserum. Flagellin contains at least three antigenic determinants, two of which must differ from each other (Ada, Parish, Nossal & Abbot, 1967) and these are now known to be on fragment A (C. R. Parish & J. Pye, unpublished work). (3) On a molar basis, fragment A in several tests was as strong an inhibitor as flagellin, suggesting that the conformation of the A portion of the flagellin molecule, after separation from the rest of the molecule, remained essentially unchanged. (4) By the same token, there was some evidence that the antigenic activity of the A portion of flagellin

differed slightly from the A portion in polymer. Despite this difference, these determinants were exposed in the polymer, a finding consistent with the proposition in the preceding paper (Parish & Ada, 1969*a*) that the portion of the flagellin molecule exposed in polymer consisted mainly of fragment A. (5) Of the other portions of the flagellin molecule, fragment B alone seemed to possess some antigenic activity. In some tests, this activity may well have been because of slight contamination of the preparation with fragment A. In the haem-agglutination-inhibition test, however, the activity of this fragment was higher than could be readily explained by contamination with fragment A. This finding was reproducible and suggests that this procedure measures a minor antigenic activity not detected by the other three procedures used.

Although possibly explained by their smaller size, the low immunogenicity of fragments B, C and D may alternatively have been a reflection of an amino acid sequence that was less foreign to the rat or rabbit than was the amino acid sequence in fragment A. Presumably, portions of the sequence in fragments C or D may be concerned with the polymerization process or in the movement of the flagella during locomotion.

Crumpton (1967) has reviewed the literature pertaining to structural features of antigenic sites in protein antigens. Perhaps the work most relevant to the present investigation is that on bovine and human serum albumins (Lapresle & Webb, 1965; Press & Porter, 1962). In this case, enzyme digests yielded fragments with molecular weights between 11000 and 20000, which were serologically active provided that the conformation was intact. Smaller fragments were less active serologically. In the investigation of flagellin, an obvious future step is the controlled breakdown of fragment A and the biological examination of the smaller peptides. A study of the immunogenic properties of fragment A of flagellin after injection into rats without adjuvant has yielded notable results, some of which have been published in a preliminary form (Parish, Lang & Ada, 1967). These results are presented in detail elsewhere (Ada & Parish, 1968; Parish & Ada, 1969*b*).

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