STUDIES ON THE EFFECT OF STAPHYLOCCOCCAL CULTURE FILTRATES ON ISOLATED RABBIT GUT

J. C. KELSEY AND BETTY C. HOBBS

The Food Hygiene Laboratory, Central Public Health Laboratory (Medical Research Council), Colindale

(With 6 Figures in the Text)

INTRODUCTION

Anderson (1953) found that filtrates from twelve presumptive food-poisoning strains of staphylococci produced on isolated rabbit gut a characteristic increase in tone which he failed to detect in a control series of twelve filtrates from pyogenic strains, not associated with food poisoning.

Despite the efforts of many workers, briefly reviewed by Anderson in the introduction to his paper, a satisfactory test for staphylococcal enterotoxin remains to be found. It was therefore decided to try to repeat Anderson's work and if possible to develop from it a test for the routine examination of staphylococci for enterotoxin production.

Preliminary trials using Anderson's original strains failed to show any difference between the food-poisoning and pyogenic groups, the response of the gut being apparently related to the haemolysins present in the filtrate rather than to the source of the organism. In view of this discrepancy a larger series of freshly isolated strains from widely varying sources was examined.

METHODS AND MATERIALS

Freshly isolated strains of coagulase-positive staphylococci from human and animal lesions and from well-substantiated outbreaks of food poisoning were used, together with a small group of coagulase-negative strains. Each strain was tested for haemolysins by the method of Elek & Levy (1950) and was typed by bacteriophage (Wilson & Atkinson, 1945; Williams & Rippon, 1952; Williams, Rippon & Dowsett, 1953), and serologically (Hobbs, 1948) (Table 1). For certain experiments dried cultures of classic strains were used, e.g. Wood 46.

Filtrates were prepared by the method of Dolman & Wilson (1938), 100 ml. of medium being used in a Roux bottle fitted with a perforated screw cap; 10% of carbon dioxide was introduced through the rubber cap by means of a hypodermic needle. The bottles were incubated for 40 hr. at 37° C. Cultures were harvested by filtration through filter-paper after the agar gel had been destroyed by freezing overnight at −10° C. Filtrates were cleared by centrifugation and were generally used fresh. If not used at once they were kept frozen at −10° C. Each filtrate was titrated for α- and β-lysins, the end-points being taken as 50% lysis of 1% rabbit and sheep cells respectively, after 1 hr. incubation at 37° C. The α-titre was read

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at once, the β-titre after overnight storage at 4° C. The β-titration was made in the presence of α-antitoxin when the α-titre was high. Antitoxins used were:

(*a*) Commercial staphylococcus antitoxin. $\alpha = 1200$ units/ml.

(*b*) A pure β-antitoxin obtained from Prof. C. E. Dolman with a β-titre of approximately 85 units/ml.

Table 1. *Strains used and gut responses obtained, together with bacteriophage and serological groups represented and α-titres*

No. of strains examined	Bacteriophage groups*	Serological groups	α-titre	Gut responses obtained					
				0	α	α–0	α–β	β	β–0
		A. Pyogenic strains							
2	III, N.R.	I, III	256	−	+	−	+	−	−
11	I, II, III, Misc., N.R.	I, II, III	128	−	+	+	−	−	−
7	I, III, Misc., N.R.	I, III	64	+	+	+	−	−	−
4	I, N.R.	I, II, III	32	+	−	−	−	−	−
2	I	I, II	16	+	−	−	−	−	−
2	III, N.R.	I, III	8	+	−	−	−	−	−
2	I	I, II	0	+	−	−	−	−	−
		B. Food-poisoning strains							
5	III	III	512	−	−	+	−	−	−
4	III	III	128	−	−	+	−	−	−
2	III	III	64	−	−	+	−	−	−
1	III	III	32	+	−	−	−	−	−
		C. Animal strains							
4	IV	III	512	−	−	+	+	−	−
1	III	III	256	−	−	+	−	−	−
2	I	II, III	32	−	−	−	−	+	+
1	IV	III	16	−	−	−	−	−	+
1	IV	III	8	+	−	−	−	−	−
3	I, N.R.	II, III, N.T.	0	+	−	−	−	−	−
		D. Coagulase-negative strains							
8	N.R.	N.T.	0	+	−	−	−	−	−

Key to responses:

0 = no response.

α = α response, not tested for abolition by α-antitoxin.

α–0 = α response, abolished by α-antitoxin.

α–β = α response, replaced by β response in presence of α-antitoxin.

β = β response, not tested for abolition by β-antitoxin.

β–0 = β response, abolished by β-antitoxin.

* *Note.* The strains are grouped by their phage reaction as defined by Williams *et al.* (1953). Group IV has been formed to include those phages lysing predominantly bovine strains, e.g. 42D and 42F. Misc. indicates strong lysis by phages of more than one group. N.R. indicates no reactions. N.T. indicates not typed.

Gut was obtained from rabbits exsanguinated after stunning. The first 2 or 3 ft. only were used, and after washing through with Tyrode solution were held in more Tyrode solution at 4° C. until required. Segments approximately 4 cm. long were suspended in Tyrode solution at 37° C. and aerated by a current of air; the bath

contained 60 ml. and the standard dose was 4 ml. of filtrate. Fresh gut was used for each day's work and a new segment was used for each filtrate tested. A smoked drum moving at 23 mm./min. recorded the gut movements. A dose of acetylcholine (0·2 ml. of 10^{-5} dilution) was used as a test of the reactivity of the gut segments.

RESULTS

(*a*) *Specific responses*

Apart from certain inconstant effects which will be discussed later the characteristic and most frequently found response was one in which the tone increased, the amplitude decreased, and eventually movements ceased with the gut in irreversible spasm. In this state it failed to respond to acetylcholine or to larger doses of filtrate (Figs. 1, 2). This kind of response was given only by filtrates showing α-lytic activity, irrespective of the source of the staphylococcus.

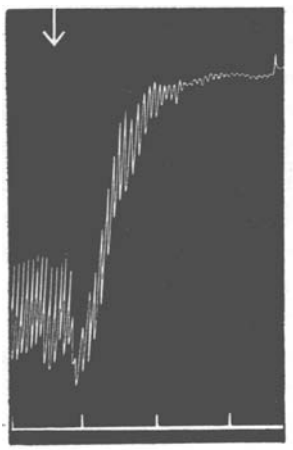

Fig. 1. α response (α-titre = 1/512).

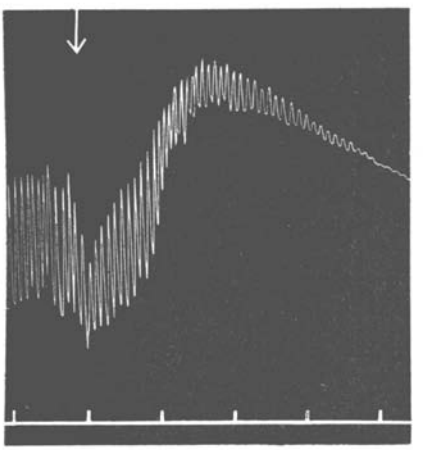

Fig. 2. α response (α-titre = 1/64).

A minimum dose of α-lysin seemed to be required, the response being invariably given by filtrates with α-titres of 1/128 or over and never by those with titres of 1/16 or less. Of the nine filtrates with a titre of 1/64, six were positive. The individual responses are listed in Table 1 and summarized in Fig. 6.

As these results suggested that the response was due to α-lysin, the effect of antitoxin was tried. Of seven 'pyogenic', four 'animal' and eleven 'food-poisoning' filtrates tested, all were inactive if 100–120 units of commercial α-antitoxin was added to the bath previously or was incubated with the filtrate before addition to the bath. In control experiments, tetanus and botulinum antitoxin failed to abolish the effect.

The experiments were essentially qualitative, and gave only a rough quantitative measure of the relation between response and α-titre of the filtrate tested. The increase in tone produced was measured by graded doses of acetylcholine, but given enough time, a maximal increase of tone was produced by all positive filtrates.

With a filtrate made from the classic Wood 46 strain—a powerful producer of

α-lysin without β-lysin—the time between addition of the filtrate to the bath and cessation of movements varied inversely as the titre. A similar general relationship was found between the titre of the other filtrates and the time to the cessation of gut movement, but a smooth curve could not be obtained.

When the α-titre was reduced by heating or by partial neutralization with antitoxin the time interval was again increased.

Another reproducible but less common response was a progressive decrease in tone and amplitude leading to a relaxed, immobile gut which failed to respond to acetylcholine or to larger doses of filtrate (Fig. 3). This response was given only by the few filtrates showing β-lysin activity, most of which were from animal strains. It was not abolished by α-antitoxin, but was abolished by Dolman's β-antitoxin in the two filtrates tried. The minimum effective dose seemed to be higher than with the α-lysin, for positive reactions were found only when the β-titre was 1/256 or over. Two filtrates from strains, in which α- and β-lysins were present together in high titre, gave an α response, the β response being obtained only when α-antitoxin was added to the bath before the addition of the filtrate. No attempt was made to correlate β-titre and reaction time.

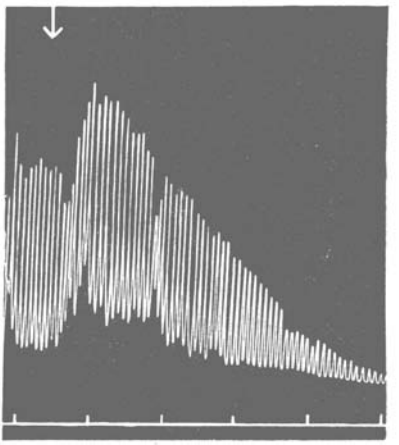

Fig. 3. β response (α-titre = 1/32, β-titre = 1/256).

(*b*) *Non-specific stimulation*

A third type of response consisted of an early increase in tone or amplitude or both, with or without a transient preliminary depression of activity. Gut movements continued and sensitivity to acetylcholine or α-lytic filtrate was unimpaired. Figs. 4 and 5 show two kinds of this response, which was given not only by filtrates from all sources but also by control filtrates made from uninoculated medium. The same filtrate did not produce this effect from each segment of gut examined. With four filtrates, tried on several consecutive pieces of gut, 2/3, 3/5, 1/3 and 2/2 positive reactions were obtained. In one experiment of five tests two were negative, one showed an increase in tone, one an increase in amplitude and one an increase in both together. The variability seemed to be in the gut; one segment gave a positive response to six successive doses of filtrate, with washing between each. The effect was not abolished by α- or β-antitoxin, by boiling for 30 min. or by adding 250 μg. of atropine to the bath, which was sufficient to inhibit the response to a maximal dose of acetylcholine.

As this response was given by filtrates from all sources, including coagulase-negative strains and uninoculated medium, it was termed non-specific stimulation (N.S.S.). The N.S.S. was liable to occur when α- or β-lysin were absent or had been neutralized by antitoxin. It was generally overshadowed by a typical α response, but occasionally it was strong enough to distort a characteristic response and produce an atypical tracing, especially in conjunction with the less potent β-lysin.

In an attempt to find the causal agent of the N.S.S., various fractions of Dolman's medium were tried on the gut. N.S.S. was obtained from the peptone, but not from the salts or agar, and it was therefore assumed that peptone was responsible. Media containing other peptones or casein digests were tried, but whenever 'peptone' (i.e. the product of the partial hydrolysis of protein, as shown by a pink biuret reaction) was present N.S.S. was liable to occur. It is possible that synthetic media containing amino-acids and no peptone might not give the N.S.S. A little practice made the distinction between the response characteristic of α- or β-lysins and a N.S.S. quite easy, as the latter never stopped gut movements or reduced acetylcholine sensitivity.

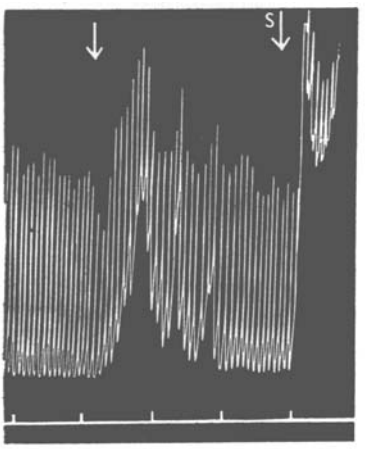

Fig. 4.

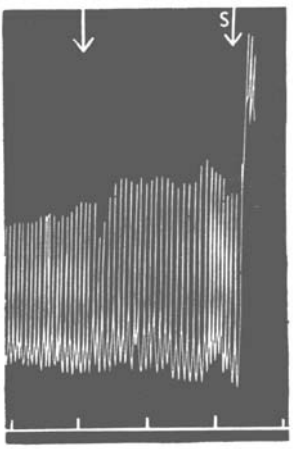

Fig. 5.

Figs. 4 and 5. Two types of non-specific response obtained from contiguous segments of gut by successive application of the same filtrate (α-titre = 0, coagulase-negative strain). In all traces the time scale represents 1 min. intervals. The first arrow marks the addition of the filtrate, a second that of the standard test dose of acetylcholine.

The only gut responses demonstrated therefore were the two specific ones, associated with α- and β-lysins and the N.S.S. Nothing attributable to enterotoxin was found.

(c) *Additional experiments*

The effect of heat was tried on α-lytic filtrates. The results of a typical experiment are shown in Table 2. It will be seen that heating at 100° C. and at 56° C. for various periods reduced the α-titre and increased the time taken to immobilize the gut, the effect being rather greater at 56° C. than at 100° C.

Incubation periods of 1, 2, 3, 4 and 5 days were tried with cultures of one strain: in each case the same α-titre of 1/256 and the same gut responses were obtained.

The Wood 46 strain was grown for 40 hr. in CO_2 concentrations of 0, 10, 20, and 30%. In each case the same α-titre of 1/1024 and the same gut responses were obtained. As closed Roux bottles were used the staphylococci may have produced enough CO_2 in the air space to make the added CO_2 unnecessary.

With filtrates of Wood 46 strain grown in Robertson's cooked meat medium, no α-lysin was demonstrated, nor was any gut response given.

DISCUSSION

Our results do not confirm those obtained by Anderson (1953), but are in agreement with the findings of Anderson, James & Marks (1954) who examined the response of isolated rabbit gut to the individual staphylococcal toxins, α-, β-, γ- and δ-. These workers found characteristic responses to α- and β-lysins, similar to those found in the present series, but no response to γ- and δ-lysins; they also observed the N.S.S. They could find no significant difference between strains from food-poisoning and other sources, apart from the general tendency of the former to produce more α-toxin. They considered that Anderson (1953) had failed to notice the α-toxin effect because the greater speed of his drum did not allow its full effect to be displayed. We agree, and would add that the N.S.S. may also have confused the

Table 2. *The effect of heating a filtrate* (*Wood* 46) *upon titre, gut response and time taken to gut immobilization*

Temperature at which heated (° C.)	Duration of heating in minutes	α-titre	Gut response	Time (sec)
56	0	1024	α	30
	5	512	α	180
	15	128	α	290
	30	32	0	—
100	0	1024	α	30
	5	512	α	130
	15	256	α	170
	30	64	α	330

picture, especially if the significance of the cessation of gut movement was not appreciated and the response to acetylcholine was not examined.

Richmond, Reed, Shaughnessy & Michael (1942) reported an increased tone and/or amplitude in 75 % of 108 trials with 'enterotoxin' compared with 17 % of 80 trials with control filtrates, and concluded that the effect was due to staphylococcal enterotoxin, acting as a non-specific irritant. However, a very limited number of enterotoxic strains seem to have been used, the control was an airborne strain of staphylococcus, no haemolysin titres are given, the fluids tested were the result of Berkefeld filtration and 'generally, the control material was applied to strips of gut which had also been subjected to the enterotoxin'. No mention is made of any response in which gut movements ceased, and acetylcholine was not used. We agree with Anderson that the conclusions to be drawn from these experiments are limited.

Any investigation into the effect of staphylococcal filtrates on gut *in vitro* presupposes that enterotoxin may act partly if not entirely by local irritation. There is little evidence for this, however, and Bayliss (1940) using whole cat preparations concluded that enterotoxin probably produces emesis by a reflex action, with afferent impulses arising in the viscera passing to the vomiting centre and thence efferent impulses activating the diaphragm, trunk muscles and enteron.

Though a reliable and convenient method of demonstrating staphylococcal enterotoxin remains an urgent need, the work now presented has convinced us that

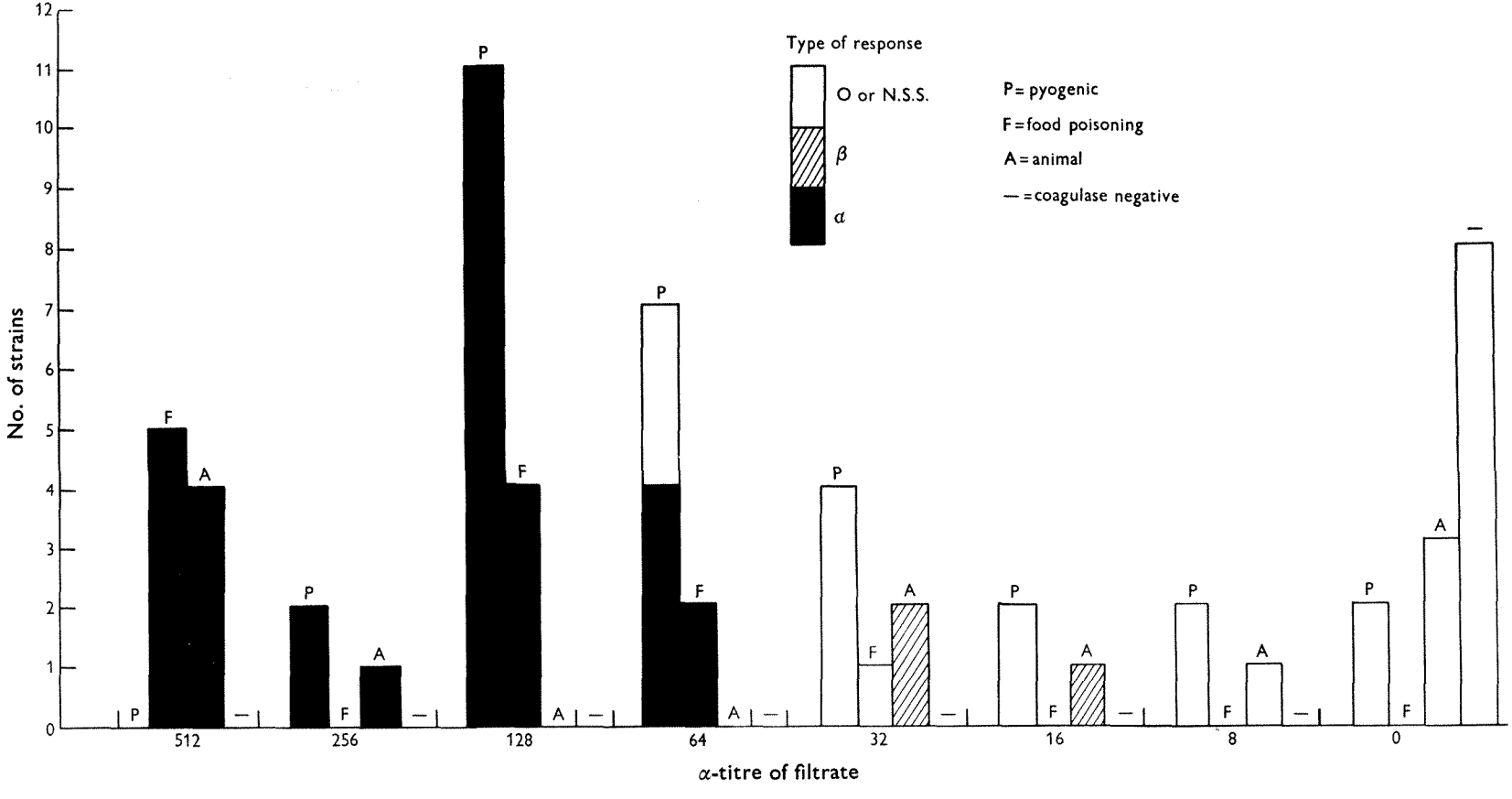

Fig. 6. Distribution of gut responses and α-titres.

further observations on isolated strips of rabbit gut are likely to prove both unrewarding and frustrating, and that search for a reliable test will most profitably be made along other lines.

SUMMARY

1. When toxic filtrates derived from coagulase-positive staphylococci including twelve food-poisoning strains, thirty pyogenic strains of human and twelve of animal origin were applied to isolated rabbit gut *in vitro*, two characteristic responses were obtained, associated with the α- and β-lytic titres respectively, and abolished by the corresponding antisera. They were independent of the origin of the strains.

2. A non-specific response is also described which is thought to be due to the peptone in the medium.

3. No effect that could be attributed to enterotoxin, such as was reported by Anderson (1953), was observed and it is concluded that isolated rabbit gut is unlikely to prove a useful indicator of its presence.

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