The Alpha, Beta and Gamma Antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916)

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SUMMARY: Three antigenic components (α , β , γ) are present in toxic culture-filtrates of *Clostridium histolyticum*. Of these α is the lethal and necrotizing toxin, β is a collagenase, and γ is a cysteine-activated proteinase which attacks altered collagen (e.g. hide-powder or azocoll) but not native collagen. The β and γ -enzymes both attack gelatin. By methods based on the properties of these antigens, *Cl. histolyticum* antisera can be tested for the corresponding antibodies.

Weinberg & Séguin (1916) described as *Bacillus histolyticus* an obligate anaerobe isolated by them from patients with gas gangrene. The organism tended to occur in pairs, was rather small ($8-5\mu \times 0.5-0.7\mu$), richly flagellate, vigorously motile in young cultures and in effusions, had large subterminal endospores and was actively proteolytic. It was pathogenic for guinea-pigs, mice and rabbits, less so for rats; intramuscular injection of culture led to extensive local destruction, splitting of the skin, extrusion of a haemorrhagic muscle pulp, denudation of the bone and sometimes to auto-amputation through a joint. Neither putrid changes nor gas formation occurred in the liquefying area, which frequently spread far beyond the injected limb.

There have been few subsequent records of the isolation of the organism, now called *Clostridium histolyticum* (see Peterson & Hall, 1922-3; Hall, 1923-4; Torrey, 1925). Other work has shown that it digests native and denatured proteins to amino-acids with production of ammonia, that it clots milk and later digests the clot (Blanc & Pozerski, 1920*a*) and haemolyses sheep red cells (Kahn, 1922). There is some question whether *Cl. histolyticum* is saccharolytic; Kahn (1922) found that it fermented maltose, lactose, xylose and inulin with production of acid; Hall (1922-3) found that all five strains examined by him failed to ferment glucose, fructose, maltose, lactose, sucrose, salicin, glycerol or inulin. These differences may perhaps be explained by Hoogerheide's work (1937) on the dissociation of *Cl. histolyticum* into saccharolytic non-pathogenic strains resembling *Cl. sporogenes*, and typical non-saccharolytic pathogenic *Cl. histolyticum*.

Descriptions of the microscopic changes produced by infection with *Cl. histolyticum* are given by Nasta (1922), who remarked on the extension of oedema far beyond the infecting organisms, and by Beckwith & MacKillop (1923-4).

Products detected in culture filtrates

The lethal and necrotizing toxins. Weinberg & Séguin (1916) observed that the culture-filtrates were toxic for guinea-pigs and rabbits; intravenous injection of sufficiently strong filtrates killed the animal in a few minutes; paralysis was followed by respiratory arrest. No author appears to have described a necrotic

lesion following intracutaneous injection of *Cl. histolyticum* filtrates, but Pasternack (1940) showed that the necrotic and degenerative changes produced in the internal organs by infection with the organism could be almost completely duplicated by intravenous injection of filtrates. Subcutaneous injection of filtrate led to boggy oedema and purplish discoloration of the skin in 3–4 days, followed by linear rupture of the skin and discharge of 'beet-soup fluid'; most animals then recovered, though some suffered exposure of bone or spontaneous amputation. Pozerski & Guélin (1938*b*) noted the production of an 'escarre' after subcutaneous injection of *Cl. histolyticum* filtrate into guinea-pigs. Gildemeister & Schlossberger (1932) showed that filtrates injected into the cornea led to swelling of the cornea and to formation of bubbles in it. No attempt seems to have been made to decide whether all these effects are due to the same substance.

Proteinases. Blanc & Pozerski (1920*a*) showed that filtrates of *Cl. histolyticum* digested proteins less readily than cultures did, and that, except in the case of gelatin, which was digested to amino-acids, the products of digestion were peptones. Filtrates of *Cl. sporogenes* were similarly proteolytic, but their activity was inhibited by normal serum and by ovalbumin, neither of which had any effect on the proteolytic effect of *Cl. histolyticum* filtrates (Blanc & Pozerski, 1920*b*; Pozerski & Guélin, 1938*a*). Bessey & King (1934) showed that *Cl. histolyticum* filtrates attacked gelatin much more vigorously than did filtrates of *Cl. sporogenes*; no pepsin or papain type enzymes or polypeptidases were present in filtrates of 72 hr. cultures. Weil & Kocholaty (1937) found that filtrates of 3-day cultures of *Cl. histolyticum* contained a proteinase, active against casein, clupein, Witte peptone and egg-albumin, that was activated by ferrous ions, by cysteine (better by both together) by manganese, nickel, cobalt and copper ions. All sulphhydryl compounds used activated it (even the relatively insoluble thio- β -naphthol) but not hydrogen sulphide or cyanide. Oxygenation reversed the ferrous ion + cysteine effect; iodoacetic acid did not; high concentrations of hydrogen peroxide decreased the activity irreversibly. According to Kocholaty, Weil & Smith (1938) the proteinase appeared 6 hr. after inoculation of the medium, and was at its maximum after about 15 hr.; its concentration ran roughly parallel with the amount of bacterial growth, and there was no evidence that it was an intracellular enzyme. Kocholaty & Weil (1938) contended that by repeated subculture on gelatin or casein, *Cl. histolyticum* could be 'trained' to produce enzymes which attacked gelatin but not casein, and vice versa. This remarkable specificity of the proteinase could be abolished, either by growing the organism in gelatin plus the amino-acids necessary to make gelatin 'equivalent' to casein, or by activating the filtrates from either casein or gelatin cultures with ferrous ions and cysteine; the filtrates then attacked gelatin or casein indifferently (see also Kocholaty & Krejci, 1948). The proteinase was neutralized by *Cl. histolyticum* antisera.

On the other hand, Maschmann (1938) found that filtrates of *Cl. histolyticum* contained extracellular proteinases not activated by cysteine, and that only in the later stages of growth was a proteinase secreted that was activated by cysteine. van Heyningen (1940) claimed to have reconciled these discrepancies

by showing that colonies of *Cl. histolyticum* varied greatly in the proportions of two proteinases produced by them, and that it was possible to obtain strains which produced in early growth an extracellular proteinase activatable by cysteine and inhibited by iodoacetic acid, while older cultures (after 12 hr. incubation) contained an intracellular enzyme inhibited by cysteine.

A fibrinolysin has been described by Carlen (1939) and by Reed, Orr & Brown (1948).

Collagenases. It was noted by Weinberg & Séguin (1916) that while injection of cultures of *Cl. histolyticum* produced extensive destruction of muscle, filtrates attacked the connective tissue only (see also Hall & Peterson, 1922-3). This difference must, however, be relative, for Pasternack (1940) found that subcutaneous injection of filtrates led not only to extensive oedema with swelling and pseudo-colloid degeneration of collagen and elastin fibres, with hydropic degeneration of the epidermis and dissociation of epithelial glandular and endothelial cells, but also to loss of striation of muscle cells and of their normal staining capacity, with subsequent myolysis and haemorrhage. There was no change in the nuclei.

The presence of a collagenase in *Cl. histolyticum* cultures was first recorded by Jennison (1945) who showed that they disintegrated heated *tendo Achillis* of cow, and also Highberger's purified steer-hide collagen sterilized by heating; their activity was enhanced by thiolacetate. Later (1947), Jennison provided more convincing evidence by showing that filtrates attacked fine strands of clean unprocessed beef tendon, whether it was sterilized by dry heat or not; he followed the process of digestion by determining the loss of weight in the collagen used. Evans (1947) observed that *Cl. histolyticum* produced a substance which attacked hide-powder and was distinct from the lethal toxin. This substance might be a collagenase or one of the proteinases that attacks degraded collagen.

EXPERIMENTAL

Cultural methods and production of toxins. Eight strains of *Cl. histolyticum* were obtained as desiccates from the Culture Collection of these Laboratories. It seems reasonably certain that five of these are distinct strains; no evidence can be provided to support the distinctness of the others. Smooth colonies on agar plates were selected from all the strains and grown in 50 ml. quantities of Brewer's medium for 24 hr., or in larger volumes of nutrient broth or a papain digest of horse muscle for 16-24 hr. The cultures were largely freed from bacteria by the addition of 'Hyflo' Supercel (Johns, Manville; 5 g./l.) and filtration through paper, and if necessary sterilized by candling. In general, the methods were similar to those found satisfactory for producing the toxic culture filtrates of *Cl. oedematiens* (Oakley, Warrack & Clarke, 1947).

Sera. We had only six sera, produced some years before by Mr A. T. Glenny, F.R.S., by immunizing horses with toxic culture-filtrates of *Cl. histolyticum* and with toxoids made from them. Testing for antibodies was carried out by conventional methods, using serum RR 2001 (100 units) as standard.

Examination of culture-filtrates for toxins

Lethal toxins. Filtrate + serum mixtures were allowed to stand for 30 min. at room temperature, then injected intravenously in 0.5 ml. volumes into mice weighing 16–20 g. Level of test, 0.25, 0.5 or 1 unit injected, according to strength of filtrate; standard indicating effect, death within 48 hr. of half the mice injected.

Table 1. *Values of sera in lethal tests against Clostridium histolyticum filtrates*

Serum	Serum values against filtrate		
	CN 647	CN 919	CN 949
RR 2001	100	100	100
RR 2005	230	240	260
RR 2008	25	30	25
RR 2035	530	570	600
RR 8003	55	50	60
RR 8006	70	75	75

Only three filtrates were lethal in sufficiently small amounts to make reasonably accurate testing possible. Table 1 shows that the serum values in lethal tests were, within the rather considerable experimental error, the same whichever filtrate was used. The lethal toxin for which the end-point was determined was therefore probably the same for all three filtrates; there was no evidence of a second lethal antigen.

Necrotizing toxins. Filtrate + serum mixtures were allowed to stand for 30 min. at room temperature, then injected intracutaneously in volumes of 0.2 ml. into guinea-pigs. Level of test 0.2, 0.4 or 0.8 unit injected, according to strength of filtrate; standard indicating effect, a small necrotic lesion. (In under-neutralized mixtures a characteristic circular oedematous purple necrotic lesion was produced.)

Table 2. *Comparison of anti-lethal and anti-necrotizing values of sera, to show that the lethal toxin of Clostridium histolyticum has necrotizing properties*

Serum	Serum values in lethal tests	Serum values in necrotizing tests against filtrate		
		CN 647	CN 919	CN 949
RR 2001	100	100	100	100
RR 2005	240	240	250	250
RR 2008	27	25	24	25
RR 2035	570	550	550	530
RR 8003	55	55	52	46
RR 8006	73	65	65	60

Table 2 shows that serum values in necrotizing tests against all the three filtrates used were constant for any particular serum and equal to the anti-lethal values; thus the lethal toxin is also necrotizing.

Collagenases. Filtrate+serum mixtures were allowed to stand at room temperature for 30 min. in tubes 6 cm. long $\times$ 0.8 cm. internal diameter (Lambeth tubes); they were then incubated overnight with pieces of fresh guinea-pig muscle or collagen paper (Oakley, Warrack & van Heyningen, 1946; Delaunay, Guillaumie & Delaunay, 1949). Level of test 1 unit; standard indicating effect: a well-marked softening of muscle or disintegration of collagen paper.

Table 3. *Comparison of anti-lethal values of sera with those obtained in muscle-softening and collagen paper tests to show that the collagenase (β) is distinct from the lethal toxin (α)*

Serum	Serum values in lethal tests	Serum values against filtrate					
		NX 729 using as indicator		NX 730 using as indicator		CN 647 using as indicator	
		Fresh muscle	Collagen paper	Fresh muscle	Collagen paper	Fresh muscle	Collagen paper
RR 2001	100	100	100	100	100	100	100
RR 2005	240	330	350	320	250	310	350
RR 2008	27	370	370	330	370	320	370
RR 2085	570	220	240	200	210	250	230
RR 8003	55	180	180	160	200	—	260
RR 8006	73	170	170	170	160	150	180

Table 3 shows that the serum values against collagen paper or guinea-pig muscle as indicators are independent of the filtrate used and agree with one another, though they bear no relationship to the values obtained in lethal tests. The collagenase (β) is therefore distinct from the lethal and necrotizing toxin (α).

Oakley, Warrack & Warren (1948) showed that *Cl. welchii* collagenase (κ -toxin) has lethal and necrotizing activities; unfortunately the very low anti-lethal values of our *Cl. histolyticum* antisera, as compared with their anti-collagenase values, have made it impossible so far to decide whether *Cl. histolyticum* collagenase is lethal and necrotizing.

All the *Cl. histolyticum* antisera contained small amounts of *Cl. welchii* κ -antitoxin; comparison of the *Cl. welchii* anti- κ values with the *Cl. histolyticum* anti- β values shows that there is no relationship between them; the collagenases of *Cl. welchii* and *Cl. histolyticum* are antigenically distinct.

Other proteinases. Filtrate serum mixtures were allowed to stand for 30 min. at room temperature, and were then incubated overnight in a water-bath at 37° with 'azocoll' (Oakley *et al.* 1946); this material is attacked by several proteolytic enzymes which do not attack collagen (Todd, 1947; Oakley *et al.* 1948). Level of test one unit; standard indicating effect, diffusion of a small amount of colour from the azocoll.

Table 4 shows that serum values against azocoll as indicator are divisible into two groups, those corresponding to the anti- β values, and those that are much lower. The lower values are characteristic of two sera RR 2005 and RR 2085, and it is noticeable that though their values differ according to the filtrate used for test, the values for the two sera are substantially equal against any given

Table 4. *Comparison of anti-collagenase values of sera and those found in azocoll tests, to show evidence of an enzyme (γ) attacking azocoll but not collagen*

Serum	Anti-collagenase values*	Serum values in azocoll tests against filtrate			
		CN 647*	CN 919*	NX 729†	NX 730†
RR 2001	100	100	100	100	100
RR 2005	350	160	160	140	140
RR 2008	380	370	450	300	250
RR 2035	250	160	210	150	170
RR 8003	210	320	230	225	210
RR 8006	160	190	180	190	190

* All the values in these columns were obtained in 1946.

† Values in these columns obtained in 1949.

filtrate. In other words, the end-point given by these two sera in azocoll tests is for a proteinase (γ) capable of attacking azocoll but not collagen; the anti- γ values of RR 2005 and RR 2035 are approximately equal, their anti- β values are not.

Addition of 0.05M cysteine to one of the culture filtrates (from *Cl. histolyticum* CN 647) had no effect on its test dose or on its standard indicating dose against collagen paper; its activity against azocoll was slightly increased, and its test dose against this material was diminished to about one-half, using serum RR 2001 as standard. As the test dose against collagen paper was unaffected by addition of cysteine, one may reasonably assume that the collagenase test dose against azocoll was unaffected also; therefore, since the test dose of the cysteine activated filtrate was diminished to one-half in azocoll tests, any serum giving the collagenase end-point would appear to double in value when tested against it. Table 5 shows that sera RR 8003 and RR 8006

Table 5. *Comparison of anti-collagenase values of sera with those obtained in azocoll tests, to give further evidence of the existence of γ , and of its activation by cysteine. The relevant end-points are indicated in brackets*

Serum	Anti-collagenase values	Serum values in azocoll tests against filtrate CN 647		
		Unmodified	+ 0.05M cysteine	+ 0.01 M iodoacetate
RR 2001	100	100 (β or γ)	100 (γ)	100 (β or γ)
RR 2005	320	160 (γ)	160 (γ)	150 (γ)
RR 2008	360	370 (β or γ)	360 (γ)	345 (β)
RR 2035	225	160 (γ)	180 (γ)	140 (γ)
RR 8003	260	320 (β)	550 (β)	290 (β)
RR 8006	180	190 (β)	410 (β)	190 (β)

have apparently doubled their values when tested against cysteine-activated filtrate in azocoll tests; thus they are giving the collagenase (β) end-point. When all the serum values are divided by two, so that the values of RR 8003 and RR 8006 are decreased to their usual anti- β values, all the other sera show values less than their anti- β values; their end-points are thus clearly for an antigen other than β . It cannot certainly be stated what this antigen is, but

since sera RR 2005 and RR 2035 give equal values against cysteine-activated filtrate, it is probable that the end-point given by sera other than RR 8003 and RR 8006 is the γ -end-point, and that γ -antigen is activated by cysteine. To look at it in another way, if before activation with cysteine the β : γ -antigen ratio in CN 647 was 1:1, after activation it was 1:2, with corresponding effects on the values of sera titrated against it (see Oakley, 1943).

It follows, moreover, that if the standard serum RR 2001 is given an anti- γ value of 100 units, the anti- γ values of the remaining sera will be those found in azocoll tests against cysteine-activated culture filtrate of CN 647, except for RR 8003 and RR 8006, whose anti- γ values will be greater than 550 units and greater than 410 units respectively.

For filtrate CN 647 neither the test dose against collagen paper nor the test dose against azocoll was affected by 0.01M iodoacetate; presumably neither β nor γ -antigen is activated or inhibited by it. Serum values against iodoacetate-treated filtrate did not differ from those against untreated filtrate. This filtrate therefore appeared to contain an enzyme (γ) similar to the cysteine-activated proteinase described by Weil & Kocholaty (1937) and by Maschmann (1938); no evidence has been obtained of enzymes inhibited by cysteine or iodoacetic acid; we hope to carry out a more thorough examination of filtrates for such proteinases in the near future.

Gelatinases. Serum values were determined in parallel in azocoll and gelatin-agar tests (Oakley *et al.* 1948) against two filtrates NX 729 and NX 730. Level of test in gelatin agar tests 1 unit; standard indicating effect, a clear zone of convenient size. The serum values (Table 6) show that in azocoll tests these

Table 6. *Comparison of anti- β and anti- γ values of sera with those obtained in azocoll and gelatin-agar tests to show that both β and γ attack azocoll and gelatin*

Serum	Anti- β values	Anti- γ values	Serum values against filtrate			
			NX 729 using as indicator		NX 730 using as indicator	
			Azocoll	Gelatin-agar	Azocoll	Gelatin-agar
RR 2001	100	100	100	100 (β or γ)	100	100
RR 2005	320	160	140	170 (γ)	140	160
RR 2008	360	360	300	360 (β)	250	350
RR 2035	225	150	150	160 (γ)	130	170
RR 8003	260	> 550	225	240 (β)	210	270
RR 8006	180	> 410	190	230 (β)	190	230

filtrates behaved very much like the previous non-activated filtrates: RR 2005 and RR 2035 gave the γ -end-point, RR 8003 and RR 8006 the β -end-point, while RR 2001 and RR 2008 gave either the β or γ -end-point. Serum values in gelatin agar tests were, as compared with the standard, slightly higher than the values in azocoll tests. The difference was small, and will need much more work to confirm. In general the results are of the same order in gelatin-agar and in azocoll tests, and the simplest explanation is that the same end-point is determined in both. If this is so, both β and γ attack gelatin.

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