The Relation of Erythritol Usage to Virulence in the Brucellas

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SUMMARY

Erythritol stimulated the growth of virulent strains of *Brucella abortus* more than that of attenuated strains. No similar correlation existed between virulence and response to erythritol with strains of *B. melitensis* and *B. suis*; these were stimulated indiscriminately by erythritol.

Erythritol was not detected in extracts of bovine white blood cells and hence is unlikely to be connected with the primary invasive process of intraphagocytic survival and growth of *B. abortus*. However, ultrafiltrates from extracts of bovine white blood cells stimulated the growth of *B. abortus* in laboratory media.

INTRODUCTION

The presence of erythritol—a growth stimulant for brucellas—in susceptible tissues appears to explain the localization of brucellas in the placenta and male genitalia of susceptible animal species; those animal species which do not suffer intense placentitis in brucellosis had no detectable erythritol in their placentas (Smith *et al.* 1962; Pearce *et al.* 1962; Williams, Keppie & Smith, 1962; Keppie, Williams, Witt & Smith, 1965). In these studies, single virulent strains of *Brucella abortus*, *B. melitensis* and *B. suis* were used. The effect of erythritol on the growth of a number of strains has been investigated to see whether a connexion exists between erythritol usage and virulence. McCullough & Beal (1951) had already shown that the growth of four different virulent strains of each *Brucella* species was stimulated by erythritol when it was present as the sole carbon and energy source in a simple basal medium. In the present studies, we have examined the effect of erythritol on the growth of several virulent and avirulent strains of *Brucella* in media containing adequate glucose. Some correlation was found between the virulence of various strains of *B. abortus* and their growth response to erythritol. These findings could not be extended to strains of *B. melitensis* and *B. suis* but they prompted the investigation reported in this paper.

The ability of various strains of *Brucella abortus* to survive phagocytosis and multiply within bovine phagocytes is correlated with their virulence and appears to rely to some extent on protective cell-wall materials which may be produced more by virulent than by avirulent cells (Smith & Fitzgeorge, 1964; Macrae & Smith, 1964). However, avirulent cells produce some protective material and a factor which may also contribute to the different behaviour of avirulent and virulent strains on phagocytosis might be a superior ability of the latter to exploit the growth conditions of the host's phagocytes, while the bactericidal mechanisms of the latter are neutralized by the cell-wall materials. This hypothesis would be supported if the phagocytes contained small amounts of erythritol which could stimulate the growth of virulent rather than avirulent strains of *B. abortus*. Hence, extracts of white blood cells and other constituents of blood were examined for the presence

of small amounts of erythritol and for the ability to stimulate the growth of virulent and avirulent strains of *B. abortus* in laboratory media.

METHODS

Strains of Brucella. The virulence of the *Brucella* strains was determined in guinea pigs as follows; a dose of 3×10^6 for *Brucella abortus* and *B. melitensis*, and 1×10^3 for *B. suis* was injected intramuscularly into guinea pigs (400 ± 50 g.). Three weeks later the average count in the spleens of these animals was determined by the culture of suitable dilutions of macerated spleen. The strains were designated virulent when the spleen counts were greater than 3×10^4 , attenuated when greater than 1×10^3 and less than 3×10^4 , and avirulent when less than 1×10^3 (cf. Table II of Smith & Fitzgeorge, 1964):

<i>B. abortus</i> strains	Virulent	2308, BL 297, B 265, BF 79, A 5, 544 and 1503
	Attenuated	205/19, s 19, 58/20 and 45/20
	Avirulent	11, 99, 45/0
<i>B. melitensis</i> strains	Virulent	6015, 1374 and ether
	Attenuated	61/115 and Rev. 1
	Avirulent	B 115, 61/118
<i>B. suis</i> strains	Virulent	KG 25, 8973/56, 1830, 15 LA, and 63/171
	Attenuated	63/166, 61/59, 61/66, and Thomsen
	Avirulent	63/260 and 61/60

Erythritol was supplied by G. T. Gurr Ltd. and recrystallized from moist acetone.

Tests for ability of materials to stimulate the growth of Brucella in vitro

(a) *Viable count method.* The method has been described previously (Williams *et al.* 1962). The basal medium was a mixture of tryptic digest meat broth (1 part) and isotonic saline (2 parts); the inoculum was 100 organisms/ml.

(b) *Turbidimetric methods.* Shake cultures in cuvette-flasks were used (Copeland, 1955). Optically matched Pyrex test-tubes were fixed to the sides of 50 ml. conical flasks. Medium, inoculated with the test organism (see below) was distributed into the cuvette-flasks (10 ml. per flask). Experimental flasks received erythritol in Locke solution (0.2 ml.). Control flasks received Locke solution (0.2 ml.). The flasks were incubated on a horizontal shaker at 37°. Strains requiring added CO₂ for growth were incubated in sealed aluminium boxes gassed with air containing 5% CO₂. When turbidity readings were required the flasks were turned through approximately 120°, thus transferring the culture to the side-arm, and turbidimetric readings were obtained directly in the Coleman spectrophotometer. The flasks were then returned to the shaker when subsequent readings were required for the preparation of growth curves. The optical densities of duplicate flasks varied by less than 3%. When required (see Table 2) regression coefficients for the log phase of growth were calculated from the curves between 17 and 25 hr and the ratios of the growth rates with and without erythritol were obtained.

Two media were used in this work.

Medium (1): a mixture of tryptic digest meat broth with Locke solution (1+1 by vol.) with an inoculum of $1-2 \times 10^6$ /ml. Turbidimetric readings were made at 570 m μ .

Medium (2): a modified form of that used by Rode, Oglesby & Schuhardt (1950).

A glutamate-free modification of this medium (Anderson & Smith, 1965) would not support the growth of avirulent strains of *Brucella abortus*; hence 100 μ g. L-glutamic acid/ml. was added. The modified medium differed from that of Rode *et al.* (1950) as follows. D-glucose 7.0 mg./ml. (instead of 4.0 mg./ml.); ammonium sulphate 1.35 mg./ml. (instead of nil); L-glutamic acid 100 μ g./ml. (instead of 3000 μ g. DL-glutamic acid/ml.). A concentrated ($4\times$) stock solution, minus the biotin, glucose and glutamic acid, was stored at -20° . A supply of medium for 2 weeks was prepared from the stock solution by dilution, addition of biotin and filtration through a 'Millipore' filter. The filtered solution was stable at $0-4^{\circ}$ for 14 days. Glucose and glutamate were added before each experiment and the medium refiltered. The medium was inoculated with 2×10^7 /ml. and extinction measurements on cultures were made at 430 m μ .

Estimation of erythritol. The method has been described by Williams *et al.* (1962).

Preparation of cell extracts from bovine blood for biological and chemical analysis

Differential centrifugation of blood yielded large quantities of white blood cells which were purified from red blood cells. The white blood cells were disrupted ultrasonically and the ultrafiltrates of the extracts were examined. Red blood cells were also obtained and disrupted by freezing and thawing.

Citrated (0.25 %) bovine blood (10 l.) was centrifuged for 60 min. at 2° in an M.S.E. Major centrifuge. The plasma was removed and a 200 ml. sample frozen at -20° ; a 200 ml. sample of the red blood cells also was frozen at -20° . The 'buffy coat' (300-750 ml.) containing the majority of white blood cells was removed by pipette and centrifuged at 27,000 rev./min. for 60 min. in the 30 rotor of a Spinco ultracentrifuge. The plasma from the deposit was decanted revealing the white blood cells as a coherent 'skin' (2-5 mm. thick) on the surface of the remaining erythrocytes. This skin was tipped from each tube on to a microscope slide ($3\times 1\frac{1}{2}$ in.), and the slide inverted on a white enamel tray. Careful removal of the slide showed a contaminating layer of erythrocytes on the lower surface of the leucocyte skin. By gently drawing the edges of two other slides across this exposed surface (beginning at the centre and moving in opposite directions) the majority of these erythrocytes were removed. These manipulations were carried out at $0-4^{\circ}$. The 'pure' leucocyte preparation was frozen at -20° . A yield of $2-4\times 10^{10}$ leucocytes was obtained from 10 l. of bovine blood. Haemocytometer counts showed that samples prepared by this method had w.b.c.:r.b.c. ratios which varied from 2:1 to 1:5. Dye-exclusion tests with eosin indicated that 50-75 % of leucocytes in the final sample were viable.

The three fractions were treated as follows:

Leucocyte fraction. This was thawed and homogenized by maceration in an M.S.E. overhead macerator with the minimum quantity (about 20 ml.) of isotonic phosphate saline buffer (NaCl 7.5, Na_2HPO_4 1.42, KH_2PO_4 1.36 g./l.; pH 7.4 with 40 %, w/v, NaOH). The cooled cell suspension was stirred magnetically and treated ultrasonically for 15 min. with the M.S.E. Mullard ultrasonic disintegrator as described by Smith, Keppie, Cocking & Witt (1960). Haemocytometer counts showed that 99.9 % of the leucocytes had disintegrated. The extract was centrifuged at 27,000 rev./min. for 60 min. in the 30 rotor of a Spinco ultracentrifuge. The supernatant fluid was ultrafiltered at $0-2^{\circ}$ and the ultrafiltrate stored at -20° .

Erythrocyte fraction. This (1×10^{10} red cells/ml. disrupted by the freezing) was thawed and centrifuged at 27,000 rev./min. for 60 min. in the 30 rotor of a Spinco ultracentrifuge. Diffusates, prepared by dialysing the supernatant fluid (200 ml.) against de-ionized water (3×400 ml. over 3 days), were freeze dried. To estimate the total amount of diffusible material in the extracts (so that freeze-dried diffusates could be redissolved at a strength $\equiv$ the original extracts) the losses in dry weight (105° , 16 hr) of small samples of the extracts were obtained after exhaustive dialysis.

Plasma. A diffusate was obtained from the plasma by treating it as described for the extract of erythrocytes.

RESULTS

The effect of erythritol on the growth of strains of Brucella abortus after 24 hr in a broth medium in vitro

The results in Table 1 show that there was some correlation between degree of virulence for guinea pigs and enhancement of growth by erythritol in medium (1), i.e. tryptic digest meat broth + Locke solution (1 + 1). At erythritol concentrations higher than those shown in Table 1 (2–10 μ mole/ml.) some stimulation of the growth of the relatively avirulent strains was observed but the degree of stimulation was smaller than that occurring with the more virulent strains.

Table 1. *The relation between the virulence of Brucella abortus strains and growth enhancement by erythritol*

Strain	Erythritol concentration	
	1 μ mole/ml.	0.1 μ mole/ml.
Virulent		
2808	90*	36
BL 297	88	39
B 265	59	30
BF 79	55	15
A 5	49	18
544	47	19
1503	39	17
Attenuated		
205/19	24	5
45/20	3	–15
s 19	–2	–2
58/20	–7	–2
Avirulent		
11	20	3
99	0	–1
45/0	6	6

* Growth enhancement by erythritol in medium (1), i.e. tryptic digest meat broth + Locke solution (1 + 1) after 24 hr incubation measured as

$$\frac{\text{Optical density of (sample – control)}}{\text{Optical density of control}} \times \frac{100}{1}.$$

All strains grew at approximately the same rate in the basal medium. Results are averages of several experiments.

The effect of erythritol on the growth rate of strains of Brucella abortus in a defined medium

The growth rates of various strains of *Brucella abortus* were measured in medium (2) with and without erythritol. The weighted means of the ratios of the growth rates from several experiments (Table 2) again indicated the greater response of the virulent strains to erythritol.

Table 2. *Growth rate response to erythritol by Brucella abortus strains in a defined medium (medium (2))*

Strain	Weighted mean*
Virulent	
BL 297	1.82
544	1.47
Attenuated	
205/19	1.01
S 19	0.96
58/20	1.11
Avirulent	
11	1.08
99	1.08
45/0	1.22

All strains grew at approximately the same rate in the basal medium.

* $\frac{\text{Gradient log phase with erythritol (2 } \mu\text{mole/ml.)}}{\text{Gradient log phase without erythritol}}$

The growth response to erythritol of strains of Brucella melitensis and B. suis of differing virulence

The effect of erythritol on the growth of strains of *Brucella melitensis* and *B. suis* was investigated under two sets of growth conditions: (1) low erythritol concentrations and small inoculum of organisms—growth being measured by colony count; and (2) high erythritol concentration and large inoculum of organisms—growth being measured turbidimetrically. The results in Table 3 show that all the *B. melitensis* and *B. suis* strains were stimulated to a greater or lesser extent by erythritol irrespective of their virulence for the guinea pig.

The erythritol content of fractions from bovine blood and their growth-promoting activity for Brucella abortus

Diffusates or ultrafiltrates prepared from white blood cells, red blood cells and plasma obtained from several batches of bovine blood were examined chemically for the presence of erythritol. Parallel biological tests were also carried out to see whether these extracts stimulated the growth of virulent and avirulent strains of *Brucella abortus* in a broth medium. The results are shown in Table 4. Erythritol was not detected ($< 5 \mu\text{g./ml.}$ extract) in any of the leucocyte, erythrocyte or plasma samples. However, the four leucocyte extracts, even when considerably diluted, caused significant stimulation of the growth of virulent and avirulent strains of *B. abortus*. In the same experiments, erythritol stimulated only the virulent strains (544 and 2308) and an equivalent amount of glucose had no effect on any

Table 3. *The growth response to erythritol of strains of Brucella melitensis and B. suis*

Strain	Growth enhancement by erythritol measured	
	By colony counts at 27 hr*	By turbidimetric readings at 22 hr†
<i>B. melitensis</i>		
Virulent		
6015	2.5‡	1.7‡
1374	1.7	4.4
Ether	4.0	5.1
Attenuated		
61/115	3.8	4.8
Rev 1	3.3	—
Avirulent		
B. 115	—	4.2
61/118	3.6	—
<i>B. suis</i>		
Virulent		
KG 25	1.2	1.4
8973/56	1.6	1.6
1380	1.4	2.4
15 LA	1.5	2.4
63/171	—	2.0
Attenuated		
63/166	—	1.1
61/59	—	1.2
61/66	—	1.2
Thomsen	—	5.5
Avirulent		
63/260	—	1.4
61/60	—	1.9

* Medium: tryptic digest meat broth (1 vol.) and isotonic saline (2 vol.); inoculum 100 organisms per ml.; erythritol 0.065 μ mole/ml.

† Medium (2) was used; inoculum 2×10^7 /ml.; erythritol 2 μ mole/ml.

‡ These results are the means of several similar experiments measured as

$$\frac{\text{population of treated sample}}{\text{population of control}}$$

Table 4. *The growth-enhancing effect on Brucella abortus of diffusates, from extracts of the white blood cells and other fractions of bovine blood, all with an erythritol content of less than 5 μ g./ml.*

White blood cell diffusates		Highest dilution enhancing growth (twofold) at 24 hr*			
		Virulent		Attenuated	
w.B.C. count per ml.	r.B.C. count per ml.	544	2308	45/0	58/20
8×10^8	1.7×10^9	1/60	1/60	1/60	1/60
5×10^8	2.6×10^9	1/60	1/60	1/60	1/60
5×10^8	2.7×10^8	—	1/60	1/30	1/30
6×10^8	—		1/60	1/30	1/30
Four red blood cell diffusates		All inactive 1/10 for 2308			
Four plasma diffusates		All inactive 1/10 for 2308			

* Growth measured by viable counts; medium tryptic digest meat broth (1 vol.) and isotonic saline (2 vol.); inoculum 100 organisms/ml.

strains. The factor(s) capable of stimulating both virulent and avirulent strains of *B. abortus* was apparently confined to leucocytes as all erythrocyte and plasma fractions were inactive.

DISCUSSION

The finding that erythritol stimulates virulent more than attenuated strains of *Brucella abortus* is interesting in view of the previous investigations (Williams *et al.* 1962) suggesting that the presence of foetal erythritol is a cause of the localization of *B. abortus* in bovine foetal tissue. However, the lack of erythritol in bovine white blood cells suggests that this differential effect of erythritol on virulent *B. abortus* strains probably has no relevance to the primary invasive process of intraphagocytic survival and growth. In contrast to these results with *B. abortus* there seems to be no correlation between growth stimulation by erythritol and the virulence of various strains of *B. melitensis* and of *B. suis*.

The fact that extracts of the mixed cell population of bovine 'buffy coat' stimulate the growth of *Brucella abortus* is interesting. If the same growth-stimulating activity were found in extracts of phagocytes purified from the buffy coat, it might have some relevance to the intracellular growth of virulent *B. abortus*.

A factor that may be involved in the phenomenon of intracellular survival and growth in bovine phagocytes is glutamic acid metabolism. Olsen (1951) showed that there was a correlation between virulence and high oxidative rate of glutamic acid in *Brucella abortus* and we have shown the influence of L-glutamic acid on the growth of avirulent strains in a synthetic medium. Furthermore, Anderson & Smith (1965) have shown that stimulation of growth of a virulent strain by erythritol is inhibited by high concentrations of glutamic acid. Wilson & Dasinger (1960) have discussed the influence of glutamic acid in relation to intracellular growth. They suggest that the rapid removal of intracellular glutamate by avirulent strains might produce conditions inimical to the organisms' growth.

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