Metabolite-Resistance and Virulence of Smooth *Brucella* Variants Isolated after Prolonged Cultivation. (18633)

ROBERT J. GOODLOW, WERNER BRAUN, AND LEONARD A. MIKA.
From Camp Detrick, Frederick, Md.

Recent studies have shown that population changes in synthetic broth cultures of smooth *Brucella abortus*, 6232, are affected by the concentration of alanine accumulating in the culture medium as a product of normal metabolism(1). The addition of D-alanine to synthetic broth cultures(2) of smooth cells was found to inhibit the growth of the original inoculum and to accelerate the establishment of alanine-resistant nonsmooth (*e.g.*, rough, mucoid) variants or mutants(3). Recently it was observed that after prolonged (30 days) incubation (37°C) of originally smooth cultures in which nonsmooth types had established themselves, a renewed increase in viable cell counts occurred coincidental with the progressive establishment of a smooth type that has been labeled S′. By acriflavine test (4) and general consistency, colonies of this smooth type appeared identical with the original smooth inoculum, but when cell suspensions of the two were mixed and plated on "2-1" agar(5) the S′ type gave rise to colonies distinctly smaller than those of the original S type. After 60 days of incubation another smooth variant appeared and rapidly increased in numbers. Colonies of this type, labelled "dwarf smooth" or dS were extremely small, but morphologically, tinctorially, and antigenically (by agglutinin absorption and acriflavine test) the individual cells were indistinguishable from the original S or the S′ type.

In order to determine further the extent of similarities between these various S types, studies of their relative virulence for guinea pigs were undertaken. Since the establishment of nonsmooth variants in similar cultures was shown previously to be due to their relative resistance to the inhibitory effects of the metabolite alanine, the alanine resistance of the different smooth types was determined. Susceptibility of the original S type to the toxicity of alanine has been well established (1,2). Demonstration of increased alanine resistance or altered virulence of the derived smooth types (S′ and dS) that established themselves upon prolonged cultivation would indicate that the observed population changes do not represent reversions to the original S type but involve mutants that merely simulate many characteristics of the smooth stem type.

Results. Nine respective groups of 10 guinea pigs were infected subcutaneously with graded doses of the original S, the S′, or the dS strains of *Br. abortus*, 6232, each group receiving a particular dose of the designated strain (Table I). Animals were sacrificed 5 weeks after infection, and the regional lymph nodes, spleen, and liver were cultured on modified tryptose agar plates. Results of these virulence titrations are summarized in the table. Probit analysis of these data indicates that the virulence of the S′ and dS mutants is at least comparable to that of the original smooth type.

In order to determine the relative resistance of the original S, the S′, and the dS types to the inhibitory effects of alanine, a series of flasks was prepared, each containing 100 ml of synthetic medium and to each flask were added 100 μg or 500 μg DL-alanine per ml. These media were inoculated with 1 ml of turbidimetrically adjusted saline suspensions of S, S′, or dS. The cultures were incubated at 37°C, and the number of viable cells (plate count) was determined at regular intervals throughout the period of observation. The results, shown in Fig. 1, revealed significant differences in the resistance of the three S types to the inhibitory effects of 500 μg of

1. Goodlow, R. J., Mika, L. A., and Braun, W., *J. Bact.*, 1950, v60, 291.

2. Goodlow, R. J., Braun, W., and Mika, L. A, *Arch. Biochem.*, 1951, v30, 402.

3. Braun, W., *Bact. Revs.*, 1947, v11, 75.

4. Braun, W., and Bonestell, A. E., *Am. J. Vet. Res.*, 1947, v8, 386.

5. Braun, W., *In "Brucellosis" AAAS Symposia*, 1950, v10, 26.

TABLE I. Virulence for Guinea Pigs of S, S′, and dS Types of *Brucella abortus*, 6232.

Strain	Avg No. organisms inj. per animal	Infection ratio (infected over total)	Estimate of ID_{50}	95% confidence limits	
				Lower	Upper
S	8.5	5/10			
	39.0	5/10	9.9	1	96
	78.0	9/10			
S′	8.4	7/10			
	40.2	9/10	1.3	.02	95
	80.4	9/9			
dS	8.4	6/10			
	39.6	5/10	11.7	1	133
	79.2	9/10			

alanine per ml. Similar but not as marked differences were noted with 100 μg DL-alanine per ml. Thus, despite similarity in many properties, the S, S′, and dS types proved to be dissimilar in their alanine resistance.

Discussion. These results demonstrate that in the course of progressive population changes in a closed system, mutants may establish themselves that simulate the parent type in many characteristics but differ from it in those properties responsible for increased selective value in the presence of inhibitory metabolites. It was shown that the 3 smooth types, isolated at different times in the culture's history, were similar in virulence, antigenic properties, and many other characteristics, yet differed significantly in their resistance to alanine, a compound which is known to accumulate in these cultures as an end product of metabolism. This difference in alanine resistance permitted the S′ and dS mutants to establish themselves at periods when alanine levels of the cultures were high and when the S parent type would have failed to find a favorable environment. Thus, what superficially appeared to involve reverse population changes, or the establishment of reverse mutants, actually involved the establishment of novel mutants with phenotypes similar to that of the original smooth cells. Many past observations recorded as reverse changes(3) may represent other examples of progressive mutational changes. Similar results have been noted recently in studies involving phenotypic changes from streptomycin-dependence to streptomycin-resistance in *E. coli*(6). Such observations raise the questions whether true reverse mutational changes occur at all and if so at what frequency. In order to determine the occurrence of true reverse mutants, an environment free of inhibitory metabolites would be required, at least for *Brucella*, in which the role of metabolites in the determination of selective values now has been recognized.

It is noteworthy that the progressive establishment of novel smooth types in cultures of

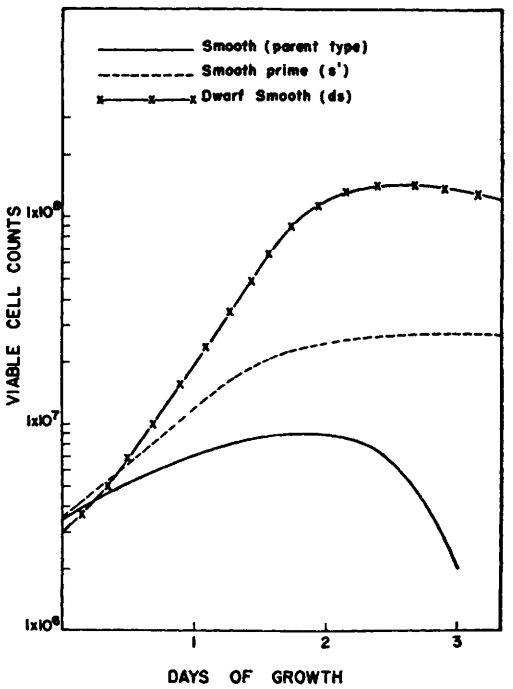

FIG. 1.
Viable cells (plate counts) of S, S′, and dS on various days in synthetic medium containing 500 μg of DL-alanine per ml.

6. Demerec, M., *Trans. N. Y. Acad. Sci.*, 1950, v12, 186.

Brucella after prolonged cultivation has involved reversions to virulence in mutants originating from relatively avirulent nonsmooth types. Thus, it would appear that in most instances the potentiality for virulence is not really lost* but its manifestation is merely suppressed and may be reestablished by various types of mutational change. As frequently suspected in the past(3), this ability of relatively avirulent cells to yield new mutants with high virulence, capable of establishing themselves under proper environmental conditions, may be of significance to problems of pathogenesis and epidemiology.

Summary. Following prolonged incubation of originally smooth *Brucella abortus* broth cultures, relatively avirulent nonsmooth variants established themselves but were eventually replaced by a smooth type, labelled S′, in turn replaced later by still another smooth type, labelled dS. These three smooth types were found to be similar in many characteristics, including virulence, but differed significantly in their resistance to alanine, an inhibitory metabolite which accumulates in the culture fluid. These differences in alanine resistance permitted the S′ and dS mutants to establish themselves at periods when alanine levels of the cultures were high. Therefore, what superficially appeared to be an establishment of reverse mutants actually involved the establishment of novel mutants which simulated the original smooth type in many characteristics.

* Strain 19 of *Br. abortus* seems to be one exception.

Received March 27, 1951. P.S.E.B.M., 1951, v76.

Influence of Alpha-Naphthyl Thiourea on Gastric Evacuation.* (18634)

SALLY H. DIEKE. (Introduced by C. P. Richter)

From the Psychobiological Laboratory. Johns Hopkins Medical School, Baltimore, Md.

In the course of earlier experiments performed in this laboratory(1), it was noted that the stomachs of rats dying from acute poisoning with alpha-naphthyl thiourea (ANTU) were frequently hugely distended with food, and since the deaths occurred some hours after the rats had eaten, this suggested the possibility that the ANTU had in some way interfered with the normal process of gastric evacuation. The experiments described in the present paper were, therefore, planned to investigate the possible effect of various doses of ANTU on gastric emptying time.

Methods. The experiments were performed with adult albino rats, both male and female, of the Wistar strain or a strain derived from it. The acute median lethal dose (LD_{50}) of ANTU, as administered to these rats, was roughly 5 mg/kg of body weight; doses of 2 mg/kg killed none, while 10 mg/kg was almost invariably fatal within 7 to 17 hours (2). The rats were trained to eat a sizable meal in one hour or less; ANTU suspended in olive oil was then administered by intraperitoneal injection, and the weights of the stomach contents at varying intervals afterwards were then compared with those of control rats that received only olive oil. In one experiment banthine† was given to a group of rats to compare the effect of this drug with that of ANTU in delaying gastric evacuation. Data on a few rats receiving epinephrine‡ were also obtained. The rats were trained to eat a substantial amount of food in a short time by offering food for only a stated 2-hour

* Carried out under a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1. Richter, C. P., *J.A.M.A.*, 1945, v129, 927.

2. Unpublished data from this laboratory.

† Diethylaminoethyl xanthine-9-carboxylate methobromide, provided through the courtesy of Dr. Irwin C. Winter of G. D. Searle & Co.

‡ 1:500 suspension in peanut oil, from Abbott Laboratories.