OBSERVATIONS ON THE PROBLEM OF BRUCELLA BLOOD CULTURES[1]

M. J. PICKETT AND ERIC L. NELSON[2]

Department of Bacteriology, University of California, Los Angeles, California

Received for publication September 26, 1950

Of the various laboratory data that are sought to support a tentative clinical diagnosis of chronic brucellosis, none is given more emphasis than a positive culture. Cultures, particularly from blood, are frequently negative, however, even when taken daily for a week or more. These failures have been variously ascribed to nutritional inadequacies of the culture media employed and to the absence of brucellae in the blood stream. Some credence, indeed, may be given to the latter proposal in view of the positive cultures obtained from biopsy specimens by various investigators (Spink, 1948; McVay, Guthrie, Michelson, and Sprunt, 1948, 1950; Weed and Dahlin, 1950). A third possibility, and one that does not appear to have been adequately explored, may be expressed as follows: Brucellae may well be present in a 5- to 10-ml blood specimen (and probably intracellular), but their growth, when commonly employed cultural procedures are followed, will be suppressed by concomitant antibody, even though the latter may not be manifest in the agglutinin titer. Accordingly then, a "washed" specimen should be used and should realize not only removal of inhibitory antibody but also a significant concentration of the brucellae.

We have examined some 50 febrile bloods (from hospitalized patients with clinical symptoms, though with no bacteriological evidence, of brucellosis) in an attempt to evaluate this "antibody-inhibition" hypothesis. All specimens examined yielded bacterial growth, although in only two instances were these identified as smooth, specifically agglutinable brucellae. These unexpected results led to an examination of approximately one hundred normal human bloods, which, also, were uniformly positive in respect to bacterial growth. Attempts to identify these bacteria by means of the various microscopical, cultural, and biochemical criteria commonly employed in diagnostic bacteriology were met with little success. The cultures did show, however, a marked colonial and microscopical similarity to *Brucella* variants that we were obtaining from stock smooth *Brucella* cultures. This suggested that most if not all of our positive cultures were those of nonsmooth *Brucella* variants and that, therefore, a serological examination might disclose significant antigenic similarities between the known *Brucella* variants and the organisms derived from blood cultures. This was a fruitful procedure and left little room to doubt the nature of the cultures derived from both normal and febrile bloods. The working hypothesis that led to the

[1] Presented in part before the Society of American Bacteriologists, Baltimore, May 15, 1950.

[2] With the technical assistance of R. G. Bleier, D. R. Erickson, L. D. Franzen, D. H. Howard, J. D. Liberman, M. H. Samuels, and W. A. Zavenelli.

discovery of these *Brucella* variants in normal bloods has also disclosed the presence of *Brucella* bacteriophage in many of the bloods, and it now appears that it is the latter, rather than specific antibody, which is largely removed from washed specimens.

EXPERIMENTAL METHODS

Media. Several commercial peptones were examined, by means of two strains of smooth *Brucella abortus*, in respect to their ability to give visible growth from minimal inocula in broth media. With each of these, sodium chloride and glucose were added at levels of 0 to 1.0 per cent in an attempt to determine the optimal concentration of each of these compounds for each peptone tested. Difco tryptose was early discarded because of its failure to give growth from relatively large inocula (1,000 to 2,000 organisms); these results were in keeping with those obtained by Schuhardt, Rode, Foster, and Oglesby (1949). Sheffield NZ-case gave growth in 3 to 4 days from an inoculum of 1 to 10 organisms but, in 2 per cent concentration, did not yield a sparklingly clear medium. Both Albimi C and Albimi M peptones were satisfactory in respect to early growth from minimal inocula, and the former was selected as the peptone of choice for this study.[3] It was incorporated in the following media:

(1) CTN broth: Albimi C peptone, 2.0 g; NaCl, 0.5 g; glucose, 0.2 g; thiamine hydrochloride, 0.1 mg; nicotinamide, 0.1 mg; and distilled water, 100 ml.

(2) Citrated broth: CTN broth containing 1 per cent $Na_3C_6H_5O_7 \cdot 2H_2O$.

(3) CTN agar: CTN broth containing 2 per cent Difco agar.

The thiamine and nicotinamide were added in order to provide these growth factors at levels compatible with those reported by McCullough, Mills, Herbst, Roessler, and Brewer (1947). All glassware was acid-cleaned since irregular results were obtained with glass that had been washed with soap.

Preparation of specimens. Blood was drawn from the radial vein in 5-ml amounts and added to 5 ml of citrated (2 per cent) saline in 25-ml screw-cap tubes. This was centrifuged 30 minutes at 2,500 rpm (1710G), after which the plasma was removed for use in agglutination and precipitation tests. The sedimented cells were then resuspended in 20 ml of sterile distilled water and again centrifuged 30 minutes. The washings were discarded, and the sediment was again washed with 20 ml of distilled water. The second washings were also discarded, and 0.1 ml of the sediment was spread uniformly over half of a CTN agar plate. The second half of the plate was streaked with a loop, the first half being used as a source of inoculum. This method for seeding the plates was followed in order to effect maximum dilution of a large inoculum, and in several instances it appeared to effect a streaking of the bacteria beyond residual contaminating bacteriophage.

The blood sediment remaining in the specimen tube received 10 ml of citrated

[3] Since this study was completed, the C peptone has been found suboptimal for two strains of *B. melitensis* and has been replaced in the formulae by Albimi M peptone. Still other peptones and commercially compounded media are now being examined in respect to their relative efficacy for the brucellae and their activation of *Brucella* phage.

broth, and 0.1-ml subcultures were made from this, in the manner described above, to CTN agar plates after 2, 4, 7, and 10 days' incubation. All cultures were incubated at 35 C in a candle jar.[4] The cultures were examined daily for at least 2, and sometimes 3, weeks before being discarded, although in no instance was evidence obtained that incubation need be carried beyond 7 days.

Colonies were examined with the aid of a dissecting scope at 20 × magnification by methods similar to those described by Henry (1933), replated once for purity, then held at 4 C on CTN agar slants for further cultural and serological examination. Approximately 15 colonial forms, including the R and SR of Henry (1933), were recovered during the course of this investigation. Many of these were, however, too unstable culturally to permit exact definition, and others were autoagglutinable. Four colonial forms were selected for serological examination because of their frequency of occurrence, cultural stability, and specific agglutination in homologous antisera. These were designated, on the basis of colonial appearance, smooth brown (SB), mucoid pink (MP), and mucoid (16M and 33M). The homologous antisera (rabbit) were prepared against a "type" strain of each and against a stock smooth *Brucella abortus* (NIH no. 345) by immunization with phenolized suspensions.

Serological tests. Each organism isolated from a blood specimen was prepared in 0.1 per cent suspension in 0.5 per cent phenolized saline for use as an agglutinogen in quantitative titrations against anti-S, -SB, -MP, -16M, and -33M sera and homologous human plasma (or serum) at final dilutions of 1:20 to 1:2,560. In addition, quantitative agglutination tests were run with each human plasma (or serum) against the type S, SB, MP, 16M, and 33M antigens. Two antigen control tubes, saline and normal rabbit serum at a final dilution of 1:40, were included in each titration. No bacterial suspension was considered suitable for use if more than 2 weeks old because of the "titer-lowering" effect of old preparations. (The menstruum from a month-old stock 10 per cent suspension of smooth *B. abortus*, for example, gave a strong precipitation reaction with homologous antiserum.) Each titration series was incubated 2 hours at 56 C, followed by 18 to 24 hours at 4 C, before final reading. The end point recorded was the last tube showing slight but definite agglutination.

Qualitative precipitation tests were run by layering the antigen, consisting of the supernatant fluid obtained from a phenolized 10 per cent bacterial suspension that had stood 24 hours at 4 C before centrifugation, over undiluted serum or plasma in a 3-mm tube. The tubes were incubated at room temperature and examined up to 60 minutes before discarding.

RESULTS

The SB *Brucella* variant was that one most commonly encountered on primary plates and, when these were negative, was almost the sole form recovered from secondary plates inoculated with 2- to 7-day citrated broth cultures. The

[4] Candle jar incubation has recently been found inadequate for two strains of smooth *B. abortus*, and Brewer jars containing 10 to 15 per cent CO_2 are now used for all blood specimens and for CO_2-dependent stock cultures.

33M variant, a relatively large and distinctly mucoid colonial form, occurred rather infrequently, though it apparently possessed an *in vivo* as well as *in vitro* stability since it was again found, 2 months later, in the bloods of those humans from whom it had been recovered at initial bleeding. The MP variant was only occasionally recovered on primary plates and rarely on secondary plates but did, however, appear as a relatively common variant arising from SB, from M, and from known-stock smooth *Brucella* cultures.

As indicated above, attempts to identify these bacteria on the basis of cultural characteristics, Huddleson (1943) and Cruickshank (1948) differential dye sensitivity, or biochemical tests were met with little success. Microscopically, the MP, 16M, and 33M type strains were gram-negative coccobacillary forms approximately 0.5 by 2 μ in size. The SB type strain was a gram-intermediate to gram-positive coccal form, 0.5 to 0.7 μ in diameter.[5]

TABLE 1

Homologous and heterologous agglutinin titers of specific antisera for five type strains of Brucella

TYPE	SOURCE	AGGLUTININ TITERS				
		S	SB	MP	16M	33M
S	Stock smooth abortus	3,240	20	40	80	80
SB	Febrile blood	320	1,280	80	160	160
MP	From a smooth suis	80	20	1,280	40	20
16M	Normal blood	40	80	160	320	160
33M	Febrile blood	20	40	40	80	1,280

The first step toward a serological examination of these cultures consisted of selecting a representative "type strain" for each colonial form and preparing the homologous antisera (rabbit). The homologous and heterologous titers of these sera are given in table 1, from which it may be seen that two of the type strains were obtained from stocks, the S type strain being smooth abortus no. 345 and the MP being a variant derived from a stock smooth *Brucella suis*. It was found impossible to obtain high titer agglutination with the 16M variant, a particularly mucoid form. These results, however, were similar to those reported for the mucoid *Brucella* variants examined by Plastridge and McAlpine (1930).

Figure 1 represents a graphic summary showing the number of strains of each agglutinable variant that were isolated from normal human blood, and the agglutination titer that was obtained for each of these against its corresponding antiserum. The titer in each instance, in figure 1, is expressed as the percentage of the specific, homologous titer of the serum. Those strains that agglutinated

[5] This appears to be a phage-infected *Brucella* variant; this and other gram-positive, phage-infected *Brucella* variants have recently been obtained by adding *Brucella* phage to stock cultures of smooth *B. abortus*.

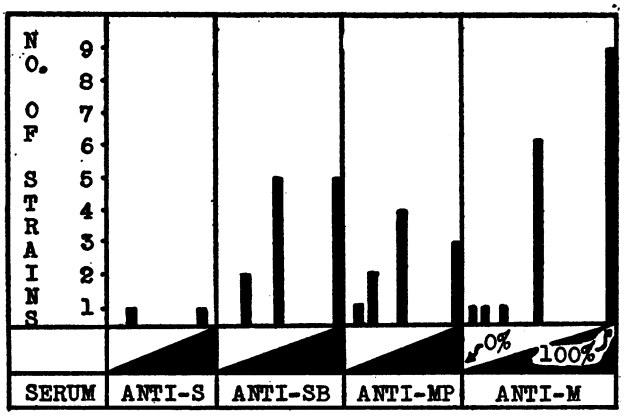

Figure 1. Number of strains of each variant and their titers against specific antisera.

TABLE 2

Precipitation tests with specific antisera and normal rabbit serum (NRS) against bacteria isolated from normal and febrile bloods and from air

VARIANT ISOLATED	ANTISERUM					
Normal blood	S	SB	MP	16M	33M	NRS
SB	16+ 2−	21+ 2−	7+ 16−	19+ 4−	20+ 3−	0+ 23−
MP	3+ 2−	2+ 3−	3+ 2−	2+ 3−	4+ 1−	0+ 5−
16M	0+ 1−	1+ 0−	0+ 1−	1+ 0−	1+ 0−	0+ 1−
33M	1+ 2−	0+ 3−	0+ 3−	1+ 2−	3+ 0−	0+ 3−
Febrile blood						
S	2+ 0−	1+ 1−	0+ 2−	1+ 1−	0+ 2−	0+ 2−
SB	5+ 3−	8+ 0−	3+ 5−	6+ 2−	6+ 2−	0+ 8−
MP	1+ 1−	3+ 0−	1+ 2−	1+ 2−	2+ 1−	0+ 3−
16M	2+ 1−	1+ 2−	0+ 3−	3+ 0−	2+ 1−	0+ 3−
33M	0+ 1−	0+ 1−	0+ 1−	0+ 1−	1+ 0−	0+ 1−
Air flora	0+ 9−	0+ 9−	0+ 9−	0+ 9−	0+ 9−	0+ 9−

in saline or normal rabbit serum were not included in the data summarized here. The SB variants, 12 culturally stable strains of which were isolated from as many humans, were quite homogeneous serologically. Only two of these agglutinated at 25 per cent of serum titer, whereas five agglutinated to 50 per cent of the titer, and five agglutinated to full titer. All the variants showed some tendency to cross-agglutinate with heterologous sera, although cross reactions were more noticeable in the precipitation tests. Anti-S serum, for instance (table

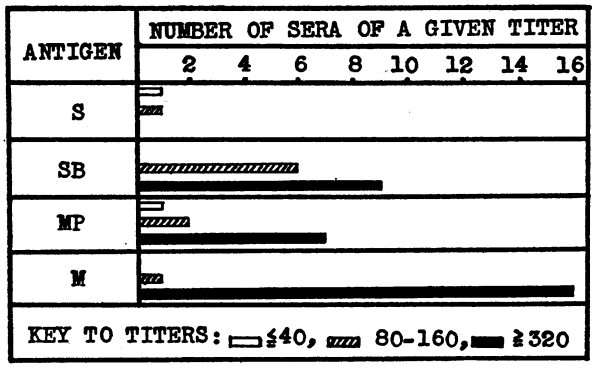

Figure 2 Titers of 44 normal human sera against their homologous variants.

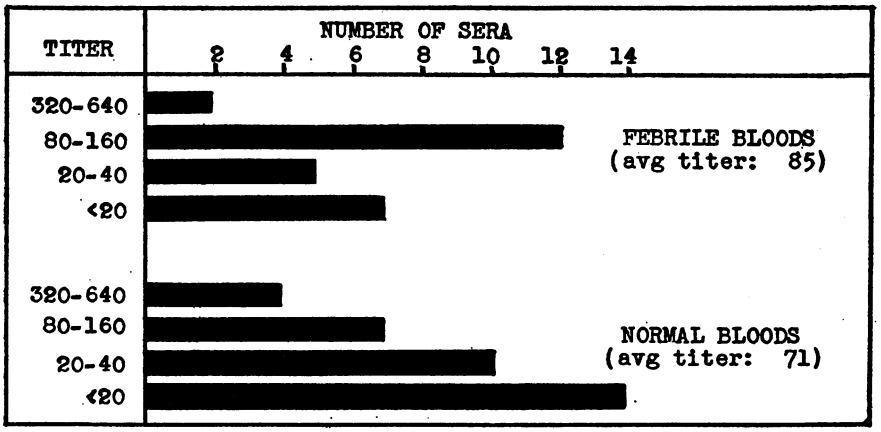

Figure 3. Agglutination titers of normal and febrile sera against stock smooth *Brucella* antigen.

2), gave a strongly positive precipitation test with most of the SB variants that were isolated from normal human bloods.

The data in table 2 include results obtained from precipitation tests with specific antisera against organisms from blood cultures and also against selected air-borne contaminants. The latter represent those bacteria which were obtained by exposing CTN plates to air for 60 minutes prior to routine incubation and which bore at least a superficial colonial resemblance to the *Brucella* variants obtained from blood cultures. The fact that none of these known air contam-

inants gave positive tests with the specific antisera serves as additional evidence that the organisms recovered from blood cultures did not represent normal flora of the air.

Quantitative agglutination tests were also made with the blood isolates against their homologous human sera (figure 2) and also with stock smooth *B. abortus* antigen against all normal and febrile sera (figure 3). The majority of these sera agglutinated their homologous strains at significant titer, and in addition several of the sera contained agglutinins at significant titers for the stock smooth *B. abortus* antigen. The data in figure 3, like those presented by Stoenner, Jenkins, and Bramhall (1949), Magoffin, Kabler, Spink, and Fleming (1949), and Sherwood, Downs, Miller, and Canuteson (1950), re-emphasize the fact that there is no specific titer that can be accepted as diagnostic for clinical brucellosis.

DISCUSSION

The procedures and interpretations that evolved during the course of our attempts to evaluate the extent of antibody inhibition in *Brucella* blood cultures have varied extensively since the work was initiated. Blood clots washed with saline were first employed, this procedure being similar to that recommended by West and Borman (1945) and Mickle (1949). It was then found that cultures became positive earlier when the broth was enriched with hemolyzed (distilled water) normal human erythrocytes. The growth enhancement produced by the hemolyzate was first thought to be referable to a nutritional factor but was soon traced to the presence of microorganisms in this supplement. This immediately indicated not only that hemolyzed specimens were preferable to clots, but also that normal bloods were not necessarily bacteriologically sterile. Accordingly, the cultural procedures were altered such that citrated specimens were taken, and these were washed with distilled water rather than saline. The effect of this change in procedure is indicated by the results obtained when four 5-ml citrated blood specimens, obtained from one of us, were cultured simultaneously: (1) specimen washed twice with saline, then overlaid with broth—negative culture at 14 days; (2) washed twice with distilled water, overlaid with broth—negative at 14 days; (3) washed twice with saline, overlaid with citrated broth —positive in 12 days; and (4) washed twice with distilled water, overlaid with citrated broth—strongly positive in 1 day (the SB variant was recovered from each positive culture).

Upon our adoption of citrated specimens washed with distilled water, cultures became uniformly positive with both normal and febrile bloods. The primary plates, if positive, yielded a variety of variants in small numbers, with the SB variant being somewhat the more commonly encountered. Secondary plates from 4- to 7-day citrated broth cultures, on the other hand, were usually strongly positive for the SB, and sometimes for the 16M or 33M, variant. These variants, though colonially quite atypical for smooth brucellae, were antigenically related to *Brucella abortus*, as indicated in the data presented above. Furthermore, microscopically, colonially, and antigenically similar variants have since been derived from stock smooth *Brucella abortus*, *Brucella suis*, and *Brucella*

melitensis. Additional evidence that these blood cultures represented variants of smooth brucellae was derived from the recovery of typical smooth brucellae from one guinea pig and from one of six chick embryos that had been experimentally infected with an SB variant.

The recovery of brucellae from clinically normal bloods introduced an entirely new aspect to the problem of *Brucella* blood cultures, and the problem was made more complex by the discovery at this time of several distinct phage plaques on a mucoid *Brucella* variant recovered from a febrile blood. This led to a search for *Brucella* phage from other sources, and the virus was found in several pools of culturally unstable *Brucella* variants obtained from normal and febrile bloods, in a pool of variants derived from a stock *Brucella abortus*, and in two normal human sera (Pickett and Nelson, 1950).

Details of the *Brucella*-phage system have not yet been elucidated, but our data at the moment suggest that this virus is only feebly lytic, that its activity is markedly affected by the concentration of calcium ion, that it effects the development of mucoid *Brucella* variants from known smooth brucellae, and that it is present in relatively high titer (10^2 to 10^3 in some normal human sera that contain no smooth *Brucella* agglutinins but is present in SC only in low titer (10^0 to 10^1) in other normal human sera that contain smooth *Brucella* agglutinins at titers of 640 to 1,280. It should be noted here too that, although the discovery of *Brucella* phage in normal bloods at first furnished an explanation for positive cultures from clinically normal humans, we now have considerable evidence that phage is also present in febrile (brucellar?) bloods. It is evident that recovery of this virus from normal but not from brucellar bloods would permit the construction of an attractive epidemiological hypothesis concerning this host-parasite-phage interrelationship.

It now appears likely that the washing of citrated blood specimens accomplishes primarily the removal of *Brucella* phage rather than of *Brucella* antibody, although the latter function has not been excluded. It is also clear that diagnostic media should contain a phage inhibitor, and we now have some evidence that either crystal violet or acriflavine may satisfactorily fulfill this requirement. The latter agent, particularly, has effected considerable reduction of both naturally occurring and experimentally induced phage infection in several of our stock smooth *Brucella* cultures.

SUMMARY

Citrated human blood specimens were washed with distilled water prior to culturing for brucellae. From such specimens microorganisms were routinely recovered, and these were identified, on the basis of their microscopical, antigenic, and pathogenic characteristics, as *Brucella*. With but few exceptions, however, all were nonsmooth variants. This procedure for handling *Brucella* blood cultures was discussed in respect to the results presented here and to the presence of concomitant *Brucella* antibody and *Brucella* phage in blood specimens.

REFERENCES

CRUICKSHANK, J. C. 1948 A simple method for testing dye sensitivity of *Brucella* species. J. Path. Bact., **60**, 328–329.

HENRY, B. S. 1933 Dissociation in the genus *Brucella*. J. Infectious Diseases, **52**, 374–402.

HUDDLESON, I. F. 1943 Brucellosis in man and animals. The Commonwealth Fund, New York.

MCCULLOUGH, W. G., MILLS, R. C., HERBST, E. J., ROESSLER, W. G., AND BREWER, C. R. 1947 Studies on the nutritional requirements of *Brucella suis*. J. Bact., **53**, 5–15.

MCVAY, L. V., GUTHRIE, F., MICHELSON, I. D., AND SPRUNT, D. H. 1948 Isolation of brucella from apparently healthy individuals. Proc. Soc. Exptl. Biol. Med., **69**, 607–608.

MCVAY, L. V., GUTHRIE, F., MICHELSON, I. D., AND SPRUNT, D. H. 1950 The isolation of brucella organisms from apparently healthy individuals. Yale J. Biol. Med., **22**, 661–673.

MAGOFFIN, R. L., KABLER, P., SPINK, W. W., AND FLEMING, D. 1949 An epidemiologic study of brucellosis in Minnesota. Public Health Repts., **64**, 1021–1043.

MICKLE, F. L. 1949 Culturing of blood clots for brucella organisms. Conn. State Dept. Health, Bureau of Laboratories, Division of Diagnostic Microbiology Laboratory Methods, Approved Method BI-3.

PICKETT, M. J., AND NELSON, E. L. 1950 *Brucella* bacteriophage. J. Hyg. *In press*.

PLASTRIDGE, W. N., AND MCALPINE, J. G. 1930 Microbic dissociation in the *abortus-melitensis* group. J. Infectious Diseases, **46**, 315–327.

SCHUHARDT, V. T., RODE, L. J., FOSTER, J. W., AND OGLESBY, G. 1949 An antibrucella factor in peptones. J. Bact., **57**, 1–8.

SHERWOOD, N. P., DOWNS, C. M., MILLER, W. R., AND CANUTESON, R. I. 1950 Incidence and titres of agglutinins for *Brucella abortus* and *P. tularensis* in the sera of 843 students. J. Kans. Med. Soc., **51**, 6–10.

SPINK, W. W. 1948 Pathogenesis of human brucellosis with respect to prevention and treatment. Ann. Internal Med., **29**, 238–258.

STOENNER, H. G., JENKINS, A. A., AND BRAMHALL, E. H. 1949 Studies of brucellosis in Utah. J. Infectious Diseases, **85**, 213–224.

WEED, L. A., AND DAHLIN, D. C. 1950 Bacteriologic examination of tissue removed for biopsy. Am. J. Clin. Path., **20**, 116–132.

WEST, D. E., AND BORMAN, E. K. 1945 The culturing of blood clots for *Brucella* organisms. J. Infectious Diseases, **77**, 187–192.