THE PROTECTION OF INTRACELLULAR BRUCELLA AGAINST THERAPEUTIC AGENTS AND THE BACTERICIDAL ACTION OF SERUM

By JAMES M. SHAFFER, M.D., CARRELL J. KUCERA, AND WESLEY W. SPINK, M.D.

(From the Division of Internal Medicine, University of Minnesota Hospitals and Medical School, Minneapolis)

PLATE 4

(Received for publication, August 25, 1952)

The ability of brucella to localize and remain viable within the cells of an infected host must be considered an important factor in explaining the relapses following therapy and the tendency toward chronicity of this disease. Experimental evidence supporting this concept has been provided by the observation of Magoffin and Spink (1) that intracellular brucella are protected *in vitro* against the lethal effect of therapeutic agents. In order to extend these observations a method has been developed which permits a quantitative *in vitro* study of the basic unit involved in brucellosis. This basic unit is a viable host cell containing viable intracellular brucella. Exudative leukocytes containing large numbers of intracellular brucella have been maintained *in vitro* in a synthetic tissue culture medium, or in human or animal serum. The methods used in these studies have permitted an evaluation of the effect of therapeutic agents and the bactericidal action of animal or human serum on intracellular brucella. In addition the metabolic status of the host cell has been correlated with its ability to protect the intracellular organisms. The metabolic activity of both host cells and intracellular organisms has been investigated by direct microscopic observation of the reduction of tetrazolium salts to pigmented formazans.

Methods

Adult, male white rats were injected intraperitoneally with 8 ml. of sterile sodium caseinate to produce a peritoneal exudate. The sodium caseinate was prepared from pure casein by the method of Martin *et al.* (2). 8 hours later 5 billion brucella organisms in 5 ml. of physiological saline solution were injected into the peritoneal cavity containing the exudate and *in vivo* phagocytosis allowed to proceed for 16 hours. Preliminary studies indicated that at the end of this time practically all the leukocytes in the exudate contained brucella and very few organisms remained in an extracellular position. The animal was then anesthetized and exsanguinated by cutting the large neck vessels. The skin of the abdomen was prepared with tincture of zephiran 1:1000 and the peritoneal cavity opened aseptically. 20 or 30 ml. of sterile heparinized (1:5000) Simms -X7 solution[1] was introduced into the peritoneal cavity through a

[1] All tissue culture media used in these studies were obtained from Microbiological Associates, Inc., Bethesda.

3 cm. to 4 cm. incision. The Simms solution was mixed with the peritoneal exudate by gentle agitation of the animal and the suspension of leukocytes in Simms solution removed from the peritoneal cavity by aspiration. 2 ml. of leukocyte suspension was distributed into each of two series of sterile, cotton-plugged, 15 ml. pyrex centrifuge tubes. The first series of tubes, designated as series A, was centrifuged at 1100 R.P.M. for 3 minutes, the supernatant decanted and discarded. The packed leukocytes were then washed by resuspending them in 2 ml. of heparinized Simms solution and again centrifuged for 3 minutes at 1100 R.P.M. After discarding the supernatant the washed, packed leukocytes were resuspended in 2 ml. of a synthetic tissue culture medium,[2] either as such or containing the therapeutic agent or agents to be tested for activity against intracellular brucella. After thorough mixing of the leukocytes containing intracellular brucella in series A with the tissue culture medium, incubation was carried out at 37°C. for 24 hours with gentle agitation at frequent intervals.

The second series of tubes, designated as series B, was handled in the same manner as series A except that the tubes were immersed in a water bath at 60°C. for 30 seconds before the leukocytes were washed and resuspended in the tissue culture medium. This exposure to heat was sufficient to injure the leukocytes but left most of the intracellular brucella in a viable state. series B consisting of living brucella in heat-injured leukocytes served as a control for series A which consisted of viable brucella in viable leukocytes. "Zero-time brucella counts" to determine the number of viable brucella per milliliter of the final suspension of leukocytes, prior to exposure to test agents, were obtained immediately before the tubes were incubated. These brucella counts were made with an agar pour plate method in which 0.5 ml. of tenfold dilutions of the leukocytic suspension in V-614 alone, from both series A and series B, were incorporated in trypticase soy agar[3] or Albimi brucella agar[4].

At the completion of the 24 hour period of incubation 1.0 ml. portions were removed from each tube in both series A and series B and transferred to sterile, cotton-plugged centrifuge tubes. The leukocytes in these samples were washed several times by centrifuging and resuspending them in sterile physiological saline to remove the test agents from the extracellular fluid. The washed leukocytes were finally resuspended in 1.0 ml. of sterile physiological saline. This suspension was then used to determine the "24 hour brucella counts" by the same agar pour plate method used to obtain the "zero-time brucella counts." After the period of incubation of 24 hours, portions of the remaining 1.0 ml. of leukocytic suspensions in each tube of series A and series B were used to determine the per cent of viable leukocytes remaining in each tube, and to prepare heat-fixed spreads on glass slides for Macchiavello stains. A neutral red vital staining method as described by Diggs (3) was used to determine the per cent of viable leukocytes in each tube. Finally, the remaining leukocytes and the brucella contained within them were exposed to neotetrazolium chloride[5] in concentrations of 0.003 to 0.0003 per cent for 12 to 24 hours at 37°C. Following exposure to neotetrazolium the leukocytes were examined microscopically as either air-dried, heat-fixed spreads on glass slides or by placing a small drop of the leukocytic suspension, which had been exposed to tetrazolium, on a clean glass slide and covering it with a coverslip.

The brucella organisms used in these studies were smooth, virulent strains originally isolated from the blood of patients with brucellosis and maintained in this laboratory for several years. The brucella suspensions injected into rats were prepared from the 24 hour growth of brucella on trypticase soy agar or Albimi brucella agar.

The therapeutic agents tested singly or in combinations against intracellular brucella in-

---

[2] Fischer V-614 medium, Microbiological Associates, Inc.

[3] Baltimore Biological Laboratory, Baltimore.

[4] Albimi Laboratories, Brooklyn.

[5] Montclair Research Corporation, Montclair.

cluded: streptomycin, aureomycin, terramycin, chloramphenicol, sodium sulfadiazine, sulfamerazine, cortisone, BAL (2,3-dimercaptopropanol), trypan blue, neopenil (penicillin G diethylaminoethyl ester hydriodide), and erythromycin (ilotycin). Each agent was tested over a range of concentrations from those attainable in human subjects to the highest concentration which could be utilized *in vitro*. This upper limit was determined by the solubility of the agent, by its toxicity for the leukocytes, or by unphysiological shifts in pH in the tissue culture medium.

Studies on the bactericidal effect of human or animal serum on intracellular and extracellular brucella were carried out in a slightly different manner. Leukocytes containing intracellular brucella were obtained from the peritoneal exudate of mice instead of rats. The exudate was produced by the intraperitoneal injection of 1.0 ml. of sterile sodium caseinate, followed 8 hours later by the intraperitoneal injection of one billion brucella. The suspension of intracellular brucella was collected 16 hours later as previously described. The sera used for these studies were obtained from rabbits or human subjects within 1 hour before the experiments were begun. The serum to be tested for bactericidal action was divided into four sterile, cotton-plugged centrifuge tubes. The intracellular brucella organisms contained in the mouse leukocytes were added to and mixed with the serum in two of the tubes. Control suspensions of extracellular brucella organisms were prepared by adding bacteria from a 24 hour growth on an agar slant to physiological saline solution and then mixing the preparation with the serum in the other two tubes. The number of viable intracellular brucella per milliliter of leukocytic suspension could not be determined prior to each experiment. However, preliminary studies indicated that 50 to 100 leukocytes per ml. of serum would, at "zero time," yield approximately the same number of viable brucella as the addition of 0.05 ml. of a saline suspension of brucella containing 50,000 organisms per ml. to each milliliter of serum. Aliquots from each tube for agar pour plate brucella counts were removed immediately after the mixing of the serum with the leukocytes or the serum with extracellular brucella, and at intervals up to 48 hours. The tubes were incubated at 37°C. during each experiment.

RESULTS

Vital staining with neutral red or tetrazolium indicated that in Fischer's V-614 tissue culture medium 60 to 80 per cent of the leukocytes were still viable after 24 hours *in vitro* at 37°C. The leukocytes did not survive as well in Simms solution or in a gelatin Tyrode solution. The survival time of leukocytes containing intracellular brucella was essentially the same as that observed with leukocytes which did not contain brucella. The addition of therapeutic agents to the tissue culture medium had variable effects on the survival of leukocytes *in vitro*, depending on the agent used and the concentration of drug employed. In general, it was possible to test most of the currently available therapeutic agents in concentrations considerably above the levels which could be obtained in human subjects, without producing a marked toxicity for the leukocytes. Macchiavello stains revealed that the peritoneal exudate produced by sodium caseinate consisted of approximately 60 to 70 per cent of polymorphonuclear neutrophile leukocytes with the rest of the cells consisting of macrophages, monocytes, and lymphocytes. The monocytes and macrophages consistently contained larger numbers of intracellular brucella per cell than did the polymorphonuclear leukocytes.

Effect of Therapeutic Agents on Intracellular Brucella

None of the therapeutic agents tested *in vitro*, whether used singly or in combinations, was capable of killing all the intracellular brucella within 24 hours. However, the most effective reduction in the number of living brucella present within viable leukocytes was accomplished by a combination consisting of streptomycin plus one of the bacteriostatic antibiotics, such as aureomycin, terramycin, or chloramphenicol. A remarkable protection of intracellular brucella against the lethal action of streptomycin is demonstrated by the data in Table I. Organisms from this strain of *Brucella suis* suspended in a cell-free medium were killed by streptomycin in a concentration of 2 μg. per ml.; yet considerable numbers of streptomycin-sensitive brucella were recovered from leukocytes when exposed to streptomycin in concentrations of 50,000 μg. per

TABLE I

Effect of Streptomycin on Intracellular Brucella suis

Zero time brucella counts: Series A—7,930,000
Series B—2,020,000

Streptomycin concentration	Per cent viable leukocytes (series A)	24 hr. brucella counts	
		Series A	Series B
μg./ml.			
100	75	910,000	20
1000	50	151,000	50
10000	26	19,400	26
50000	6	13,300	12

ml. for 24 hours. The data in Table I also demonstrate that this protection of intracellular brucella is dependent upon the presence of viable and intact leukocytes. Very few viable brucella could be recovered from the tubes in series B in which the leukocytes had been injured by heat prior to exposure to streptomycin. None of the leukocytes in series B was viable after 24 hours of exposure. In series A, 75 per cent of the leukocytes were viable after exposure to 100 μg. per ml. of streptomycin for 24 hours, indicating that this concentration of drug was not demonstrably toxic to the leukocytes. The higher concentrations of streptomycin were progressively more toxic to the leukocytes so that few cells appeared to be viable after 24 hours' exposure to 50,000 μg. per ml. Data are presented in Table II demonstrating the superiority of a combined type of therapy against intracellular brucella. Streptomycin plus terramycin or chloramphenicol was much more effective in reducing the number of viable intracellular brucella than was streptomycin alone or streptomycin plus sulfamerazine. Here again, injury to the leukocytes by heat markedly reduced the protection afforded the intracellular brucella. The survival of leukocytes in

series A was essentially the same for all four groups so that the increased effectiveness of the combined therapy could not be attributed to any obvious differences in toxicity of the drugs for the host cell.

Attempts to augment the action of streptomycin or other therapeutic agents against intracellular brucella were unsuccessful. Streptomycin plus trypan blue was little more effective than was streptomycin alone. The addition of BAL in concentrations of 5 μg. per ml. to streptomycin or chloramphenicol did not alter the activity of these drugs against intracellular brucella. Cortisone in concentrations of 25 μg. or 50 μg. per ml. had no significant effect on the kill-

TABLE II

Effect of Streptomycin Alone and When Combined with Other Drugs on Intracellular Brucella suis

Zero time brucella counts: Series A—3,380,000
Series B—2,150,000

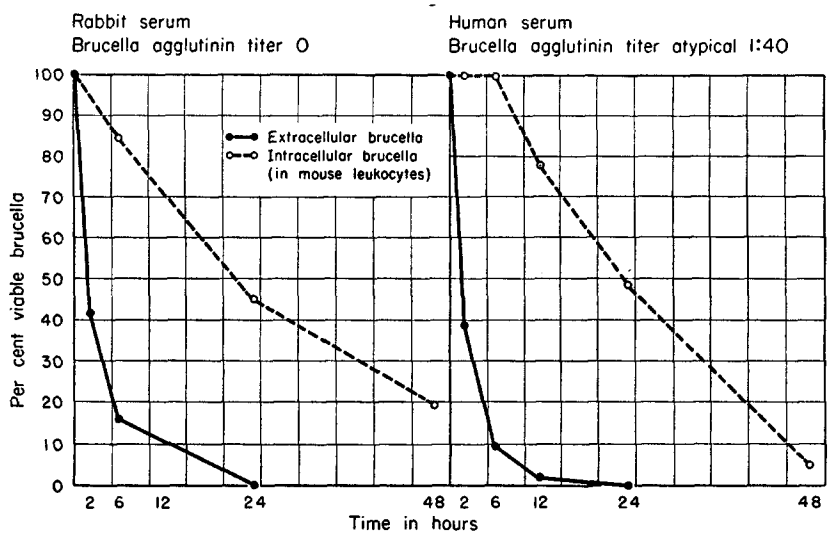
Drug and concentration	Per cent viable leukocytes (series A)	24 hr. brucella counts	
		Series A	Series B
μg./ml.			
Streptomycin—50	76	389,000	910
Streptomycin—50 Terramycin—50	76	79,000	1430
Streptomycin—50 Chloramphenicol—50	72	50,000	870
Streptomycin—50 Sulfamerazine—500	86	221,000	2570

ing of intracellular brucella by streptomycin. Neopenil was no more effective against a penicillin-sensitive strain of intracellular *Brucella abortus* than an equivalent concentration of crystalline penicillin G. Erythromycin in concentrations up to 100 μg. per ml. was no more effective against intracellular brucella than was streptomycin. Erythromycin was quite toxic for leukocytes *in vitro* in concentrations above 100 μg. per ml. Aureomycin, terramycin, or chloramphenicol alone in concentrations of 50 μg. to 100 μg. per ml. had some lethal activity against intracellular brucella. However, this antibrucella effect was much less pronounced than equivalent concentrations of streptomycin alone, and the essential action of these drugs was to suppress multiplication of the bacteria rather than to kill them. The methods used in these studies did not permit an evaluation of the effect of prolonged bacteriostasis on intracellular brucella. However, other studies *in vivo* (4) have shown that viable brucella

may be recovered from the tissues of mice treated continuously for 3 weeks with aureomycin or terramycin.

Bactericidal Effect of Serum on Intracellular and Extracellular Brucella

The protection of intracellular brucella against the bactericidal action of fresh rabbit serum or human serum is demonstrated in Text-fig. 1. With the sera used in these experiments extracellular brucella were rapidly killed so that only about 10 to 15 per cent of the organisms survived 6 hours' exposure to the sera *in vitro* at 37°C. No viable extracellular brucella could be recovered



TEXT-FIG. 1. Bactericidal effect of serum on intracellular and extracellular *Brucella abortus in vitro.*

from these sera after exposure at 37°C. for 24 hours. On the other hand, intracellular brucella were killed by these sera much more slowly. After 6 hours, about 80 to 85 per cent of the intracellular brucella were still viable. After 24 hours, between 40 and 50 per cent of the intracellular brucella were still alive. The human serum used in this experiment was obtained from the blood of an individual who was considered to be relatively immune to brucella infection. He had never had clinical evidence of brucellosis but he had been repeatedly exposed to infection in the laboratory over a period of 3 years. At one time his serum had shown a brucella agglutinin titer of 1:160. The bactericidal power of human serum was found to differ from one individual to another. Serum from another individual who had had brucellosis about 3 months previously was found to have a very weak bactericidal effect against either extracellular or intracellular brucella. The brucella agglutinin titer of this serum at the time

of testing was 1:160. With serum from this convalescent human case of brucellosis 80 per cent of the added extracellular brucella survived exposure for 12 hours at 37°C. No bactericidal effect on intracellular brucella could be demonstrated with this serum when leukocytes containing the organisms were exposed to the serum *in vitro* for 24 hours. At the end of 48 hours, 70 per cent of the intracellular brucella were still viable. Hall (5) has noted similar variations in the bactericidal action of human serum on extracellular brucella as well as a reduced bactericidal action of undiluted serum following the acute phase of human brucellosis. The observations that intracellular brucella are protected against the bactericidal action of serum shed some light on the mechanism whereby a brucella infection can persist despite the presence of a highly effective humoral defense mechanism.

Reduction of Tetrazolium Salts by Leukocytes and Brucella

Preliminary investigations indicated that neotetrazolium chloride (NT) was more satisfactory for the purposes of this study than was 2,3,5-triphenyltetrazolium hydrochloride (TPT). The intense purple-black formazan produced by the reduction of NT facilitated the microscopic observation of leukocytes and brucella. The orange-red formazan resulting from the reduction of TPT was found to be less stable and more lipoid-soluble. In addition the pigmentation of both leukocytes and brucella by the TPT formazan was much less intense than by the NT formazan. For these reasons the use of TPT was abandoned and subsequent studies performed only with NT.

Viable exudative leukocytes maintained in V-614 tissue culture medium rapidly reduced NT *in vitro* at 37°C. This reduction of NT by a suspension of viable leukocytes was visible in the gross as a red-purple color which became purple-black with time. The rapidity with which this gross color change occurred depended upon the concentration of NT and the per cent of viable leukocytes. It was found that when a concentration of NT of 0.003 per cent was in contact with bacteria or leukocytes for 12 to 24 hours, a maximum reduction of NT occurred and in this manner the metabolic activity of leukocytes by direct microscopic observation could be assessed. Microscopically, viable leukocytes produced intense purple-black granules within the cytoplasm or long sheaf-like crystals (Fig. 1). The ability of leukocytes to reduce NT decreased with time as the cells were maintained *in vitro*. Over 95 per cent of the fresh exudative leukocytes exposed to 0.003 per cent NT *in vitro* produced the characteristic formazan granules within the cytoplasm. After 24 hours *in vitro* from 60 to 80 per cent of the leukocytes were still capable of producing formazan granules. With these and other intermediate time intervals the per cent of leukocytes manifesting metabolic activity, as reflected by purple-black formazan production, closely corresponded to the per cent of leukocytes interpreted to be viable by the neutral red vital-staining method.

Heat-injured leukocytes did not reduce NT, nor did exposure to neutral red indicate viability. These observations revealed that NT reduction by leukocytes *in vitro* provided a reliable method for assessing the metabolic activity of an individual leukocyte. If a leukocyte on microscopic examination contained formazan granules it was considered to be exhibiting metabolic activity and to be viable. If the leukocyte did not exhibit formazan granules it was considered to be non-viable.

There was a variable reduction of NT by brucella populations. A 24 hour broth culture of brucella exposed to 0.003 per cent NT for 12 to 24 hours and subsequently examined microscopically exhibited all degrees of formazan production from no evidence of reduction to large purple-black granules within the bacterial cells, associated with a bizarre, swollen, and distorted appearance (Fig. 2). Perhaps the most characteristic appearance of brucella organisms exposed to NT was a cell containing one large, round, black unipolar granule. Heat-killed or phenol-killed brucella did not reduce NT. Some brucella exposed to lethal concentrations of streptomycin for as long as 48 hours retained their ability to reduce NT (Fig. 3). This reducing power was exhibited by relatively few cells in a bacterial population in which there were no viable organisms as determined by the usual cultural methods. Exposure of brucella to high concentrations of aureomycin, terramycin, or chloramphenicol for as long as 48 hours had little effect on the ability of the bacteria to reduce NT (Fig. 4). Exposure of brucella *in vitro* to streptomycin plus one of the broad spectrum antibiotics decreased but did not completely prevent NT reduction by some of the bacteria. Phagocytosis of brucella did not significantly alter their ability to reduce NT. Viable leukocytes containing large numbers of intracellular brucella when exposed to NT for 12 to 24 hours and then examined microscopically presented a characteristic appearance (Fig. 5). In such leukocytes the larger black granules resulting from the metabolic activity of the host cell could usually be easily distinguished from the smaller black granules within the cell bodies of individual intracellular brucella. Not all intracellular brucella reduced NT however, and it was not possible to assess the metabolic activity of the individual intracellular organism on the basis of formazan production alone.

Reduction of NT by leukocytes and brucella has provided a method applying both to the host cell and the intracellular parasite. From the available evidence it would appear that formazan production by a leukocyte is closely related to those vital processes which are indicated by the characteristic appearance of viable leukocytes stained with neutral red. On the other hand it has not been possible to demonstrate a consistent, clear cut relationship between the viability of brucella and their ability to reduce NT. The tetrazolium technique as applied in these studies has permitted microscopic examination of individual leukocytes containing intracellular brucella. From the appear-

ance of the leukocyte it has been possible to estimate the metabolic activity of the host cell, but it has not been possible to distinguish a viable from a non-viable intracellular organism with any degree of certainty.

DISCUSSION

The ability of brucella to localize and remain viable within the cells of an infected host appears to explain the tendency toward chronicity of this disease. It also explains the difficulty experienced in completely eradicating these organisms from the tissues by the administration of therapeutic agents. These studies and the observations of Magoffin and Spink (1) have shown that intracellular brucella are much less susceptible to the lethal effect of drugs than are extracellular brucella. These studies have also provided additional evidence supporting the clinical and experimental observations that the most effective therapy against brucella infections is a combined type of therapy with streptomycin plus aureomycin, terramycin, or chloramphenicol. It has also been possible to demonstrate that intracellular brucella are protected against the bactericidal action of serum, one of the few known mechanisms in brucellosis by which animals or man may prevent invasion of the tissues by brucella or overcome the infection once it has become established. This protective phenomenon of intracellular organisms is not unique for brucella. In 1916 Rous and Jones (6) demonstrated such protection with intracellular typhoid bacilli and more recently Barski (7), Mackaness (8, 9), and Suter (10) have shown that intracellular tubercle bacilli are protected against the lethal action of streptomycin. The studies reported here deal only with brucella contained within leukocytes maintained *in vitro* and cannot be assumed to reflect the role of the cells of the reticulo-endothelial system in brucellosis. The role of a cellular mechanism in the ultimate eradication of a brucella infection, if such exists, has not been demonstrated. From the present studies it would appear that phagocytosis of brucella by leukocytes did not kill the bacteria, nor did the presence of large numbers of organisms within the leukocytes injure the host cell. From these and other studies in experimentally infected animals the following concept of brucella infection can be derived:—

(*a*) Intracellular localization of brucella by phagocytosis favors the host by clearing the blood stream and localizing the infection.

(*b*) This intracellular localization of brucella favors the bacteria by maintaining them in a viable state and protecting them against attempts to eradicate the infection from the tissues.

(*c*) This biologic phenomenon accounts, at least in part, for the relative ineffectiveness of therapeutic agents or humoral defense mechanisms in brucellosis and the tendency toward chronicity of the disease.

The tetrazolium aspect of this study was stimulated by the reports indicating that the tetrazolium salts are reduced to pigmented formazans by a variety of

living tissues including leukocytes and bacteria (11–14). It was anticipated that viable leukocytes could be differentiated from non-viable leukocytes, and living from dead intracellular brucella by direct microscopic observation. This anticipation was not completely realized, but the tetrazolium studies did yield some provocative results relative to the effects of the antibiotics on brucella. Streptomycin, which is bactericidal for brucella *in vitro*, produced a quantitative reduction in the number of individual organisms capable of reducing neotetrazolium. Nevertheless, there were always a considerable number of brucella which, though non-viable on culture, apparently killed by streptomycin, were still able to produce intensely dark formazan granules in the presence of NT. The bacteriostatic antibiotics, aureomycin, terramycin, and chloramphenicol produced little alteration in the ability of brucella to reduce NT. These observations suggest that if the bacteriostatic mechanism of these drugs acts through inhibition of an enzyme system within the bacterial cell, that enzyme system is not the one involved in the reduction of NT. Evidence that bacterial viability may be destroyed before the ability of the organism to reduce the tetrazolium salts is lost has also been demonstrated by Bielig, Kausche, and Haardick (12). These investigators showed that formazan production by bacteria was not reduced by heat until a temperature 10° above the thermal death point for almost all the organisms was reached. They also showed that in old, resting cultures of *Escherichia coli* and *Salmonella* 50 to 80 per cent of the organisms showed no visible formazan granules. That these organisms, which did not reduce tetrazolium, were still viable was demonstrated by transferring the bacteria to fresh media in which active mitosis occurred. It therefore appears that the ability or inability of bacteria to reduce the tetrazolium salts is not necessarily synonymous with viability or nonviability of the organisms.

Brucellosis in man or animals is usually a self-limiting disease and the majority of brucella infections will eventually be controlled or eradicated by the host without specific therapy. The mechanisms by which this clearance of brucella is accomplished are still not clear. Humoral and cellular mechanisms both may be involved. It is known that the bactericidal action of blood or serum can be increased by an infection or immunization of animals (15–16). However, unless brucella cells become accessible to the action of blood, such an increase in the bactericidal action of the humoral defenses would be relatively ineffective. Circulating phagocytes appear to have little, if any, lethal effect on the brucella which become localized within these cells. But, Braude (17) has noted that in experimentally infected animals it was difficult to demonstrate brucella in the cells of the granulomas after the 5th day, indicating that within a relatively short time most of the brucella had been disposed of by the host. It was also his impression that a predominance of mononuclear cells in the granulomas was associated with a favorable course of the disease in the animals. These observations suggest that the cells of the reticulo-endothelial

system may exert a lethal effect on brucella *in vivo*. On the other hand, brucella can commonly be cultured from tissues in which no organisms can be seen microscopically, suggesting that only a balance between host and parasite is achieved rather than complete eradication of the parasite. Another possibility is that cells of a certain type may be primarily involved in perpetuating brucella infections. Goodpasture (18) has implicated the plasma cell as a factor in the chronic or carrier-type of typhoid infection. There is at present, however, no evidence that such a specific host-cell-brucella relationship exists as an explanation for the tendency toward chronicity in brucellosis. Investigations to date, then, have not clarified to any great extent the relative importance of humoral or cellular mechanisms in the control and eradication of brucella infections. The studies reported here only indicate that the effectiveness of extracellular factors is reduced by the protection afforded intracellular brucella.

It was repeatedly observed in these studies that streptomycin would kill many, but not all, of the intracellular brucella contained within exudative leukocytes. It was also observed that, within the range of concentrations nontoxic for leukocytes, increasing the concentration of streptomycin reduced the number of surviving intracellular organisms. These observations would indicate that streptomycin does penetrate to some extent into the leukocytes. Why then are some intracellular brucella protected against much higher concentrations of streptomycin? Our observations suggest two possible explanations. First, it could be postulated that streptomycin penetrates one or more types of leukocytes more effectively than other types. For example, the organisms within polymorphonuclear leukocytes might be killed by streptomycin more readily than those contained within monocytes. The only evidence supporting this hypothesis is the observation that the few leukocytes which survived exposure to the highest concentrations of streptomycin tested appeared to be monocytes, and from those few surviving leukocytes considerable numbers of living brucella were recovered. A second possible explanation is that when brucella localize within a cell both physical and chemical changes may occur which alter the ability of streptomycin to exert a lethal effect on the organisms. It was also frequently observed in these studies that brucella were not uncommonly localized within compartments in the leukocytes. Braude has observed a similar phenomenon within the histiocytes of experimentally infected animals (19). It would seem possible that such a "cell within a cell" situation could protect those brucella within a compartment more effectively and decrease the activity of an agent such as streptomycin.

The observation that streptomycin plus aureomycin, terramycin, or chloramphenicol was more effective in reducing the number of viable intracellular brucella organisms than any one drug alone is in agreement with a number of experimental and clinical reports (19–23). Earlier studies from this laboratory (24) suggested that streptomycin and sulfadiazine exerted a synergistic effect

on brucella in experimentally infected chick embryos. The results of these studies on intracellular brucella also indicate that the combined action of streptomycin and one of the bacteriostatic agents is more than just an additive effect.

SUMMARY AND CONCLUSIONS

A method for the *in vitro* study of intracellular brucella has been described. Exudative leukocytes containing intracellular brucella have been maintained *in vitro* in a synthetic tissue culture medium or in human or animal serum.

Intracellular brucella are protected *in vitro* against the lethal action of therapeutic agents or the bactericidal action of serum. This protection of intracellular brucella is dependent upon the presence of an intact, viable host cell.

None of the currently available therapeutic agents, whether used alone or in combinations, were capable of killing all intracellular brucella *in vitro* in 24 hours. A remarkable protection of intracellular brucella against streptomycin has been demonstrated. The most effective reduction in the number of viable intracellular brucella was accomplished by exposure of the host cells to streptomycin plus aureomycin, terramycin, or chloramphenicol.

The available evidence suggests that the ability of brucella to localize and remain viable within the cells of an infected host is an important biologic factor in establishing and perpetuating brucella infections, despite therapeutic measures or the operation of the host's humoral defense mechanisms.

Reduction of neotetrazolium by leukocytes and brucella *in vitro* provides a method for assessing the metabolic status of the host cell, but does not discriminate with any degree of certainty a viable from a non-viable intracellular organism.

BIBLIOGRAPHY

1. Magoffin, R. L., and Spink, W. W., *J. Lab. and Clin. Med.*, 1951, **37**, 924.
2. Martin, S. P., Pierce, C. H., Middlebrook, G., and Dubos, R. J., *J. Exp. Med.*, 1950, **91**, 381.
3. Diggs, L. W., A Textbook of Clinical Pathology, (F. P. Parker, editor), Baltimore, Williams & Wilkins Co. 3rd edition, 1948, 73.
4. Shaffer, J. M., Kucera, C. J., and Spink, W. W., *J. Immunol.*, in press.
5. Hall, W. H., 3rd Inter-American Congress on Brucellosis, Washington, D. C., 1950, 87.
6. Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, **23**, 601.
7. Barski, G., *Ann. Inst. Pasteur*, 1948, **74**, 1.
8. Mackaness, G. B., *J. Path. and Bact.*, 1952, **64**, 429.
9. Mackaness, G. B., and Smith, N., *Am. Rev. Tuberc.*, 1952, **66**, 125.
10. Suter, E., *Am. Rev. Tuberc.*, 1952, **65**, 775.
11. Antopol, W., Glaubach, S., and Goldman, L., *Pub. Health Rep.*, 1948, **63**, 1231; *Tr. New York Acad. Sc.*, 1950, **12**, 156.

12. Bielig, H. J., Kausche, G. A., and Haardick, H., *Z. Naturforsch.*, 1949, **4b,** 80.
13. Narahara, H. T., Quittner, H., Goldman, L., and Antopol, W., *Tr. New York Acad. Sc.*, 1950, **12,** 160.
14. Smith, F. E., *Science*, 1951, **113,** 751.
15. Irwin, M. R., and Ferguson, L. C., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38,** 451.
16. Huddleson, I. F., *Am. J. Vet. Research*, 1947, **8,** 374.
17. Braude, A. I., *J. Infect. Dis.*, 1951, **89,** 87.
18. Goodpasture, E. W., *Am. J. Path.*, 1937, **13,** 175.
19. Heilman, F. R., *Proc. Staff Meet. Mayo Clin.*, 1949, **24,** 133.
20. Herrell, W. E., and Barber, T. E., *J. Am. Med. Assn.*, 1950, **144,** 519.
21. Werner, C. A., and Knight, V., *J. Immunol.*, 1950, **65,** 509.
22. Spink, W. W., Hall, W. H., and Magoffin, R. L., *Arch. Int. Med.*, 1951, **88,** 419.
23. Kelly, E. H., and Gorelick, A. N., *J. Infect. Dis.*, 1952, **90,** 249.
24. Shaffer, J. M., and Spink, W. W., *J. Immunol.*, 1948, **60,** 405.

EXPLANATION OF PLATE 4

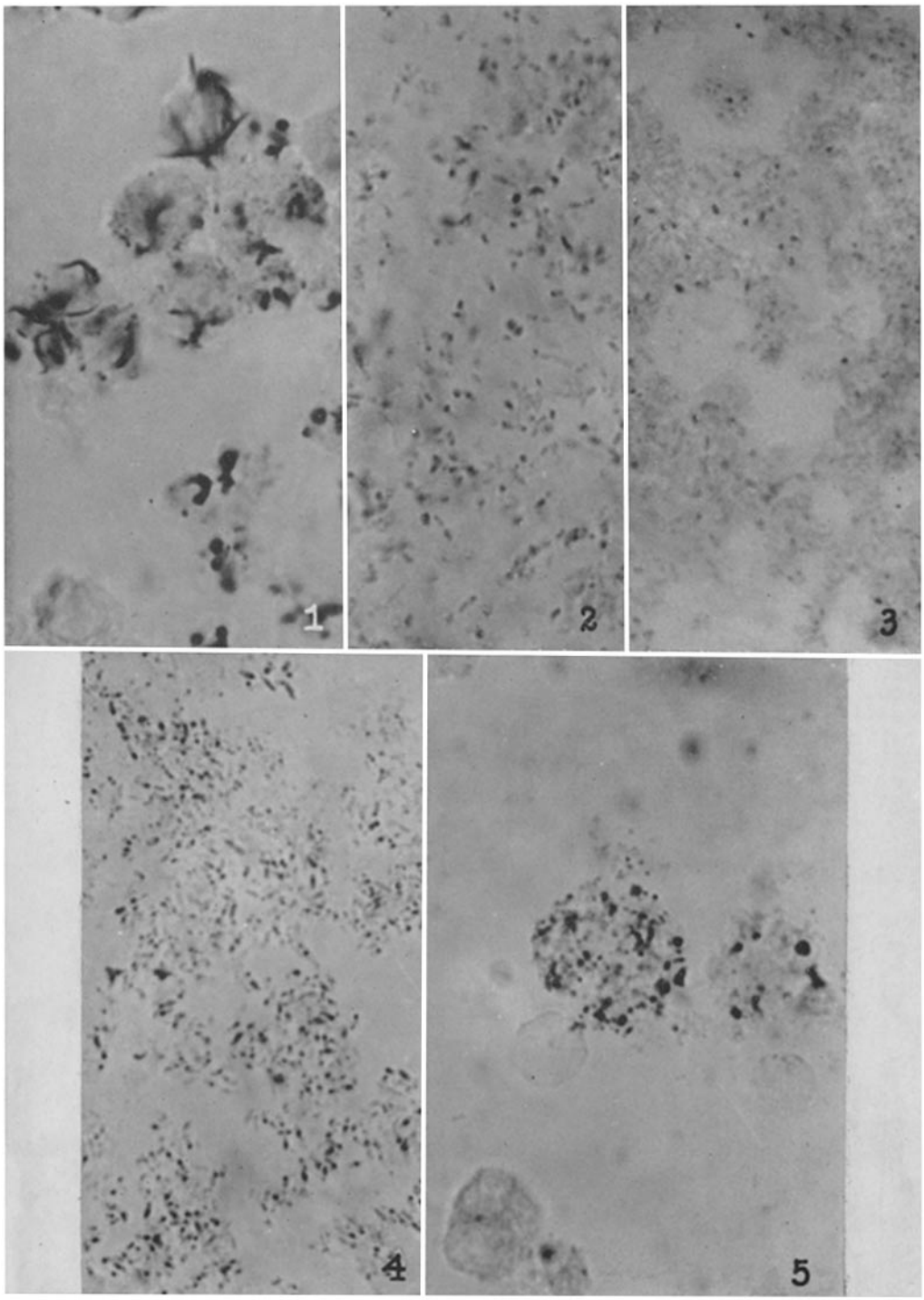
FIG. 1. Formazan granules within the cytoplasm and crystals produced by exudative leukocytes which were maintained in a synthetic tissue culture medium for 12 hours before exposure to neotetrazolium chloride. × 500.

FIG. 2. Reduction of neotetrazolium by a 24 hour broth culture of *Brucella suis*. Note variation in the shape, size, and intensity of staining of individual organisms. × 950.

FIG. 3. Reduction of NT by *Brucella suis* exposed to streptomycin (100 μg. per ml.) for 48 hours. No viable brucella could be recovered from this preparation before exposure to NT. × 950.

FIG. 4. Reduction of NT by *Brucella suis* exposed to chloramphenicol (100 μg. per ml.) for 48 hours. Viable brucella recovered from this preparation before exposure to NT. × 950.

FIG. 5. NT formazan production by a large leukocyte containing many intracellular brucella (center). Adjacent leukocytes show less formazan production indicating fewer intracellular organisms (lower center) and less metabolic activity (left upper). × 500.



(Shaffer *et al.*: Protection of brucella against therapeutic agents)