SYNERGISTIC ACTION OF STREPTOMYCIN WITH OTHER ANTIBIOTICS ON INTRACELLULAR *BRUCELLA ABORTUS* IN VITRO[1]

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ABSTRACT

RICHARDSON, MARVIS (Michigan State University, East Lansing) AND JANE N. HOLT. Synergistic action of streptomycin with other antibiotics on intracellular *Brucella abortus* in vitro. J. Bacteriol. **84**:638–646. 1962.—It has been found that streptomycin acts synergistically with penicillin and tetracycline to inhibit the growth of *Brucella abortus* within bovine cells cultured in vitro. Extracellular penicillin at 10 to 50 μg/ml was essentially bacteriostatic for intracellular brucellae. A combination of 5 to 50 μg/ml of streptomycin, which alone did not prevent the multiplication of intracellular brucellae, and 50 μg/ml of penicillin was bactericidal. Tetracycline at 0.5 to 1.0 μg/ml, which permitted growth or was bacteriostatic for brucellae within tissue cells, proved markedly bactericidal when combined with 10 μg/ml of streptomycin. Within certain limits, the synergistic effect of streptomycin and tetracycline did not appear to be a function of concentration. When the addition of streptomycin was delayed until 24 hr after the addition of tetracycline, the antibiotics acted synergistically on intracellular brucellae. To achieve intracellular synergism, five to ten times the effective extracellular concentration of streptomycin was required. Penicillin and tetracycline acted at the same concentration on extracellular and intracellular brucellae. These results suggest that tissue cell systems may prove useful for study of multiple drug therapy to be used in vivo against intracellular bacteria.

Antibiotics are commonly used to inhibit extracellular growth of bacteria during the period of intracellular bacterial multiplication in vitro. This serves to limit continued invasion of the tissue cells and to eliminate effects due to external bacterial products. After the demonstrations by Mackaness (1952) and Suter (1952) with mycobacteria, many workers studying intracellular bacteria of several other genera found that multiplication proceeds within tissue cells in a medium which contains ten to several hundred times the concentration of streptomycin that inhibits extracellular growth. For this reason, it has been assumed that streptomycin in low concentration does not penetrate tissue cells. Evidence to the contrary is accumulating. Freeman and Vana (1958) found that brucellae did not multiply within tissue cells under their experimental conditions when streptomycin at 10 μg/ml was present in the medium. Jenkin and Benacerraf (1960) reported growth of *Salmonella* in mouse macrophages with 1 μg/ml but not with 2 μg/ml of streptomycin. Showacre et al. (1961) demonstrated by phase microscopy that streptomycin at 20 μg/ml promptly inhibits intracellular multiplication of *S. typhosa*. Also, streptomycin at 10 μg/ml inhibits the growth of *Pasteurella tularensis* in strain L cells (Merriott, Shoemaker, and Downs, 1961) and in rat monocytes (McElree, *personal communication*), and inhibits the growth of *Shigella* in tissue cultures (Gerber and Watkins, 1961).

In this laboratory, streptomycin at 10 μg/ml has been found to act synergistically with penicillin against intracellular *Brucella abortus* in vitro (Richardson, 1959*a*). In the beginning of our study of parasitization, a combination of penicillin and streptomycin was used as an expedient to inhibit the extracellular growth of brucellae. Penicillin penetrates tissue cells in vitro (Eagle, 1954), but brucellae are considered resistant to penicillin. It was assumed that streptomycin in low concentration would affect only the extracellular organisms. A few brucellae were identifiable within tissue cells but extensive multiplication did not occur. Investigation of the factors responsible for failure of intracellular multiplication revealed inhibition by the com-

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bined antibiotics. Additional work, using the *Brucella*-cell system subsequently developed for quantitative studies (Richardson, 1959*b*), showed the action of streptomycin to be synergistic with penicillin. Although streptomycin alone at 10 μg/ml did not inhibit growth, the synergism with penicillin against intracellular brucellae seemed evidence of its presence within tissue cells. As indicated in a preliminary report (Richardson and Holt, 1960), the work has been extended to include a study of the action of tetracycline with streptomycin in low concentration, since streptomycin and the tetracyclines in combination have proved more effective than either alone for the treatment of brucellosis (McCullough, 1958; Spink, 1960). Also, several factors which might influence the intracellular effect of streptomycin have been examined.

MATERIALS AND METHODS

The materials and methods used were essentially those previously described (Richardson, 1959*b*). In brief, suspensions of primary fetal skin, adult uterine, and adult testicular cells were prepared by trypsinization of bovine tissues with 0.05% trypsin for 30 to 60 min at 37 C. Generally, streptomycin and penicillin at 25 μg/ml were used during the cell suspension and the first day of cell growth. Approximately 10^5 tissue cells in 1 ml of medium were grown as a monolayer in screw-cap tubes in an atmosphere of 5% CO_2 in air. Flying cover slips were included in some tubes. During a 4- to 8-day growth period, the medium was changed two or more times, usually at 24 hr and at intervals up to 72 hr thereafter. This ensured reduction of antibiotic concentrations to levels ineffective on brucellae. To minimize physiological shock to tissue cells and to maintain uniform bacterial intracellular growth conditions, a small number of tubes at a time was handled (at room temperature), changing the medium and gassing the tubes as rapidly as consistent with aseptic technique.

If microscopic observation of stained tissue cells on cover slips indicated that the cells were in good condition and proliferating, they were exposed to 10^3 to 10^8 brucellae 1 day after the medium had been renewed. This was done by addition of 0.1 ml of bacterial suspension to the medium in each tube. It was not necessary to regas the tubes if brucellae were added to the medium rapidly and the tubes sealed immediately. The tissue cells were exposed to extracellular brucellae for 5 to 24 hr at 37 C. The exposure period was terminated by replacement of the brucellae-containing medium with one containing the antibiotics to be tested, designated as zero hr in the experiments. If the test extended beyond 72 hr, the antibiotic-containing medium was renewed every 48 hr. Three or more tubes of tissue cells were employed at each concentration of antibiotic tested. To determine the number of intracellular brucellae per tube, the tissue cells were washed with Tryptose (Difco) diluent and lysed by shaking for 1 hr with distilled water containing 0.05% Tryptose. Serial dilutions in Tryptose diluent were prepared and the brucellae enumerated by colony count.

A lactalbumin hydrolyzate medium and a supplemented medium of Eagle (Richardson, 1959*b*) were used in the experiments on intracellular action of streptomycin and penicillin. An unsupplemented basal medium of Eagle in the Saline F of Puck, Cieciura, and Robinson (1958) was used in the experiments with streptomycin and tetracycline. Calf serum for this medium was obtained from a commercial source.

The antibiotics employed were: penicillin "G"-sodium (not less than 1,625 units per mg); streptomycin sulfate (not less than 740 μg per mg); and tetracycline hydrochloride (980 units per mg). The concentrations of antibiotics are expressed on a weight basis. Antibiotic stock solutions were prepared at a concentration of 5 mg per ml, stored at -10 C, and diluted in medium at the time of use.

The majority of the experiments were performed with CO_2-independent, smooth, virulent *B. abortus* strain 2308. Similar data were obtained with recently isolated CO_2-dependent strains.

RESULTS

To study intracellular multiplication of bacteria, it is necessary to limit the invasion of tissue cells by viable bacteria from the medium. Streptomycin at 10 to 100 μg/ml is commonly used for this purpose. Preliminary investigation showed that streptomycin *alone* at 2 to 50 μg/ml inhibited growth of *B. abortus* in the medium and at the same time allowed intracellular multiplication to proceed. Multiplication within the cells appeared independent of streptomycin concentration. However, it was recognized that streptomycin at a low concentration within the cell might be limiting. To provide a control for the study of the effect of antibiotics on intra-

cellular growth, various means were tried to inhibit the extracellular growth of brucellae. An antibiotic-free medium could not be used because *B. abortus* increased 100-fold in 24 hr in the tissue culture medium. When specific anti-*Brucella* sera with agglutination titers up to 1:5,120 were included at several percentages in the medium, growth of brucellae continued. At this time, the normal calf serum, which was present at 16% in the tissue cell culture medium, was obtained from a local slaughter house, filtered, and stored at 4 C until the supply was exhausted. Complement could have been limiting for bactericidal action of specific serum. In retrospect, it seems that the use of specific serum should have been examined also with complement added to the system. Changing the medium at frequent intervals after the exposure period reduced the number of extracellular brucellae but did not suffice to hold bacterial growth at a low level. Minimal conditions which would allow tissue cells to survive but reduce the growth rate of brucellae were not examined since our goal was the study of intracellular growth in metabolizing and proliferating tissue cells. Insofar as an antibiotic-free control was not available during this study, the number of intracellular brucellae in the presence of combined antibiotics has been compared with the number obtained with streptomycin alone.

Effect of streptomycin and penicillin on intracellular growth. The first experiments were designed to examine the effect of streptomycin alone, penicillin alone, and the two in combination. The tissue cells were exposed to 10^3 to 10^6 brucellae for 24 hr. Intracellular multiplication was followed by determining the number of brucellae when the antibiotics were added and at 12- to 48-hr intervals for periods up to 7 days. Figure 1 illustrates the results of one experiment with cells which contained 6×10^3 brucellae at the time the antibiotics were added. During a 6-day period, intracellular multiplication proceeded at the same rate with streptomycin alone at 2, 10, or 50 μg/ml in the medium. Multiplication proceeded with 2 μg/ml of penicillin, but penicillin at 10 μg/ml was bacteriostatic, the number of brucellae remaining close to the 0-hr count throughout the 6 days. With penicillin at 50 μg/ml, the number of brucellae dropped one log unit in 24 hr, but continued exposure did not reduce the count further. In combination,

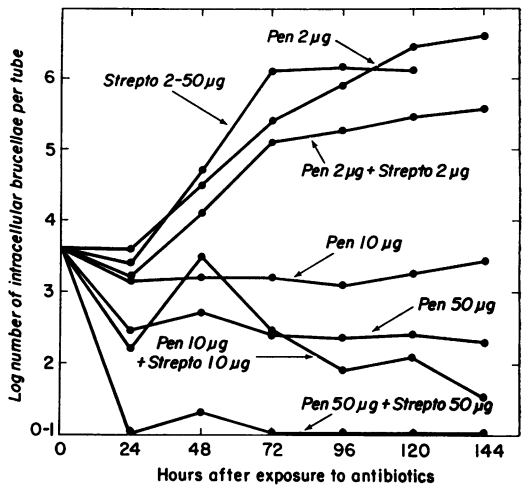

FIG. 1. *Effect of streptomycin and penicillin, alone and combined, on the intracellular growth of Brucella abortus. Bovine uterine mucosal cells in lactalbumin hydrolyzate medium with 5% CO_2 in air were exposed for 24 hr to 2 × 10^3 brucellae; 6 × 10^3 intracellular brucellae were present at 0 hr.*

streptomycin and penicillin appeared to act synergistically. The difference between streptomycin and penicillin at 2 μg/ml of each and penicillin alone probably is not significant. Prolonged exposure to 10 μg/ml of each antibiotic was more inhibitory to intracellular brucellae than was penicillin alone, and streptomycin and penicillin at 50 μg/ml of each proved bactericidal. Other experiments showed that a combination of 10 μg/ml of streptomycin and 50 μg/ml of penicillin was equally effective in sterilizing the culture. Thus, streptomycin at concentrations which permitted intracellular growth and penicillin at bacteriostatic concentrations appeared to act synergistically on the intracellular growth of *B. abortus*.

On the premise that cells from fetal and adult tissues might differ in permeability to antibiotics, several experiments were performed in parallel with cells from adult uterine mucosa and fetal skin cells. Similar results were obtained with fetal skin cells, primary and subcultured, and with uterine cells in primary culture or from a line maintained in vitro over 18 months. Apparently, antibiotics in low concentration penetrate bovine cells from adult uterine tissue as readily as fetal skin cells.

To exclude the possibility of genetic changes or

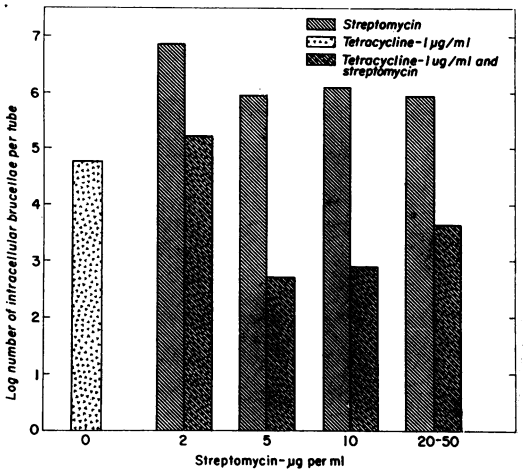

FIG. 2. *Effect of concentration of streptomycin, alone and combined with tetracycline at 1 µg/ml, on intracellular growth of Brucella abortus. Bovine fetal skin cells in Eagle basal medium with 5% CO_2 in air were exposed to 2 × 10^5 brucellae for 5 hr; 5 × 10^4 intracellular brucellae were present at 0 hr. Number of brucellae per tube represents the mean at 42 to 44 hr after addition of antibiotics.*

selection of tissue cells induced by the presence of antibiotics during the suspension of cells or during the first days of growth, primary uterine mucosal cells and fetal skin cells never exposed to antibiotics were employed in several tests. The cell preparation used to obtain the data in Fig. 1 was one of these. Eliminating antibiotics during cell suspension made no difference in the results.

Although the results obtained with streptomycin and penicillin were revealing and consistent, experimental conditions limited quantitative study. The bovine cells had been exposed to brucellae over a 24-hr period to ensure establishment within the cells at the time of addition of the antibiotics. Microscopic examination at 48 hr showed that the cytoplasm of some cells was filled with brucellae while other cells contained only a few organisms. Since penicillin acts only on dividing bacteria, it seemed probable that the antibiotic pair would affect brucellae phagocytized late in the 24-hr exposure period (and possibly in the lag phase of intracellular growth) at a different rate from those in the log phase when the antibiotics were added. In addition, if bacterial growth within the cell was unimpeded, the tissue cells ruptured in 2 to 3 days. Liberated brucellae invaded other cells to initiate another growth sequence. Progressive intracellular invasion and prolonged exposure to antibiotic approximated in vivo conditions, but the model complicated quantitative study of antibiotic synergism. For the next experiments, the exposure period was shortened to obtain synchronized intracellular multiplication, and the number of intracellular brucellae was determined only during the first growth sequence.

Effect of streptomycin and tetracycline on intracellular growth. For the experiments with streptomycin and tetracycline, the exposure period was 5 hr. Exploratory determinations had shown that a plateau in the uptake of brucellae was attained at 4 to 5 hr. Bovine tissue cells exposed to 10^5 *B. abortus* for 5 hr phagocytized about 10^4 brucellae under the test conditions. This number provided a sensitive system for study of antibiotic effects, and the results were reproducible. Except as noted, the number of intracellular brucellae was determined 42 to 44 hr after addition of antibiotics, prior to rupture of the tissue cells.

Preliminary investigation with streptomycin and tetracycline demonstrated synergistic action against intracellular brucellae. Ten µg/ml of streptomycin, which by itself did not inhibit intracellular growth, and 1.0 µg/ml of tetracycline, which alone was bacteriostatic, proved to be bactericidal in combination. Experiments were conducted to examine the effect of various concentrations of streptomycin in the presence of a constant concentration of tetracycline. Figure 2 represents the means obtained with approximately 15 tubes at each antibiotic concentration. It is apparent that streptomycin at 5 to 50 µg/ml acted synergistically with tetracycline at 1.0 µg/ml to inhibit intracellular growth. Occasionally, the combined antibiotics were not bactericidal, i.e., nine of the tubes with 20 to 50 µg/ml of streptomycin and tetracycline contained less than 10 brucellae but two tubes contained 10^4 brucellae. The graph indicates that streptomycin at 2 µg/ml did not enhance the activity of tetracycline. However, several tubes contained less than 10^3 brucellae, and a high percentage of the colonies were of "pinprick" size, indicating inability to multiply at a normal rate. A parallel series of tests was conducted, using tetracycline at 0.5 µg/ml with streptomycin at 2 to 50 µg/ml. The pattern of multiplication, bacteriostasis, and bactericidal

action essentially duplicated that shown in Fig. 2. From these and other similar experiments, it was concluded that the synergistic effect of streptomycin with tetracycline on intracellular *B. abortus* was not a function of streptomycin concentration within the 10 to 50 μg/ml limits. With 5 μg/ml of streptomycin, the results were less uniform than at higher levels. The results varied considerably with 2 μg/ml of streptomycin but synergistic effect was frequently evident.

Another series of experiments concerned the synergistic effect of various concentrations of tetracycline in the presence of 10 μg/ml of streptomycin. With tetracycline alone at 0.1 μg/ml, brucellae multiplied within the cells as rapidly as with streptomycin alone (Fig. 3). As the concentration of tetracycline increased to 1.0 μg/ml, intracellular multiplication decreased. Little or no multiplication occurred with 1 to 10 μg/ml of tetracycline alone, the intracellular numbers remaining near the 0-hr level. At each concentration of tetracycline, the streptomycin-tetracycline pair proved a more effective inhibitor than tetracycline alone. Increasing the tetracycline concentration beyond 1.0 μg/ml did not appreciably enhance the synergistic action.

The frequent occurrence of tubes in which little or no inhibition occurred necessitated summation of data from several experiments to determine the effect of variables. For example, it was not evident in single experiments whether

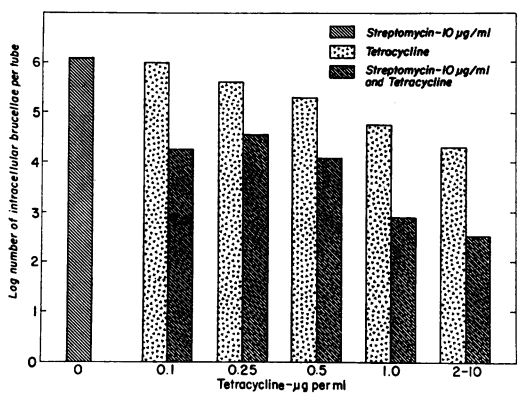

FIG. 3. *Effect of concentration of tetracycline, alone and combined with streptomycin at 10 μg/ml, on intracellular growth of Brucella abortus. Cells and conditions as in Fig. 2.*

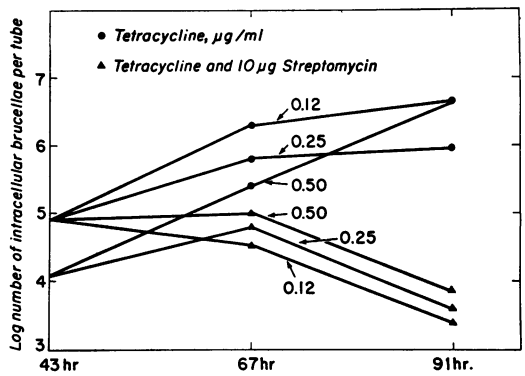

FIG. 4. *Effect of streptomycin added 24 hr after tetracycline on the intracellular growth of Brucella abortus. Cells and other conditions as in Fig. 2.*

0.5 μg/ml of tetracycline was as effective as 1.0 μg/ml when combined with 10 μg/ml of streptomycin. Summation of data showed that tetracycline was equally effective at 0.5 and 1.0 μg/ml. Thirty-six of 54 tubes tested with 0.5 μg/ml and 29 of 42 tubes tested with 1.0 μg/ml contained less than 2×10^2 brucellae; 44 of the 54 and 35 of the 42 contained less than 2×10^3 brucellae. Thus, combined antibiotics reduced the number of intracellular brucellae from 2×10^4 to less than 2×10^2 in 68% of the tubes and to less than 2×10^3 in 83% of the tubes. Statistically, on the basis of 96 observations, the chance that a tube would contain less than 10^2 brucellae was 56 to 80%.

Several experiments were carried out with duplicate cell cultures which contained 10^4 and 10^6 intracellular organisms at the time the antibiotics were added. With streptomycin at 10 μg/ml and tetracycline at 1 μg/ml, the results were less uniform with brucellae at the 10^6 level. However, after 2 days in the presence of combined antibiotics, the number of brucellae per tube was generally 1 to 2 log units less than in the presence of either antibiotic alone. Adult uterine and testicular cells as well as fetal skin cells were included in this series of experiments. The results were similar.

Effect of streptomycin added 24 hr after tetracycline. It seemed possible that streptomycin did not penetrate the tissue cell to exert its effect but that it acted only on brucellae at the cell surface when the antibiotics were added. The presence of tetracycline alone inside the cell might result in bactericidal action on strepto-

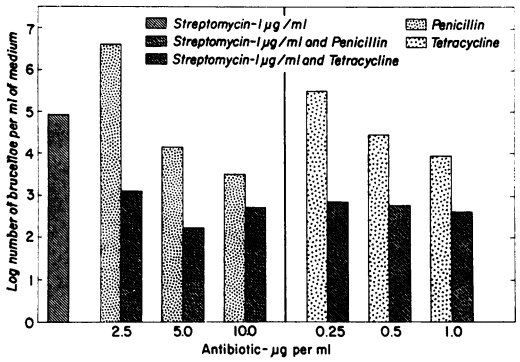

FIG. 5. *Effect of streptomycin, alone and with other antibiotics, on the growth of Brucella abortus in liquid medium. Eagle basal medium in saline F with 16% serum and 5% CO_2 in air; 2 × 10^4 brucellae/ml at 0 hr. Number of brucellae/ml determined at 48 hr; 10^9 brucellae/ml at 48 hr in medium without antibiotics.*

mycin-altered bacteria. To investigate this possibility, medium containing tetracycline *alone* was added after the initial exposure of cells to brucellae. Approximately 24 hr later, this medium was removed, the tissue cells rinsed, and the medium replaced with one containing (i) 10 μg/ml of streptomycin; (ii) 0.12, 0.25, or 0.5 μg/ml of tetracycline; or (iii) streptomycin and tetracycline combined. Since tetracycline is bacteriostatic for 48 hr at 1.0 μg/ml under these conditions, it was necessary to employ it at lower concentrations and to follow intracellular multiplication for several days to obtain a differential.

Figure 4 shows the results of one of six satisfactory tests. When streptomycin was added 24 hr after termination of phagocytosis, it acted synergistically with tetracycline on intracellular brucellae. With streptomycin alone, brucellae multiplied to 10^6 by 67 to 91 hr (not shown). In combinations, the antibiotics were bactericidal. Again this provides evidence that streptomycin at 10 μg/ml penetrates the bovine cell in active form in sufficient concentration to exert synergistic action with tetracycline.

Effect of the antibiotics on growth of extracellular brucellae. Figure 5 illustrates the effect of the antibiotics on the growth of *B. abortus* in one of the media used for the growth of tissue cells. An inoculum of 10^4 brucellae/ml was selected to approximate the number of intracellular organisms present when antibiotics were added to the cell system. The conditions employed in the tissue-cell system were adhered to, i.e., 1 ml of medium in 5% CO_2 in air, incubation at a 5° angle, etc. The number of brucellae was determined by colony count.

With no antibiotic present, brucellae increased in this medium to 10^8 to 10^9 in the 48-hr period (not shown). Streptomycin alone at 1.0 μg/ml was bacteriostatic. This contrasts markedly with progressive multiplication of intracellular brucellae which occurred with up to 50 μg/ml of streptomycin in the medium. Moreover, it is evident that streptomycin at 1.0 μg/ml was capable of acting with penicillin or tetracycline to reduce the number of viable brucellae in the medium. To achieve a synergistic effect regularly on brucellae within tissue cells required 10 μg/ml of streptomycin.

Penicillin at 5 to 10 μg/ml and tetracycline at 0.5 to 1.0 μg/ml were bacteriostatic for brucellae in the medium. At these concentrations, penicillin and tetracycline were effective against intracellular organisms both in bacteriostasis and acting synergistically with streptomycin. Whether the effect demonstrated in the medium with combined antibiotics is an additive or a synergistic one has not been examined carefully. Synergism would be more apparent with each antibiotic of the pair at a level which permits rapid growth in the medium. Under our experimental conditions, uniform results were not obtained when concentrations of streptomycin less than 1.0 μg/ml were tested in combination with the other antibiotics. However, penicillin at 2.5 μg/ml, which permitted rapid growth of brucellae, proved bactericidal in combination with 1.0 μg/ml of streptomycin. Results of the same order were obtained regularly with 1.0 μg/ml of penicillin (not shown) and frequently with 0.1 μg/ml of tetracycline in combination with streptomycin. This would indicate synergism of the antibiotic pairs in the medium. Designation of the effect of the combined antibiotics on intracellular *B. abortus* seems justified insofar as growth proceeded at all concentrations of streptomycin alone and a bactericidal effect resulted from the combined antibiotics when the second drug was present in bacteriostatic concentration or less.

DISCUSSION

Only limited use has been made of tissue-cell techniques to study synergism of antibiotics

within the cell. Two of the three specific infections of man which are more effectively treated by combined drugs than by one alone are brucellosis (streptomycin and the tetracyclines) and tuberculosis (streptomycin, isoniazid, and *p*-aminosalicylic acid). Although the causative agents of both diseases are intracellular parasites, in vitro infected cells have not been exploited as experimental models. No doubt this stems from the early recognition of protection of intracellular brucellae and tubercle bacilli from high concentrations of streptomycin, the common drug in synergistic drug combinations. Suter (1952) found that it required 80 to 100 μg/ml of streptomycin to inhibit intracellular tubercle bacilli, whereas 0.5 μg/ml was effective in the medium. Shaffer, Kucera, and Spink (1953) investigated the effect of streptomycin on *B. suis* in leukocytes. Although streptomycin at 2 μg/ml killed *B. suis* of this strain in liquid medium, 50 or 100 μg/ml of the antibiotic only reduced the number of brucellae in the leukocytes one log unit. This remarkable protection of intracellular bacteria from streptomycin alone led to the general assumption that streptomycin at levels which can be maintained in vivo (10 μg/ml for short periods in humans) does not penetrate cells. Mackaness and Smith (1953) found that tubercle bacilli in macrophages were resistant to streptomycin at 10 μg/ml and to isoniazid alone, but they were killed by subinhibitory concentrations of the two acting simultaneously. The correlation of these data with clinical findings is well known. The fact that intracellular synergism could be demonstrated with 10 μg/ml of streptomycin seems to have been ignored. Intracellular synergism with low levels of streptomycin has been demonstrated in our work with *Brucella*. Also, Hopps et al. (1961) were able to eradicate typhoid organisms from 2 of 19 cultures of strain L fibroblasts exposed for at least 3 weeks to streptomycin at 20 μg/ml combined with other antibiotics. Singly, these agents only reduced the number of intracellular bacteria.

Reviewing multiple-drug therapy, McDermott (1958) emphasized that synergism is a highly specialized phenomenon and that more effort should be devoted to testing drug susceptibilities of individual species under conditions simulating their environment in vivo. *B. abortus* within bovine cells seems to provide such a system, in that cattle are the natural host. The synergistic action of streptomycin, with tetracycline or penicillin, on intracellular brucellae in vitro offers presumptive evidence of intracellular synergism in vivo. Although streptomycin-tetracycline remains the therapy of choice for treatment of brucellosis, particularly in severe cases (McCullough, 1958; Spink, 1960), a high and similar incidence of relapses occurs with either streptomycin-tetracycline or with tetracycline alone (Farid et al., 1961). Streptomycin is known to act only on growing bacteria. It may not function in vivo with agents such as tetracycline which are primarily bacteriostatic. On the other hand, the concentration of streptomycin may be a critical factor. To achieve synergism of streptomycin with tetracycline on intracellular brucellae in vitro required five to ten times the concentration of streptomycin which was effective outside the cell.

The superiority of streptomycin-penicillin over either drug alone for treatment of enterococcal endocarditis provides the outstanding example of synergism in vivo. Recently, Fisher et al. (1960) found that penicillin alone and streptomycin alone were only moderately effective against *Streptococcus faecalis* in mice, but when both agents were given together the infecting organisms, which cause pyelonephritis in this species, were eliminated. The streptomycin-penicillin drug pair has not been investigated with brucellae in vivo. Brucellae are considered resistant to penicillin. But this is a relative term. Kempe (1958) pointed out that infections by gram-negative organisms resistant to the usual antibiotics have been successfully treated by massive doses of penicillin. It might prove effective in multiple-drug therapy for gram-negative organisms.

Why does streptomycin alone not kill intracellular brucellae, as it does in cell-free medium? Under our experimental conditions, intracellular brucellae multiplied with 50 μg/ml of streptomycin outside the cell. This antibiotic seems only to pave the way for damage which occurs with the synergistic pair. It has been suggested that streptomycin might act on bacteria still on or close to the cell surface at the time antibiotics are added. The presence of only the other drug of the synergistic pair inside the cell could result in bactericidal action on the streptomycin-altered bacteria. Our demonstration of intracellular synergism when streptomycin was added

24 hr after tetracycline seems to preclude this possibility. The findings of Anand and Davis (1960) indicate that streptomycin damages the cell membrane of the susceptible bacterium. They suggest that this mechanism could explain the synergism between streptomycin and other bactericidal agents, in that the injury to the permeability barrier would permit entry of other drugs. The effect of streptomycin alone may be of little consequence to brucellae in the tissue-cell milieu. Discussing intracellular infections in the light of the findings of his group, Smadel (1960) theorized that microorganisms reversibly damaged by antibiotics might survive in cells, as a protoplast does in the proper osmotic solutions, but not in ordinary media. He believes that the original injury may be the same but that the reversible state of the organisms within the cell is of longer duration. Our finding of intracellular synergism with streptomycin at 5 to 10 μg/ml in the medium rules out tissue-cell impermeability as the cause of failure of streptomycin to act on intracellular brucellae. However, if the tissue cell is not completely permeable, the concentration attained in the cell may be inadequate for bactericidal action or growth inhibition alone, although sufficient to potentiate the effect of the synergistic pair.

Streptomycin at 0.5 to 1.0 μg/ml exhibits synergism against brucellae in nutrient medium. It is possible that it acts at this concentration within the cell. Mackaness and Smith (1953) stated that with 10 μg/ml of streptomycin in the medium the effective concentration in macrophages was of the order of 0.5 μg/ml. Whether this was based on the minimal concentration required for equivalent activity in the extracellular medium or on chemical analysis is not apparent. Eagle (1955) found that radioactive streptomycin enters certain mammalian cells in tissue culture but data on concentration are not available. Regardless of the explanation for the failure of streptomycin alone to inhibit intracellular multiplication, it is apparent that streptomycin at 5 to 10 μg/ml penetrates the bovine cell in tissue culture in sufficient concentration to exert synergistic action with other antibiotics.

ACKNOWLEDGMENT

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