GROWTH OF *BORDETELLA PERTUSSIS* IN TISSUE CULTURE

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The use of tissue culture for cultivation of bacteria has received comparatively little attention in contrast to its extensive use in propagating viral agents. Intracellular multiplication of brucellae has been reported in both normal and immune mononuclear phagocytes (Pomales-Lebron and Stinebring, 1957) and in chick embryo fibroblasts (Holland and Pickett, 1956). Investigations of the mycobacteria in explants dates from the earliest days of tissue culture. Recently, with the use of monolayer techniques, Shepard (1957*a*, *b*) has demonstrated multiplication of several members of this genus in cultures of HeLa cells, monkey kidney cells, and in human amnion cells. Growth of tubercle bacilli has also been reported in rabbit and guinea pig macrophages (Mackaness, 1952; Suter, 1952) maintained in tissue culture.

The cell damaging effect of *Bordetella pertussis* on human and kitten brain tissue explants was shown by Felton *et al.* (1954). These investigators found no evidence of bacterial multiplication with this system. They also found that the damage phenomenon did not occur if HeLa cells were used in place of the tissue explants. In their work, emphasis was on cell damage, and the presence of antibiotics in the system may have prevented bacterial multiplication.

During the course of experiments with tissue cultures in this laboratory, it was noticed that monolayers of monkey kidney cells in a medium free of antibiotics became heavily infected within a few days after the inoculation of *B. pertussis*. This observation seemed worthy of further investigation since such a system offered a potential method for additional studies of the effect of bacteria on host cells and of the effect of host cells on the growth of the microorganism. With emphasis on the latter aspect, the results of experiments designed to extend the observation of the growth of *B. pertussis* in tissue culture are presented in this report.

MATERIALS AND METHODS

Strain 18323 of *B. pertussis* was prepared for tissue culture inoculation as described in the National Institutes of Health booklet, *Minimum Requirements: Pertussis Vaccine* (1952). Briefly, this consisted of 3 transfers of the bacteria from the freeze-dried state on Bordet-Gengou (B-G) medium; the growth of the final transfer was suspended in a solution of an enzymatic digest of casein (protolysate) and adjusted to 10 opacity units in a colorimeter. This suspension, containing approximately 2.5×10^9 bacteria per ml as determined by colony count, was used within 1 hr after preparation.

The kidneys from rhesus monkeys were prepared for tissue culture following trypsinization by the method of Youngner (1954). Cells were grown and maintained in medium 199 (Morgan *et al.*, 1950) containing 2 per cent calf serum. The cell suspension containing approximately 4.0×10^5 cells per ml, which was used for the preparation of tissue cultures, was dispensed in amounts of 1.5 ml in tubes, and 6.0 ml in 2-ounce prescription bottles.

KB and HeLa seed cells[3] were purchased and were adapted for growth and maintained in medium 199 containing 10 per cent calf serum. The volumes of cell suspensions used in the preparation of these tissue cultures were the same as used with monkey kidney cells, and contained 175,000 KB or HeLa cells per ml.

A line of nasal epithelial cells grown from human nonmalignant tissue and designated DMB was also used.[4] This line was initially grown in Hanks salt solution containing 40 per cent human serum, and was maintained in medium 199 with

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10 per cent calf serum. The inocula for preparing these tissue cultures contained 175,000 cells per ml, and were used in volumes as indicated above.

Tissue cultures of all cell types were 5 to 7 days old when inoculated with bacteria, and were incubated at 34 to 35 C during the course of the experiments. The initial pH of the tissue culture fluids in all experiments was approximately 7.4. The tissue cultures were prepared in media containing 100 units of penicillin and 100 μg of streptomycin per ml. Unless otherwise indicated, the cultures were washed twice and maintained in medium 199 with no antibiotics 24 hr before use and washed twice again, immediately before use.

Two methods were used for the demonstration of multiplication of *B. pertussis* in tissue culture, and for the dependence of growth upon presence of actively metabolizing epithelial cells: (a) observations were made of the multiplication of *B. pertussis* in tissue cultures as indicated by bacterial plate counts on B-G medium, and, (b) stained tissue culture preparations were made and observed at various intervals following the inoculation of the microorganisms.

Three nutrient media were used in these studies, none of which contained antibiotics unless otherwise indicated:

Medium 1 consisted of medium 199 with 2 per cent calf serum.

Medium 2 consisted of medium 199 collected from cultures of uninoculated monkey kidney cells after 5 to 7 days of growth in medium 199 containing no antibiotics. This was designated "old" medium 199.

Medium 3 consisted of an extract prepared by the sonic disruption of packed monkey kidney cells in a 10,000 kc Raytheon oscillator for 15 min. Following centrifugation at 3000 rpm in an International centrifuge size 2 model V (2000 $\times$ G) for 30 min, the supernatent fluid was combined with an equal volume of medium 199 containing 4 per cent calf serum, and was filtered through a Seitz sterilizing pad.

Cultures of the various cell types were grown on cover slips in Leighton tubes for those experiments showing bacterial multiplication by microscopic examination of stained tissue culture preparations. The tissue culture preparations growing on cover slips were inoculated with *B. pertussis* and divided into 2 groups. Group A received antibiotics following a 4-hr exposure to the microorganism; group B was not exposed to antibiotics at any time after the 4-hr exposure to the microorganisms. The tubes of group A, following 16 hr of antibiotic treatment, were again divided into A1 and A2. Those of A1 remained on antibiotics for the duration of the experiment. The exposed, antibiotic treated preparations of A2 were carefully washed after the 16 hr antibiotic treatment to remove the antibiotics, and the fluid was replaced with medium 1 free of antibiotics. The tubes of all groups received a homologous change of media on the 4th and 7th days following inoculation with bacteria. Cover slips were removed from the Leighton tubes at various intervals, fixed with methyl alcohol, stained with Giemsa stain, and examined for bacterial multiplication.

RESULTS

Infection and antibiotic exposure time. The time necessary, within the four intervals studied, for the bacteria to remain in contact with the cells in order to produce infection is shown in table 1. (Infection here indicated localization of bacteria within or on the epithelial cells.) These data also show the time required, within the three intervals studied, for antibiotics to kill all bacteria in the liquid medium. Following the inoculation of tube cultures of monkey kidney cells with an arbitrarily selected dose (0.03 ml) of *B. pertussis*, the period of infection was terminated at various intervals by decanting the fluid medium, washing once, and replacing with medium 1 containing antibiotics. Again at various intervals, this antibiotic-containing medium was carefully washed from the cultures and replaced with medium 1 containing no antibiotics. From this point, 0.1 ml aliquots of the nutrient fluid were removed from each tube daily and plated on B-G medium. Control tubes not containing kidney cells were treated in exactly the same manner. Media in all tissue culture tubes and controls were replaced on the 4th day of incubation.

It may be noted that an exposure of the cells to bacteria for a period of 40 min did not insure a uniform infection of the cell sheet since cellular degeneration did not occur and recovery of *B. pertussis* was erratic. Therefore, a 4 to 6-hr infecting time was selected for use in subsequent experiments. An antibiotic exposure time of 16 hr was chosen as a matter of convenience since it was apparent that all of the times tested were suffi-

TABLE 1

Relation of infecting and antibiotic exposure times to growth of Bordetella pertussis in monkey kidney tissue cultures

Time Incubated with Bacteria*	Antibiotic Exposure Time	No. of Colonies from 4-Day Plate Counts of *B. pertussis* Recovered per 0.1 ml Tissue Culture Fluid					
		Immediately after antibiotic treatment	Days in tissue culture				
			1	3	5	7	9
	hr						
B. Pertussis plus monkey kidney:							
40 min	3	4	0	0	0	0	—†
40 min	8	0	0	0	0	0	0
40 min	20	0	0	0	0	0	0
4 hr	3	0	0	0	0	400	TN‡
4 hr	8	0	0	0	0	100	TN
4 hr	20	0	0	0	0	600	TN
20 hr	3	0	0	0	0	550	TN
20 hr	8	0	0	0	0	550	TN
20 hr	20	0	0	0	0	93	TN
9 days	0		40	TN	TN	TN	TN
B. pertussis plus 199 only:							
40 min	3	12	0	0	0	0	0
40 min	20	0	0	0	0	0	0
20 hr	20	0	0	0	0	0	0
9 days	0		TN	TN	TN	TN	TN

* All tubes were infected with 1.5×10^6 organisms in 0.03 ml.

† Occasional recovery of *B. pertussis* was noted under these experimental conditions.

‡ TN = Too numerous to count, and the first evidence of epithelial cell degeneration.

cient to kill the bacteria present in the liquid medium. The data summarized in table 1 also show that epithelial cell degeneration did not occur until the organisms were abundantly present in the liquid medium. The bacteria recovered from the control shown in table 1 (pertussis in medium 199 for 9 days) were from the inoculum. The recovery of these bacteria did not represent growth of pertussis in medium 199. It was evident that the supporting epithelial cells were necessary for survival of *B. pertussis* following antibiotic exposure.

Similar studies were made comparing the growth of the microorganism, with and without antibiotic treatment, in the other 3 epithelial cell types. The results were directly comparable with those obtained with monkey kidney cells.

Growth characteristics as evidenced by bacterial plate counts. To determine the effect of medium 199 alone on the multiplication of *B. pertussis*, and to determine whether or not the epithelial cells were merely supplying a required growth factor, culture vessels with and without monkey kidney cells (and containing medium 1), were inoculated and assayed at various intervals for bacterial multiplication. Similarly, culture vessels containing monkey kidney extract (medium 3) were inoculated and assayed at various intervals. The results of these experiments are shown in figure 1.

Also shown are the results of attempts to grow *B. pertussis* under 3 other conditions. (a) Tissue cultures that had been killed by autoclaving at 118 C for 15 min were overlaid with medium 1, inoculated, and assayed for bacterial multiplication. (b) A 32-ounce bottle culture of monkey kidney cells containing a sterile bag of Visking tubing was prepared. *B. pertussis* was suspended in medium 1 and placed in the bag through glass connections. The bag was submerged in medium 1 and remained in direct contact with the monkey kidney cells; aliquots of the contents of the bag were plated daily on B-G medium. (c) Medium 2 ("old" medium 199) was inoculated with *B.*

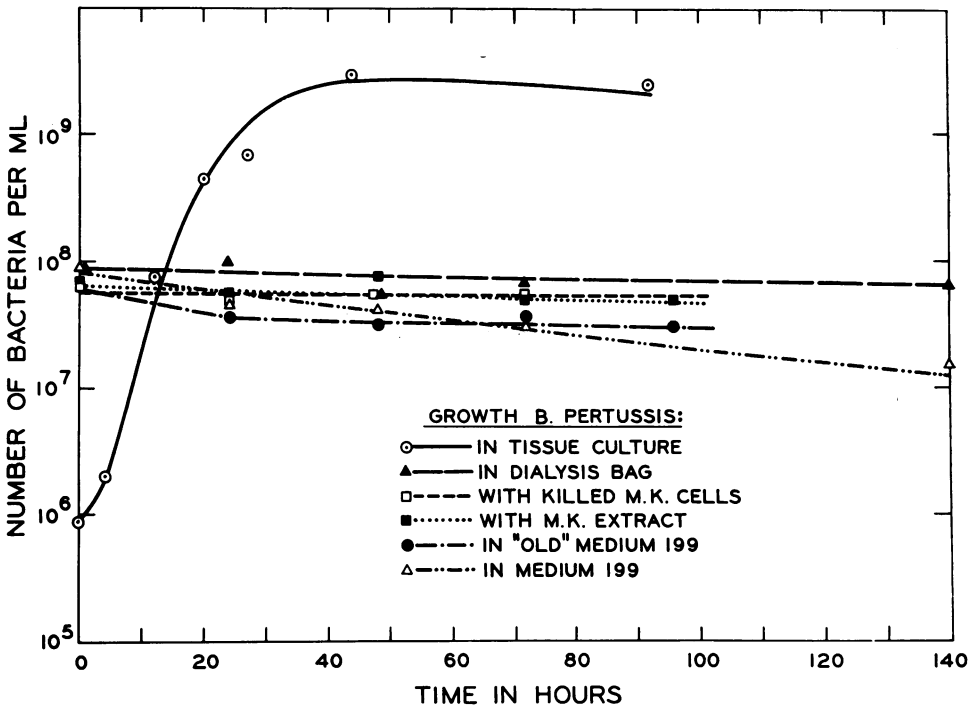

Figure 1. Growth characteristics of *Bordetella pertussis* in tissue culture

pertussis and assayed for bacterial multiplication at intervals.

These data show that medium 199 alone did not support the growth of *B. pertussis*. The failure of bacterial multiplication to occur in medium 2 or medium 3 alone, in the presence of killed monkey kidney cells, or when separated from the cells by a semipermeable membrane, would indicate that bacterial multiplication was not dependent upon an essential growth substance contained within the cells nor upon a diffusible metabolite produced by the tissue culture cells.

The results given in figure 1 are based upon two amounts of inoculum. The 10^6 inoculum was included to demonstrate a typical growth curve. In the absence of viable tissue cells, no apparent multiplication of *B. pertussis* was noted, regardless of the size of the initial inoculum.

Microscopic examination of infected tissue cultures. Leighton tube preparations of epithelial cells were infected with *B. pertussis* and, following treatment with and without antibiotics, the appearance of the bacteria in tissue culture systems was noted by microscopic examination of the cover slips which were removed from the tubes at various intervals and stained. Typical results of these experiments are shown in figures 2 to 4.

Figure 2 indicates the degree of infection of monkey kidney cells after 4 hr of exposure to the bacteria, and, in the absence of antibiotics, the gross multiplication which occurred within the 3-day interval. Bacteria were present in or on the cells after 12 days of continuous exposure to antibiotics following the initial 4-hr period of infection. Cellular degeneration was not evident at this time as shown by comparison with the uninfected tissue culture.

Figure 3 is a representative series of monkey kidney cells which were exposed to the bacteria for 4 hr and to antibiotics for 16 hr. The media of all tubes were changed at this time with one half receiving antibiotics and the other half no antibiotics. Cover slip preparations were stained and examined at various intervals following this change. It was noted in all of the epithelial cells examined that, after the 16 hr of antibiotics and regardless of the antibiotic content of the replacement medium, there was a gradual decrease in the number of bacteria in the cells. This condition prevailed until the 7th day in the cultures which contained media free of antibiotics. Between the

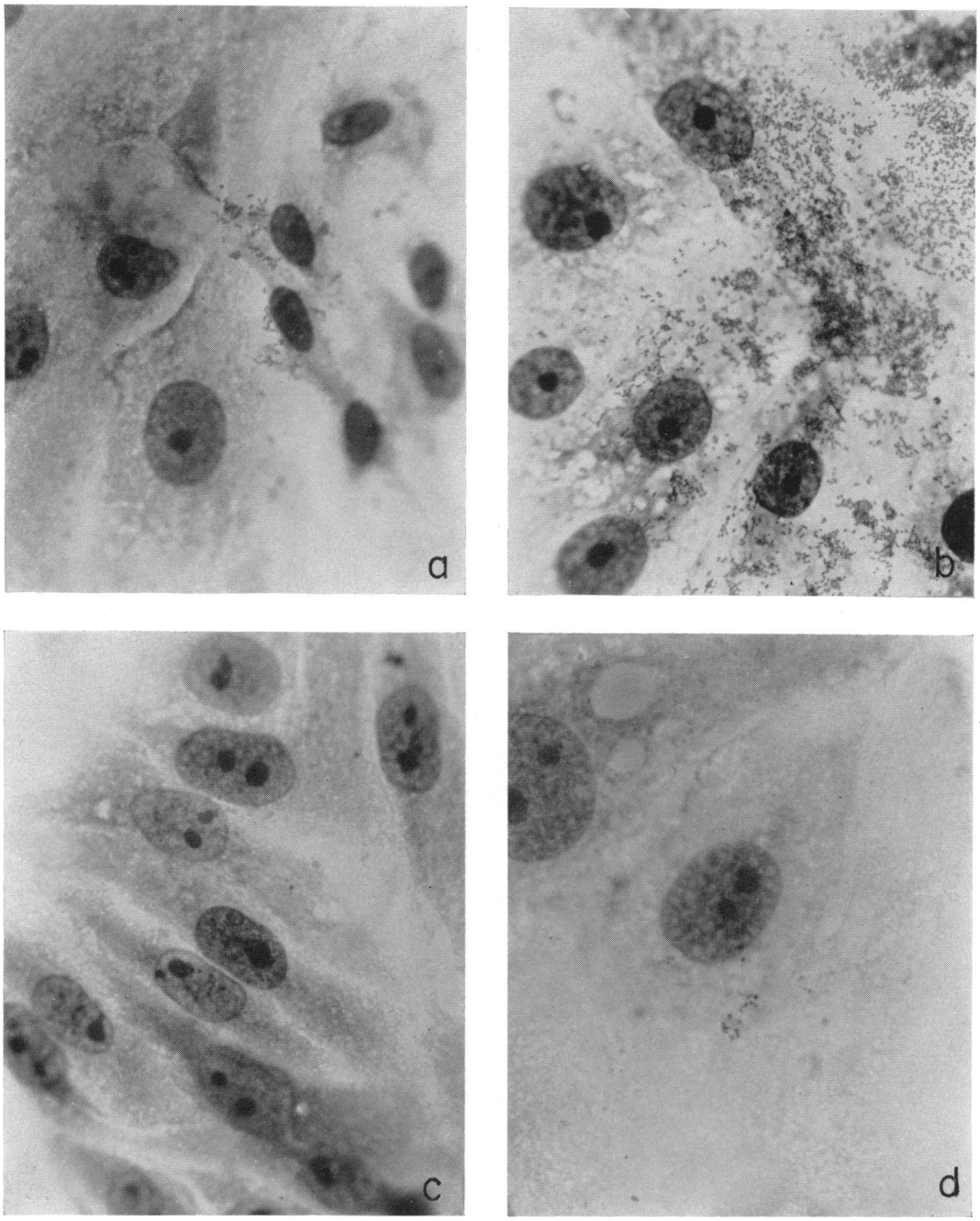

Figure 2. Bordetella pertussis in tissue culture (×1386). *a.* Monkey kidney cells after 4 hr infection. *b.* Monkey kidney cells after 3 days infection. *c.* Noninfected monkey kidney cells. *d.* Monkey kidney cells infected for 4 hr followed by 12 days of antibiotic treatment.

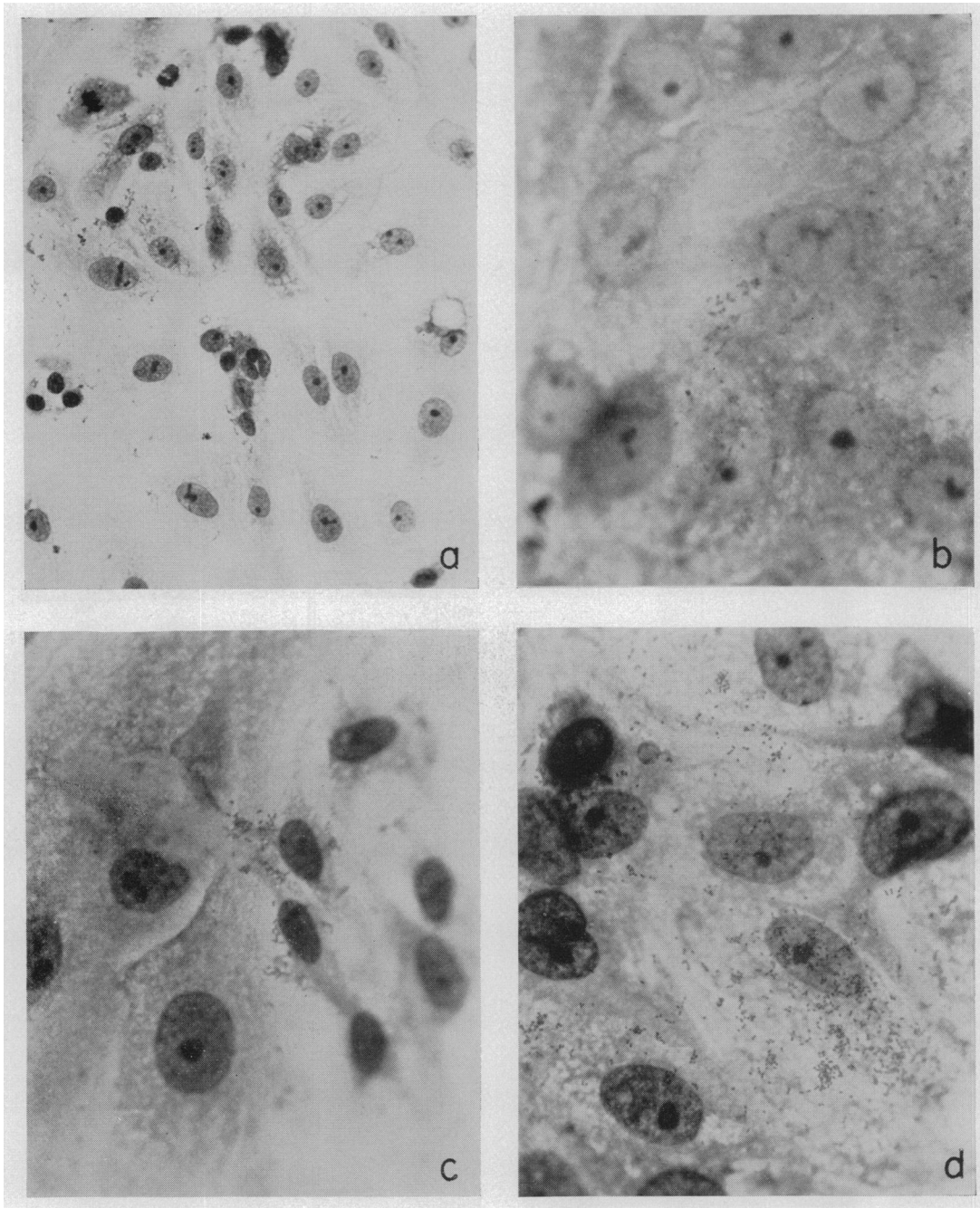

Figure 3. Bordetella pertussis in tissue culture. *a*. Monkey kidney cells infected for 4 hr followed by 16 hr antibiotic treatment (×532). *b*. Monkey kidney cells infected for 4 hr followed by 16 hr antibiotic treatment, then by 4 days without antibiotic treatment (×1368). *c*. Monkey kidney cells infected for 4 hr followed by 16 hr antibiotic treatment, then by 6 days without antibiotic treatment (×1368). *d*. Monkey kidney cells infected for 4 hr followed by 16 hr antibiotic treatment, then by 7 days without antibiotic treatment (×1368).

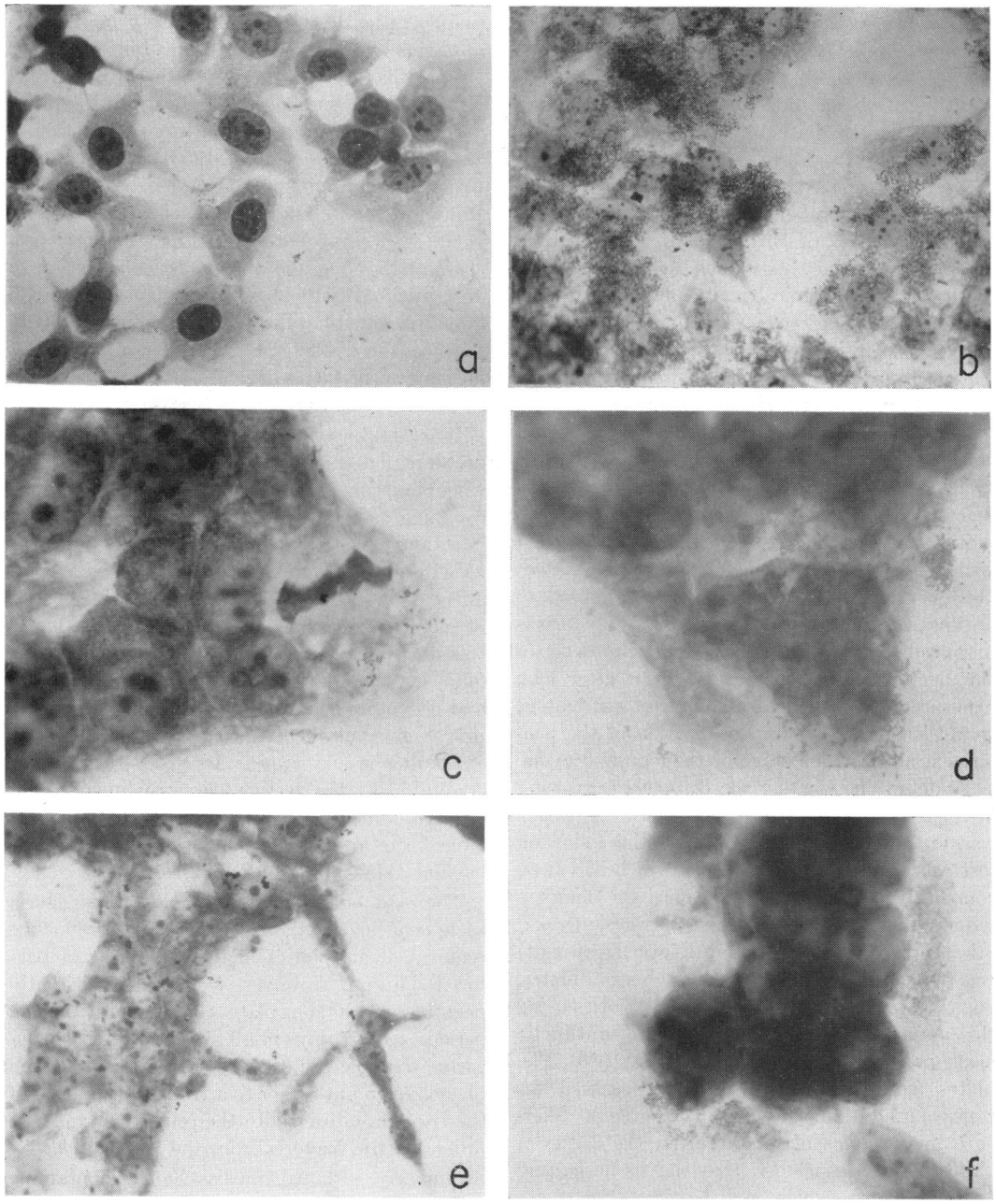

Figure 4. Bordetella pertussis in tissue culture. *a*. HeLa cells after 4 hr infection (×532). *b*. HeLa cells after 3 days infection (×532). *c*. DMB cells after 4 hr infection (×1368). *d*. DMB cells after 3 days infection (×1368). *e*. KB cells after 4 hr infection followed by 16 hr antibiotic treatment (×532). *f*. KB cells after 4 hr infection followed by 16 hr of antibiotic treatment, then by 8 days with no antibiotic treatment (× 1368).

7th and 9th days, gross multiplication of the bacteria occurred. This observation is correlated with the results of the experiment shown in table 1. Multiplication of the microorganism did not occur while the epithelial cells were in contact with antibiotics; at least, not within the experimental limit of 12 days.

The same general growth pattern was observed when HeLa, KB, or DMB cells were substituted for monkey kidney as shown in figure 4. The epithelial cells became heavily infected within 2 to 3 days following the bacterial inoculation in the absence of antibiotics. However, when the infected cells were treated with antibiotics, suppression and delay of growth was observed.

These results demonstrated that all of the cell types used in the present study supported the multiplication of *B. pertussis* and, moreover, all reacted in much the same manner. With no antibiotic treatment, bacterial multiplication occurred within 2 to 3 days after the infection was initiated. However, if the infected cells were exposed to antibiotics, the same delay as noted in table 1 was manifested in all of the cell types. The presence of the bacteria in tissue cultures even after long exposure to bactericidal amounts of antibiotics, would suggest a cellular localization of the bacteria in at least one stage of their growth cycle.

To determine whether the *B. pertussis* growing in tissue culture fluids after antibiotic treatment was an antibiotic resistant form, the following test was performed. *B. pertussis* which had been consecutively passaged 10 times in monkey kidney tissue cultures (2 of the passages, 4th and 8th, were recovered after antibiotic treatment) was plated on B-G medium. On each set of plates, cups were placed and filled with antibiotics using this modification of a standardized antibiotic assay procedure (Grove and Randall, 1955). The plates were then incubated and observed for zones of inhibition. These results showed no difference in the areas of inhibition on the 2 sets of plates. It was concluded that the tissue culture grown bacteria which included the 2 passages propagated after antibiotic treatment were as sensitive to antibiotics as the nontissue culture grown, nonantibiotic exposed, *B. pertussis*.

DISCUSSION

The cultivation of an organism, such as *B. pertussis* in tissue culture under conditions where bacterial growth depends upon the presence of living epithelial cells, suggests new methods of approach to many of the problems concerned with the host-parasite relationship. Other investigators have used this method to demonstrate the intracellular protection of brucella from the action of antibiotics (Shaffer *et al.*, 1953) and antiserum (Magoffin and Spink, 1951). As far back as 1916, Rous and Jones found that phagocytized typhoid bacilli were protected against the lethal action of chemical poisons and the bactericidal action of antiserum with the protection dependent upon living phagocytic cells. The intracellular localization of these bacteria may be an important mechanism of their survival and may be related to clinical manifestations of disease.

It is well known that chemotherapeutic measures are not as successful in the treatment of whooping cough after the onset of paroxysmal coughing as they are with many other bacterial infections. The appearance of a stage in the growth of *B. pertussis* where the bacteria are not affected by antibiotics may explain the variance of success in the chemotherapy of disease due to this microorganism. The recovery of *B. pertussis* following the short-term exposure to penicillin and streptomycin and the persistence of the microorganism when continually exposed to the antibiotics would appear to make this system adaptable to the testing or screening of other chemotherapeutic agents. Tissue culture methods have been so used in the case of the tubercle bacillus (Mackaness, 1952).

The 7- to 9-day period required for the bacteria to appear in the tissue culture fluids following removal of antibiotics, may be a result of bacterial dilution. However, from the consistently negative results on plate counts made during periods up to 7 days in all experiments, it is suggested that the results were not due to a dilution phenomenon but rather to a true incorporation of the bacteria into or onto the cells which in turn, protected the bacteria from the antibiotics.

Similarly, although media changes containing no antibiotics were made after the 16-hr exposure time, and again on the 4th day, it is possible that the delay of 7 to 9 days in the appearance of the bacteria in the fluids was caused by minute amounts of antibiotic that was in the nutrient media or adsorbed to the cells. The apparent difference in the bacterial count shown in table 1 (40 colonies recovered on the first day) and the growth curve in figure 1 may have been due to

residual antibiotic in the cultures of the monkey kidney cells.

In addition to the theoretical aspects, the ability to cultivate *B. pertussis* in tissue culture may have certain practical advantages. The selection of the smooth form of the bacteria is generally believed essential for the preparation of pertussis vaccine for human use. It may be important, therefore, that any medium used for production purposes must be able to support the growth of this bacterial phase. It is interesting to note that the bacteria recovered after the 10th serial transfer in tissue culture mentioned above, retained characteristics of the smooth form; they were highly hemolytic when cultured on B-G medium, failed to grow on nutrient agar, were virulent for mice (although to a lesser degree) via the intracerebral route of inoculation, and were agglutinated with specific immune serum.

The necessity of incorporating blood (Bordet-Gengou), charcoal (Ensminger *et al.*, 1953), or starch (Pollock, 1947; Cohen and Wheeler, 1946) makes the usual media scarcely less complex than a well-established tissue culture system.

The selective activity of cells in tissue culture as a means to differentiate virulent and nonvirulent strains of bacteria has been reported by other investigators (Rogers and Tompsett, 1952; Goodman *et al.*, 1956; Shepard, 1957*a*, *b*). It has also been observed (Holland and Pickett, 1956) that, when a mixture of smooth and nonsmooth forms of brucella was incubated in the presence of fibroblasts, the smooth form multiplied preferentially whereas the nonsmooth form was suppressed and failed to survive. Further investigation of this nature on the behavior of *B. pertussis* in tissue culture may be of interest. This system may also lend itself to a study of host sensitivity at a cellular level. It may be of interest to exploit tissue culture systems for the selection and stabilizing of strains for vaccine production.

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SUMMARY

Multiplication of *Bordetella pertussis* was found to occur in tissue culture preparations of monkey kidney, HeLa, KB, and DMB strains of tissue cells. The bacteria failed to grow in the nutrient medium 199 alone, in medium 199 containing monkey kidney extract, in the presence of killed supporting cells, or when separated from these cells by a semipermeable membrane. *B. pertussis* infected tissue cultures, incubated up to 12 days in the presence of antibiotics, were overgrown and destroyed by the bacteria 7 to 9 days after removal of the antibiotics.

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