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Growth of *Leptospira pomona* and Its Effect on Various Tissue Culture Systems[1]

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ABSTRACT

MILLER, ROBERT E. (University of Nebraska College of Medicine, Omaha), NORMAN G. MILLER, AND ROBERTA J. WHITE. Growth of *Leptospira pomona* and its effect on various tissue culture systems. J. Bacteriol. **92**:502–509. 1966.—*Leptospira pomona* strain 3341 was grown in association with primary fetal bovine kidney (PBK) and human embryonic skin-muscle fibroblastic (HE) cells in Eagle's minimal essential medium (MEM) with 5% sheep serum. Growth curves of leptospires in PBK and HE cell cultures showed no substantial increase in growth above that obtained in Eagle's MEM in the absence of tissue culture cells. This suggested that no stimulatory growth factors for leptospires were produced by the tissue cells. Fibroblastic cells of the PBK monolayer showed separation, deterioration, and, finally, complete disintegration. Epithelial-like cells remained unaffected. HE cells showed the same cytopathic effect as PBK fibroblastic cells, indicating that this effect was not limited to PBK fibroblastic cells. Warthin-Starry stains of PBK and HE cell monolayers showed masses of leptospires adhering to fibroblastic cells, whereas only a few were seen on epithelial-like cells. Large numbers of leptospires on the surface of fibroblastic cells are very likely associated with the cytopathic effect. Dislodgment of leptospires from fibroblastic cells did not increase the total number of spirochetes in the culture. This indicated that leptospiral growth did not occur on the surface of these cells.

The mechanism by which pathogenic leptospires produce deleterious effects on various tissues and specific cells in man and animals is poorly understood at the present time. Since tissue culture monolayers have been found to be of value in studying the relationship of a variety of bacteria to tissue cells, it was thought that this technique might also be applied to leptospires.

Boyd (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1959) attempted to grow *Leptospira pomona* in embryonic chick liver, but he was unsuccessful because of difficulty in maintaining cell cultures in media devoid of antibiotics. Faine, Shahar, and Aronson (3) demonstrated phagocytosis of *L. icterohaemorrhagiae* by macrophages, and Rose, Eveland, and Ellinghausen (Bacteriol. Proc., p. 70, 1965) showed phagocytosis of *L. pomona* by monocytes in tissue culture systems. Ellison et al. (2) reported that there was no appreciable increase in *L. canicola* in canine kidney tissue cultures, nor was there any evidence of a cytopathic effect (CPE) on these cells. Vosta and Polednikova (15) were successful in producing a CPE on swine kidney monolayers with intact and disrupted leptospires. Harrington and Sleight (5) also reported a similar CPE on fetal bovine monolayers inoculated with *L. pomona*. However, there is no indication of growth of leptospires in either of these tissue culture systems.

This paper describes the growth of *L. pomona* in two tissue culture systems, as well as its relationship to and CPE on the cell monolayers of both systems.

MATERIALS AND METHODS

Test organism. L. pomona strain 3341, obtained from A. D. Alexander of the World Health Organization Reference Laboratory, Walter Reed Army Institute of Research, Washington, D.C., was used in this study. This organism was maintained in Fletcher's and Stuart's media (Difco) with 10% rabbit serum until it was found that it could be grown in Eagle's minimal essential medium (MEM) with 10% ovine serum. It was subsequently subcultured in the latter medium throughout this study.

[1] Taken in part from a thesis submitted by R. E. Miller in partial fulfillment of the requirements for a M.S. degree at the University of Nebraska College of Medicine, Omaha.

Test sera. A number of animal sera were tested for their stimulation of growth of tissue culture cells as well as leptospires. These included rabbit-1, pooled sera obtained from Pel-Freez Biologicals, Inc., Rogers, Ark., used routinely for growing leptospires; rabbit-2, ultrafiltered pooled serum recommended for tissue culture (Pel-Freez Biologicals, Inc.); pooled calf and fetal calf serum obtained from Corvel, Inc., Omaha, Neb.; pooled goat, horse, and sheep sera, as well as individual sheep sera 1 through 10 also obtained from Corvel, Inc. (Table 1).

All sera were tested for leptospiral agglutinins by the method of Wolff (16).

Tissue culture. Primary cell cultures were prepared from fetal bovine kidneys according to the method described by Madin, Andriese, and Darby (10). The minced cortex of the kidneys was trypsinized, and Leighton tubes containing cover slips were inoculated with 1 ml of a 1:200 dilution of cells in growth medium. Growth medium consisted of 0.5% lactalbumin hydrolysate (LAH) in Earle's balanced salt solution plus 10% sheep serum. Antibiotics were not added to the growth medium. The cell cultures were incubated at 37 C until a complete monolayer was formed.

A serially propagated strain of human embryonic skin-muscle fibroblastic (HE) cells developed in the virology Laboratory of the University of Nebraska College of Medicine was also used in this study. These cells were grown at 37 C in Eagle's MEM with Hanks' balanced salt solution (BSS) and 10% calf serum.

Inoculation of cell monolayers with leptospires. All cells were washed three times with Hanks' BSS before inoculation with leptospires. A 1.8-ml amount of Eagle's MEM with 5% sheep serum without antibiotics was added to the Leighton tubes. The tubes were also inoculated with 0.2 ml of leptospires at the same time. On several occasions, leptospires were washed in Hanks' BSS and resuspended in Eagle's MEM with sheep serum to minimize the transfer of toxic materials in the inoculum. Several inoculum sizes were used, giving a final concentration of 10^5, 10^6, and 10^7 leptospires per milliliter in the medium above the cell monolayers. All cultures were incubated at 30 C.

Measurement of growth. The growth of leptospires was determined by direct count by use of a dark-field microscope at × 430 magnification and a Petroff-Hausser counting chamber. Cultures were diluted 1:2 with 0.5% formaldehyde in 0.85% saline, and the chamber was charged by use of a capillary pipette. The total number of spirochetes was determined according to the method of Kolmer, Spaulding, and Robinson (9).

Staining methods. Cover slips containing monolayers were removed at 24-hr intervals and washed with Dulbecco's phosphate buffer solution (PBS). The monolayers were either fixed in 0.4% formaldehyde prior to staining with the Warthin-Starry technique (1) to demonstrate leptospires or in absolute methanol before staining with the May-Gruenwald Giemsa method (4) to show cell morphology. The stained cover slip preparations were mounted in Canada Balsam on glass slides and photographed with a Leitz Ortholux Microscope with plano-objective lens and a Leica M-1 camera containing Panchromatic X film.

RESULTS

Medium for the maintenance of tissue culture cells and growth of leptospires. To study the effects of *L. pomona* on tissue culture cells, it was necessary to develop a medium which would simultaneously maintain the cell monolayer as well as support the growth of leptospires. LAH medium, Eagle's MEM, and medium 199 were tested with 13 sera in 10% concentration to determine which combination was most suitable for this purpose. Eagle's MEM with rabbit-1, sheep-5, and 10 sera supported good growth of leptospires comparable to that obtained in Stuart's and Fletcher's leptospiral media with the same three sera (Table 1). However, leptospires did not grow well in Hanks' BSS, used as a basal salt solution in Eagle's MEM, after the addition of the three sera (not seen in Table 1). Also, poor growth was obtained in LAH medium with all of the 13 sera (Table 1) even when thiamine was added to this medium. Thiamine, present in both Eagle's MEM and medium 199, has been shown to stimulate the growth of *L. pomona* (6). Further testing of medium 199 was discontinued after five sera were found to be unsatisfactory for leptospiral growth. In addition, medium 199 was found to be inferior to LAH medium and Eagle's MEM for growing PBK cells.

Rabbit-1 and sheep-5 and 10 sera in Eagle's MEM were tested for supporting growth and maintaining PBK and HE cells. Both sheep sera were satisfactory for this purpose, whereas rabbit serum was not. Consequently, Eagle's MEM con-

TABLE 1. *Growth of leptospires in media supplemented with various animal sera*

Serum	Media				
	Stuart's	Fletcher's	0.5% LAH	Eagle's	Medium 199
Rabbit-1	++[a]	++	+	++	−
Rabbit-2	+	+	−	−	−
Sheep	−	+	−	−	ND
Sheep-1	+	+	−	−	−
Sheep-2	−	+	−	−	ND
Sheep-3	+	+	ND	+	−
Sheep-4	−	+	−	−	ND
Sheep-5	++	++	+	++	ND
Sheep-8	−	+	−	−	ND
Sheep-10	++	++	−	++	ND
Calf	−	−	−	−	−
Fetal calf	+	−	−	−	ND
Horse	−	−	ND	−	ND
Goat	−	−	−	−	ND

[a] Symbols: ++ = good growth; + = poor growth; − = no growth; ND = not done.

taining either of the two sheep sera was used throughout this study.

Growth of leptospires in bovine kidney cell cultures. Growth curves for *L. pomona* 3341 were done in triplicate to compare quantitatively the growth of strain 3341 in PBK cell cultures with growth produced in Eagle's MEM containing 5% sheep serum. Figure 1 (A, B, and C) represents growth curves obtained when the initial concentration was 10^7, 10^6, and 10^5 organisms per milliliter, respectively.

Regardless of the initial concentration, leptospires reached a maximal concentration of approximately 10^8 spirochetes per milliliter whether they were grown in association with PBK cells or in Eagle's MEM alone. Nearly identical growth curves were obtained when HE cells were used.

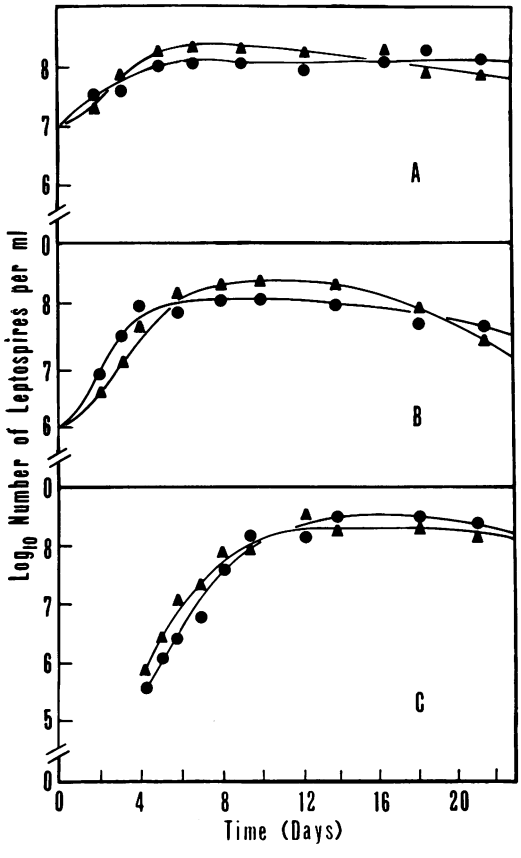

FIG. 1. *Growth of Leptospira pomona 3341 in PBK cell cultures in Eagle's MEM as compared with growth in Eagle's MEM alone. The initial concentration of leptospires in graph A was 10^7 per milliliter; graph B, 10^6 per milliliter, and graph C 10^5 per milliliter. Symbols: ●, growth in cell cultures; ▲, growth in Eagle's MEM only.*

These results indicated that the growth of leptospires was neither suppressed nor enhanced when grown in either of these tissue culture systems.

Serial transfers of leptospires in PBK cell cultures were made over a period of 5 months to determine whether continuous exposure to these cells would affect the growth of leptospires. Subcultures were made regularly at 3-week intervals. Leptospires reached optimal concentrations (10^8 per milliliter) after each subculture, indicating that these organisms could be transferred for long periods of time in the presence of PBK cell cultures.

CPE of leptospires on PBK and HE cell monolayers. Typical uninoculated control cover glass cultures of PBK cells showed fibroblastic cells surrounding epithelial-like cells in a confluent monolayer (Fig. 2). A CPE involving only fibroblastic cells was routinely observed when PBK monolayers were infected with leptospires. There was a loss of intercellular adhesion and condensation of the cytoplasm which ultimatey resulted in the majority of the fibroblastic cells sloughing off the cover glass while the epithelial cells remained intact (Fig. 3).

Figure 4 shows degeneration of HE cells after 7 days of incubation with leptospires. Uninoculated controls (Fig. 5) remained intact when held for the same length of time. The cytoplasm of infected HE cells was often vacuolated, and the chromatin within the nuclei became condensed. The nuclei often appeared quite bizarre (Fig. 6).

The initial concentration of leptospires in the inoculum determined when the CPE was first seen. This varied from the 4th postinoculation day, in the case of monolayers inoculated with 10^7 leptospires per milliliter, to the 13th day, for monolayers inoculated with 10^5 leptospires per milliliter. However, in all cases, the CPE was not observed until leptospires had reached maximal concentrations.

Relationship of leptospires to cell monolayers. Warthin-Starry stains of PBK cell monolayers showed masses of spirochetes adhering to fibroblastic cells, whereas only few leptospires were seen on the surface of epithelial-like cells (Fig. 7). Figure 8 shows the majority of the spirochetes attached to the long filamentous processes of the fibroblastic cells. The primary position of the leptospires in relation to the cell appeared to be extracellular, since most of the organism protruded from the surface of the cytoplasmic strands.

HE cells also showed masses of leptospires adhering to their surface. However, in this case, many more clumps of spirochetes could be seen attached to the HE cells than to fibroblastic cells in PBK monolayers.

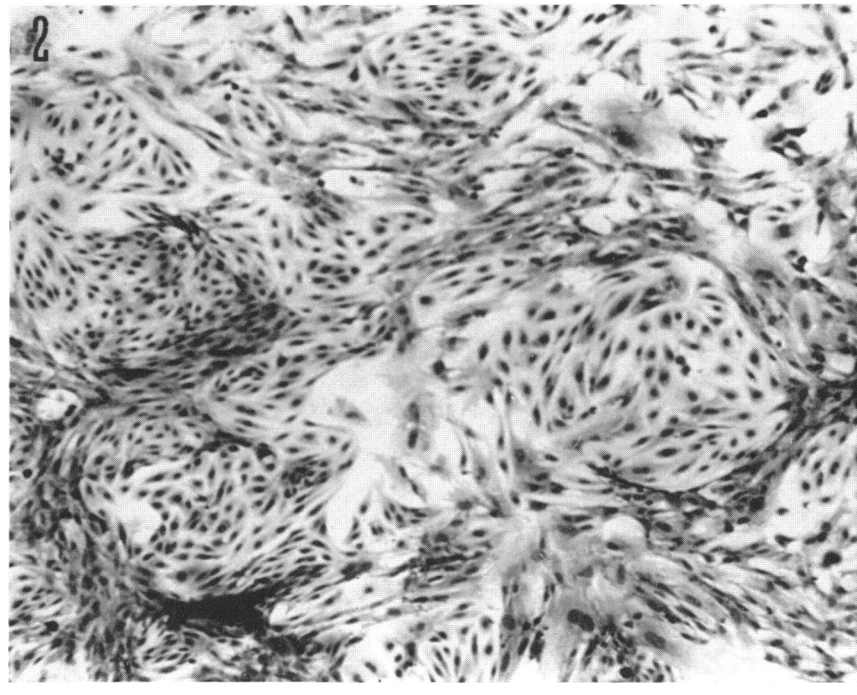

FIG. 2. *Control PBK cell monolayer at 6 days. Intact fibroblastic cells are seen surrounding epithelial-like cells forming a confluent monolayer. May-Gruenwald Giemsa stain.* × *130.*

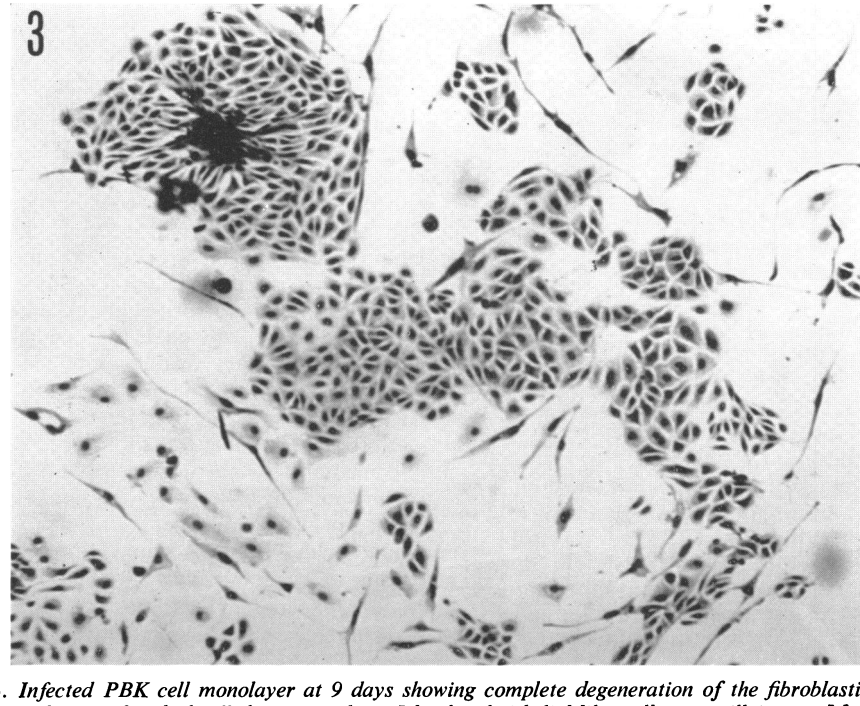

FIG. 3. *Infected PBK cell monolayer at 9 days showing complete degeneration of the fibroblastic cells, wtih the majority having sloughed off the cover glass. Islands of eithelial-like cells are still intact. May-Gruenwald Giemsa stain.* × *130.*

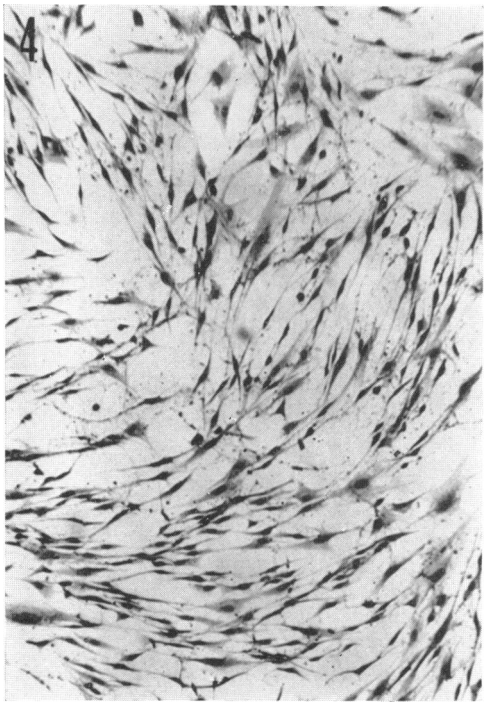

FIG. 4. *Infected HE fibroblastic cells at 7 days showing separation and degeneration of the cells. May-Gruenwald Giemsa stain. × 130.*

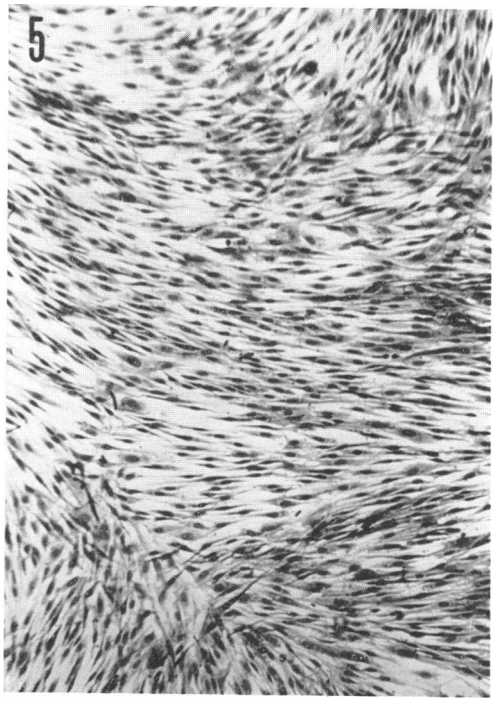

FIG. 5. *Control HE fibroblastic cells at 7 days showing a typical confluent monolayer. May-Gruenwald Giemsa stain. × 130.*

Nature of the concentration of leptospires on fibroblasts. When maximal concentrations (10^8 per milliliter) of leptospires were added to the cell monolayers, the spirochetes adhered to the fibroblastic cells in great numbers within 8 hr. Also, when leptospires were allowed to multiply in the tissue culture system, the number of organisms adhering to the fibroblastic cells increased as the number of leptospires increased in the supernatant medium above the monolayer. These observations indicated that leptospires were attaching to the surface of fibroblastic cells.

Further experiments were done to determine whether multiplication actually occurred on the cell surface by dislodging the leptospires, and then determining whether significant increases could be detected in the supernatant medium. This was done by treating the monolayers with 2 ml of 0.002% sodium hypochlorite (NaOCl) in 0.85% NaCl as described by Kent et al. (8), for dispersing clumps of treponemes. The total count after dislodgement of the leptospires did not appreciably exceed the number of leptospires in the tissue culture medium above the monolayer prior to the removal of the spirochetes from the cells. This suggested that multiplication did not occur on the surface of fibroblastic cells.

DISCUSSION

Eagle's MEM with 5% serum from two sheep satisfied the growth requirements of leptospires and maintained both PBK and HE cells for a sufficient period of time to permit the study of the effects of these spirochetes on cell monolayers.

It is difficult to say with certainty whether the lack of leptospiral growth in LAH medium and medium 199 was the result of the absence of essential growth factors or the inhibitory activity of certain substances in these two media. Since all three tissue culture media contained the basic nutrients necessary for the growth of leptospires, it seems most likely that inhibitory substances were responsible for the failure of LAH medium and medium 199 to support the growth of these organisms. Ellison et al. (2) were also unable to show any appreciable increase in leptospires in a canine kidney culture system maintained in LAH medium. The amino acids and thiamine in Eagle's MEM appeared to be necessary for leptospiral growth, since only poor growth was obtained in Hanks' BSS containing ovine sera. Several of these amino acids and thiamine have been shown to have a stimulatory effect on the growth of leptospires (6, 7, 12, 13, 14).

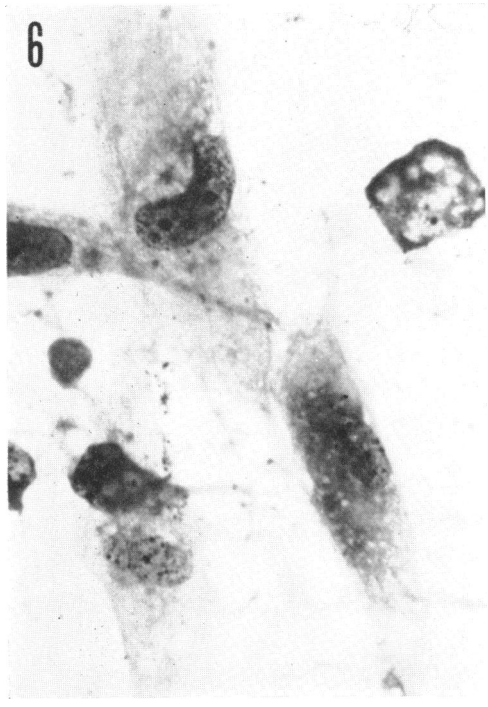

FIG. 6. *Infected HE fibroblastic cells at 7 days showing vacuolated cytoplasm and bizarre nuclei with condensed chromatin. May-Gruenwald stain.* × *520.*

Leptospires reached concentrations of approximately 10^8 cells per milliliter in Eagle's MEM regardless of the size of the inoculum and whether tissue cells were present or not, indicating that growth occurred primarily in the medium above the monolayers. The failure to demonstrate an increase in leptospires in the supernatant medium after dislodgement from fibroblastic cells, and the adherence of large masses of spirochetes of fibroblastic cells only 8 hr after inoculation of large numbers of organisms, favors the concept of attachment to rather than growth on fibroblastic cells. Recently, Harrington and Sleight (5) also showed masses of leptospires attached to fetal bovine kidney cells and Rose et al. (Bacteriol. Proc., p. 70, 1965) demonstrated adherence to leukemic monocytes in vitro. It is quite possible that attachment of leptospires to certain cells may be associated with some substance on their surface. Acid mucopolysaccharides have been shown to be deposited in large quantities on the surface of fibroblastic tissue culture cells (11). It is possible that a similar mechanism of attachment may also occur in vivo, since large numbers of leptospires were observed by electron microscopy to be closely associated with the surfaces of liver parenchymal and proximal tubule cells of the hamster (N. G. Miller and R. B. Wilson, *in press*).

This study also indicated that the close association of leptospires with the surface of fibroblastic

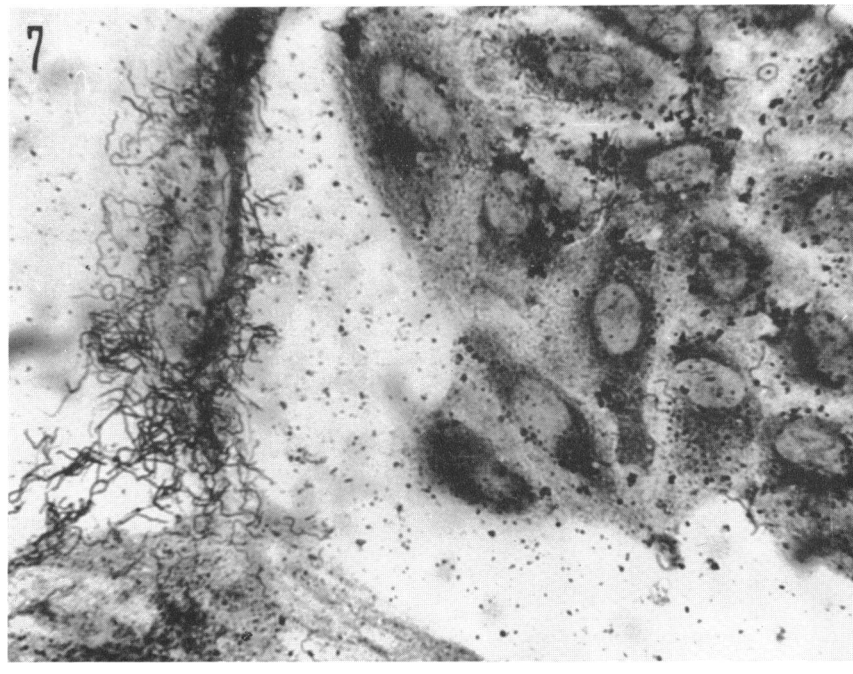

FIG. 7. *Infected PBK cells with masses of leptospires adhering to the fibroblastic cell at the left, whereas very few spirochetes are seen associated with the epithelial-like cells at the right. Warthin-Starry stain.* × *520.*

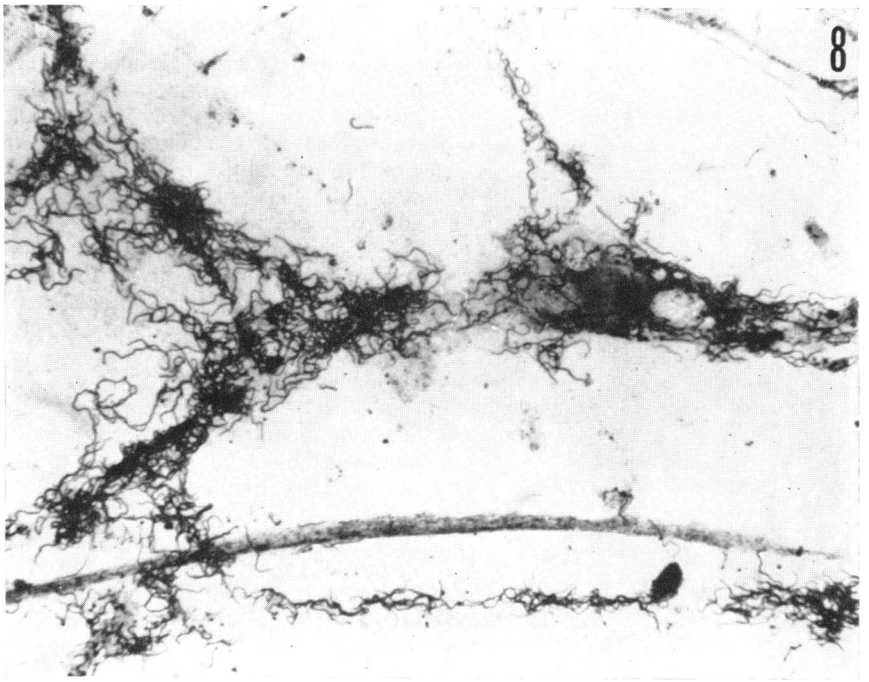

FIG. 8. *Large numbers of leptospires adhering to the filamentous cytoplasmic processes of a PBK fibroblastic cell. Warthin-Starry stain.* × *520.*

cells is in some way responsible for the degeneration of these tissue cells. This could be the result of interference by leptospires with the absorption of essential nutrients by fibroblastic cells or the effect of "toxic" substances closely associated with the leptospiral cell. Some evidence for the latter hypothesis was presented by Vosta and Polednikova (15), who produced a CPE on pig kidney cell monolayers with disintegrated leptospires.

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