Free Living Amoebae as Contaminants in Monkey Kidney Tissue Culture. (23515)

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During routine use of trypsinized monkey kidney tissue culture for viral research, "spontaneous" contamination of the tissue culture (TC) with a free living amoeba occurred twice in 1956 in our laboratory. Such a rare contamination, to the best of our knowledge, has never been reported. The isolation and the characteristics of the organism in TC are described in this paper.

Materials and methods. Tissue culture. Primary cultures of trypsinized monkey kidney cells were prepared by the method of Dulbecco and Vogt(1) as modified(2) by Youngner. Serial passage cultures of HeLa cells, Chang's liver cells and bovine kidney cells(3) were purchased from Microbiological Associates, Inc. Primary cultures of human amnion cells were kindly provided by Dr. Kenneth Takemoto(4) and a serial passage culture of human skin cells was obtained from Dr. Wilton R. Earle(5). Primary cultures of trypsinized chick embryo cells and rabbit kidney cells were prepared in a manner similar to the monkey kidney tissue culture. All these cell lines were cultured in roller tubes. The media used for these cell lines were either Eagle's basal medium with i-inositol(6.7) or Medium 199, except for monkey kidney cells, for which 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution was used(8). Two per cent calf serum was added to all the media. For passage usually 0.2 ml of TC fluid containing the amoebic cysts or both cysts and trophozoites was transferred to each culture tube containing 1.8 ml of medium. Infected tissue cultures kept either stationary or in a rotating drum were incubated at 35°C. *Photography.* Photographs of unstained specimens were taken from the roller tubes. For stained specimens, the amoebae were propagated in TC on cover slips lying on the flat wall of Leighton tubes. After incubation the medium was poured off, 10% formalin was poured into the tube for fixation, and then the cover slip was removed for staining.

Results. Isolation of the Amoebae. Two strains of apparently the same amoeba were isolated from monkey kidney tissue cultures which showed cellular degeneration spontaneously on routine microscopic examination. Subcultures were made on monkey kidney tissue cultures and the same phenomenon was noted. Under low power (30X) microscopic examination, rounded bodies were observed, which were similar in appearance to cells manifesting changes induced by certain simian viruses (Fig. 1). When the specimen was examined under higher magnification (150X) these round bodies appeared not to be cells but cysts (Fig. 1), some of which were found to be intracellular. A hanging drop preparation was made on a slide from the TC fluid and examined microscopically with high magnification (450X). Moving amoebae were found and identified as free living amoebae belonging to the genus *Acanthameba.*†

Culture characteristics. The amoebae multiplied very well in cultures of HeLa cells, Chang's liver cells, human amnion cells, human skin cells, chick embryo cells, beef kidney cells, rabbit kidney cells and monkey kidney cells. Monkey kidney cells were least susceptible, while HeLa cells and chick embryo cells were most susceptible. After inoculation with the amoebae, the various cell lines did not undergo any specific degeneration that we could see. They became granular and gradually disintegrated and disappeared. When the cysts were inoculated into monkey kidney tissue culture, trophozoites were not seen until 5 to 8 days after inoculation. In cultures of HeLa cells or chick embryo cells, trophozoites were found 24 hours

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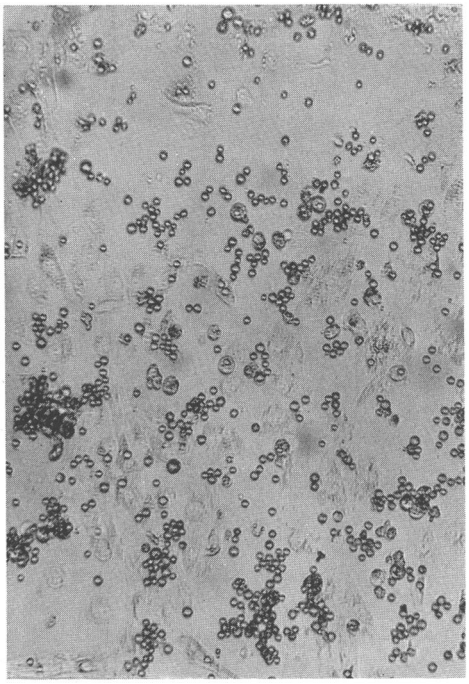

FIG. 1. Free living amoebae in monkey kidney tissue culture, early stage, showing cysts. × 90.

after inoculation, and the cells were destroyed in 24 to 48 hours. The sensitivity of the cells also depended on the age of the cells and the size of the inoculum. Older cells were more susceptible. Various culture media including Eagle's basal medium, Medium 199, and lactalbumin hydrolysate medium were used for cultivation and no difference was observed. The amoebae did not multiply in the absence of cells regardless of the media used. (When the TC fluid containing the amoebae was poured into boiled tap water to which rice powder was added and in which bacteria were present, the amoebae multiplied therein.)† The usual titer for cytotoxic changes in TC was 10^4 or 10^5 per 1.0 ml. By direct counts of the cysts in the TC fluids, there were usually 200,000 or more amoebae per 1.0 ml. One strain was transferred consecutively in monkey kidney tissue culture for 16 passages, and the other strain for 12 passages. Both strains were transferred in HeLa cells for more than 10 passages.

In some experiments 10-fold serial dilutions were made from the monkey kidney tissue culture fluid containing the amoebae, and the dilutions were inoculated into fresh monkey kidney tissue culture tubes, which were examined from day to day for presence of cysts and trophozoites. As shown in Table I, cysts appeared first, and they were gradually replaced by trophozoites. Toward the end of the observation period, as the tissue cells were all destroyed, all the trophozoites again became encysted.

In the monkey kidney tissue culture the cysts were found both outside and inside of the cells. In the cells they were found in the cytoplasm usually pushing the nucleus of the cell to the side (Fig. 2). From 1 to 28 cysts could be found in a single cell. In HeLa cells or chick embryo cells, intracellular amoebic

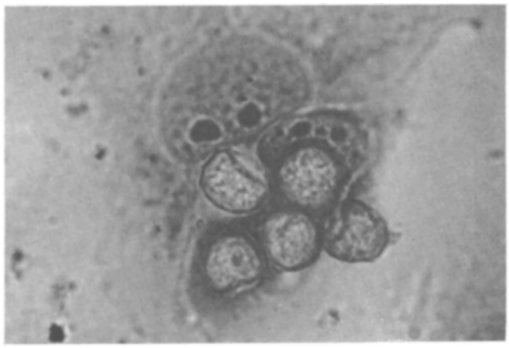

FIG. 2. Free living amoebae in monkey kidney culture, stained with azure eosin, showing wall of intracellular cysts. × 840.

TABLE I. Multiplication of a Free Living Amoeba in Monkey Kidney Tissue Culture.

Conc. of T.C. fluid inoc.	Observation of cysts and/or trophozoites on different days after inoculation													
	3		6		8		9		11		16		20	
	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr	Cy	Tr
10^{-1}	+	—	++	—	++	±	+++	++	—	+++	+	+++	++++	—
10^{-2}	+	—	++	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-3}	+	—	++	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-4}	±	—	+	—	++	±	++	++	—	+++	+	+++	++++	—
10^{-5}	—	—	—	—	—	—	++	—	—	+++	+	+++	++++	—

+, ++, etc., indicate relative No. of amoebae found. — = amoebae not found. ± = questionably found.

Cy = amoebic cysts. Tr = trophozoites.

cysts were occasionally found. The cyst had a heavy double wall, the inner one rugose. The cytoplasm of the cyst was granular. No nucleus of the cyst could be seen when it was examined in TC tubes. The cyst varied in size, usually from 10 to 21μ in diameter, as measured from material taken from the monkey kidney tissue culture.

In one of the experiments the cysts were treated with 10% formalin and were then washed and inoculated into monkey kidney tissue culture tubes. These cysts could be found inside the cells. This observation suggested that the monkey kidney cells could phagocytize the cysts.

The trophozoites varied from 7 x 12 to 16 x 43 μ in diameter. They were sluggish in movement. A trophozoite produced many small pseudopodia. Many granules and food vacuoles were found in the cytoplasm with one or more contractile vacuoles. A contractile vacuole appeared when the amoeba was put into tap water and examined in hanging drop preparations microscopically. This was the chief reason why it was identified as a free living amoeba. Intracellular trophozoites were not observed.

Stability. The trophozoites were very sensitive to temperature. As soon as they were placed in the refrigerator at 4°C they became encysted. The cysts were very resistant to low temperatures. Culture fluids containing the cysts kept at −50°C for 8 months still yielded trophozoites when inoculated into TC tubes, although the titer was greatly reduced. The amoebae remained viable in the pH range 5.0-9.0 and were not affected by streptomycin and penicillin in TC.

Filtration experiment. Fluids of monkey kidney tissue culture and chick embryo tissue culture containing the cysts and trophozoites were filtered through an ultra-fine sintered glass filter or a S-3 Seitz filter. Ten-fold serial dilutions were made from the filtrate, and the dilutions, from 10^{-0} to 10^{-7}, were inoculated into monkey kidney tissue culture tubes and HeLa cell TC tubes. No cytopathogenicity or amoebae were found after seven or more days of incubation. The unfiltered fluid yielded positive cultures of amoebae at the concentration of 10^{-5}. These experiments are evidence that a virus was not present together with the amoebae.

Mixed infection of the amoebae and poliovirus in tissue culture. Monkey kidney tissue culture and chick embryo tissue culture were infected with both the amoebae and type 3 poliovirus. There was no significant influence of the presence of one on the growth of the other. The infection of the cultures with amoebae did not make chick embryo cells susceptible to the poliomyelitis virus. The amoebic cysts also failed to support the growth of the poliomyelitis virus.

Staining reactions of the amoebae. The staining reactions of the amoebae were quite variable in both cystic and trophozoite stages. There was also great variability in staining coincident with intermedate stages between encystment and decystment.

Cysts. Typical cysts were very difficult to stain with any of the common stains such as azure eosin or hematoxylin (iron or alum), frequently remaining unstained. However, very good staining could be effected with the Bauer reaction(9) whereby the entire cyst stained red. They usually stained red or pink with the periodic acid-Schiff reaction, thereby indicating carbohydrate. They were yellow or unstained with the Van Gieson stain. The dinitrofluorobenzene H-acid method(10) for proteins stained the entire cyst red.

Trophozoites. With azure eosin, the karyosome and nuclear membrane stained blue and the nucleus faint blue. The cytoplasm stained a moderate irregular blue throughout because of numerous vacuoles and granules. Some of these granules stained with phosphotungstic acid hematoxylin (Fig. 3), Hale(9) periodic acid-Schiff (Fig. 4), dinitrofluorobenzene H-acid (Fig. 5) and oil red 0. It is apparent therefore that some of these granules contain protein, fat or carbohydrate. The oil red 0 method(9) also demonstrated fine droplets of fat on the trophozoite limiting cytoplasmic membrane.

Discussion. These observations reveal the adaptability of TC and its allied technics for the culture and study of certain amoebae. Shaffer and Sienkiewicz(11) and Shaffer(12) developed a method using chick embryo tissues suspended in TC nutrient fluid for cultivation of *Endamoeba histolytica.* Baernstein, Rees and Bartgis(13) grew *E. histoly-*

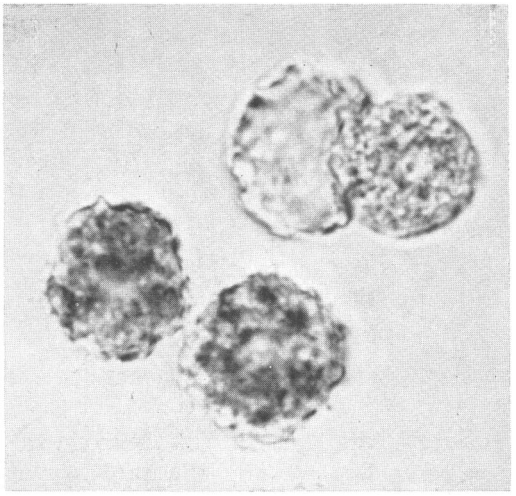

FIG. 3. Cyst and trophozoite-like forms of free living amoebae stained with phosphotungstic acid-hematoxylin. In stages that appear to be intermediate between cysts and trophozoites, some granules stain with phosphotungstic acid-hematoxylin and others do not. Cysts are usually unstained. × 1935.

tica in a suspension of fine particles from chick embryo homogenate. In the present study the amoebae grew very well in a TC system which was simple and free from bacteria. It is interesting to note that the amoebae failed to grow in Eagle's basal medium or Medium 199 which supported the growth of mammalian cells. The finding of amoebic cysts inside the cells suggests that further studies *in vitro* might throw new light on the interaction between the amoebae and the host cell. The cytolytic effect of the amoebae in HeLa cells offers another potential oncolytic agent for possible trial *in vivo*.

Summary. Two strains of apparently the same free living amoebae were found to be spontaneous contaminants in monkey kidney tissue culture. The TC technic proved to be a suitable method for cultivation of the amoebae in the absence of bacteria. Pure cultures of the amoeba were obtained in many cell lines. Both cysts and trophozoites were found in TC. In monkey kidney tissue culture, cysts were often found in the cytoplasm of the cells. One strain was subjected to 16 consecutive passages in monkey kidney tissue culture while the other had 12. The average CPE titer of the TC fluid was 10^4 to 10^5 per 1.0 ml. The cysts in the TC fluid survived at −50°C for eight months, although the titer was greatly reduced.

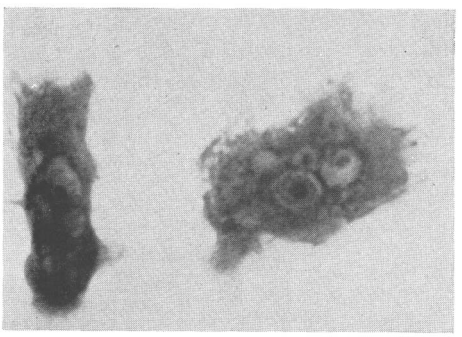

FIG. 4. Trophozoites of free living amoebae stained with periodic acid-Schiff and Mayer's hematoxylin. The cytoplasm is generally reactive for carbohydrate throughout. × 1650.

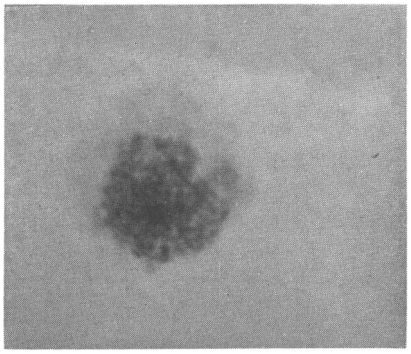

FIG. 5. Trophozoite of free living amoebae stained with dinitrofluorobenzene H-acid method for proteins. The cytoplasmic granules, karyosome and nucleus are strongly reactive. × 1800.

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Detection of Incomplete Enterobacterial Antibodies in Cord Blood by Means of Antiglobulin Hemagglutination Test.* (23516)

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The passive transfer of antibodies through the placenta from the mother to the fetus depends upon two main factors, namely, the structure and function of the placenta and the characteristics of the antibody. In some animal species, notably rodents, apes, and man, certain antibodies readily pass the placental barrier. In other species, such as the pig and ruminants, placental transmission does not take place, but antibodies are transferred to the offspring through colostrum. The role of the antibody molecule in the transmission through the placenta is illustrated by the fact that, for as yet poorly understood reasons, incomplete Rh antibodies pass the human placenta far more readily than complete antibodies of identical serologic specificity. Recently, it was shown by means of the indirect bacterial hemagglutination test, which is considerably more sensitive than the conventional bacterial agglutination method, that antibodies against the somatic (O) antigens of enteropathogenic *Escherichia coli* are not quantitatively transferred from mother to newborn infant. In a series of 26 cord blood specimens antibodies could be demonstrated in only 3, although the maternal serum of all contained such antibodies(1). Similarly, Stulberg and associates(2) found 77% of 45 cord serum specimens lacking in such antibodies. Further, when antibodies were present in cord blood, their titer was substantially lower than that of the corresponding maternal antibodies. The possibility was considered that incomplete antibodies to enterobacteriaceae may pass the human placenta, provided that the mother has such antibodies. Weil and Felsen(3) demonstrated incomplete *Shigella flexneri* agglutinins in approximately half of 30 human sera by combining the conventional bacterial agglutination method with the antiglobulin technic. To determine whether the hypothesis just mentioned is correct, maternal and cord serum specimens were examined by means of a hemagglutination-antiglobulin test for complete and incomplete enterobacterial antibodies.

Material and methods. Fifty paired maternal and cord blood specimens from healthy individuals were obtained through the cooperation of the Obstetrical Department of the Children's Hospital. The serum specimens were stored in the deep freeze until used. Data on blood groups and Rh antibodies were available. The hemagglutination test was carried out as described previously(4,5). As O antigens supernates of the following agar-grown cultures were employed: *E. coli*, serogroups 111 and 55, *Shigella flexneri* (2 freshly isolated and serologically different types), *S. sonnei*, *Salmonella paratyphi* B (group B), *S. montevideo* (group C_1), *S. muenchen-oregon* (group C_2), and *S. enteritidis* (group D). These supernates (refrigerated centrifuge) were prepared from heated (1 hour 100°C) suspensions and were employed in a dilution of 1:10. Erythrocytes obtained from blood donors (blood group O, Rh negative) were washed 3 times, treated with the antigens in a waterbath at 37°C for 30 minutes, and again washed three times. Serial two-fold dilutions of the sera (0.2 ml) were mixed with the modified red blood cell suspensions (0.2 ml). Non-

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