Examination of Adult Primary Human Kidney Cell Cultures for Adventitious Viral Agents[1] (35767)

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Recent studies utilizing improved virological detection techniques have demonstrated the presence of latent viral agents in a wide variety of mammalian avian tissues (1–3). Hsiung, in her review of latent infections in primates, emphasized the high incidence of such agents in the kidneys of overtly healthy nonhuman primates (1). In two studies, over 50% of primary simian kidney cell cultures were found to contain adventitious agents (1, 4).

Comparable data on the incidence of such agents in adult human kidneys are limited. Reports on the isolation of viruses from primary human kidney cell cultures have been concerned primarily with newborn or young children (5–7). The present study was undertaken in an effort to determine whether adult humans without renal diseases harbor latent agents. The importance of establishing whether such agents are present is apparent when one considers their possible role in human renal disease, as well as their potential role as passengers in renal transplant operations.

Materials and Methods. Autopsy specimens, consisting of a wedge-shaped transverse section through the entire width of one kidney, were obtained from adults undergoing routine postmortem examination at the Walter Reed General Hospital. Individuals with obvious renal pathology were excluded from the study; otherwise, no attempt was made to select the patient population. Causes of death varied from acute traumatic episodes to terminal malignancies.

During the period from November, 1968 to June, 1969, 27 specimens were obtained for cell cultivation. Cell cultures from six of these specimens became grossly contaminated with bacteria within 48 hr and were discarded. The remaining 21 specimens were obtained from 15 males and 6 females, ranging in age from 13 to 71 years. Primary cell cultures were initiated 5–24 hr after death. Portions of each specimen were trypsinized, suspended in Eagle's No. 2 medium with 10% fetal calf serum, and seeded into 250-ml Falcon plastic flasks. After forming confluent sheets, the cells were maintained on Eagle's No. 2 medium with 1–2% fetal calf serum. Neomycin (20 μg/ml) and Aureomycin (20 μg/ml) were added to all media. Cell cultures were examined at 3–4-day intervals for cytopathic effects (CPE), and the medium was changed every 7 days.

Fluorescence microscopy. The methods employed in the fluorescence studies have been described previously (8, 9). During the early phases of this study, primary cultures that did not show gross CPE in 30–40 days were trypsinized and planted into plastic petri dishes. After a confluent monolayer was formed (7–9 days), the cells were fixed, stained, and examined by the indirect fluorescent antibody technique. Later cultures, constituting the majority of the specimens, were fixed in the original flasks 4–7 weeks after the initiation of the cultures. The top halves of the flasks were then removed, and 6–8 individual staining wells were constructed utilizing melted dental wax to form retaining walls.

Undiluted pools of 2–3 human sera, obtained from healthy blood donors, pooled hu-

[1] Presented at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., April 26–May 1, 1970.

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man gamma globulin (Lederle) diluted 1:5 with phosphate buffer, and the patient's own undiluted serum, when available, were used as the antibody sources. After exposure to these preparations, the cells were treated with horse antihuman globulin, conjugated with fluorescein isothiocyanate (Progressive Laboratories, Inc.). Conjugated antihuman globulin alone was run in parallel with each experiment as a negative control.

The cells were examined with a Bausch and Lomb microscope fitted with a darkfield condenser, a 5–58 exciter filter, and T-2 barrier filters. An Osram HBO 200 high-pressure mercury arc lamp was used as the source of the UV light.

Acridine orange. Fixed cells from all primary cell cultures were stained with acridine orange at pH 5.2 and examined under the darkfield fluorescence microscope for viral inclusions.

Electron microscopy. Replicate primary cell cultures were pelleted and fixed for thin sectioning at the same time that fluorescent antibody and acridine orange studies were performed. The details of this procedure are described elsewhere (10). Sections were stained with a saturated solution of uranyl acetate in 50% ethyl alcohol, followed by 0.3% lead citrate. Negative staining was done by a method described previously (11). All preparations were examined with an RCA emu 3-G electron microscope at 50 kV.

Serological reactions. The adenovirus isolate was typed as follows: approximately 10^4 infectious units of the adenovirus isolate were mixed with 1:10 dilutions of various reference adenovirus antisera (obtained from the Research Reference Reagent Branch, NIAID, NIH), incubated for 1 hr at room temperature, and inoculated on petri plates containing human amnion (HA) cells (strain FL). After 1 hr, the medium was replaced on the cell cultures and the cultures were incubated at 37° in a 5% CO_2 atmosphere for about 24 hr. The cell monolayers were then washed, fixed, and stained by the indirect immunofluorescence method for adenoviruses. These preparations were examined in the fluorescence microscope for foci of infected cells and neutralization was recorded if the number of foci was suppressed by more than 80%.

The titer of adenovirus antibody in the patient's serum was determined by tissue culture neutralization (13).

Passage attempts. Portions of primary kidney cell suspensions that had been frozen (—70°) and thawed at least once were inoculated into both primary (human embryonic kidney [HEK]) and continuous (HA [strain FL], WI-38, Vero) cell lines. Substrate cell cultures were maintained for 1 to 4 weeks and examined at 3–4-day intervals for CPE. The cultures were then fixed and examined by the indirect fluorescent antibody method, using the same sources of antibody as were employed in the testing of the original primary cell cultures.

Results. Primary cell cultures. Nine of the 21 primary cell cultures were classified as either early or late failures. In four instances, at least one flask showed gross contamination within 48 hr; companion flasks revealed either no growth at 7 days or limited proliferation with eventual contamination 2–3 weeks later. Four other specimens in this group of nine showed no growth after 7 days. The remaining specimen in the group of failures yielded cultures that initially were comprised of small isolated colonies, but did not proliferate beyond this stage.

The remaining 12 cell cultures were maintained for a 4–7-week observation period. Successful propagation of these cultures appeared to be relatively independent of the age of the patient or the time interval between the death of the patient and the initiation of the cultures. However, the eventual outcome of the cell cultures was influenced by the cause of death: all 8 specimens received from patients dying relatively suddenly from acute illnesses or trauma were successfully cultivated; whereas, only 4 out of 11 specimens obtained from patients dying of various malignancies were successfully propagated.

The 12 cultures that were maintained for the 4–7-week observation period were examined at 3–4-day intervals for cytopathic effects. One kidney cell culture, obtained from a 58-year-old male who died shortly

after a myocardial infarction, developed obvious CPE 28 days after initiation. Electron microscopic examination of negative stains of the supernatant from the original flask and thin sections of a cell pellet from a companion flask showed typical adenovirus particles. Typing of this virus, utilizing a standard neutralization test combined with the indirect fluorescent antibody method, identified the isolate as a type 2 adenovirus. A sample of the patient's serum, obtained at necropsy, had a neutralizing antibody titer of 1:40 to this type.

Observation of the remaining 11 cell cultures for CPE was hampered by the development of focal rounding and vacuolization of cells in virtually all flasks 2–3 weeks after initiation. These changes were probably due to nutritional deficiences. Characteristic viral CPE was not observed in any of the 11 cultures. All cultures were tested for mycoplasma and were found to be negative.

Fluorescent antibody, acridine orange, and electron microscopic studies. At the conclusion of the observation period, each of the 11 primary cell cultures was examined by the indirect fluorescent antibody technique. Seven of the 11 cell cultures exhibited fluorescent foci with one or more of the antibody sources. The staining was focal, and consisted of both perinuclear and cytoplasmic fluorescence. Four to six pools of 2–3 human sera were applied to each cell culture. In five cultures, all pools yielded fluorescent foci; in two other cultures, 3 out of 4 and 4 out of 6 pools, respectively, showed fluorescence. In those instances where the patient's own serum was used as an antibody source, 3 out of 4 cell cultures exhibited fluorescent staining. However, when pooled human gamma globulin was used as the antibody source, only 1 out of 7 cell cultures showed any reactivity (Table I summarizes these results).

The four other cell cultures that were tested by this technique did not show any staining with any of the pools, the gamma globulin, or their own serum. Similarly, all negative controls run in parallel with the above failed to exhibit any fluorescent staining.

TABLE I. Primary Cell Cultures Exhibiting Fluorescent Foci with One or More Antibody Sources in Indirect Fluorescent Antibody Technique.

Patient	Pooled human sera (no. reactive/ total no.)	Patient's own serum	Pooled human gamma globulin
E.B.	4/6	NA[a]	—
D.D.	6/6	+	—
D.S.	6/6	+	—
M.B.	6/6	+	—
C.W.	4/4	NA	+
A.B.	4/4	NA	—
W.M.	3/4	—	—

[a] NA = not available.

Acridine orange staining, performed at the same time as the indirect fluorescence studies on each of the 11 cultures, failed to reveal any inclusions. Negative stain preparations of cell suspensions from 5 of the cell cultures with fluorescent foci did not demonstrate any virus particles. Thin sections of cell pellets from each of the 11 cultures were also examined with the electron microscope, but again, no virus particles were observed.

Passage attempts. No CPE was observed in any of the cell cultures during the 1–4-week observation period. At the end of this time the indirect fluorescent antibody technique failed to demonstrate any foci of fluorescence in the continuous HA, WI-38, and Vero cells. Passage attempts in HEK cells were terminated when three different lots from two commercial suppliers exhibited focal fluorescent staining in the uninoculated control dishes.

Discussion. Very little information is available on the incidence of latent viruses in adult human kidneys. There have been a number of reports of viruses isolated from urine, occasionally in conjunction with clinical evidence of altered renal function, in a variety of human viral illnesses (14–18). However, the relationship of these findings to the actual replication, or persistence, of viruses in renal tissue is unknown.

The few studies that have reported isolation of viruses from human kidney cell cultures have been concerned primarily with newborn or young children. Benyesh-Melnick

and Rosenberg isolated nine agents from 84 primary cell cultures successfully propagated from autopsy specimens obtained from children 1 year of age or under (7). These isolates consisted of three cytomegaloviruses, three adenoviruses (types 1, 2, and 7), and one each of measles, varicella, and coxsackie virus type B1. Klein and Huang isolated five adenoviruses from over one hundred primary kidney cell cultures obtained from prematurely born infants (6). Hsiung, utilizing cell cultures from both adults and children, recovered 7 viruses from 124 specimens. These included 2 measles viruses, 2 foamy viruses, 1 reovirus, 1 adenovirus, and 1 myxovirus (1).

The isolation of only one adenovirus from our series of 12 long-term adult human kidney cell cultures is consistent with the overall recovery rate in these studies (Benyesh-Melnick *et al.*, 11%; Hsiung, 6%; Klein and Huang, 5%). In addition to the adenovirus isolations reported by these authors, adenoviruses have been recovered from the urine of patients with various diseases (16–18). However, there is no direct evidence that they are responsible for renal diseases in humans. In various other species, adenoviruses are known to cause both overt (dog, mouse) and occult (monkeys, chickens, swine) kidney infections (15). In the patient from whom we isolated an adenovirus, the infection was apparently latent because there was no clinical or pathological evidence of renal disease.

The significance of the fluorescent staining present in seven of our long-term primary cultures, as well as that seen in the three HEK lots, is unclear at the present time. We employed multiple detection techniques in our search for adventitious agents, but each method has its limitations. For instance, electron microscopic examination of cell cultures, utilizing either negative staining or thin sections, is relatively insensitive, since over 10^6 particles/ml, or 100 particles/cell, are required for easy detection of medium-sized viruses with cubic symmetry (11). Similarly, light microscopic examination of acridine orange stained cells reveals only those viruses which produce characteristic inclusions in a significant proportion of the cultivated cells.

Attempts to pass latent agents into cell cultures may be hampered by several factors. Some types of viruses (varicella-zoster, simian cytomegalovirus) are passeed with difficulty, or not at all, if infected cells are frozen and thawed (21, 22). In addition, some viruses are known to be extremely specific in their requirements for cell substrates. We felt that the most logical choice of cells for passage of primary adult human kidney material would be primary HEK. Unfortunately, this proved to be impossible with the three HEK lots which we examined because they themselves contained foci of fluorescing cells.

The indirect fluorescent antibody technique, as employed in these studies, has proved to be a relatively sensitive technique for detecting adventitious viral agents (4, 8, 9). Therefore, the possibility exists that noncytopathic latent agents, or viral antigens, were present in seven of our long-term primary cell cultures and were detected only by this technique. However, nonspecific reactions, due largely to a charge effect, have been encountered with this method (20). Therefore, we believe that positive FA results alone do not strongly support the conclusion that other adventitious viruses, or viral antigens, were present in our cell cultures. Before this interpretation can be made, other evidence must be obtained, including successful serial passage, direct observation of viral particles and specific immunologic reactions with appropriate viral antibody preparations.

Summary. Twelve primary adult human kidney cell cultures were examined by several techniques for the presence of adventitious viral agents. Although no definite cytopathic effects were observed in 11 of the cell cultures, 7 of these exhibited suspicious fluorescent foci after staining by the indirect fluorescent-antibody technique 4 to 7 weeks after initiation of the cultures. Electron microscopy (EM) studies, utilizing negative staining and thin sections, were done on several of the cell cultures, but no virus particles were observed. Passage attempts in primary and continuous cell cultures did not reveal the presence of transmissible viral agents in any of the 11 cell cultures. One cell culture, obtained from a 58-year-old male

who died shortly after a myocardial infarction, showed cytopathic changes 28 days after initiation. EM examination of the cell pellet showed typical adenovirus particles which were identified as a type 2 adenovirus. In contrast to observations with subhuman primate tissues, latent or adventitious agents do not appear to be readily detected in adult human kidney cell cultures.

The authors express their appreciation to Col. David M. Robinson, Chief, Department of Pathology, Walter Reed General Hospital, for his assistance in obtaining the autopsy specimens.

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Received April 1, 1971. P.S.E.B.M., 1971, Vol. 137.