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# Replication of Cytomegalovirus in Human Arterial Smooth Muscle Cells

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**Cytomegalovirus (CMV) strain AD-169 replicated in smooth muscle cell (SMC) cultures derived from human umbilical arteries, producing enveloped infectious virions. However, unlike the effects of CMV on fully permissive human lung fibroblasts, the effects of strain AD-169 on SMC cultures were delayed and prolonged, resulting in extended survival of a fraction of the starting population. This period of survival did not exceed the life-span of the control SMC cultures. Infectious CMV continued to be isolated from the surviving SMC cultures after extinction of the original inoculum by dilution and after treatment of the cultures with CMV neutralizing antibody. The implications of these findings for the pathogenesis of atherosclerosis are discussed.**

The findings of an etiologic role of an oncogenic avian herpesvirus in avian atherosclerosis (7, 14, 16, 17) and of the monoclonality of individual human atheromatous nodules (3, 18) prompted interest in the possible role of herpesviruses in human atherosclerosis. Cytomegalovirus (CMV) antigen was observed by immunofluorescence in only the cytoplasm of cells cultured from human atherosclerotic lesions and in cells cultured from normal portions of the vessels of patients with atherosclerosis, but no evidence for CMV replication was detected by electron microscopy (13). Benditt et al. (2) did not detect the CMV genome by in situ hybridization in the abnormally thickened intima of the arteries from patients undergoing bypass surgery, and neither the CMV genome nor CMV cytopathic effects were detected in human fetal arterial smooth muscle cell (SMC) cultures within the 48-h experimental interval after inoculation with CMV. This report describes the replication of CMV strain AD-169 at longer intervals after infection of human SMC cultures derived from the umbilical arteries of two different donors.

## MATERIALS AND METHODS

**Cell cultures.** Preparation of SMC cultures from the arteries of umbilical cords from two different individuals was begun 1.5 to 2.5 h after parturition, including storage for approximately 30 min to 1 h at 5°C in Dulbecco modified Eagle medium (one cord) or RPMI 1640 medium (the other cord) with 400 μg of streptomycin and 400 U of penicillin G per ml, respectively. Short segments of artery were removed and stripped of adventitia, slit lengthwise, minced finely, and placed in a solution of 2 mg of type I collagenase (Worthington Diagnostics, Freehold, N.J.) per ml of Dulbecco modified Eagle medium at room temperature. The suspension was digested for 1.5 to 2 h at 37°C with gentle agitation; gross fragments were removed by filtration through gauze, and the remaining cells were separated from the collagenase by centrifugation. The cell pellet was suspended in Dulbecco modified Eagle medium with 30% bovine newborn calf serum or bovine fetal serum containing 400 μg of streptomycin and 400 U of penicillin G per ml, respectively, and cells were cultivated in plastic flasks. All sera were inactivated at 56°C for 30 min before use in culture media. Antibiotic levels were reduced fourfold within 2 weeks. Cells were prepared for subculture with a combination of 0.25% trypsin and 0.1% EDTA in $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline. For comparative purposes, human lung fibroblasts (IMR-90) (15) were obtained from the American Type Culture Collection, Rockville, Md., and used between passages 14 and 23. IMR-90 cells were cultivated in Eagle minimum essential medium with 10% bovine newborn calf serum. To test for mycoplasma, the cell suspensions were inoculated onto a solid medium (12) and incubated anaerobically at 37°C for 3 weeks; thin sections of SMC pellets and of IMR-90 cell pellets were examined also by electron microscopy.

Cells were fixed with Formalin and glutaraldehyde and stained with Giemsa (9) for examination by light microscopy. For electron microscopy, cell pellets were fixed and postfixed, respectively, in phosphate-buffered 3% glutaraldehyde and 1% osmium tetroxide, stained with 0.1% aqueous uranyl acetate, dehydrated in increasing concentrations of ethanol, exposed to propylene oxide (Eastman Kodak Co., Rochester, N.Y.), and embedded in Araldite 502 (CIBA-GEIGY Corp., Summit, N.J.). Thin sections were stained with 2% uranyl acetate and lead citrate (20). Specimens were examined with the Siemens Elmiskop IA (Siemens America Inc., New York, N.Y.) at 80 kV.

**Immunofluorescence.** For immunofluorescence studies, SMCs and IMR-90 cells were grown in Lab-Tek tissue culture chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). At intervals after infection with CMV, slides were washed once in phosphate-buffered saline fixed in methanol-acetone (1:1) for 7 min at 5°C. To avoid nonspecific reactions with immunoglobulin G Fc receptors induced by CMV infection (11), CMV antigens were detected by the indirect anti-complement immunofluorescence test with guinea pig complement and anti-guinea pig complement C3 conjugated with fluorescein isothiocyanate (U.S. Biochemical Corp., Cleveland, Ohio). Slides were incubated for 30 min at 30 to 31°C after the addition of each reagent. Hanks balanced salt solution without phenol red was used to dilute all reagents and to wash slides after incubation with antiserum, complement, and conjugated anti-C3 in sequence.

After testing many human sera for the presence or absence of antibody against CMV, Epstein-Barr virus, and herpes simplex virus types 1 and 2 by immunofluorescence and by complement fixation (herpes simplex viruses), the sera of two donors were selected for use in these experiments. One donor serum reacted only against CMV and contained CMV

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neutralizing antibody. The other donor serum reacted against Epstein-Barr virus and herpes simplex viruses but not against CMV. The positive and negative reactions of the two respective indicator sera were identical 5 to 6.5 h and 24 h after infection of IMR-90 cells with the AD-169 strain of CMV that was obtained from T. Albrecht (University of Texas Medical Branch, Galveston, Tex.) and the American Type Culture Collection. Uninfected SMCs or IMR-90 cells did not react specifically with the indicator sera that were used routinely at a dilution of 1:10.

The reactivity of SMCs and of IMR-90 cells with human anti-smooth muscle sera (ASMA) obtained from two commercial sources (Immuno-Products Industries, South Bound Brook, N.J., and Bioware Products, Whippany, N.J.) also was tested. Cells in Lab-Tek chamber slides were treated with 0.5% Triton X-100 as described by Danto and Fischman (5), but HEPES (*N*-2-hydroxyethypiperazine-*N'*-2-ethanesulfonic acid) hydrochloride was substituted for PIPES (piperazine-*N*-*N'*-bis(2-ethanesulfonic acid) hydrochloride in the suspending buffer, and after incubation with ASMA, cells were exposed to anti-human globulin conjugated with fluorescein isothiocyanate (Electro-Nucleonics, Inc., Columbia, Md.). The reactivity of ASMA was confirmed with sections of rat stomach (Clinical Sciences Inc., Whippany, N.J.) by the same indirect fluorescence technique. Cells of the inner and outer SMC layers and of the muscularis mucosae fluoresced brightly. The submucosal layer, which contains many fibroblasts, did not fluoresce except for the muscular walls of arterioles. This was true whether or not sections were pretreated with Triton X-100.

**Infection of cell cultures.** A single lot of the AD-169 strain of CMV, which was obtained from T. Albrecht, grown and quantified in IMR-90 cells, and stored at −80°C, was used in these studies. IMR-90 cells infected with AD-169 reacted by immunofluorescence with only the CMV-positive, Epstein-Barr virus-negative, herpes simplex virus-negative serum but not with the CMV-negative, Epstein-Barr virus-positive, herpes simplex virus-positive serum. Nearly confluent SMC cultures in passage 3 and IMR-90 cultures between passages 14 and 23 were prepared in Lab-Tek chamber slides, infected with a multiplicity of infection of three to five 50% tissue culture infective doses per cell, incubated at 37°C, and at intervals thereafter were fixed, along with uninfected SMCs and IMR-90 cells, and reacted with the indicator sera to detect CMV antigens. Larger cultures (25 $cm^2$) in the same passage were infected with the same multiplicity of infection when approximately 50% confluent and were maintained and observed for the length of their survival in parallel with uninfected SMC cultures and with uninfected IMR-90 cultures. Infected IMR-90 cultures were maintained in Eagle minimum essential medium containing 10% bovine newborn calf serum for about 14 days, until destruction of the cultures was complete as judged by detachment of virtually all cells. SMCs were maintained in Dulbecco modified Eagle medium containing 10% bovine newborn calf serum for about 20 days after infection, and then the serum supplement was changed to 30% bovine fetal serum. Cultures were examined by electron microscopy at intervals after infection. After subcultivation, the cultures were sampled at different intervals for recoverability of infectious CMV by transfer of spent media to IMR-90 cultures.

## RESULTS

**Identity of cultures derived from the umbilical artery.** The characteristics of proliferating SMC cultures were similar to human SMC cultures derived from blood vessels of the umbilical cord, as described by Gimbrone and Cotran (10). Endothelial cells, readily recognized by their morphology and organization, did not grow in these cultures as a result of prolonged collagenase digestion of tissue initially and of sensitivity to trypsin exposure for at least 5 to 10 min during subcultivation procedures (10). SMC cultures maintained beyond confluence eventually developed a distinctive topography unlike that of IMR-90 fibroblast cultures. Multilayered accumulations of cells were surrounded by parallel, generally contiguous, elongated cells traversed by wavy bands. (Fig. 1) (10). These cells resembled SMCs undergoing "dedifferentiation," a presumed prerequisite modification before proliferation is possible (4). When examined by electron microscopy, cytoplasmic dense bodies within a filamentous network characteristic of SMCs (10) could be found readily (Fig. 2); such structures were not observed in IMR-90 cells. Interspersed between this dense growth in less populated areas were foci of flattened SMCs with prominent cytoplasmic strands or filaments (Fig. 3) (10). These latter groups were relatively small in number in passage 3 but predominated eventually as dividing cells dwindled and focal accumulations or hillocks disappeared at passages 5 to 6. Surviving cells could be maintained for months with no apparent increase in cell numbers. Eskin et al. (6) reported a similar course of increase and decline of SMCs derived from human atherosclerotic plaques and from the uninvolved vessel wall.

SMCs derived from two different umbilical arteries, but not IMR-90 cells, reacted positively (Fig. 4) with ASMA. Although SMCs differed in numbers of fluorescent filaments or strands and in the intensity of fluorescence, more than 90% of the cells were estimated to react positively with ASMA. That a small minority of cells did not react with ASMA might have been because of differences in expression or accessibility of antigen resulting from differences in cell cycle or viability, or heterogeneity in the level of dedifferentiation of SMCs, atypical behavior resulting from adaptation to an in vitro milieu, variation among cells in susceptibility to the effects of Triton X-100 extraction, or test sensitivity.

**Patterns of immunofluorescence.** During the first 6 days after infection, differences in the presence, intensity, and temporal development of CMV immunofluorescence were noted in SMC cultures infected in passage 3 compared with infected IMR-90 fibroblast cultures. These differences supported, in still another way, conclusions about the identity of the umbilical artery cultures as SMCs rather than fibroblasts based on topography, ultrastructure, and immunofluorescence with ASMA.

At 6 h after infection of IMR-90 cultures with CMV, nuclear fluorescence was detectable in virtually all nuclei, many of which were brightly fluorescent, whereas in SMC cultures a minority of nuclei were fluorescent and fluorescence was dimmer generally than in infected IMR-90 cells. Immunofluorescence increased progressively in SMC cultures 24 to 48 h after infection. The intensity of nuclear fluorescence increased, and fluorescing nuclei were detectable in greater numbers of cells than at 6 h after infection. Cytoplasmic fluorescence began to be discernible in SMC cultures during this interval but was present in almost all IMR-90 cells. By days 4 to 6 after infection, cytoplasmic fluorescence began to predominate over nuclear fluorescence in SMC cultures, but intact, well-spread SMCs with only intranuclear fluorescence were still observed. These might have been cells with delayed synthesis of CMV antigen or cells with arrested replication of CMV at the stage

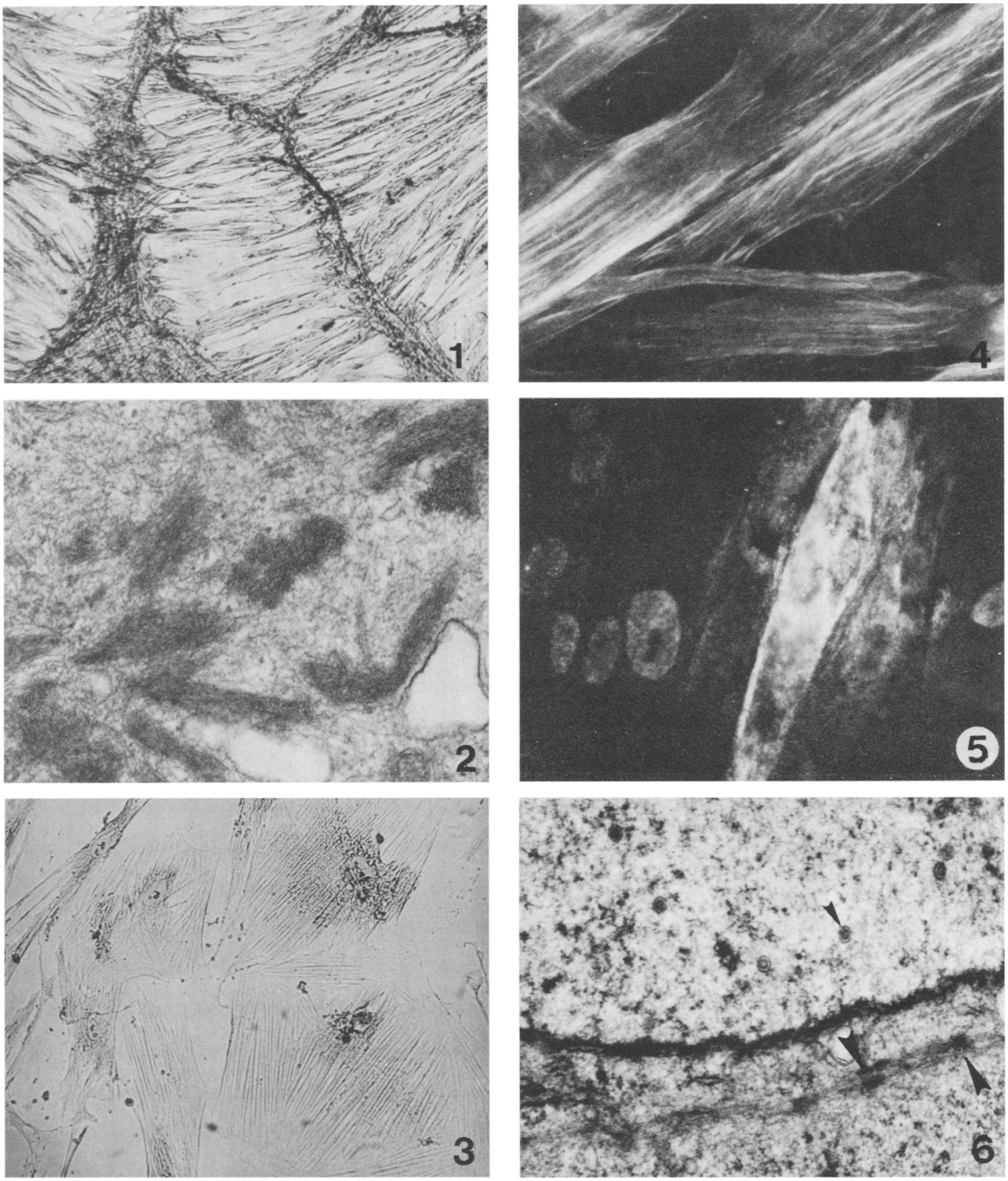
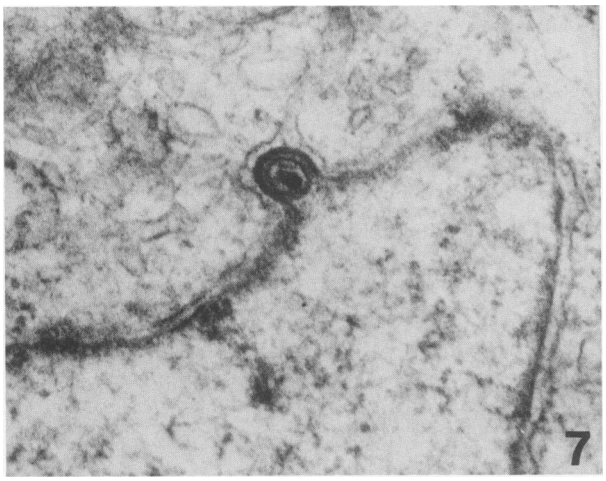
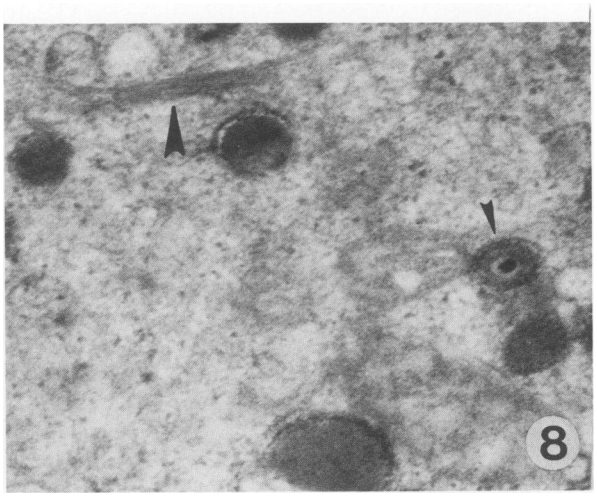
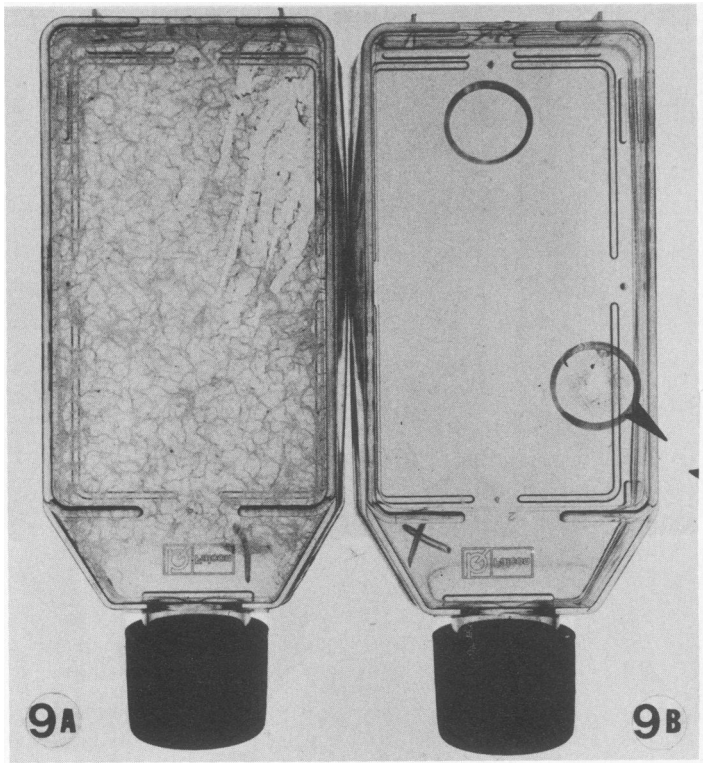
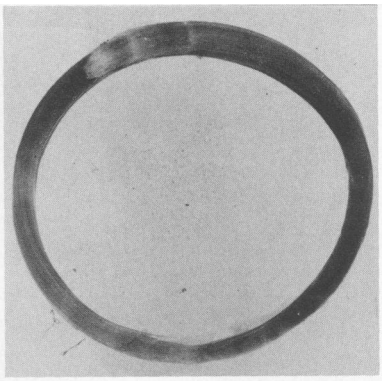


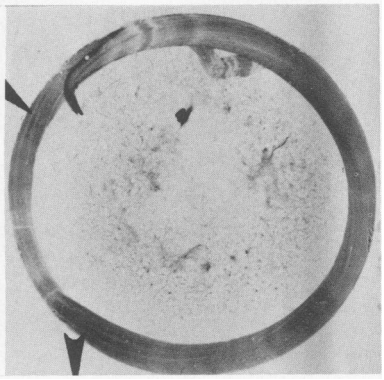

FIG. 1. Typical postconfluent topography of dedifferentiated SMC cultures. Magnification, ×150.
FIG. 2. Dense bodies within filamentous network in cytoplasm of SMCs. Magnification, ×52,700.
FIG. 3. Focus of SMCs outside of actively proliferating areas. Magnification, ×370.
FIG. 4. Fluorescent filaments in SMCs reactive with ASMA. Magnification, ×1,600.
FIG. 5. Nuclear and cytoplasmic CMV immunofluorescence in cells of SMC cultures. Magnification, ×1,200.
FIG. 6. SMCs containing several CMV capsids within the nucleus (small arrow) and dense bodies (large arrows), with associated filaments within the cytoplasm. Magnification, ×29,700.

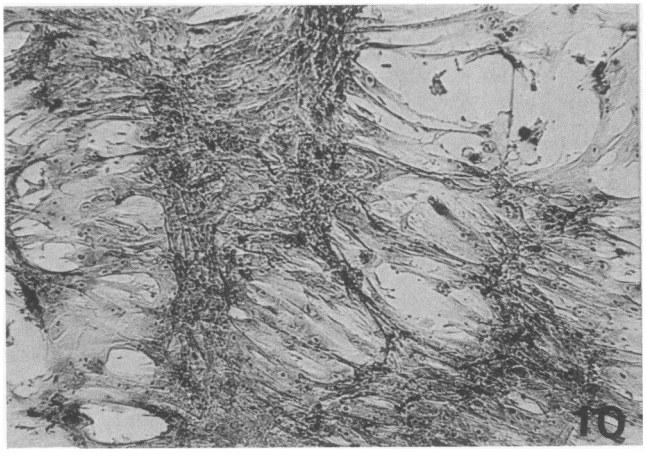












of early intranuclear antigen development. No fluorescence of any kind was detectable in approximately 50% of adherent, intact cells on day 6 after infection of SMC cultures. A typical focus of nuclear and cytoplasmic fluorescence on day 4 after infection is shown in Fig. 5. The presence of CMV-specific fluorescence in many more cells than were unreactive (<10%) with ASMA indicates that CMV antigen was being synthesized in some SMCs.

**Replication of CMV.** The cytopathic effect of CMV in SMCs paralleled the pattern of CMV immunofluorescence. Cytopathic effects were delayed compared with infected IMR-90 fibroblasts, and complete destruction of SMC cultures followed a protracted course. IMR-90 cultures showed rounding or retraction of most cells about 48 h after infection; cells gradually detached until almost none remained adherent approximately 2 weeks after infection. In contrast, the cytopathic effects in SMC cultures were delayed and then prolonged, appearing to involve the culture incrementally over a period of 3 to 4 months, at which time few surviving cells remained. CMV nucleocapsids were found readily in some of the nuclei of SMCs. CMV capsids within the nucleus of a cell containing cytoplasmic dense bodies and associated filaments characteristic of SMCs 6 days after infection are shown in Fig. 6; enveloped virions were also found in such cells. A CMV nucleocapsid being enveloped at the nuclear membrane 6 days after infection of an SMC culture is shown in Fig. 7. Approximately 1 month after infection of SMC cultures, when control, infected IMR-90 fibroblast cultures were no longer viable, enveloped virions were found readily in surviving SMC cultures. An enveloped virion is shown within a cytoplasmic vacuole of an SMC along with a remnant of the dense body-filament system (Fig. 8).

Intact SMCs, apparently normal in appearance when examined by light microscopy, could be found during much of the protracted life-span of infected SMC cultures. Some surviving foci in SMC cultures infected during passage 3 showed a capacity for growth as judged by the development of multilayering (Fig. 9B and 10) and the expansion of the colony in passage 3. Flasks of uninfected and infected cultures are shown approximately 6 weeks after infection, with a focus of surviving layered cells and a separate area of cells with no apparent tendency to form layers in the infected culture (Fig. 9). Growth and cytopathic effects appeared to occur simultaneously in the infected cultures, but growth was insufficient to repopulate the culture to its initial density. Multilayering was not observed in subsequent passages of infected SMC cultures. Infected SMCs from one cord were subcultivated twice and those from the second cord once, when it was judged that insufficient replicative capacity remained for further passage.

Infectious CMV continued to be isolated from infected SMC cultures near the end of their existence after a greater than $10^{20}$-fold dilution of the initial inoculum ($2 \times 10^6$ 50% tissue culture infective doses) by washing and by continual replacement of the nutrient medium during maintenance of cultures. This finding also indicates that some SMCs permitted CMV replication, because at such late intervals permissive fibroblasts could no longer have been the origin of infectious CMV in the SMC cultures. Generally, several foci of infection could be observed in an IMR-90 culture after transfer to it of the entire volume of spent medium from an infected terminal SMC culture. Total destruction of the IMR-90 culture ensued. An SMC culture producing several focus-forming units still yielded infectious CMV after a 90-min treatment at 37°C with a dilution of anti-CMV serum with the capacity to neutralize at least 10 50% tissue culture infective doses of CMV, suggesting further the formation of new infectious virus. Infectious virus from terminal SMC cultures transferred to IMR-90 cells reacted in the same way by immunofluorescence with the two indicator sera as CMV infecting IMR-90 cells initially. Although subcultured infected SMC cultures persisted for relatively lengthy periods, their life-spans did not exceed those of subcultured uninfected control SMC cultures. Mycoplasma were not detected in the infected SMC cultures. The comparative effects of CMV on human lung fibroblasts and on SMC cultures are summarized in Table 1.

TABLE 1. Comparative effects of CMV on smooth muscle and on fibroblast cell cultures of human origin

| Cell culture | Cytopathic effect | Time of complete destruction of culture (approx) | CMV antigens detected by immuno-fluorescence | Formation of enveloped nucleocapsids |
|---|---|---|---|---|
| IMR-90[a] | + | 14 days | + | + |
| SMC[b] | +; Delayed, extensive, prolonged | 3–4 mo | +; Delayed, not detected in many cells | + |

[a] Fibroblasts from fetal lung.
[b] SMCs from umbilical artery.

## DISCUSSION

In studies of SMC cultures initiated from both atheromatous and uninvolved arterial tissue of atherosclerotic patients, Melnick et al. (13) used the immunofluorescence technique with mouse or guinea pig antisera against human CMV strains AD-169 and C87, respectively. They found only cytoplasmic fluorescence in some cultures but no nuclear fluorescence and no evidence of viral replication by electron microscopy or by cocultivation with permissive cells.

In the present study, both nuclear fluorescence and cytoplasmic fluorescence were observed in human SMC cultures derived from umbilical arteries and infected with CMV. Enveloped CMV nucleocapsids also were detected by elec-

FIG. 7. CMV nucleocapsid being enveloped at the nuclear membrane of a cell in an SMC culture. Magnification, ×52,700.

FIG. 8. SMC containing an enveloped virion within a cytoplasmic vacuole (small arrow). Remnant of dense body-filament system( large arrow) also visible. Magnification, ×50,500.

FIG. 9. Uninfected control and infected SMC cultures approximately 6 weeks after infection. (A) Uninfected control SMC culture. (B) SMC culture after infection with CMV in passage 3. Lower circle delineates a single surviving focus of multilayered cells, the center of which has detached. Higher magnifications of this focus are shown at right and in Fig. 10 (arrows). Upper circle delineates area of surviving cells showing no tendency to accumulate in layers, with higher magnification to the right.

FIG. 10. Single focus of multilayered SMC growth approximately 6 weeks after infection with CMV in a culture otherwise markedly depopulated by CMV cytopathic effect (Fig. 9B). Magnification, ×150.

tron microscopy in cells containing dense bodies and associated filaments, structural characteristics of SMCs. Months after destruction of the infected control fibroblast cultures and extinction of the original inoculum by dilution, infectious virus was demonstrated in surviving SMC cultures by transfer to permissive IMR-90 cells with ensuing cytopathic effects and CMV specific immunofluorescence. The latter evidence suggests that some SMCs are permissive for replication of infectious CMV. However, the delayed nature of CMV-specific immunofluorescence, the presence of only nuclear fluorescence relatively late after infection in some SMCs, and the absence of fluorescence in other SMCs suggest the possible existence of some nonpermissive SMCs. Nonpermissive systems, i.e., hamster or guinea pig fibroblast cultures supporting CMV antigen development as detected by immunofluorescence but not permitting infectious CMV production have been described (1, 8).

This study, describing an essentially similar protracted course of cytopathic effects and delayed development of CMV immunofluorescence in SMC cultures from two different donors, may explain the failure of Benditt et al. (2) to detect either the CMV genome by in situ hybridization or cytopathic effects in human fetal SMC cultures examined only 24 and 48 h after infection. It remains to be established whether the eventual death of all SMCs surviving the acute phase of CMV infection was because of viral effects or whether some portion of the surviving population was refractory to infection from the outset and eventually ceased to divide, apparently entering a phase of senescent crisis, and died, just as the uninfected SMCs.

Melnick et al. (13) suggest that because they detected CMV antigen but not replicating CMV by electron microscopy, the artery wall may be a site of CMV latency. Although we demonstrated replicating CMV in human arterial SMC cultures by both electron microscopy and infectivity and our infected cultures failed to outlive our control SMC cultures, this does not preclude the possibility of latency of CMV in human arterial SMCs. Indeed, the much less permissive nature of SMCs compared with fibroblasts for rapid CMV replication and cytopathic effect and the finding of an occasional focus of proliferating SMCs in long-infected cultures (Fig. 10) suggest SMC cultures as useful for further studies of CMV with reference to the possibility of both latency and transformation. Among other possibilities, the establishment of latency may depend on individual differences in the susceptibility of SMCs, on the CMV strain (19), on the influence of humoral and cell-mediated immunity, and on chance infection or recrudescence during critical phases of vessel repair. To the extent that CMV replicates in and destroys proliferating SMCs, it may interfere with the repair or resolution of lesions dependent on the division of SMCs. Both atherosclerotic lesions and lesions caused by the mechanical denudation of endothelial cells in monkeys are characterized respectively by SMCs with higher rates of DNA synthesis (22) and by hyperplastic proliferation of modified SMCs (21). CMV-mediated interference with vessel wall repair may be mitigated by immune factors arising after primary infection of the host or recalled upon secondary infection or upon recrudescence of latent infection. If CMV becomes latent at such sites, a vascular lesion may undergo intermittent resolution and dissolution as latent CMV is activated and destroys intimal cells, in turn provoking division of SMCs and more damage to the modified or differentiated SMCs, with resolution once again as immune factors reestablish homeostasis. The occurence of atypical proliferation of intimal SMCs and of degenerative processes in the evolution of atherosclerosis is well known. Thus, in addition to or instead of its possible role as a tranforming agent, CMV may play a role in atherogenesis compatible with the "injury" theory of atherogenesis, by virtue of its ability to replicate in human arterial SMCs.

## ACKNOWLEDGMENTS

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