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Replication of *Chlamydia pneumoniae* In Vitro in Human Macrophages, Endothelial Cells, and Aortic Artery Smooth Muscle Cells

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Chlamydia pneumoniae* has recently been associated with atherosclerotic lesions in coronary arteries. To investigate the biological basis for the dissemination and proliferation of this organism in such lesions, the in vitro growth of *C. pneumoniae* was studied in two macrophage cell lines, peripheral blood monocyte-derived macrophages, human bronchoalveolar lavage macrophages, several endothelial cell lines, and aortic smooth muscle cells. Five strains of *C. pneumoniae* were capable of three passages in human U937 macrophages and in murine RAW 246.7 macrophages. Titers were suppressed in both macrophage types with each passage, as compared with growth titers in HEp-2 cells. Both human bronchoalveolar lavage macrophages and peripheral blood monocyte-derived macrophages were able to inhibit *C. pneumoniae* after 96 h of growth. Eleven *C. pneumoniae* strains were capable of replicating in normal human aortic artery-derived endothelial cells, umbilical vein-derived endothelial cells, and pulmonary artery endothelial cells. Infection in human aortic artery smooth muscle cells was also established for 13 strains of *C. pneumoniae*. The in vitro ability of *C. pneumoniae* to maintain infections in macrophages, endothelial cells, and aortic smooth muscle cells may provide support for the hypothesis that *C. pneumoniae* can infect such cells and, when infection is followed by an immune response, may contribute to atheroma formation in vivo. More studies are needed to investigate the complex relationship between lytic infection and persistence and the potential for *C. pneumoniae* to influence the generation of atheromatous lesions.

Chlamydia pneumoniae has been established as an important respiratory pathogen associated with 5 to 10% of community-acquired cases of pneumonia, pharyngitis, bronchitis, and sinusitis (3, 5, 7, 11–14, 25). In addition, *C. pneumoniae* infection has been associated with asthma, acute chest syndrome of sickle cell anemia, human immunodeficiency virus infection, Guillain-Barré syndrome, endocarditis, and otitis media and with patients with immunosuppressive diseases (1, 4, 8, 16, 17, 26–28). Cases of chronic persistent respiratory infection, in which antibiotic therapy failed to eradicate the organism, have also been reported (18). There is also evidence that *C. pneumoniae* can be cultured infrequently (2 and 4.5%, respectively) from asymptomatic and selectively healthy individuals (10, 20, 21).

More recently, *C. pneumoniae* has been associated with coronary artery disease (24, 30, 31, 33, 34). The earliest serological association was reported by Saikku et al., who demonstrated an association of increased antibody titers to *C. pneumoniae* in men with acute myocardial infarction and chronic coronary heart disease (31). In another prospective study, they demonstrated that chronic *C. pneumoniae* infection may be a risk factor for the development of coronary heart disease (30). In the United States, Thom et al. reported a relationship between antibody to *C. pneumoniae* and angiographically demonstrated coronary artery disease (34). A study of autopsy cases from South Africa detected *C. pneumoniae* organisms by electron microscopy in the coronary arterial fatty streaks and atheromatous plaques in seven autopsy subjects (33). Immunoperoxidase staining using monoclonal antibodies for *C. pneumoniae* was positive for five of the subjects, demonstrating organisms in the cells around the plaque and in the smooth muscle layer. Since other chlamydiae have been reported to be capable of growth in macrophages and monocytes, a hypothesis of venous and arterial transmission from the respiratory tract to coronary lesions has been postulated for *C. pneumoniae* (15, 29, 38). To explore the biological basis for such a route of dissemination and local infection at an initial site of injury in a coronary artery, we investigated the ability of *C. pneumoniae* to infect macrophage cells as well as its capability to infect endothelial cells of venous and arterial origin. In addition, we explored its ability to replicate in aortic artery smooth muscle cells.

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MATERIALS AND METHODS

Cell lines. RAW 264.7 cells (termed RAW cells), a murine macrophage cell line (ATCC CRL-24), and HEp-2 cells were maintained in Dulbecco medium supplemented with 10% fetal calf serum, glutamine, and antibiotics (19). U937 cells, a nonadherent human macrophage line, and McCoy cells were maintained in RPMI 1640 supplemented as described above. U937 cells were adhered by treatment with myristate phorbol acetate (10 ng/ml). Human pulmonary alveolar macrophages obtained by bronchoalveolar lavage (BAL) and peripheral blood monocyte-derived macrophages (PBMCs) were obtained from normal human male donors. Macrophages and monocytes were washed in RPMI 1640 and plated at 10^6/ml. BAL macrophages were allowed to adhere for 3 days before inoculation. PBMCs were allow to adhere and transform into macrophages for 1 week, with daily changes of supplemented RPMI 1640 before inoculation. Normal human umbilical vein-derived endothelial cells (HUVEC), normal human aortic artery-derived endothelial cells (HAEC), and normal human pulmonary

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artery cells were obtained from Clonetics, Inc. (San Diego, Calif.), and maintained in endothelial cell growth medium (EGM; Clonetics), which was supplemented with human recombinant epidermal growth factor, hydrocortisone, fetal bovine serum, gentamicin, amphotericin, and bovine brain extract (Clonetics). The cells were propagated in either shell vials or 96-well tissue culture plates. Cell viability was monitored by trypan blue exclusion. Quality control for the macrophage lines consisted of staining with fluorescein isothiocyanate (FITC)- or phycoerythrin-labeled anti-CD14 monoclonal antibodies (Coulter Electronics, Inc., Hialeah, Fla., and Becton Dickinson, San José, Calif., respectively). HUVEC and HAEC were identified as endothelial cells by staining with FITC-labeled anti-factor VIII antibody (INC Star Atlantic Antibodies, Stillwater, Minn.) and human DiI-acetyl low-density lipoprotein (Biotech Research Institute, Rockville, Md.) uptake.

Aortic artery smooth muscle cells were obtained as cryopreserved tertiary cultures from a single male donor from Clonetics, Inc. The smooth muscle cells were grown in either 96-well plates or shell vials in smooth muscle growth medium (Clonetics) supplemented with recombinant epidermal growth factor, fibroblast growth factor, dexamethasone, fetal bovine serum, gentamicin, and amphotericin. For quality control, smooth muscle cells were also stained with anti-α actin monoclonal antibody (Sigma Chemical Co., St. Louis, Mo.). The anti-α actin antibody was visualized by use of either fluorescein-conjugated or rhodamine-conjugated goat anti-mouse immunoglobulin G antibody (Cappel Research Products, Organon Teknika Corporation, Durham, N.C.). For negative controls, anti-α actin antibody was used on HEp-2 cells and fluorescein-conjugated anti-factor VIII antibody was applied to smooth muscle cells to rule out the presence of endothelial cells.

HEp-2 cells and McCoy cells were used as control cell lines to assess inoculum titer, and HEp-2 cells were used to determine titers after the macrophage inhibition assays.

Organisms. Strains of *C. pneumoniae* used were TW 183, AR 39, AR 388, VR 1310, 2023, 2043, T2364, BAL 15, BAL 16, BAL 37, CM1, CWL 011, and FML 16 (9). The *Chlamydia trachomatis* serovars used were B, Ba, C, E, F, G, H, and L_2. The *Chlamydia psittaci* strains used were 6BC and SM006.

Growth and staining. Organisms were grown in cells in either shell vials (200 μl) or 96-well microtiter plates (100 μl). After centrifugation for 1 h at 37°C at 800 × *g*, the inoculum was removed and growth medium containing 1 μg of cyclohexamide per ml was added. For McCoy cells and HEp-2 cells, growth medium was supplemented RPMI 1640 or Iscove's Dulbecco medium (19). For smooth muscle cells, smooth muscle growth medium (Clonetics) was used. EGM was used for endothelial cells. After growth for 48 or 72 h and fixation with methanol, inclusions of *C. pneumoniae* and *C. psittaci* were stained with genus-specific FITC-labeled monoclonal antibody (Kallestad, Chaska, Minn.) and inclusions of *C. trachomatis* were stained with species-specific FITC-labeled monoclonal antibody (Syva, San José, Calif.).

Passage of chlamydiae in U937 and RAW macrophages. The growth kinetics of *C. pneumoniae* in U937 and RAW macrophages was studied with five strains (TW 183, AR 39, 2023, BAL 15, and CM1). The same size inocula (10^3 inclusion-forming units [IFU]/ml) were used for each strain for the two macrophage cell types and for HEp-2 cells. Each strain was inoculated simultaneously in replicate 10-fold serial dilutions in 96-well plates. At the end of each passage (72 h), one replicate dilutional series of wells were stained and the inclusions were enumerated to determine the titer, while the other replicate unstained wells were passaged to fresh cell layers of the same type for the next passage. The infected cells in each well of the dilutional series to be passaged were removed each time by complete scraping of the well with a pipette tip before the inoculation of the fresh cell layers. The inoculum volume was maintained at a constant of 200 μl each time. Growth titers were expressed as mean titers of the five strains of *C. pneumoniae* in inclusion-forming units per milliliter. Similarly, *C. trachomatis* serovars B, G, and H and *C. psittaci* 6BC were also passaged in RAW and U937 cells.

Viability studies of *C. pneumoniae* in U937 and RAW macrophages. To assess the maintenance of viability after growth in macrophages for different time periods and the decrease or increase in infectious progeny titers over time, scrapings of replicately inoculated wells were passaged back to HEp-2 cells in the following manner. Identical inocula (10^3 IFU/ml) of each of three strains of *C. pneumoniae* (AR 39, 2023, and BAL 15) and strain TW 183 (2×10^2 IFU/ml) were simultaneously inoculated into each of the three cell types, U937, RAW, and HEp-2 (control). At time points from 48 to 96 h, replicate wells of infected cells in 96-well plates were scraped and frozen at −70°C, and growth titers were determined subsequently in HEp-2 cells. Specifically, inclusion counts were determined after thawing and sonication of the scraped wells in a water bath ultrasonic sonicator by making 10-fold serial dilutions of the sonicate in supplemented RPMI 1640 and inoculating 100 μl of each dilution in duplicate into fresh HEp-2 cell layers. Growth titers were averaged to obtain the mean titers of the four strains of *C. pneumoniae* and were expressed as inclusion-forming units per milliliter.

Viability studies of *C. pneumoniae* in human BAL and PBMC macrophages. Only strain 2023 of *C. pneumoniae* was tested in the viability assay with human BAL and PBMC-derived macrophages because of the availability of the cells. For the BAL macrophages and corresponding HEp-2 control cells, the inoculum titer was 10^4/ml. For both the PBMC-derived macrophages and their control HEp-2 cells, the inoculum titer, determined at the time of inoculation, as described above, was 4.6×10^4/ml. At time points from 24 to 96 h, infected cell layers were scraped, frozen at −70°C, and used subsequently for determination of the titer. The titers of the thawed and sonicated specimens were determined as described above for infectious progeny in control HEp-2 cells. Growth titers were expressed as inclusion-forming units per milliliter.

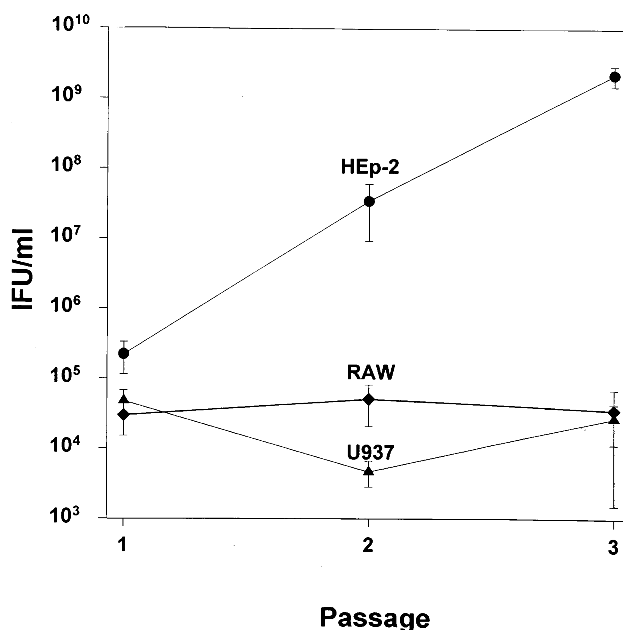

FIG. 1. Successive passage of five strains of *C. pneumoniae* in U937, RAW, and HEp-2 cells. Titers are expressed as the mean titers and standard errors of five strains of *C. pneumoniae*. Growth titers are expressed as inclusion-forming units per milliliter.

Growth of *C. pneumoniae* in endothelial cells. In general, comparative growth studies were performed by placing the same dilutions of sonicated inocula simultaneously into the test cell line and reference cell line (HEp-2). Titers were determined in duplicate, and counts of inclusions were averaged. Growth was defined in terms of visible fluorescent-stained inclusion bodies, which were enumerated per well and calculated as inclusion-forming units per milliliter. Ten strains of *C. pneumoniae* were each inoculated into HAEC and HEp-2 cells from frozen stock at 10^4 IFU/ml. In a separate experiment, the 10 strains of *C. pneumoniae* were each inoculated into HUVEC and HEp-2 cells from frozen stock originally titered at 10^3 IFU/ml. The titer of each strain of *C. pneumoniae* inoculated into HAEC or HUVEC was determined simultaneously in HEp-2 cells to eliminate any changes in inoculum titer due to freezing or experimental effects. Growth titers were compared for HEp-2 and HAEC or HUVEC with identical inocula administered from the same sonicated vial at the same time. Titers were calculated from duplicate wells and subsequently averaged to obtain mean titers for all of the strains that grew in each cell type. For growth in HPAEC and HEp-2 cells, inocula of both 10^4 and 10^3 IFU/ml for 11 strains of *C. pneumoniae* were used. The resulting numbers of inclusions were assessed by semiquantitative count, rather than by actual titer (by serial dilution), of inclusions per milliliter.

Growth of *C. pneumoniae* in smooth muscle cells. Thirteen strains of *C. pneumoniae* were each used to simultaneously infect smooth muscle cells and HEp-2 cells as controls with inocula of both 10^4 and 10^3 IFU/ml. The numbers of inclusions were assessed semiquantitatively. In a second experiment, three strains (AR 388, 2023, and FML 16) were diluted to a low inclusion count (10^2 to 10^3 IFU/ml), and the titers of the identical inocula were determined simultaneously in smooth muscle cells and HEp-2 cells, with the exact inclusion counts enumerated and expressed as inclusion-forming units per milliliter. In addition, these same three strains were passaged two or three more times in smooth muscle cells to assess the maintenance of viability in the smooth muscle cells over time.

RESULTS

Passage of *C. pneumoniae* in U937 and RAW macrophages. Of five *C. pneumoniae* strains (TW 183, AR 39, 2023, BAL 15, and CM1) tested at an inoculum titer of 10^3 IFU/ml, all were capable of three passages in U937 cells (Fig. 1). The titer of this inoculum from the freezer into the macrophage cells is

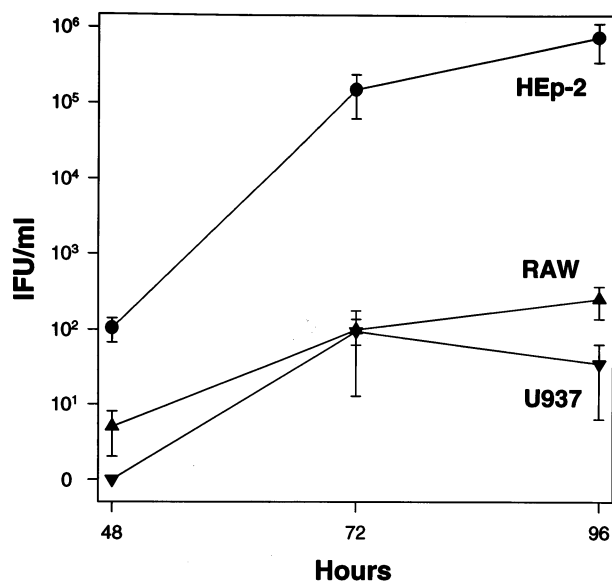

FIG. 2. Viability of *C. pneumoniae* after growth by U937 and RAW macrophage cells. Inocula of identical titer of each of four strains of *C. pneumoniae* were inoculated simultaneously into each of the three cell types, i.e., U937, RAW, and HEp-2 (control). At specific time points, replicate wells of infected cells in 96-well plates were scraped, and growth titers were determined subsequently in HEp-2 cells to assess production of infectious progeny by the U937 and RAW macrophages. Growth titers represent the mean titers of four strains of *C. pneumoniae* and are expressed as inclusion-forming units per milliliter with standard errors.

termed passage one. Titers of new inclusions, representing the results of a growth cycle from elementary body through the reticulate body form to the formation of an inclusion, generally decreased slightly in the second passage, while remaining the same or increasing marginally in the third passage. All five *C. pneumoniae* strains were also able to sustain passage three times in RAW cells, with mean titers remaining approximately the same for each passage (Fig. 1). The titers of the same inocula increased 2 to 3 logs each time when passaged at the same time in HEp-2 cells. Upon reinoculation from macrophage cells back into HEp-2 cells, each strain of macrophage-passaged organisms appeared to grow as well as unpassaged organisms (data not shown). *C. trachomatis* serovars B, G, and H were all completely inhibited by the third passage in both U937 and RAW cells. *C. psittaci* was inhibited at passage 2 in U937 cells but survived three passages in RAW cells.

Viability in U937 and RAW macrophages. When chlamydial strains were assessed for viability in HEp-2 cells at various time points after growth in U937 and RAW cells, three of four *C. pneumoniae* strains were producing infectious progeny by 72 h but at a titer 2 to 4 logs lower than that when strains were passaged through HEp-2 cells (Fig. 2). *C. pneumoniae* TW 183, inoculated at a lower initial concentration (2×10^2 IFU/ml) than the other strains, was completely inhibited by the macrophages. The other strains (AR 39, 2023, and BAL 15), which were inoculated at 10^3 IFU/ml, were also able to sustain viability in HEp-2 cells after passage through the macrophage cell lines. *C. trachomatis* serovars B and E behaved like *C. pneumoniae* strains did, producing viable organisms at titers lower than those observed after passage through McCoy cells. For example, comparable titers for serovar B, passage 1, were 6.2 $\times 10^3$ IFU/ml in U937 cells and 4.0×10^3 IFU/ml in McCoy cells. For *C. psittaci*, the corresponding titers were 1.0×10^1 and 8.0×10^3 IFU/ml, respectively. The titers in RAW cells were 7.1×10^3 IFU/ml for serovar B and 1.4×10^2 IFU/ml for *C. psittaci*. By passage 2, the titer for serovar B dropped to 1.6 $\times 10^3$ IFU/ml in U937 cells and to 4.8×10^3 IFU/ml in RAW cells, and there was no growth in passage 3. For *C. psittaci*, in passage 2, there was no growth in U937 cells, but for RAW cells, the titers were 1.4×10^4 IFU/ml for passage 2 and 2.4×10^2 IFU/ml for passage 3.

Viability studies of *C. pneumoniae* in human BAL and PBMC macrophages. After 96 h of growth in the BAL macrophages, the infectious progeny, as determined in HEp-2 cells, had decreased in titer to 6×10^2 IFU/ml. However, the same inoculum had increased in titer to 2.8×10^6 IFU/ml after 96 h of growth in the control HEp-2 cells. The growth kinetics of *C. pneumoniae* in both cell types are shown in Fig. 3A. The same experiment performed with PBMC-derived macrophage cells from the same donor demonstrated the same phenomenon of partial, not complete, inhibition. After 96 h of growth in the PBMC-derived macrophages, the viable growth titer, as determined in HEp-2 cells, had decreased to 2.0×10^2 IFU/ml, whereas the titer of the same inoculum in HEp-2 cells had markedly increased to 1.2×10^7 IFU/ml. A similar titer increase in HEp-2 cells and an inhibition of viable progeny occurred in PBMC-derived macrophages over the time points from 24 to 96 h (Fig. 3B).

Comparison of growth in endothelial cells. Eight of ten strains of *C. pneumoniae* grew in HAEC at a mean titer of 3.2 $\times 10^1$ IFU/ml as compared with growth in HEp-2 cells at a mean titer of 9.5×10^3 IFU/ml ($P = 0.085$) (Fig. 4). The inclusions formed in the HAEC were somewhat smaller in size and always fewer in number but were well formed and able to be scraped and passaged to fresh HAEC layers, with an increase in numbers of inclusions (data not shown).

The HUVEC also supported growth of 9 of 10 strains of *C. pneumoniae*, with a mean titer of 4.3×10^2 IFU/ml, as compared with growth in HEp-2 cells at a mean titer of 9.5×10^3 IFU/ml ($P = 0.099$) (Fig. 4).

All 11 strains of *C. pneumoniae* tested were able to replicate in normal human pulmonary artery cells when an inoculum of 10^4 IFU/ml was used. The numbers of inclusions, as assessed semiquantitatively, and inclusion size and shape were similar to those seen in HEp-2 control monolayers. Reduced numbers of inclusions in the normal human pulmonary artery cells relative to that in the HEp-2 cells were seen in 5 of the 11 strains tested when the inoculum size was reduced to 10^3 IFU/ml (data not shown).

Comparison of growth in smooth muscle cells. Initially, 12 of 13 strains of *C. pneumoniae* grew in the smooth muscle cells. The one strain which did not grow (FML 16) was able to replicate when inoculated in a second experiment. When the three strains AR 388, 2023, and FML 16 were passaged in smooth muscle cells, all were passaged successively. AR 388 was maintained through three passages. Six of eight *C. trachomatis* serovars (including L_2) and two of two strains of *C. psittaci* also grew in smooth muscle cells. Inclusions in smooth muscle cells were of comparable size but generally fewer in number than those grown in McCoy and HEp-2 cells from identical inocula. For the three strains of *C. pneumoniae* which were diluted to a low inclusion count (10^2 to 10^3 IFU/ml), the titers were approximately 1 log lower for the smooth muscle cells than for the HEp-2 cells (Fig. 5).

Morphology. *C. pneumoniae* inclusion bodies of strains growing in the various cell types as well as quality control strains of cell types are shown in Fig. 6. In comparison with *C. pneumoniae* grown in HEp-2 cells, *C. pneumoniae* grown in human BAL macrophages often formed smaller inclusions and occasionally two inclusions per cell were seen (Fig. 6B). When

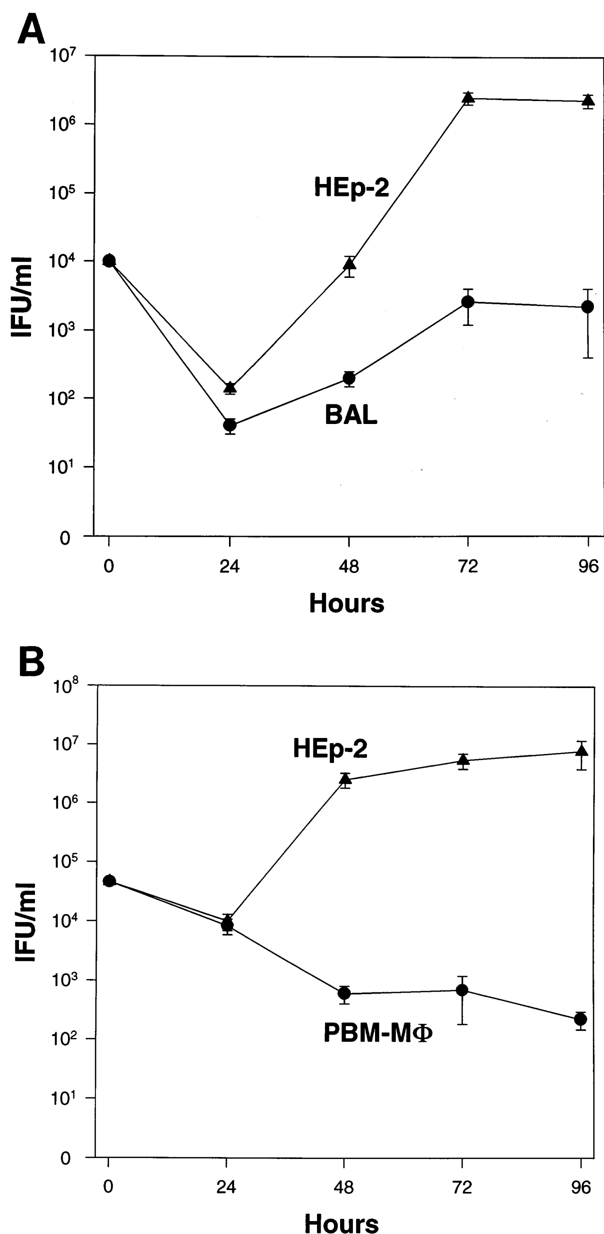

FIG. 3. (A) Viability of *C. pneumoniae* in human BAL macrophages. *C. pneumoniae* 2023 (10^4 IFU/ml) was used to inoculate replicate monolayers of BAL cells and HEp-2 cells as control cells. At specific time points, cells were scraped and subsequently inoculated in HEp-2 cells to determine the titer of infectious progeny. Growth titers represent mean titers of inclusion-forming units per milliliter with standard errors. (B) Viability of *C. pneumoniae* in PBMC-derived macrophages (PBM-MΦ). PBMCs were allowed to adhere to and transform into macrophages for 1 week in 96-well plates before being inoculated with 4.6×10^4 IFU of *C. pneumoniae* 2023 per ml. Control plates of HEp-2 cells were infected simultaneously. Infected cells were scraped from replicate wells of both cell types at specific times, and growth titers of infectious progeny were subsequently determined in HEp-2 cells. Growth titers are expressed as inclusion-forming units per milliliter with standard errors.

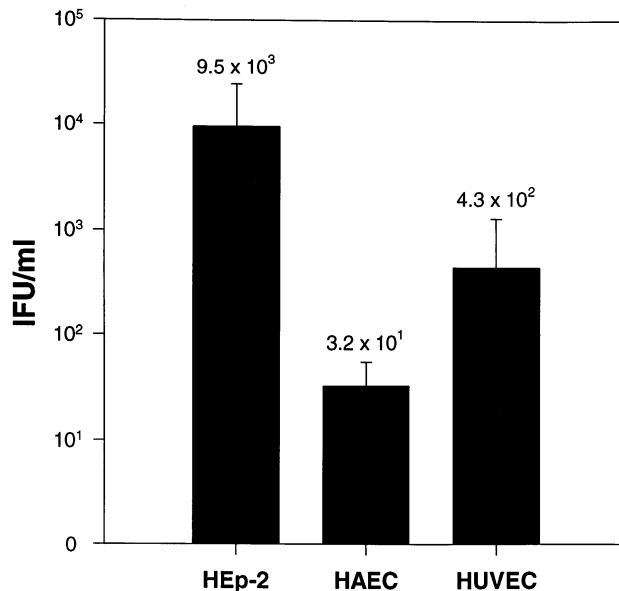

FIG. 4. Comparison of growth of *C. pneumoniae* in HAEC and HUVEC with growth in HEp-2 control cells. Ten strains of *C. pneumoniae* were each inoculated simultaneously into HAEC and HEp-2 cells. The 10 strains were each inoculated simultaneously into HUVEC and HEp-2 cells in a separate experiment. The mean titers of the strains of *C. pneumoniae* that grew are expressed as inclusion-forming units per milliliter with standard errors.

C. pneumoniae was grown in U937 and RAW macrophages, the inclusions formed were generally smaller than those of *C. pneumoniae* grown in HEp-2 cells and often appeared to be aberrant and atypical in shape (Fig. 6C). In HAEC and HUVEC, the strains often formed typical inclusions, but there was also some atypical fluorescent-staining amorphous material, which was not organized into typical inclusion bodies, as well as some tiny inclusions (Fig. 6D and E). When *C. pneumoniae* was grown in smooth muscle cells, the inclusions were much more typical in shape but smaller than the inclusions in HEp-2 cell-grown *C. pneumoniae* (Fig. 6F).

The results of the quality control staining of the various cell types was as expected (Fig. 6G to I). Endothelial cells always

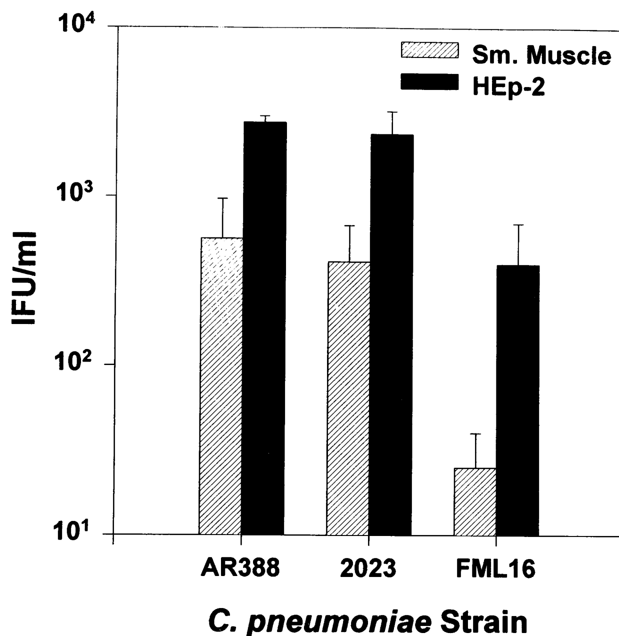

FIG. 5. Comparison of titers of three strains of *C. pneumoniae* after growth in aortic artery smooth muscle cells and HEp-2 cells. The mean titers of duplicate wells are expressed as inclusion-forming units per milliliter with standard errors.

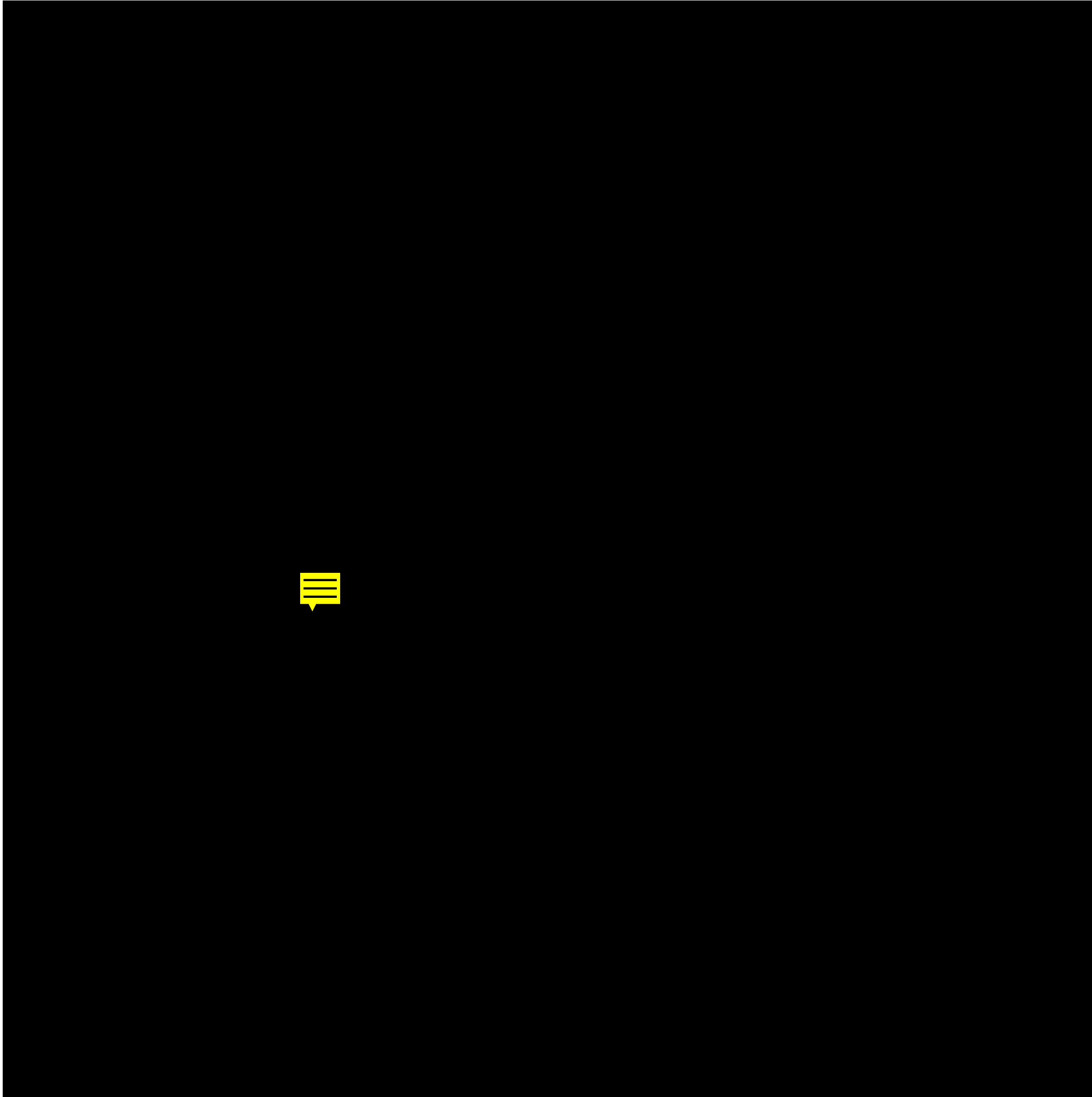

FIG. 6. Photomicrographs (magnification, ×400) of *C. pneumoniae* inclusion bodies growing in the various cell types including quality control. (A) Strain CM1 in HEp-2 cells; (B) strain 2023 in a human BAL macrophages; (C) strain BAL 15 in RAW macrophages; (D) strain CWL 011 in HAEC; (E) strain CWL 011 in HUVEC; (F) strain 2023 in smooth muscle cells; (G) HAEC stained with FITC-conjugated anti-factor VIII, visualizing the cells as endothelial cells; (H) HUVEC demonstrating DiI-acetyl low-density lipoprotein uptake, characteristic of endothelial cells; (I) smooth muscle cells stained with FITC-conjugated anti-α actin monoclonal antibody.

stained positive with FITC-conjugated anti-factor VIII, indicating that the cells were producing factor VIII and were endothelial cells. Additionally, the endothelial cells demonstrated DiI-acetyl low-density lipoprotein uptake, which is characteristic of endothelial cells. The smooth muscle cells stained with FITC-conjugated anti-α actin monoclonal antibody, indicating that there were α-actin molecules present. As expected the endothelial cells did not stain with anti-α actin antibody nor did the smooth muscle cells stain with the anti-factor VIII or take up DiI-acetyl low-density lipoprotein. The macrophage cell lines all stained positively with the anti-CD14 monoclonal antibodies.

DISCUSSION

The ability of chlamydial organisms to infect cardiac tissue has been well documented (6, 15, 22, 26, 32, 35, 36). *C. psittaci*, *C. trachomatis*, and *C. pneumoniae* have all been implicated as causes of endocarditis (6, 22, 26, 32, 35, 36). Myocarditis has been described in cases involving *C. psittaci* (32). Since chla-

mydial organisms can infect and proliferate in cardiac tissues, a logical extension would be that chlamydia could also infect the tissues of the coronary arteries, such as the endothelial and smooth muscle cells of these vessels. The first associations of *C. pneumoniae* with coronary heart disease were serological, with the risk of coronary artery disease being greater for patients with higher antibody titers or the presence of immune complexes to *C. pneumoniae* (30, 31, 34). The demonstration of chlamydial elementary bodies by electron microscopy in atheroma plaques from South African autopsy cases increased interest in this association (33). These findings were strengthened by a positive PCR for *C. pneumoniae* in 13 of 30 additional autopsy cases and positive immunoperoxidase staining in 15 of 36 cases (24). Campbell et al. recently reported a prospective study of 25 patients undergoing coronary atherectomy in which PCR for *C. pneumoniae* was positive in 32% of specimens and immunocytochemistry was positive for 44% of specimens (2). Another series of atheromas from 28 U.S. patients, for which cultures of atheroma, nasopharyngeal cultures, electron microscopy, PCR, and serology were performed, have not substantiated these findings to the same degree (37). Only one nasopharyngeal culture was positive and one atheroma was PCR positive for *C. pneumoniae* (37). Collectively, these results lend credence to a possible role of *C. pneumoniae* in coronary heart disease.

The development of a hypothesis for an association of *C. pneumoniae* with cardiac disease would have to entail the transportation of this respiratory pathogen from the lung to cardiac tissue, followed by infection of the cellular components of cardiac vessels. The bronchoalveolar macrophages would theoretically phagocytize the elementary bodies of *C. pneumoniae* resulting from a lytic pulmonary infection. The organisms could then gain entry to the bloodstream via the macrophage-monocyte route and hence be transported to sites of vascular injury in coronary vessels. This concept is only a theoretical possibility, since no actual data exist. An alternative hypothesis would encompass infectious elementary bodies passing through the circulatory system to directly infect susceptible cells.

Subsequently, chronic persistent infection of macrophages and endothelial cells with smooth muscle cell infection and proliferation would ensue, resulting in increasing cytokine elaboration, immune complex deposition, and vascular injury with the accumulation of lipid deposits, macrophage deposition, and thrombus formation. *C. pneumoniae* has been shown to be able to persistently infect an immortalized endothelial cell line (23). If such deposited or local macrophages were to include cells chronically infected with chlamydiae, the opportunity for local infection of a functionally altered endothelial cell layer might exist. Our data suggest support for this phenomenon since we have demonstrated the ability of *C. pneumoniae* to replicate and be passaged in two types of macrophage cell lines as well as human pulmonary alveolar macrophages and human PBMC-derived macrophages. *C. pneumoniae* potentially has the ability to gain entry into the circulatory system from pulmonary tissue via the macrophage-monocyte route or directly. Although some growth inhibition in macrophages was observed in our studies, continued passage, which produced viable progeny, demonstrates a possible mechanism for reaching distant sites such as coronary arteries.

The reasons for the decreased growth titers in the various cell types in comparison with the growth titers obtained in HEp-2 cells are not clear. It may be that there is a lower rate of growth in these cells of endothelial, macrophage, and smooth muscle origin and that sampling at the various time points did not coincide with the end of the growth cycle. Another explanation might be that there is decreased efficiency of entry into the endothelial, macrophage, and smooth muscle cells. Evidence that not only maintenance of infectivity in these cell types but also growth is occurring is demonstrated by the fact that inclusion bodies are visualized in the cells, as shown in Fig. 6. Additionally, successive passage in the cell types always resulted in the formation of inclusion bodies, indicating that the infectious replication cycle from elementary body to inclusion formation was completed.

The ability of chlamydial organisms to infect and proliferate in endothelial cells and in smooth muscle cells of aortic artery origin further supports the hypothesis of an association between the presence of chlamydia in endothelial cells and that in the intimal smooth muscle cells with atheromatous plaque formation, as reported by other investigators (24, 33). Whether growth of *C. pneumoniae* could actually occur in vivo in endothelial cells and in the smooth muscle cells in coronary arteries must await further experimental studies in animal models and more prospective studies in humans. However, the demonstration of this phenomenon in vitro emphasizes the potential of *C. pneumoniae* to infect and replicate in cardiac intimal tissues.

The role of *C. pneumoniae* in its association with coronary heart disease will be difficult to define. A potential hypothesis is that after a respiratory infection with *C. pneumoniae*, latently infected macrophages or blood-borne chlamydial elementary bodies would be attracted to the site of an initial injury, or even participate in an initial injury at the endothelial cell layer in the wall of a coronary artery, resulting in a complex interaction of host immune responses and parasite-mediated molecular factors. The etiologic significance of the presence of *C. pneumoniae* in atheromatous lesions can be ascertained only after much further study of its atherogenesis-initiating capabilities or -potentiating functions in appropriate in vitro cellular tissue culture systems and in vivo animal pathogenesis models.

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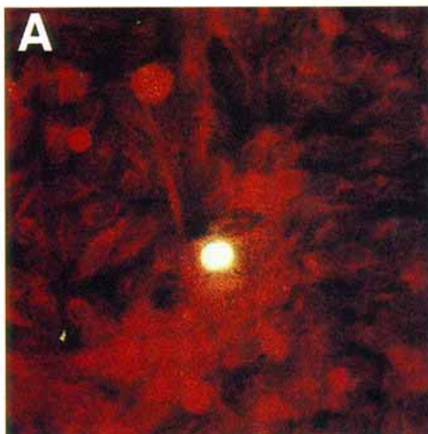
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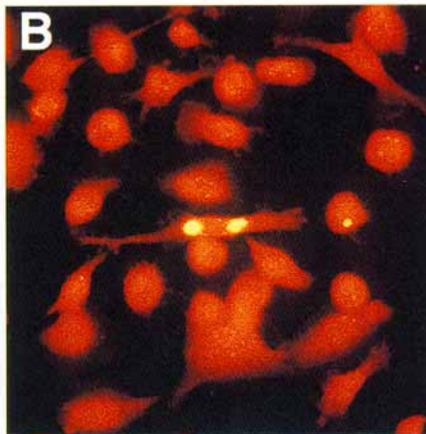
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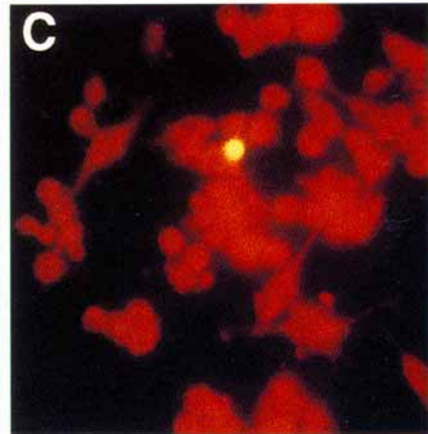
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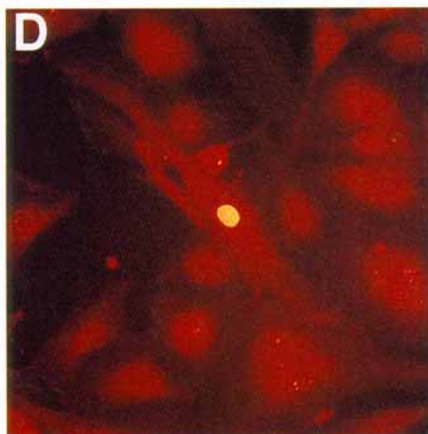
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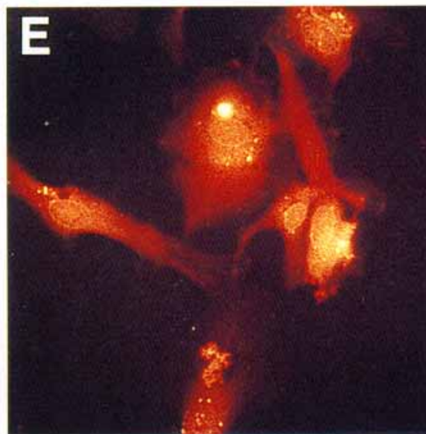
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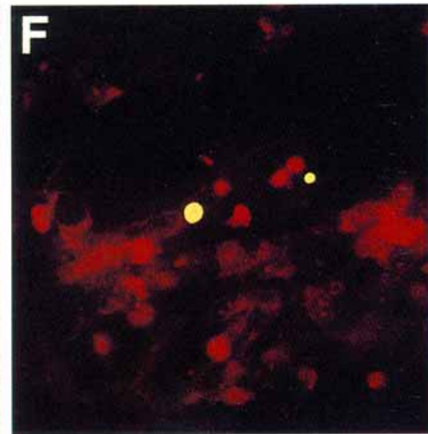
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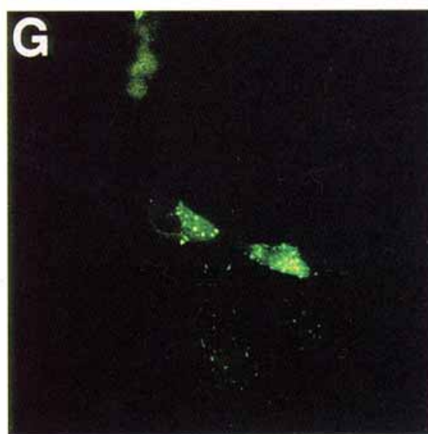
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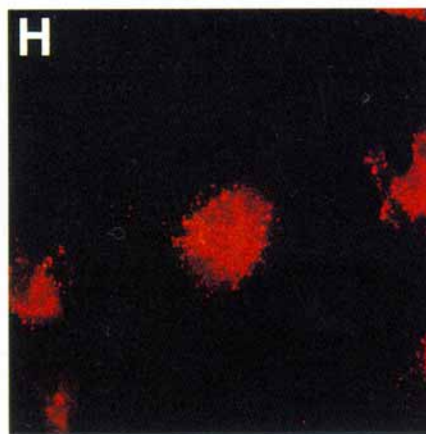
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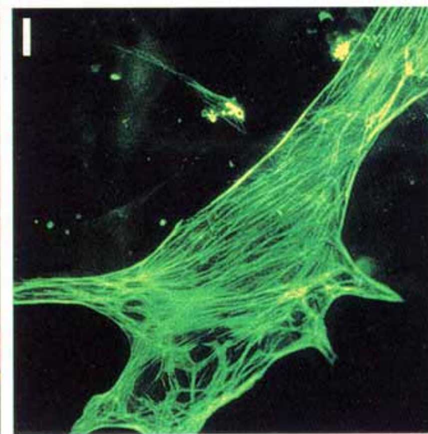
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