In Vitro Detection of Apoptosis in Monocytes/Macrophages Infected with Human Coronavirus

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Human coronavirus (HCoV) strain 229E infection, but not HCoV strain OC43 infection, of monocytes/macrophages from healthy donors and patients with multiple sclerosis in remission resulted in increased apoptosis, as measured by DNA changes and annexin V staining. Apoptosis correlated with the differential release of infectious virus. HCoV strain 229E titers were $10^{1.5}$ to $10^{6}$ 50% tissue culture-infective doses (TCID$_{50}$/mL, and HCoV strain OC43 titers were only $10^{1.2}$ to $10^{2.7}$ TCID$_{50}$/mL.

Human coronaviruses (HCoV) that cause upper respiratory illness fall into two serogroups, 229E (group 1) and OC43 (group 2). The importance of either serogroup as a cause of disease outside of the respiratory tract has not been determined. Coronavirus-like particles were observed by electron microscopy in a perivascular lesion in the brain of a patient with multiple sclerosis (MS) (2). Sequences from the nucleocapsid genes of both serogroups are found in the brain (7). Macrophage infection contributes to the pathogenesis of other members of the order *Nidovirales*, such as porcine reproductive and respiratory syndrome virus, turkey hemorrhagic enteritis virus, and murine hepatitis virus (MHV), encephalomyelitis with virus-induced demyelination, a disease showing similarities to MS (8, 13, 17). Following infection with MHV strain A59, apoptosis was detected in macrophages in brain parenchyma demyelinating lesions (14). In the MHV JHM model, macrophages infiltrating the central nervous system become activated and express chemokine receptor CCR5, inducible nitric oxide synthase, and gamma interferon-inducing factor (5, 8, 18). Macrophage activation induces resistance to apoptosis, suggesting that survival is essential for the release of inflammatory mediators and reactive free radical species (4, 12). Given that both serogroups of HCoV infect macrophages, with HCoV strain 229E causing cell rounding, detachment, and occasional syncytia, we sought to determine whether apoptosis was induced (3, 10). These studies were approved by the Institutional Review Board of the State University of New York at Buffalo.

Peripheral blood monocytes/macrophages were obtained from age-matched healthy adult donors and from patients diagnosed with MS who were in remission and not receiving medication. After 48 h in culture, the adherent cells were infected with HCoV strains 229E and OC43 at a multiplicity of 1 (3, 10). Both infectivity assays were done on the same day with pairs of normal and MS monocytes/macrophages. The HCoV strains used were obtained from the American Type Culture Collection (Manassas, Va.), plaque purified twice, and grown on L-132 (229E) or HRT-18 (OC43) cells. Infectious virus titers of samples were quantitated by the immunoperoxidase assay with antiviral monoclonal antibodies (MAb) to S (spike antigen), 1.10C.3 for HCoV strain OC43 and 5.11H.6 for HCoV strain 229E (15). Cultures were harvested on days 1 and 2 postinfection (p.i.) and evaluated. HCoV strain 229E-infected blood monocytes/macrophages produced viral titers of $10^{3.5}$ to $10^9$ 50% tissue culture-infective doses (TCID$_{50}$/mL, and HCoV strain OC43-infected cells only produced viral titers of $10^{1.2}$ to $10^{2.5}$ TCID$_{50}$/mL (Fig. 1A and B). After HCoV strain 229E infection, the cells appeared pyknotic and granular, with swelling of the plasma membrane and a few syncytia at 16 to 18 h p.i. The number of syncytia did not increase with time. The titer and cytopathic effect were similar to those previously reported (10). HCoV strain OC43 infection proceeded without a cytopathic effect and at a lower titer, comparable the amount of virus detected after 2 h of incubation at 4°C and two washes to remove the inoculum. In cultures kept for 6 days, the titer increased slightly to $10^{2.7}$ TCID$_{50}$/mL. Viability was comparable to that of uninfected controls. Previously, HCoV strain OC43 titers of $10^{2.3}$ to $10^{3.9}$ PFU/mL were obtained from umbilical cord blood monocytes/macrophages (3). For confirmation that the viral titers measured were indeed associated with infection of monocytes or macrophages, immunofluorescence microscopy was performed with MAb to S viral antigen. After blocking of Fc receptors with normal goat serum (DAKO, Carpinteria, Calif.), cells where stained for the viral S antigen expressed on the surface of paraformaldehyde (4%-fixed cells. In cultures infected with HCoV strain 229E, the majority of cells (>55%) expressed viral antigen 2 days p.i., similar to earlier work (10). Following HCoV strain OC43 infection, very few cells (<5%) expressed viral antigen, compared to 15% of cord blood-derived monocytes/macrophages positive for viral nucleocapsid antigen (3) (Fig. 1C). This suggests that replication depends on the level of differentiation of these cells (6). Preferential viral replication in less differentiated tissues was observed with primary neurons derived from dorsal root ganglia and astrocyte cultures (1, 11). Healthy donors and MS patients gave similar responses within the parameters of our experiments.

Next, apoptosis was quantified after terminal deoxynucleotidyltransferase (TdT) labeling (DeadEnd Colorimetric Apo-
proliferation of monocytes/macrophages derived from MS patients (lanes 1, 3, and 5) and healthy control subjects (lanes 2, 4, and 6). Panels A and B represent the same patients. Open bars: virus yields at 24 h p.i. Closed bars: virus yields at 48 h p.i. The virus titers after 2 h of incubation at 33°C and removal of the inoculum by two cell washings were 75 to 125 TCID50/ml. (A) HCoV strain 229E infections. (B) HCoV strain OC43 infections. The limit of detection was 25 TCID50/ml. (C) Detection of HCoV antigens by immunocytochemistry analysis of monocytes/macrophages infected for 48 h with an S antigen-specific MAb (5.11H.6 for 229E and 1.10C.3 for OC43). a and c are immunofluorescence images, b and d are phase-contrast images of the same field, a and b are 229E-infected cells; c and d are OC43-infected cells. Original magnification, ×600.

FIG. 1. Yield of infectious virus from HCoV-infected monocytes/macrophages derived from MS patients (lanes 1, 3, and 5) and healthy control subjects (lanes 2, 4, and 6). Panels A and B represent the same patients. Open bars: virus yields at 24 h p.i. Closed bars: virus yields at 48 h p.i. The virus titers after 2 h of incubation at 33°C and removal of the inoculum by two cell washings were 75 to 125 TCID50/ml. (A) HCoV strain 229E infections. (B) HCoV strain OC43 infections. The limit of detection was 25 TCID50/ml. (C) Detection of HCoV antigens by immunocytochemistry analysis of monocytes/macrophages infected for 48 h with an S antigen-specific MAb (5.11H.6 for 229E and 1.10C.3 for OC43). a and c are immunofluorescence images, b and d are phase-contrast images of the same field, a and b are 229E-infected cells; c and d are OC43-infected cells. Original magnification, ×600.

To further examine HCoV strain 229E-infected cells for viral spike antigen expression, monocytes/macrophages from healthy donors, in culture for 48 h, were infected with HCoV strain 229E at a multiplicity of 1, incubated for 2 h at 33°C, washed twice in PBS, and further incubated at 33°C in RPMI 1640 medium with 1% autologous serum. After 2 h and at various intervals thereafter, 0.5 × 10^6 to 1 × 10^6 cells, in duplicate, were preincubated with 10 μl of undiluted normal

TABLE 1. Susceptibility of monocytes/macrophages to apoptosis caused by HCoV

| Virus | % of monocyte/macrophage samples from donors by TdT assay ± SD | Normal | Normal | Normal | MS | MS | MS |
|-------|-------------------------------------------------------------|--------|--------|--------|----|----|----|----|
| 229E  | 23 ± 2.2 25 ± 3.4 34 ± 1.8 19 ± 2.8 35 ± 8 16 ± 1        | 0.6 ± 0.4 | 3.5 ± 1.2 | ND    | 81 ± 6 | 11 ± 1 | 11 ± 1 | 81 ± 6 |
| OC43  | 13 ± 3.8 2.7 ± 0.6 4.2 ± 1 11 ± 3.5 ND    | 21 ± 2  | 2.8 ± 3  | 35 ± 12| ND  | 35 ± 10 | ND  | 35 ± 10 |
| None (mock infected) | 4 ± 1.5 12 ± 1.5 4.7 ± 1.4 8 ± 2 12 ± 3.5 12 ± 3 | ND  | ND  | ND  | ND  | ND  | ND  | ND  |

a Quantitation of TdT labeling-positive cells at 48 h p.i. Two to 400 cells per slide, in duplicate, were scored.
b ND, not done.
goat serum (DAKO) for 15 min. After one washing, 20 μl of HCoV strain 229E-specific MAb 5.11H.3 or an isotypic control antibody (1/2,000 dilution) was added. After two washings, 10 μl of fluorescein-conjugated goat anti-mouse F(ab')2 (1/200 dilution; DAKO) was added. The cells were kept on ice in 400 μl of PBS and analyzed with a Coulter EPICS XL-MCL flow cytometer within 1 h after staining. Results of duplicate cultures were averaged.

A time-dependent expression of cell membrane HCoV strain 229E S antigen on monocytes/macrophages was observed (Fig. 3A). At 24 h p.i., 47% of the infected cells showed immunofluorescence. This compares well with a previous report that about half of the attached infected cells fluoresced (10). At 48 h p.i., a >0.5-log displacement increase in signal intensity for virus-specific MAb, compared to the isotype control MAb, was seen (Fig. 3B). The percentages of viable cells, as measured by trypan blue dye exclusion, were 75, 86, 52, and 34, respectively, at 1, 2, 3, and 4 days p.i., while the uninfected cells remained 82 to 90% viable throughout. Previously, 70% viability in the infected attached cells after 24 h was reported (10).

In order to demonstrate that the apoptotic cells were indeed the cells infected by the virus, double staining with virus-specific MAb (5.11H.6) to S antigen and annexin V-PE was per-
formed on monocytes/macrophages from three donors and the standard deviation was calculated for the 2- to 24-h intervals. Cytotoxic fluorometric analysis showed the presence of both markers on viable cells (exclusion of 7-AAD+ cells), with 29% showing dual fluorescence at 16 h p.i. (Fig. 3C).

Overall, the increase in apoptotic cells observed in HCoV strain 229E-infected cultures at 16 h p.i. was associated with few necrotic cells and in normal cultures, there were only 4 to 12% apoptotic cells. In the mock-infected cultures, some cells likely enter the apoptotic pathway of cell death. Monocytes normally circulate in the blood for only a few days, during which time they either migrate to tissues and differentiate to macrophages or die through apoptosis (12). Apoptosis caused by HCoV in adherent monocytes/macrophages in culture might then be due to commitment to proapoptotic signals generated by the virus. Possibly, production of cytokine mediators such as tumor necrosis factor alpha was also a factor since a difference was observed in the percent apoptosis at 16 h p.i. (47% versus 29%) between cells stained only with annexin V and cells both immunostained and annexin V stained (Fig. 2A and 3C). Studies of cytokine expression in HCoV strain 229E-infected monocytes/macrophages are necessary to clarify this point. Following infection of monocytes/macrophages by HCoV strain OC43, viability remained high over 6 days and no apoptosis was observed. The low viral titers were confirmed by demonstration of viral spike antigen on the surface of cells in infected cultures by immunofluorescence microscopy. Lack of apoptosis is likely due to the very poor ability of this virus to infect the cells. Further studies of HCoV-induced apoptosis in monocytes freshly isolated from persons with clinically active MS would be appropriate because monocyte/macrophage activation was observed (acute exacerbation and progressive MS) (9, 19).

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