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 \(10^{3.5}\) 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 perivascular 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).

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ptosis Detection System; Promega) in accordance with the manufacturer’s instructions. TdT assays were done with preparations of monocytes/macrophages different from those used in infectivity assays, and the assay results are not directly comparable. The response to HCoV strain 229E inoculation was 16 to 35% of monocyte/macrophage cells positive for TdT staining (Table 1). HCoV strain OC43 infection proceeded with no significant difference in apoptosis compared to mock-infected monocytes/macrophages. Healthy subjects and MS patients gave similar responses. To verify that HCoV strain 229E infection induces apoptotic changes in monocytes/macrophages, a flow cytometric analysis for cell membrane expression of externalized phosphatidylserine was performed by double labeling of HCoV strain 229E-infected monocytes/macrophages from healthy donors with annexin V and 7-amino-actinomycin (7-AAD; a vital dye). Monocyte/macrophage cells in culture for 48 h were infected with HCoV strain 229E at a multiplicity of 1 or mock infected, incubated at 33°C for 2 h, and washed twice to remove the inoculum. After 2 h of infection and at various intervals thereafter, 0.5 × 10^6 to 1 × 10^6 cells, in duplicate, were washed in phosphate-buffered saline (PBS) and incubated with 20 μl of 7-AAD (Pharmacia). After washing in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2), the cells were incubated with 10 μl of human annexin V-phycocerythrin (PE) (R&D Systems Inc.). A 400-μl volume of binding buffer was added. The cells were kept on ice and analyzed with a Coulter EPICS XL-MCL flow cytometer within 1 h after staining. Data from 10,000 cells was collected. Six donors were examined, and the standard deviation was calculated. Membrane externalization of phosphatidylserine, as indicated by annexin V-PE^+ 7-AAD^- cells, increased significantly at 16 h p.i. (Fig. 2 A). Forty-six percent of infected cells gave a 2-log displacement in annexin V-PE signal above the cutoff value for unstained cells, compared to 12% of mock-infected cells (Fig. 2B). The difference in apoptosis (46% versus 16 to 35%) between annexin V data and TdT data may be due to time-dependent factors. Plasma membrane annexin V is exposed earlier in apoptosis, before fragmentation (16). Also, in infected cultures, the number of attached cells is reduced by 50% after 24 h of infection, compared to a reduction of 6% in uninfected cultures (10). Presumably, cells become infected and detached throughout infection.

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

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

<table>
<thead>
<tr>
<th>Virus</th>
<th>% of monocyte/macrophage samples from donors positive by TdT assay ± SD</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>229E</td>
<td>23 ± 2.2</td>
</tr>
<tr>
<td>OC43</td>
<td>13 ± 3.8</td>
</tr>
<tr>
<td>None (mock infected)</td>
<td>4 ± 1.5</td>
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</tbody>
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* 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-

FIG. 2. (A) Percentage of monocytes/macrophages showing apoptosis at the times indicated after infection with HCoV strain 229E. Apoptosis was measured by dual-parameter flow cytometry for annexin V-PE staining and DNA fluorescence with 7-AAD. Data are from six healthy donors; each case is a comparison of infected cells with mock-infected control cells. (B) Flow cytometry profiles of annexin V-PE7-AAD-stained cells at 16 h p.i. Top, HCoV strain 229E-infected cells; middle, mock-infected cells; bottom, cellular autofluorescence.

FIG. 3. Flow cytometric analysis of cell surface expression of viral antigen. Monocyte/macrophage cultures were inoculated with HCoV strain 229E and labeled at various times with virus-specific MAb 5.11H.6 or an isotypic control antibody. (A) Percentage of cells having a fluorescence signal displacement above the cutoff established for unstained cells. (B) Flow cytometry profile of fluorescein isothiocyanate-labeled cells at 48 h p.i. Left profile, cellular autofluorescence; middle (dotted) profile, isotypic control; right profile, virus-specific MAb. (C) Percentage of viable cells having dual fluorescence for virus-specific MAb 5.11H.6 (fluorescein isothiocyanate positive) and annexin V (PE'). Error bars represent standard deviations of data from three donors.
formed on monocytes/macrophages from three donors and the standard deviation was calculated for the 2- to 24-h intervals. Cytocentrifuged 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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