Human Cytomegalovirus and Monocytes: Limited Infection and Negligible Immunosuppression in Normal Mononuclear Cells Infected in vitro with Mycoplasma-free Virus Strains

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SUMMARY

Human cytomegalovirus (HCMV) infection has previously been associated with the production of immunosuppression. The mechanism by which any such immunosuppressive effect might be mediated is unclear but previous work has implicated an effect of the virus on monocytes. We have attempted to characterize the immunosuppressive activity produced by in vitro infection of normal monocytes with HCMV strain AD169. We first examined the ability of HCMV AD169 and recent clinical isolates to infect normal peripheral blood mononuclear cells in vitro. We have found by immunofluorescence analysis that only a very limited number of peripheral blood mononuclear cells (0.2 to 0.5%) showed evidence of virus infection as demonstrated by expression of the major immediate early protein. We found that the inhibitory activity of supernatants of monocytes exposed to HCMV which suppressed mitogen-driven T cell responsiveness was associated with a protein of about 95K. Experiments to investigate the mechanism of action of this inhibitor suggested the possibility of mycoplasma contamination and we were subsequently able to isolate Mycoplasma hyorhinis from our AD169 virus stock. When a series of low passage clinical isolates of HCMV were examined for their ability to cause immunosuppression, there was a direct correlation between suppression and the presence of contaminating mycoplasmas. Using mycoplasma-free isolates of HCMV we could demonstrate no immunosuppressive effect on mitogen-mediated T cell proliferation of both unseparated human peripheral blood lymphocytes and nylon wool non-adherent T cells; these virus isolates also did not suppress accessory cell function or interleukin 1 production by monocytes infected in vitro. We conclude that the previously reported immunosuppressive effects of HCMV in vitro may be attributable to the presence of mycoplasmas and are unlikely to be due to expression of HCMV in monocytes. We suggest that mycoplasma contamination of isolates of HCMV may be a more extensive problem than is currently recognized.

INTRODUCTION

Human cytomegalovirus (HCMV) infection has previously been associated with suppression of both cell-mediated and humoral immune responses (Howard & Najarian, 1974; Howard et al., 1974; Ho, 1984). This has been attributed particularly to virus-induced dysfunction of monocytes/macrophages on the basis of experiments showing that both monocytes from patients with HCMV mononucleosis and normal monocytes infected in vitro with HCMV had an impaired ability to support mitogen-driven T cell responses (Rinaldo et al., 1977; Carney &
Hirsch, 1981). We previously reported that culture supernatants from normal human monocytes infected in vitro with HCMV strain AD169 suppress the mitogen-dependent proliferation of murine thymocytes in the in vitro assay for interleukin 1 (IL-1). This occurred in the presence or absence of exogenous IL-1 (Rodgers et al., 1985). Others have also reported reduced IL-1 production by HCMV-infected monocytes (Smith et al., 1985; Starr et al., 1983). It has recently been reported that infection of mononuclear cells with low passage clinical isolates of HCMV causes suppression of T cell proliferation, natural killer cell activity and cytotoxic T cell activity (Schrier & Oldstone, 1986; Schrier et al., 1986). In these experiments HCMV AD169 was much less effective at inducing suppression.

Despite the various reported functional effects of HCMV, there is still only limited direct evidence that HCMV infection occurs to a significant extent in monocytes or lymphocytes. It has been reported that, following infection in vitro with recent clinical isolates of HCMV, expression of the HCMV major immediate early (IE) protein could be detected by immunofluorescence in a small percentage of monocytes and lymphocytes; however, there was no evidence of virus replication (Einhorn & Ost, 1984; Rice et al., 1984). Using in situ hybridization IE RNA has also been reported to be present in 1 to 2% of peripheral blood mononuclear cells of some normal healthy HCMV-seropositive subjects, principally in monocytes and T4-positive lymphocytes (Schrier et al., 1985).

We examined the possible mechanism by which HCMV could exert its reported suppressive effects and tried to correlate this with the extent of virus expression occurring when peripheral blood mononuclear cells were infected with HCMV in vitro.

We report here that the factor(s) released by monocytes after incubation in vitro with HCMV AD169, and which caused inhibition of mitogen-dependent T cell responses, appeared to be associated with a protein(s) of approx. Mr 95K (Rodgers et al., 1985). Investigations into the mechanism of action of this inhibitor gave anomalous results suggesting the possibility of mycoplasma contamination and indeed we found that our virus stock was contaminated with Mycoplasma hyorhinis. We consequently examined the effects of isolated M. hyorhinis on T cell proliferative responses. We have further found that the ability of a variety of HCMV isolates to significantly inhibit T cell proliferation correlated directly with the presence of mycoplasma contamination. In addition we have found, by immunofluorescence analysis, that only between 0.2 and 0.4% of mononuclear cells expressed the HCMV IE gene after infection in vitro with several strains of HCMV including low passage number recent clinical isolates. Taken together these results suggest that many of the profound in vitro immunosuppressive effects that have been attributed to HCMV infection are unlikely to be a direct result of HCMV infection of monocytes.

**METHODS**

**Virus.** The AD169 strain of HCMV (ATCC) was used. Clinical isolates of HCMV were obtained from patients with active CMV infection and had not been passaged more than four or five times. Growth and titration of virus stocks were carried out as described previously (Rodgers et al., 1985). To optimize the yield of virus from clinical isolates, infected fibroblasts (2 x 10⁶ to 5 x 10⁶ cells) were resuspended in 2.5 ml supernatant from virus-infected cultures and sonicated for 30 s. Supernatant and sonicate were titrated separately.

**Mycoplasma testing.** This was initially carried out using a mycoplasma isolation kit obtained from Flow Laboratories and was performed exactly according to the manufacturers’ instructions. Samples, including cells infected with HCMV virus stocks, were also tested for mycoplasma using the Hoechst dye DNA-staining method (Chen, 1977). Cells to be tested were co-cultured on slides that had previously been coated with mycoplasma-free fibroblasts (Flow 5000 cells, primary fibroblast lines from normal HCMV seronegative donors or Vero cells). The cells were incubated at 37 °C for 24 to 48 h, washed extensively to remove non-adherent cells and cultured for a further 48 h before staining for mycoplasma DNA using the Hoechst dye. Any samples that were suspected to be mycoplasma-positive were sent for further examination at the Mycoplasma Reference Facility, NCTC, Central Public Health Laboratories, Colindale, London, U.K. (Dr R.H. Leach and Mrs D.L. Mitchelmore). The tests there consisted of confirmation of the DNA staining results on incubated Vero cells after inoculation with the test sample and culture tests on the original sample and the inoculated Vero cells using a supplemented pig serum mycoplasma agar (Gourlay et al., 1974), and also the agar medium of Friis (1975) especially suitable for M. hyorhinis.

**Cells.** Human peripheral blood lymphocytes (PBL) and monocytes were prepared from normal HCMV seronegative donors by gravity sedimentation on 3% dextran 250 (Fisons Scientific Apparatus) in phosphate-
buffered saline (PBS). The lymphocyte-rich plasma was separated by Ficoll-Hypaque density gradient centrifugation (Boyum, 1973) and the mononuclear cell layer was harvested and washed three times with PBS. Monocytes were prepared by adherence to plastic dishes in serum-free medium. The mononuclear cells were resuspended at 1 × 10^6 to 2 × 10^6/ml in RPMI 1640 (serum-free) and incubated for 1 h at 37 °C. The non-adherent cells were removed and the adherent cells were washed with PBS. Where indicated the cells were infected as described in the following section and incubated in RPMI 1640 containing 10% foetal calf serum (FCS) (RPMI 10). At the end of the culture (usually 3 to 7 days) the adherent cells could be recovered from the plastic dishes by gentle scraping with a plastic policeman. The adherent cell population comprised between 85 and 95% monocytes. This was determined by both non-specific esterase staining (Ward, 1981) and by fluorescence analysis using the monoclonal antibody MO/2 (Coulter Electronics). T cells were purified from the plastic non-adherent population by passage over a nylon wool column as described by Julius et al. (1973).

The U937 human macrophage-like cell line (Koren et al., 1979) was maintained in suspension culture in RPMI 10; it was mycoplasma-free.

**Virus infection.** Adherent monocytes were infected with virus (m.o.i. 5) or mock-infected with medium alone at 37 °C for 1 h, washed four times and incubated with RPMI 10 for 1 to 6 days. PBL and U937 cells were infected with HCMV AD169 (m.o.i. up to 5) or clinical isolates (cell : infected fibroblast sonicate 5:1 giving m.o.i. of 0-005 to 0-5) for 60 to 90 rain at 37 °C. Free virus was removed by washing three times and the cells (1 × 10^6 to 2 × 10^6/ml for PBL and 2 × 10^5/ml for U937 cells) were incubated at 37 °C in RPMI 10. Where indicated, the cells were not washed after infection but were co-cultured with virus throughout the experiment.

**IL-1 assay.** This was performed using the murine thymocyte assay exactly as described previously (Rodgers et al., 1985).

**Proliferative response of human PBL.** Unseparated PBL, and nylon wool non-adherent (NWNA) T cells (1 × 10^6 to 2 × 10^5/well) were assayed in triplicate in 96-well flat-bottomed microtitre plates (Falcon). Cells were incubated with medium alone and with autologous adherent monocytes (PBM), or autologous irradiated accessory cells (1 × 10^6 to 2 × 10^4/well). The accessory cells were E-rosette-negative cells prepared as described (Falkoff et al., 1982) and irradiated for 24 min using a Gammacell 1000 (Gamma-irradiator, Atomic Energy of Canada Commercial Products). Phytohaemagglutinin (1 or 2 μg) (PHA; Sigma) was added and the cells were incubated in a final volume of 200 μl at 37 °C for 72 h. The cells were pulse-labelled with [3H]thymidine (5 Ci/mmol; 1 μCi/well; Amersham) for the last 6 h incubation and were harvested onto filter paper, dried and counted in a Beckman LS 1800 scintillation counter.

The effect of mycoplasma-contaminated supernatants from HCMV AD169-infected monocyte cultures, and of isolated *M. hyorhinis* on T cell proliferation was measured by direct cell counting and by [3H]thymidine uptake. PBL were incubated at 10^6/ml with 0-5 μg/ml PHA. Control medium, monocyte culture supernatant or isolated *M. hyorhinis* (5%, v/v) or colchicine (1.25 μg/ml) were added at the same time as PHA. After 72 h and 96 h the number of viable cells remaining was determined by ethidium bromide/acridine orange uptake. At 72 h three 200 μl batches of cells were transferred to a 96-well flat-bottomed plate and pulse-labelled with [3H]thymidine as described above.

**Immunofluorescence analysis of PBL infected in vitro with HCMV.** PBL from normal seronegative donors were infected with HCMV AD169 and clinical isolates as described above. The infected cells were incubated for 24 h, washed three times in PBS, adjusted to 10^6 cells/ml and spotted in 10 μl aliquots on slides coated with 0.1% poly-L-lysine and air-dried. As positive control, spot slides were coated with human fibroblasts (Flow 5000 cells or primary fibroblast lines) at 10^6 cells/spot and were infected with HCMV as described above. The cells were fixed in acetone at −20 °C for 10 min, blocked in 20% HCMV seronegative human serum and then incubated for 30 min with a monoclonal antibody recognizing the major 72K IE protein (Rice et al., 1984) designated L.14 (a gift from J.A. Nelson, Department of Immunology, Research Institute, Scripps Clinic, La Jolla, Ca., U.S.A.), with control monoclonal antibody or with normal mouse serum, followed by fluorescein-conjugated anti-mouse IgG (Coulter).

**Trypsin treatment.** Monocytes were mock-infected or infected with HCMV AD169 and cultured in serum-free medium for 24 to 48 h. These serum-free supernatants were incubated with 0.05% (w/v) trypsin for 30 min. The reaction was stopped by the addition of FCS to 10% and the samples were stored at −70 °C before assay for IL-1 activity using the murine thymocyte assay.

**RESULTS**

**Partial characterization of the inhibitory activity of supernatants from monocytes infected with HCMV AD169 (mycoplasma-contaminated)**

Trypsin treatment of supernatants from monocytes infected with HCMV AD169 completely abolished their ability to inhibit T cell proliferation. In fact a slight enhancement of proliferation was seen after treatment with trypsin (Table 1).
Table 1. Effect of trypsin treatment on the IL-1 inhibitory activity of supernatants from monocytes infected with mycoplasma-contaminated HCMV AD169

<table>
<thead>
<tr>
<th></th>
<th>Untreated (d.p.m. ± s.d.)</th>
<th>Trypsin-treated (d.p.m. ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium control</td>
<td>5328 ± 482</td>
<td>5600 ± 1890</td>
</tr>
<tr>
<td>Monocyte supernatant</td>
<td>1256 ± 159</td>
<td>12792 ± 470</td>
</tr>
</tbody>
</table>

Table 2. Effect of mycoplasma-contaminated HCMV AD169 infected-monocyte supernatant, isolated M. hyorhinis and colchicine on PHA proliferation of normal human PBL: [3H]thymidine uptake and direct cell counts

<table>
<thead>
<tr>
<th>Sample</th>
<th>[3H]Thymidine uptake (d.p.m. ± s.d.)</th>
<th>Viability (%)</th>
<th>Cell count concentration (10^5/ml)</th>
<th>Viability (%)</th>
<th>Cell count concentration (10^5/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>5288 ± 740</td>
<td>90</td>
<td>6.7</td>
<td>95</td>
<td>8.4</td>
</tr>
<tr>
<td>PHA* + medium</td>
<td>216022 ± 15121</td>
<td>88</td>
<td>9.5</td>
<td>89</td>
<td>13.0</td>
</tr>
<tr>
<td>PHA + HCMV monocyte supernatant</td>
<td>9626 ± 673</td>
<td>85</td>
<td>6.8</td>
<td>90</td>
<td>13.3</td>
</tr>
<tr>
<td>PHA + mycoplasma culture medium</td>
<td>50519 ± 2021</td>
<td>88</td>
<td>5.2</td>
<td>85</td>
<td>9.0</td>
</tr>
<tr>
<td>PHA + M. hyorhinis</td>
<td>6455 ± 3098</td>
<td>88</td>
<td>5.3</td>
<td>92</td>
<td>10.3</td>
</tr>
<tr>
<td>PHA + colchicine†</td>
<td>44583 ± 1795</td>
<td>90</td>
<td>6.4</td>
<td>81</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* PHA, 1 µg.
† Colchicine, 1.25 µg/ml.

We have previously shown by size fractionation on Sephacryl S200 that a major peak of inhibitory activity eluted with an Mr of about 95K with some inhibitory activity trailing into the albumin region (65K to 70K) (Rodgers et al., 1985).

Identification of mycoplasma contamination and its effect on [3H]thymidine uptake and cell division

The apparently contradictory findings from a series of experiments to investigate the mechanism of action of the inhibitory factor from virus-infected monocyte supernatants led us to suspect mycoplasma contamination of our virus stock. These findings were as follows. (i) In kinetic studies, supernatants from HCMV AD169-infected monocytes inhibited T cell proliferation only if they were added within 24 h after addition of mitogen. (ii) Addition of high concentrations of recombinant murine IL-1 (150 units/10^5 cells) caused an apparent dose-dependent reversal of inhibition. (iii) Incubation of lymphocytes with PHA plus supernatants from HCMV AD169-infected monocytes caused inhibition of [3H]thymidine uptake but had no effect on the acquisition of T cell differentiation antigens, e.g. CD11, IL-2 receptor, transferrin receptor or major histocompatibility complex class II antigens.

Initial screening by standard mycoplasma culture techniques in our own laboratory gave negative results. However, on Hoechst dye DNA staining, supernatants from monocytes infected with HCMV AD169 were strongly mycoplasma-positive. The presence of mycoplasma was subsequently confirmed after several weeks of culture, at the Mycoplasma Reference Facility where a mycoplasma was isolated from our infected monocyte supernatants and virus stock and identified as M. hyorhinis (see Methods).

We investigated the ability of mycoplasma-contaminated monocyte supernatant and isolated M. hyorhinis to inhibit PHA-stimulated cell division as measured both by [3H]thymidine uptake and directly by cell counts (Table 2). Colchicine treatment, which causes disruption of microtubules and thus prevents cell division without affecting DNA synthesis, was used as a control. Infected monocyte supernatants and isolated M. hyorhinis caused marked inhibition of [3H]thymidine uptake in response to PHA. Both the culture medium in which the mycoplasma
Fig. 1. Effect of infection with HCMV AD169 and low passage number clinical isolates on IL-1 release by U937 cells. U937 cells were infected with HCMV AD169 (mycoplasma-contaminated; *) or clinical isolates (\(\nabla\), \(\triangle\), \(\Box\), \(\Diamond\) and \(\bigcirc\), mycoplasma-contaminated; \(\blacksquare\), \(\bullet\), mycoplasma-free). After 72 h supernatants were harvested and assayed for IL-1 using the murine thymocyte assay. Results are expressed as mean d.p.m. \(^{3}H\)thymidine uptake ± S.D.

Fig. 2. Effect of infection with mycoplasma-free HCMV AD169 or recent clinical isolates on the PHA responsiveness of normal PBL. PBL were mock-infected (○) or infected with HCMV AD169 (*)(m.o.i. 5) or clinical isolates (\(\blacksquare\) (2 × 10^8 p.f.u./ml), \(\bullet\) (10^6 p.f.u./ml), \(\triangle\) (10^5 p.f.u./ml) and \(\nabla\) (5 × 10^4 p.f.u./ml) for 3/4 or 7 days before stimulation with PHA (1 and 2 μg/ml as indicated). Proliferation was measured by \(^{3}H\)thymidine uptake after a further 72 h in culture. Medium controls (Med; no PHA) at day 3/4 were less than 2000 d.p.m. and are not shown. Results are expressed as mean d.p.m. \(^{3}H\)thymidine uptake ± S.D.

was grown and colchicine caused a slight reduction in \(^{3}H\)thymidine uptake compared with medium controls. When cells were counted microscopically at 72 h there also appeared to be inhibition of cell division by infected monocyte supernatants, *M. hyorhinis* and mycoplasma culture medium as well as by colchicine. However, by 96 h, the cells incubated with infected monocyte supernatant, *M. hyorhinis* and mycoplasma culture medium had divided normally, whereas the colchicine-treated cells had not.

**Correlation of inhibition of \(^{3}H\)thymidine uptake with the presence of mycoplasma in supernatants from U937 cells infected with clinical isolates of HCMV**

Recent reports have indicated that low passage number clinical isolates are more effective at infection of lymphocytes and monocytes than HCMV AD169, as evidenced by HCMV IE expression (Einhorn & Ost, 1984; Rice et al., 1984). We therefore examined the effects of infection of U937 cells with recent low passage number clinical isolates of HCMV that had been obtained from several different laboratories. Supernatants were harvested 72 h after virus infection and assayed for their ability to inhibit \(^{3}H\)thymidine uptake in the murine thymocyte assay. The results (Fig. 1) indicate that supernatants from two of the isolates (shown by open
Table 3. PHA responsiveness of normal human PBL, T cells and monocytes after infection with mycoplasma-free HCMV AD169

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Virus infection</th>
<th>Additions (day 4)</th>
<th>[^3H]Thymidine uptake (d.p.m. ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unseparated PBL</td>
<td>–</td>
<td>–</td>
<td>47,452 ± 3,322</td>
</tr>
<tr>
<td>Unseparated PBL</td>
<td>Mock</td>
<td>–</td>
<td>48,645 ± 2,092</td>
</tr>
<tr>
<td>Unseparated PBL</td>
<td>AD169</td>
<td>–</td>
<td>43,901 ± 2,292</td>
</tr>
<tr>
<td>T cells</td>
<td>Mock</td>
<td>–</td>
<td>129 ± 53</td>
</tr>
<tr>
<td>T cells</td>
<td>Mock</td>
<td>E-ve</td>
<td>27,832 ± 6,799</td>
</tr>
<tr>
<td>T cells</td>
<td>AD169</td>
<td>E-ve</td>
<td>30,298 ± 6,059</td>
</tr>
<tr>
<td>Monocytes</td>
<td>Mock</td>
<td>T cells</td>
<td>26,436 ± 2,115</td>
</tr>
<tr>
<td>Monocytes</td>
<td>AD169</td>
<td>T cells</td>
<td>23,171 ± 3,242</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>T cells</td>
<td>1,054 ± 73</td>
</tr>
</tbody>
</table>

* PBL and NWNA T cells were added at 10^5/well. Adherent monocytes mock- or HCMV AD169-infected 4 days previously were gently scraped from Petri dishes and seeded at 10^4/well. Autologous irradiated E-rosette-negative (E-ve) accessory cells were added at 10^4/well.

triangles and inverted triangles) caused marked inhibition of thymidine uptake to dilutions of 10^-6 as did supernatants from cells infected with HCMV AD169. Supernatants from the three remaining isolates denoted by open symbols were inhibitory up to 10^-3 dilution. The two shown in closed symbols were not inhibitory. When the isolates were examined for the presence of mycoplasmas, all those showing inhibition were found to be mycoplasma-contaminated. The two that did not inhibit [^3H]thymidine uptake were mycoplasma-free. A further four low passage number clinical isolates obtained from our own laboratory and rigorously shown to be mycoplasma-free also did not cause inhibition of [^3H]thymidine uptake by murine thymocytes in the IL-1 assay (not shown).

**Effects of mycoplasma-free HCMV AD169 and recent clinical isolates on human T cell and monocyte function**

**AD169**

We have investigated the ability of a fresh stock of HCMV AD169, recently obtained from ATCC and shown to be mycoplasma-free, to inhibit human T cell and monocyte function. Normal PBL, NWNA T cells and PBM were infected with mycoplasma-free HCMV AD169 and incubated for 4 days. The cells were then assayed for their ability to respond to PHA (Table 3). The mock- and HCMV-infected T cells were assayed both alone and in the presence of autologous irradiated E-rosette-negative cells as a source of accessory cells. The ability of the mock- and HCMV-infected adherent monocytes to act as accessory cells was tested by the addition (on day 4) of freshly prepared autologous NWNA T cells (10^5/well). The results (Table 3) indicate that infection of normal PBL, NWNA T cells or adherent monocytes with mycoplasma-free HCMV AD169 had no effect on their ability either to respond to PHA or to support the response of added T cells to PHA.

**Recent clinical isolates**

We then investigated the ability of mycoplasma-free clinical isolates to inhibit PHA-mediated proliferation of normal PBL obtained from several donors (Fig. 2). To optimize the possibility of infection, the PBL were infected with 1 ml virus-infected fibroblast sonicate for 1 h at 37 °C, giving m.o.i.s ranging from 0.005 to 0.5. After infection the cells were incubated in the presence of the fibroblast sonicate for 3, 4 or 7 days before stimulation with PHA. In only one instance did one of the clinical isolates (closed circles) cause significant reduction of the PHA response. The same isolate assayed again at day 3/4 and also on day 7 no longer gave significant inhibition (Fig. 2). The results suggest that recent clinical isolates of HCMV after only four to five passages in vitro do not cause a significant reduction of the response of normal lymphocytes to PHA.
**Human CMV and monocytes**

Table 4. *Fluorescence analysis of PBL infected in vitro with HCMV AD169 and clinical isolates*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Fluorescence analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NMS</td>
</tr>
<tr>
<td>None</td>
<td>17</td>
</tr>
<tr>
<td>AD169</td>
<td>69</td>
</tr>
<tr>
<td>Clinical isolates</td>
<td></td>
</tr>
<tr>
<td>1 supernatant</td>
<td>46</td>
</tr>
<tr>
<td>2 supernatant</td>
<td>78</td>
</tr>
<tr>
<td>2 cell sonicate</td>
<td>13</td>
</tr>
<tr>
<td>3 supernatant</td>
<td>1</td>
</tr>
<tr>
<td>3 cell sonicate</td>
<td>21</td>
</tr>
<tr>
<td>4 supernatant</td>
<td>23</td>
</tr>
<tr>
<td>4 supernatant</td>
<td>34</td>
</tr>
<tr>
<td>5 cell sonicate</td>
<td>31</td>
</tr>
<tr>
<td>6 cell sonicate</td>
<td>4</td>
</tr>
</tbody>
</table>

* The results are expressed as total number of positive cells per slide using normal mouse serum (NMS) or monoclonal antibody to the major IE protein (L14). The percentage of positive cells was calculated from \([\text{L14 - NMS}/\text{total cells per slide}] \times 100\).**

**In vitro infection of peripheral blood mononuclear cells with HCMV isolates**

In these experiments peripheral blood lymphocytes and monocytes were infected *in vitro* with HCMV AD169 and with recent clinical isolates at the multiplicities indicated; all virus stocks used in these experiments were free of mycoplasma. In the case of clinical isolates, both cell free virus-containing supernatants and cell sonicates from infected fibroblasts were used in order to optimize the possibility of infection. After 24 h infected cells were analysed by immunofluorescence using the L14 monoclonal antibody (Rice *et al.*, 1984) directed against the major 72K HCMV IE antigen. The results (Table 4) indicate that only a very small percentage of the cells showed positive fluorescence with monoclonal antibody L14, and in most cases no greater than with control normal mouse serum. In contrast, permissively infected fibroblasts gave strongly positive nuclear fluorescence in 70 to 85% of cells. The recent clinical isolates of HCMV were no more effective at infecting the PBL *in vitro* than the laboratory-passaged strains. In fact, by fluorescence analysis the highest percentage of positively staining cells was demonstrated for a highly passaged clinical isolate (isolate 1). The remaining isolates and AD169 gave between 0.05 and 0.15% positive staining.

**DISCUSSION**

These studies show that following infection with HCMV *in vitro* only a very small number of peripheral blood mononuclear cells show evidence of HCMV IE proteins even at high multiplicities of infection. They further suggest that the *in vitro* immunosuppressive effects previously attributed to HCMV may, at least in part, be attributed to the presence of mycoplasmas.

Earlier work suggested that monocytes from patients with primary CMV infection, or normal human monocytes infected *in vitro* with HCMV, exerted an immunosuppressive effect on mitogen-induced proliferation of normal human T lymphocytes (Rinaldo *et al.*, 1977; Carney & Hirsch, 1981). Our own previous findings further suggested that normal monocytes infected *in vitro* with HCMV AD169 released a potent inhibitor of IL-1-dependent T cell proliferation (Rodgers *et al.*, 1985). These findings were supported by two other groups who reported significantly reduced IL-1 production by HCMV-infected monocytes (Smith *et al.*, 1985; Starr *et al.*, 1985). Other functionally similar inhibitors of IL-1 have been described in contexts other than HCMV infection. These have been derived from U937 cells after incubation with lymphokine-containing supernatants from antigen-specific T cell clones (Amento *et al.*, 1985) and from EBV-transformed B cell lines (Scala *et al.*, 1984) but there are no reports of their having
been fully characterized. In attempting to characterize the inhibitor derived from monocytes that were exposed to HCMV, we discovered that our virus stock had become contaminated by *M. hyorhinis*.

It has previously been reported that *M. arginini* is able to cause an apparent suppression of cell proliferation by its ability to compete for growth factors in the medium (Barile & Leventhal, 1968). More recently it has also been shown to release a pyrimidine-specific nucleoside phosphorylase that can degrade the \[^{3}H\]thymidine used to measure DNA synthesis (Sinigaglia & Talmadge, 1985). The possibility that *M. hyorhinis* might be acting in a similar manner led us to investigate the ability of supernatants from monocytes infected *in vitro* with mycoplasma-contaminated HCMV AD169 and isolated *M. hyorhinis* to inhibit mitogen-mediated T cell proliferation as determined by \[^{3}H\]thymidine uptake and by direct cell counting. The results (Table 2) indicated that *M. hyorhinis* appeared to inhibit \[^{3}H\]thymidine uptake and cause a delay in cell division following mitogen stimulation but did not cause inhibition of cell division. Some reports have concluded that mycoplasma contamination is unlikely from the finding that inhibition of \[^{3}H\]thymidine uptake was associated with an apparent inhibition of cell division 72 h after stimulation with mitogen. Our finding that *M. hyorhinis* could cause a delay in cell division indicates the importance of making careful cell counts at 96 h as well as 72 h before concluding that cell division has been inhibited. Our preliminary characterization data showing that the inhibitory activity was associated with a protein of about 95K (Table 1; Rodgers et al., 1985) might suggest that the mechanism by which *M. hyorhinis* was acting could indeed be by the release of a pyrimidine-specific nucleoside phosphorylase causing degradation of \[^{3}H\]thymidine, as was found for *M. arginini* (Sinigalia & Talmadge, 1985).

Some recent reports (Schrier & Oldstone, 1986; Schrier et al., 1986) suggested that low passage number clinical isolates of HCMV show a greater ability to infect lymphocytes than the laboratory-passaged fibroblast-adapted strains such as AD169. We therefore examined the extent of HCMV IE gene expression following infection of PBL with recent clinical isolates of HCMV, and the extent to which they caused suppression of T cell proliferation. In the experiments reported here we could demonstrate little evidence for the ability of recent clinical isolates or HCMV AD169 to infect normal peripheral blood lymphocytes or monocytes to any appreciable extent *in vitro*. Usually between 0-05 and 0-15% of the cells were positive by immunofluorescence staining. The finding that one of the highly passaged clinical isolate strains (isolate 1, see Table 4) gave a high value of 1-2% positive was not a reproducible finding. Permissively infected fibroblasts consistently gave a strongly positive nuclear fluorescence of a high proportion of cells.

To test for suppression of T cell proliferation responses, several clinical isolates were used to infect U937 cells at relatively high multiplicities and the supernatants were examined for inhibition of IL-1 activity. The results (Fig. 2) showed a direct correlation between the ability to cause *in vitro* suppression and the presence of mycoplasma. We then tested a further four recent clinical isolates, that we had rigorously shown to be mycoplasma-free, for their ability to suppress IL-1 responses. None caused significant suppression. Similarly we found no evidence for the ability of mycoplasma-free HCMV AD169 or recent clinical isolates to cause suppression of human lymphocyte responses to PHA or of monocytes to present the mitogen to fresh autologous T cells (Table 3). In initial experiments to examine the ability of supernatants from monocytes infected with HCMV AD169 to suppress concanavalin A-mediated human T cell proliferation we found that the response of human PBL to concanavalin A was not reproducible. We have therefore only used PHA in the subsequent experiments reported here. However, the results obtained in the *in vitro* IL-1 assay were performed using concanavalin A as mitogen. Our results suggest that mycoplasma-free virus is unable significantly to suppress the response of T cells to either concanavalin A or PHA.

Our findings are in contrast to those of Schrier et al. who have reported that *in vitro* infection of normal lymphocytes with recent clinical isolates causes suppression of mitogen-stimulated T cell proliferation as well as of natural killer cell activity and HCMV-restricted cytotoxic T cell generation (Schrier & Oldstone, 1986; Schrier et al., 1986). We have tried to optimize the likelihood of infection by using infected fibroblast sonicates containing between 5 × 10^4 and
Human CMV and monocytes

10^6 p.f.u./ml, a 5 : 1 PBL : fibroblast ratio and co-cultivation of the cells with the virus throughout the 8 day incubation period. Using these conditions, which are comparable to those of Schrier et al. (1986) we have been unable, using mycoplasma-free virus, to reproduce their, and our, previous findings. We cannot offer an explanation for this difference in findings. However, we would emphasize that M. hyorhinis is extremely difficult to culture by the usual mycoplasma culture techniques (Barile, 1981). Our contamination was not demonstrated by our own laboratory culture procedure using a commercial mycoplasma kit but only after careful screening by Hoechst dye DNA staining followed by culture that was carried out in a specialist mycoplasma laboratory. Furthermore we have found that many HCMV clinical isolates obtained from several laboratories were, in fact, contaminated with mycoplasmas. It may therefore be that mycoplasma contamination of HCMV virus stocks is a more prevalent problem than is currently apparent.

Our results would not necessarily dispute the observation that active HCMV infection may be associated with clinical immunosuppression. However, they do appear to cast some doubt on the ability of mycoplasma-free HCMV AD169 to cause significant in vitro immunosuppressive effects especially on mitogen-dependent proliferative responses. Indeed, it is difficult to envisage a mechanism whereby such profound in vitro immunosuppressive effects as those previously shown could be caused by such limited expression of HCMV IE protein in mononuclear cells (Table 4; Einhorn & Ost, 1984; Rice et al., 1984; Schrier et al., 1985). It is possible that there may be alternative explanations for the inhibition of mitogen responsiveness previously observed in the PBL of patients with active HCMV infection. For instance there is a considerable absolute increase in the number of peripheral blood cells of the CD8-positive cytotoxic/suppressor phenotype during active HCMV infections (Schooley, 1983). It is possible that such adherent T cell populations could account for some of the observed effects. Further studies should perhaps concentrate on defining the extent of HCMV infection in lymphocytes and macrophage in vivo during active CMV disease and whether immunosuppressive effects are produced by such infection in vivo.

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