Maintenance of Marek’s Disease Herpesvirus Latency \textit{in vitro} by a Factor Found in Conditioned Medium

By Celina Buscaglia* and Bruce W. Calnek

Department of Avian and Aquatic Animal Medicine, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, U.S.A.

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SUMMARY

Chicken spleen cells latently infected with Marek’s disease virus were cultured with and without conditioned medium (CM) obtained from concanavalin A-stimulated chicken spleen cell cultures. The expression of viral internal antigen(s) (VIA), which is usually associated with cultivation, was prevented or markedly reduced by the CM. This effect required the continued presence of CM, since its removal after 48 h resulted in the subsequent appearance of VIA. Although CM contains both gamma interferon (IFN-\(\gamma\)) and interleukin 2, our studies suggest that the ‘latency-maintaining activity’ (LMF) may not be associated with either of these products of stimulated lymphocytes. However, IFN-\(\gamma\) may also have had some suppressive effect. LMF appears to have an \(M_r\) greater than 10000 and to be inactivated by heating to 90 °C for 5 min.

INTRODUCTION

Marek’s disease, a widespread lymphoid neoplasm of chickens, is caused by a cell-associated herpesvirus, Marek’s disease virus (MDV). Following exposure to MDV, an early cytolytic infection peaks at 4 to 6 days post-infection (p.i.) when large amounts of viral internal antigen(s) (VIA) can be found in the lymphoid organs. At 6 to 7 days p.i., early immune responses appear, coincident with a switch from cytolytic to latent infection. Latent infection is defined for our purposes as the condition in which the presence of the viral genome can be demonstrated in the absence of viral antigens detected by immunofluorescence (Calnek, 1986).

It has been shown that immunosuppressive treatments such as administration of cyclosporin, thymectomy coupled with cyclophosphamide treatment, or betamethasone administration, all of which can impair cell-mediated immunity and lymphokine production, affect the latent state (Buscaglia \textit{et al.}, 1988). Although it appears that there is a link between immunocompetence and initiation and maintenance of latency with MDV, the mechanism(s) is not known. In view of a possible role for lymphokines, this study was undertaken to determine the effect of conditioned medium (CM), a source of cytokines including the lymphokines interleukin 2 (IL-2) and gamma interferon (IFN-\(\gamma\)) (Schauenstein \textit{et al.}, 1982; Pusztai \textit{et al.}, 1986) on MDV latency \textit{in vitro}. Our experimental system consisted of adding CM to cultures of latently infected spleen cells, which could be expected to enter a cytolytic infection cycle within 24 to 48 h after explantation as demonstrated by VIA expression (Calnek \textit{et al.}, 1982, 1984).

METHODS

\textit{Experimental birds.} Specific-pathogen-free (SPF) chickens of the P-2a (\(B^{19}\) \(B^{19}\)) (Weinstock & Schat, 1987) and S-13 (\(B^{13}\) \(B^{13}\)) (Schat \textit{et al.}, 1986) lines were obtained from departmental flocks and were used between 1 and 14 weeks of age. Obese strain chickens (Cole, 1966) were generously provided by Dr R. K. Cole, Department of Poultry and Avian Sciences, Cornell University, Ithaca, N.Y., U.S.A.; these birds were not from an SPF flock but were hatched and raised in isolation, as were P-2a and S-13 chickens.

\textit{Viral inocula.} Clone-purified MDV isolates JM-16 (moderate oncogenicity) and RB-1B (high oncogenicity) have been described (Calnek \textit{et al.}, 1984; Schat \textit{et al.}, 1982). All inocula for these experiments were from stocks of
intra-abdominal injection.

In vitro cultivation of latently infected spleen cells. When it was expected that MDV infection of the inoculated chickens had entered latency (8 to 25 days p.i.), spleens were collected aseptically and processed as reported (Calnek et al., 1984). Lymphocytes were suspended in LM-Hahn medium (Calnek et al., 1981) at a concentration of 5 \times 10^6 cells/ml and incubated in a humidified, 5% CO_2 atmosphere at 41 °C for 24 or 48 h as described (Calnek et al., 1982, 1984). Smears of cells were prepared at 0 h and after 24 and/or 48 h of incubation and examined for VIA as reported elsewhere (Buscaglia et al., 1988). Each smear contained the cells from 0.1 ml of culture and the VIA-positive cells were counted.

Production of CM. Supernatant fluids from concanavalin A (Con A)-stimulated chicken spleen cell cultures were produced by methods modified from those described by Schnetzler et al. (1983) and Kromer et al. (1984). Briefly, spleens from 9- to 14-week-old S-13 or Obese strain chickens were collected aseptically, gently forced through an autoclavable 60 μm mesh screen (Tetco, Elmsford, N.Y., U.S.A.), washed and resuspended in pH 7.3 phosphate-buffered saline (PBS). The cells were centrifuged over Ficoll-Paque (Pharmacia) and the lymphocytes were collected from the interface. They were washed in PBS and resuspended at a concentration of 2 \times 10^7 cells/ml in LM-Hahn medium.

Con A stimulation

Two different methods were used.

Pulsing. Con A (Sigma) was added at a concentration of 75 μg/ml and cells were incubated for 3 h at 41 °C. The cells were then washed twice and resuspended at 1 \times 10^7 cells/ml in LM base or in LM-Hahn (2%). LM base consisted of a modified LM-Hahn medium without chicken and foetal bovine sera (FBS); LM-Hahn (2%) has been previously described (Schat et al., 1986) and consisted of LM-Hahn medium without FBS, but containing 2% SPF chicken serum. The cells were incubated for an additional 18 to 24 h at 41 °C, after which they were removed by centrifugation. Supernatant fluids were refrigerated after being filtered through a 0.45 μm membrane filter (Corning Glass Works).

Continuous exposure to red blood cell (RBC)-bound Con A (RBC–Con A). Heparinized blood was pooled, washed twice with PBS, diluted to twice the original volume of blood with PBS and centrifuged over Ficoll-Paque. RBC pellets were harvested, washed twice with LM base and suspended in LM-Hahn (2%) at 5 \times 10^6 cells/ml. Con A was added to a concentration of 250 μg/ml and the cells were incubated at 41 °C for 30 min to allow binding of the mitogen to the cell surface. RBCs were washed four times with LM base and diluted to 2 \times 10^8 cells/ml in LM base or in LM-Hahn (2%). Lymphocyte suspensions and Con A-treated RBCs were combined in equal volumes and incubated at 41 °C for 24 h, after which all cells were removed by centrifugation. The supernatant fluids were filtered as above. Before this final filtration, part of some batches were clarified by filtration through an 8 μm filter, concentrated 20-fold in a Minitan filter (Millipore) which retains proteins with Mr values above 10,000, and pooled. Donor chickens and preparative methods for the various batches of CM used in these experiments are detailed in Table 1. For CM controls, the following cultures were prepared in parallel with normal CM production by the pulsing and the RBC–Con A methods: (i) spleen cells alone in LM base, (ii) spleen cells and untreated RBCs in LM base and (iii) Con A–RBC alone in LM base. These cells were incubated and centrifuged from the media as described above and supernatant fluids were refrigerated after being filtered through a 0.45 μm membrane.

Separation of lymphokines. CM, as a source of IL-2 and IFN, was fractionated following a procedure slightly modified from that reported by Fredericksen & Sharma (1986, 1987). Briefly, these two lymphokines were separated by hydrophobic chromatography with the use of phenyl–Sepharose CL-4B (Sigma). Two g of ammonium sulphate and 0.2 ml of 1 M-Tris–HCl buffer pH 7.5 were added to 9.8 ml CM. When necessary, CM was clarified by centrifugation. The samples were applied to 15 ml bed columns equilibrated with 20 mM-Tris–HCl, 20% ammonium sulphate buffer. Unbound proteins were washed from the columns until the absorbance at 280 nm reached zero. This was done with a Retriever IV fraction collector and a UA-5 absorbance/fluorescence detector (Isco), and corroborated by using a spectrophotometer (DU-50; Beckman). The bound proteins were eluted first with a buffer consisting of 20 mM-Tris–HCl, 20% ammonium sulphate and 10% ethylene glycol, and then with a similar buffer containing 15% ammonium sulphate and 20% ethylene glycol. Aliquots were collected and dialysed for 1 day against PBS in 10 mm × 10 cm dialysis tubing with an M, cut-off of 10,000 (Spectra/por; Spectrum Medical Industries, Los Angeles, Ca., U.S.A.) and then overnight against LM base. Elution buffers were also dialysed and used as controls. For testing, two pools of aliquots from the unbound proteins were identified as fractions 1 and 2, and two pools of aliquots representing bound proteins eluted with the two buffers were respectively identified as fractions 3 and 4 (see Fig. 1).

Assays for latency-maintaining factor (LMF). Latently infected spleen cells from JM-16-infected P-2a chickens were harvested 8-10 days after inoculation. A single exception was with experiment 6, trial 2, in which RB-1B-infected spleen cells were used. All spleen cells were free of VIA-positive cells at the time they were used for assays. Cells were always prepared freshly except for experiment 1, trial 1 and experiment 5, trial 1, when cells stored at -196 °C were used. In all tests, cultures of 5 \times 10^6 cells/ml were prepared in LM-Hahn medium or in LM-Hahn.
Maintenance of MDV latency in vitro

![Graph](image)

Fig. 1. Curve obtained from absorbance values of sequential aliquots from CM batch D6 (C) passed through a hydrophobic column with a flow rate of 0.4 ml/min. The IL-2 and IFN peaks (based on data in Table 7) are easily recognized as being associated with aliquots collected at 0 to 1 h and 4.5 to 6 h, respectively. A third small peak of unknown significance can be seen in aliquots collected at about 3 h. The three peaks had absorbance values (by spectrophotometry) of 2.70, 0.22 and 0.09 respectively. The arrows represent the addition of the buffers to elute bound fractions. Solid lines drawn below the curve indicate the aliquots pooled to make up the four fractions saved from CM D1 + D2 (C) and D6 (C). Aliquots collected during the periods represented by dotted lines were not used in the pools.

Table 1. CM batches

<table>
<thead>
<tr>
<th>Con A treatment</th>
<th>Medium</th>
<th>Strain</th>
<th>Age (weeks)</th>
<th>CM concentration (20-fold)*</th>
<th>CM batch number</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h pulse</td>
<td>LM-Hahn (2%)</td>
<td>S-13</td>
<td>13</td>
<td>-</td>
<td>A1, A2</td>
</tr>
<tr>
<td>3 h pulse</td>
<td>LM base</td>
<td>S-13</td>
<td>13</td>
<td>-</td>
<td>B1, B2</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM-Hahn (2%)</td>
<td>S-13</td>
<td>13</td>
<td>-</td>
<td>C1, C2</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM-Hahn (2%)</td>
<td>S-13</td>
<td>13</td>
<td>+</td>
<td>C3 (C)</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM-Hahn (2%)</td>
<td>Obese</td>
<td>9</td>
<td>+</td>
<td>C4 (C)</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM base</td>
<td>S-13</td>
<td>13</td>
<td>-</td>
<td>D1, D2</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM base</td>
<td>S-13</td>
<td>13</td>
<td>+</td>
<td>D1 + D2 (C)</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM base</td>
<td>S-13</td>
<td>13</td>
<td>-</td>
<td>D3, D4, D5</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM base</td>
<td>S-13</td>
<td>14</td>
<td>-</td>
<td>D6</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>LM base</td>
<td>S-13</td>
<td>13</td>
<td>+</td>
<td>D6 (C)</td>
</tr>
</tbody>
</table>

* CM was concentrated 20-fold (+) or used unconcentrated (−).

medium containing various dilutions of CM. Duplicate or triplicate smears, each representing 0.1 ml of culture, were prepared after 24 and/or 48 h of culture at 41 °C and the VIA-positive cells were counted. LMF activity was considered to be present when the mean number of positive cells in smears from the CM-treated cultures was less than 25% of that observed in untreated control cultures.

**IL-2 microassay.** Splenic lymphocytes were incubated in LM-Hahn medium for 48 h at a concentration of 5 x 10^6 cells/ml with 25 μg/ml of Con A. The resultant blast-transformed cells were vigorously shaken, washed twice in PBS and centrifuged over Ficoll-Paque. Those at the interface were harvested, washed again in PBS and resuspended at a final concentration of 1 x 10^6 cells/ml in LM-Hahn (2%) with HEPES buffer (Buscaglia et al., 1988) and seeded in round bottomed 96-well microtitre plates (Linbro/Titer-Tek, cat. no. 76-242-05) at 0.1 ml/well. Serial dilutions of CM in the same medium were added to triplicate wells at 0.1 ml/well. Medium alone was added to three wells as a control. To provide uniformity, serum-free batches of CM were supplemented with 2% SPF chicken serum for the test although no significant differences were noted in parallel tests in which those CM batches were not supplemented. After incubation at 41 °C for 48 h the cells were labelled for 16 h with 1 μCi of [3H]thymidine (sp. act. 2.0 Ci/mmol) per well. The cells were then harvested with a multiple semi-automated sample harvester (Titer-Tek cell harvester; Flow Laboratories) on filter discs (Titertek). After drying, the amount of [3H]thymidine retained on the filters was determined by liquid scintillation spectrophotometry. Results were
expressed as the mean c.p.m. of the triplicates. Samples considered to contain IL-2 were those with mean c.p.m. values at least twice that of the control medium from each plate.

**IFN assay.** The IFN content of CM was assayed by the plaque reduction method with vesicular stomatitis virus (VSV) in chicken embryo fibroblast (CEF) cultures. Serial twofold dilutions of CM in tissue culture medium were used to treat CEF monolayers for 18 h at 38 °C. The CEF cultures were then drained and challenged with 50 to 100 p.f.u. of the Indiana strain of VSV (kindly provided by Dr. E. Dubovi, Diagnostic Laboratory, New York State College of Veterinary Medicine, Cornell University). After a 1 h virus adsorption period, the cultures were drained and overlaid with an agar medium. Neutral red dye was added to stain plaques at 20 h post-inoculation and the plaques were counted 6 h later. The arithmetic mean from duplicate cultures was calculated. The IFN titre of a given sample represented the reciprocal of the highest dilution causing a 50% reduction in the number of VSV plaques observed in non-IFN-treated controls (Wagner, 1960).

**Statistical analysis.** Data were compared for statistical significance by the two-sample rank sum test. Statements of significance are based on a probability level of at least 95% (Snedecor & Cochran, 1980).

**Experimental design**

**Experiment 1.** Six trials were carried out, each with three to five spleen samples, to test various batches of CM for LMF activity. Six batches, C3 (C), C4 (C), D1 + D2 (C), D3, D4 and D5 were examined in one or more of the trials; the three former were concentrated during preparation and therefore were tested at 1:40 to 1:80 dilutions, whereas the three latter batches were not concentrated and were tested at a 1:2 dilution. Details are given in Table 2.

**Experiment 2.** This experiment was designed to determine whether the suppressive effect of CM on the expression of VIA in latently infected cells was permanent or depended on the continued presence of CM. Latently infected spleen cells were cultured for 48 h in LM-Hahn medium or in 1:2 dilutions of CM batches D3, D4 or D5. VIA-positive cells were counted after 48 h incubation. The three cultures treated with CM were then washed twice with PBS and each split into two; one was resuspended in the original CM-containing medium and the other resuspended in fresh LM-Hahn medium. These cultures were then reincubated for an additional 24 h and again examined for VIA-positive cells.

**Experiment 3.** To determine which cells and what conditions were involved in generating LMF in the Con A-stimulated spleen cell cultures, S-13 spleen cells from 11-week-old donors were cultured alone without stimulation, or after pulsing with Con A, or were cultured with RBC-Con A or with untreated RBCs. Supernatant fluids collected from those cultures and from a separate culture of only RBC-Con A were tested at a dilution of 1:2 for LMF activity using two samples of latently infected spleen cells. VIA examinations were carried out after 48 h of cultivation.

**Experiment 4.** LMF activity in CM batch D3 was tested before and after dialysis against LM base using a 10000 Mr cut-off membrane (trial 1) or before and after heating for 5 min at 90, 75, 60 or 45 °C (trial 2). Each sample was used at a dilution of 1:2 and was tested against cells from four latently infected spleens in trial 1, or one or two spleens in trial 2. Examinations for VIA were carried out after 48 h.

**Experiments 5 and 6.** These two experiments were designed to compare levels of IL-2, IFN and LMF in various batches of unconcentrated CM or in fractions of CM collected from the hydrophobic column. Each experiment had two trials. In experiment 5, CM batches A1, A2, B1, B2, C1, C2 and D1 to D5 were compared. IL-2 titrations were done with dilutions of 1:2, 1:6, 1:18 and 1:54. IFN assays were conducted on twofold dilutions from undiluted to 1:2048. LMF activity was tested against cells from a single spleen per trial in 1:2 and 1:50 dilutions (trial 1) or only a 1:2 dilution (trial 2). For experiment 6, CM batches D1 + D2 (trial 1) and D6 (trial 2) were tested for LMF activity at 1:2 or 1:6 dilutions against cell samples from two spleens in each trial, and for IL-2 and IFN as in experiment 5. An exception was with CM D1 + D2 which was tested at 1:10 and 1:100 dilutions for IL-2 activity.

**RESULTS**

**Effect of CM on maintenance of MDV latency in vitro**

Several different batches of CM, obtained by different methods of Con A stimulation of chicken spleen cells, prevented or markedly reduced the usual expression of VIA when added to cultures of latently infected spleen cells (experiment 1, Table 2). Trypan blue dye exclusion tests showed that most of the cells cultivated either in LM-Hahn medium or in the presence of CM were alive, and it was therefore concluded that the CM batches tested were not toxic.

Further proof that the suppressive effect of CM on MDV expression was not mediated through toxicity to the latently infected cells came from experiment 2. Data in Table 3 show that spleen cells in which VIA expression was suppressed by CM for 48 h of cultivation in vitro still had the capacity to ‘turn on’, since removal of the CM and reincubation for an additional 24 h
Maintenance of MDV latency in vitro

Table 2. Effect of CM on VIA expression in MDV latently infected spleen cells after 24 or 48 h in vitro cultivation (experiment 1)*

<table>
<thead>
<tr>
<th>Trial</th>
<th>No. spleen samples</th>
<th>Time p.i. (days)</th>
<th>Cultivation period (h)</th>
<th>VIA-positive cells/10⁶ cells.</th>
<th>CM batch</th>
<th>Dilution</th>
<th>VIA-positive cells/10⁶ cells.</th>
<th>Mean percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>25</td>
<td>24</td>
<td>165 (52 to 374)</td>
<td>C3 (C)</td>
<td>1:80</td>
<td>20 (5 to 62)</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>8</td>
<td>48</td>
<td>14 (9 to 57)</td>
<td>C3 (C)</td>
<td>1:40</td>
<td>0 (0 to 6)</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>10</td>
<td>24</td>
<td>44 (4 to 75)</td>
<td>C3 (C)</td>
<td>1:40</td>
<td>0 (0 to 1)†</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>13</td>
<td>24</td>
<td>18 (12 to 28)</td>
<td>C3 (C)</td>
<td>1:10</td>
<td>0 (0 to 5)†</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>13</td>
<td>24</td>
<td>85 (28 to 146)</td>
<td>D1 + D2 (C)</td>
<td>1:50</td>
<td>1 (1 to 5)‡</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>10</td>
<td>48</td>
<td>24 (7 to 48)</td>
<td>D3</td>
<td>1:2</td>
<td>0 (0 to 0)‡</td>
<td>0</td>
</tr>
</tbody>
</table>

* All spleen cell cultures were free of VIA at 0 h.
† Range is shown in parentheses.
‡ Significantly different (P < 0.05) when compared with untreated controls.

Table 3. Effect of temporary (48 h) compared to continuous exposure to CM on VIA expression in cultured MDV latently infected spleen cells (experiment 2)*

<table>
<thead>
<tr>
<th>Spleen cell treatment†</th>
<th>VIA-positive cells/10⁶ cells (% of LMF control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 48 h</td>
<td>49 to 72 h</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>CM D3</td>
<td>CM D3</td>
</tr>
<tr>
<td>CM D3</td>
<td>None</td>
</tr>
<tr>
<td>CM D4</td>
<td>CM D4</td>
</tr>
<tr>
<td>CM D4</td>
<td>None</td>
</tr>
<tr>
<td>CM D5</td>
<td>CM D5</td>
</tr>
<tr>
<td>CM D5</td>
<td>None</td>
</tr>
</tbody>
</table>

* Spleen cells were free of VIA before cultivation.
† CM batches were used at a 1:2 dilution in LM-Hahn medium. Cells were washed twice after 48 h; continued cultivation was in the original CM-containing medium or in LM-Hahn medium alone for an additional 24 h.
‡ ND, Not done.

resulted in the appearance of VIA-positive cells.

The results of experiment 3 (see Table 4) showed that strong LMF activity in CM was the result of Con A stimulation of spleen cells. CM obtained from spleen cell cultures stimulated either by pulsing with free Con A or by co-cultivation with RBC–Con A was strongly suppressive (no VIA-positive cells could be detected in tests against two latently infected spleen cell cultures). On the other hand, supernatant fluids from cultures of spleen cells alone or spleen cells cultured with normal RBCs apparently had some suppressive effect on VIA expression (VIA expression rates were 7 to 22% of the expected rates). Supernatant fluids from cultures of RBC–Con A failed to show LMF activity.

Characterization of LMF

Data from experiment 4 (Table 5) showed that LMF was not lost when CM batch D3 was dialysed to exclude constituents with an M, below 10000, but that heating to 90 °C for 5 min did destroy activity.
Table 4. **Tests for LMF activity in supernatant fluids (CM) from spleen cells, or RBCs, or combinations of the two with or without Con A stimulation (experiment 3)**

<table>
<thead>
<tr>
<th>CM preparation</th>
<th>Spleen 1 (45 VIA-positive cells/10^6 cells)</th>
<th>Spleen 2 (408 VIA-positive cells/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen cells alone</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>RBC-Con A</td>
<td>62</td>
<td>34</td>
</tr>
<tr>
<td>Spleen cells + normal RBC</td>
<td>22</td>
<td>7</td>
</tr>
<tr>
<td>Spleen cells + RBC-Con A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen cells pulsed with Con A</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* VIA expression was assessed after 48 h of *in vitro* cultivation in LM-Hahn medium (untreated controls) or in 1:2 dilutions of the various conditioned media.

Table 5. **LMF activity in dialysed or heat-treated CM (experiment 6)**

<table>
<thead>
<tr>
<th>Trial</th>
<th>CM treatment</th>
<th>No. VIA-positive CM cells/0.1 ml.</th>
<th>Median†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (LM-Hahn)</td>
<td>4 (22 to 104)</td>
<td>44</td>
</tr>
<tr>
<td>D3</td>
<td>None</td>
<td>4 (0 to 0)</td>
<td>0</td>
</tr>
<tr>
<td>D3</td>
<td>Dialysed</td>
<td>4 (0 to 0)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>None (LM-Hahn)</td>
<td>2 (21 to 45)</td>
<td>33</td>
</tr>
<tr>
<td>D3</td>
<td>None</td>
<td>1 (0)</td>
<td>0</td>
</tr>
<tr>
<td>D3</td>
<td>90 °C</td>
<td>2 (16 to 19)</td>
<td>17</td>
</tr>
<tr>
<td>D3</td>
<td>75 °C</td>
<td>1 (2)</td>
<td>2</td>
</tr>
<tr>
<td>D3</td>
<td>60 °C</td>
<td>1 (4)</td>
<td>4</td>
</tr>
<tr>
<td>D3</td>
<td>45 °C</td>
<td>1 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

* All CM samples were tested at a 1:2 dilution. VIA expression was assessed after 48 h of *in vitro* cultivation. † Range is shown in parentheses. Figures sharing the same superscript letter were significantly different (P < 0.005).

Table 6. **IL-2, IFN and LMF activity in different batches of CM (experiment 5)**

<table>
<thead>
<tr>
<th>Trial</th>
<th>CM batch*</th>
<th>IL-2</th>
<th>IFN</th>
<th>LMF activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1:2 CM</td>
</tr>
<tr>
<td>1</td>
<td>A1</td>
<td>&lt;2</td>
<td>516</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>&lt;2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>2</td>
<td>516</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>&lt;2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>6</td>
<td>256</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>&lt;2</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>18</td>
<td>1028</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>&gt;54</td>
<td>32</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>D3</td>
<td>&gt;54</td>
<td>128</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>D4</td>
<td>&gt;54</td>
<td>32</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>18</td>
<td>64</td>
<td>+</td>
</tr>
</tbody>
</table>

* All CM batches were tested at a 1:2 dilution. † Reciprocal of the highest dilution to show activity (twofold increase in [3H]thymidine uptake for IL-2 assays; 50% reduction in VSV plaques for IFN assays). ‡ LMF activity is taken as a reduction in the mean number of positive cells by 75% or more compared with the untreated controls (untreated controls had 111 VIA-positive cells/0.1 ml culture in trial 1 and 48 VIA-positive cells/0.1 ml culture in trial 2); + denotes the presence and - the absence of activity. §* ND, Not done.

The different batches of CM varied greatly in lymphokine content (experiment 5, Table 6). Two batches (A2, B2) lacked detectable levels of IL-2, IFN and LMF activity, although this could not be attributed to the method of Con A stimulation since two others (A1, B1) also...
Table 7. IL-2, IFN and LMF activity in fractions of CM unbound or bound to a hydrophobic column (experiment 6)*

<table>
<thead>
<tr>
<th>Trial</th>
<th>CM treatment</th>
<th>Dilution</th>
<th>Spleen 1 VIA-positive cells/0.1 ml</th>
<th>Spleen 2 VIA-positive cells/0.1 ml</th>
<th>Mean % of control</th>
<th>LMF*</th>
<th>IL-2 (titre)</th>
<th>IFN (titre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (LM-Hahn)</td>
<td>1:2</td>
<td>32</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CM D1 + D2</td>
<td>1:2</td>
<td>0 (0)†</td>
<td>0 (0)</td>
<td>0 ( )</td>
<td>(+)</td>
<td>&gt;100</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>CM D1 + D2 fraction 1</td>
<td>1:6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 ( )</td>
<td>(+)</td>
<td>&lt;2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D1 + D2 fraction 2</td>
<td>1:6</td>
<td>19 (59)</td>
<td>8 (30)</td>
<td>45 ( )</td>
<td>( )</td>
<td>&gt;54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D1 + D2 fraction 3</td>
<td>1:6</td>
<td>19 (59)</td>
<td>9 (33)</td>
<td>47 ( )</td>
<td>( )</td>
<td>&lt;2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D1 + D2 fraction 4</td>
<td>1:6</td>
<td>7 (22)</td>
<td>2 (7)</td>
<td>15 ( )</td>
<td>(+)</td>
<td>&lt;2</td>
<td>512</td>
</tr>
<tr>
<td>2</td>
<td>None (LM-Hahn)</td>
<td>1:2</td>
<td>93</td>
<td>73</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>None (LM base)</td>
<td>1:2</td>
<td>33 (35)</td>
<td>20 (27)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>None (buffer)</td>
<td>1:2</td>
<td>38 (40)</td>
<td>22 (30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CM D6</td>
<td>1:2</td>
<td>4 (11)</td>
<td>1 (5)</td>
<td>8 (+)</td>
<td></td>
<td>&gt;54</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>CM D6 fraction 1</td>
<td>1:2</td>
<td>3 (18)</td>
<td>8 (36)</td>
<td>18 (+)</td>
<td></td>
<td>&lt;2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D6 fraction 2</td>
<td>1:2</td>
<td>24 (63)</td>
<td>21 (95)</td>
<td>75 ( )</td>
<td>( )</td>
<td>&gt;54</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D6 fraction 3</td>
<td>1:2</td>
<td>12 (32)</td>
<td>7 (32)</td>
<td>32 ( )</td>
<td>( )</td>
<td>&lt;2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>CM D6 fraction 4</td>
<td>1:2</td>
<td>8 (21)</td>
<td>1 (5)</td>
<td>15 (+)</td>
<td></td>
<td>&lt;2</td>
<td>512</td>
</tr>
</tbody>
</table>

* Fractions 1 and 2 were unbound; fractions 3 and 4 were bound and then eluted (see Fig. 1).
† Numbers in parentheses show the number of cells that were VIA-positive as a percentage of the control.
‡ LMF was considered to be present when the mean number of VIA-positive cells was less than 25% of that for controls treated with buffer. In Trial 1, there were no controls treated with buffer, so LMF activity could only be presumed when the mean number of VIA-positive cells was less than 25% of that for untreated controls cultures in LM-Hahn medium.

DISCUSSION

To our knowledge, this is the first report of regulated expression of MDV VIA, normally associated with a switch to cytolytic infection, following the culture of latently infected lymphocytes. The use of CM was considered as a possible way of maintaining latency in vitro when previous studies (Buscaglia et al., 1988) indicated that treatments known to cause immunosuppression delayed or prevented a switch from cytolytic to latent infection or caused reactivation of cytolytic infection in MDV-infected chickens. These same treatments (cyclosporin, betamethasone administration) are also known to impair the production of lymphokines such as IL-2 or IFN-γ (Cohen et al., 1984; Hall & Goldstein 1982; Nelson, 1984; Besedovsky et al., 1986; Hess et al., 1986).
The component of CM that is responsible, which we have named LMF, appears to be produced by spleen cells with production greatly enhanced by Con A stimulation. This soluble mediator with an Mr greater than 10000 and susceptible to heat at 90 °C should probably be considered to be a cytokine, but further characterization studies are required.

LMF can be described as having a suppressive effect which requires its continued presence in the medium, since cells could later express VIA after LMF had been removed. However, the mechanism of suppression is unclear. The only conditioned media that showed strong LMF activity were those prepared either with free Con A or with RBC–Con A (Table 4), supporting the idea that lymphokines might be involved with this phenomenon. Some LMF activity could be detected in CM from cultures of spleen cells alone; however, the explanation for this may relate to the presence of cytokines in those cultures as well (Weiler & von Bülow, 1986, 1987).

CM probably contains many cytokines. IL-2 and IFN are two lymphokines known to be present in the supernatant fluids of Con A-stimulated cultures (Schauenstein et al., 1982; Pusztai et al., 1986). Our data argue against LMF being IL-2 because their titres were independent of one another. Some batches such as A1 were free of detectable levels of IL-2, but showed LMF activity (Table 6). Furthermore, fraction 2 from the hydrophobic column was rich in IL-2 and did not contain LMF (Table 7). Also, apparently LMF cannot be IFN since LMF was present in the unbound fraction whereas IFN was bound to the hydrophobic column. It may be that in addition to LMF, IFN can also regulate MDV genome expression. IFN-containing CM or CM fractions always had LMF-like activity. Alternatively, yet another suppressive cytokine may be present, which binds to the hydrophobic column and is eluted along with IFN.

The concept of a soluble mediator (cytokine) being involved in the regulation of MDV replication has considerable support from studies on other herpesvirus infections. Establishment and maintenance of herpes simplex virus (HSV) and human cytomegalovirus latency in vitro with the use of drugs alone or in combination with IFN, and with or without changes in the incubation temperature, have been reported (Wigdahl et al., 1982, 1984; Rapp, 1984; Shiraki & Rapp, 1986; Scheck et al., 1986; Cockley & Rapp, 1986). However, there are conflicting reports on the effect of IFN and other lymphokines on herpesvirus latency. It has been demonstrated that peripheral blood lymphocytes obtained from patients with recurrent disease fail to produce detectable levels of lymphokine activity (Nahmias & Roizman, 1973; Rosenberg et al., 1974; O’Reilly et al., 1977; Rasmussen & Merigan, 1978; Green et al., 1981; Cunningham & Merigan, 1984; Sheridan et al., 1982). In a double-blind study of the ability of IFN to prevent reactivation of herpes labialis after surgery involving the trigeminal nerve root, it was concluded that IFN reduces reactivation of latent HSV infection after a potent operative stimulus (Pazin et al., 1979). Similar results were obtained in vitro and in vivo (Rheinbaben & Schneweis, 1986; Daniesescu et al., 1985). However, Iannello et al. (1987) indicated that IFN does not play a major role in the latency or recurrence of herpes genitalis.

There are suggestions that other factors may be involved in herpesvirus latency. For instance, it was suggested that the resistance of clones of mouse neuroblastoma (C1300) cells to HSV is due to the production of a non-IFN intracellular inhibitor of HSV replication (Nilheden et al., 1985). Iwasaka et al. (1983) showed that recurrent lesions from HSV type 2 are associated with the induction of suppressor cells and soluble suppressor factors. More recently Sheridan et al. (1985) suggested that reactivation of latent HSV is associated with the induction of a dialysable factor(s) interfering with IFN and/or IL-2 activity.

From our results it may be concluded that CM contains a lymphokine(s) not related to IL-2 or IFN that helps to maintain MDV latency in vitro. LMF may also regulate genome expression or, alternatively, there may be two soluble independent factors with latency-maintaining activity, both of which are unrelated to IFN. Additional studies are needed to characterize this factor(s) further.

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