Effect of Immunocompetence on the Establishment and Maintenance of Latency with Marek's Disease Herpesvirus

By C. BUSCAGLIA,* B. W. CALNEK AND K. A. SCHAT
Department of Avian and Aquatic Animal Medicine, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853, U.S.A.

(Accepted 26 January 1987)

SUMMARY

Marek's disease virus (MDV) infections normally have an early cytolytic phase in lymphoid organs at 3 to 6 days post-infection followed by a period of latent infection. Little is known about the mechanisms that govern latency with herpesvirus infections, including Marek's disease (MD). To investigate the importance of immunocompetence for either the establishment or the maintenance of latency in MD, immunosuppressive treatments were applied prior to infection with MDV or after latency was established. These included cyclosporin (Cs) or betamethasone (BM) treatments, neonatal thymectomy plus cyclophosphamide treatment (Tx/Cy), and infection at a young age before full competence. The effect of all the treatments was determined by examining tissues and spleen cells for evidence of virus replication before and after cultivation in vitro. Induced (Cs or Tx/Cy treatments) or natural (infection at a young age) incompetence resulted in prolonged and more widespread early cytolytic infection. Immunosuppression by Cs after latency had developed resulted in a reappearance of cytolytic infection in the spleen and it enhanced the cytolytic infection in the thymus and the bursa of Fabricius. After immunosuppression with Cs, cytolytic infection was found mostly in T cells, although many of the virus-positive cells did not have markers for either T cells or B cells. Immunosuppression by BM after latency had developed also resulted in the reappearance of cytolytic infection in the spleen but only at a very low level. These results suggest that immunocompetence is required for the establishment and maintenance of latency.

INTRODUCTION

Marek's disease (MD) is a disease of domestic chickens caused by a cell-associated herpesvirus and characterized principally by T cell lymphoma and a demyelinating peripheral neuropathy (Calnek, 1980). The outcome of Marek's disease virus (MDV) infection is determined by virus and host cell interactions. During the first 3 to 6 days post-infection (p.i.), an early cytolytic infection of the primary lymphoid organs (bursa of Fabricius, spleen and thymus) occurs; the predominant targets are B cells (Shek et al., 1983). This is followed by a persistent latent infection of mostly Ia-bearing T cells (Calnek et al., 1984). Lymphomas may develop in some chickens several weeks or months later depending on factors such as genetic constitution, age at infection of the host and the strain of MDV.

Herpesviruses have developed a unique relationship with the host whereby, following primary infection, they remain in a latent form and may be reactivated throughout the life of the host. In the latent phase of MD there is no evidence of virus-associated antigens or virus particles in vivo, yet the virus can be recovered in vitro and short-term culture in vitro causes the appearance of virions or viral internal antigens (VIA). Although very little is known about what factors govern the induction and maintenance of latency with any of the herpesviruses, it is reasonable to consider the possibility that there can be both intrinsic and extrinsic influences. With many systems, derepression of latent infection generally occurs following immunosuppressive treatments in vivo or after cultivation in vitro of latently infected cells. Also, it has been noted
that latency develops at the time of early immune responses in MD (Calnek, 1986). Schat et al. (1980) showed that induction of latency was not affected by embryonal bursectomy; in other words, latency develops in the absence of humoral immunity. It might be presumed, then, that cell-mediated immunity (CMI) plays a major role, or that other mechanisms are involved in latency. Therefore, the purpose of this study was to determine whether immune responses are required to induce and maintain latency.

METHODS

Experimental chickens and holding conditions. Chickens ranging in age from 1 day to 7 weeks were obtained from specific pathogen-free departmental flocks of two genetic strains: MD-susceptible P-2a (B19B10) and MD-resistant N-2a (B13B11) (Weinstock & Schat, 1987). All experimental chickens were kept in isolation units on the floor.

Viral inocula. Cloned MDV isolates CU-2 (low oncogenicity) and JM-16 (moderate oncogenicity) have been described (Smith & Calnek, 1973; Calnek et al., 1984). All inocula for these experiments were from stocks of infected chicken kidney cell cultures stored at −196 °C. The dose was 500 to 1000 p.f.u./bird; inoculation was by intra-abdominal injection.

Preparation of lymphocyte suspensions. Spleens were collected aseptically and processed as reported by Calnek et al. (1984). Briefly, spleen cells were gently forced through an autoclavable 60 μm mesh (Tetco, Elmsford, N.Y., U.S.A.), washed once in phosphate-buffered saline (PBS) pH 7.3 and centrifuged over Ficoll-Paque (Pharmacia) at 400 g for 20 min. Lymphocytes, collected from the interface, were washed in PBS and resuspended in LM-Hahn medium (Calnek et al., 1981) at a concentration of 5 × 10⁶/ml for direct culture, or in PBS modified by addition of 1% bovine serum albumin and 0.1% sodium bicarbonate for staining of surface markers.

Cultivation in vitro. Lymphocytes were suspended in LM-Hahn medium at a concentration of 5 × 10⁶/ml and incubated in a humidified, 5% CO₂ atmosphere at 41 °C for 24 or 48 h as described previously (Calnek et al., 1982, 1984).

Mitogen stimulation. Stimulation in vitro by concanavalin A (Con A; Sigma) was carried out using methods similar to those described elsewhere (Schat et al., 1978). Briefly, splenic lymphocytes were counted and seeded at 5 × 10⁶ cells/well in a microtitre plate system (Linbro, Titertek cat. no. 76-247-05). There were two sets of triplicates for each sample. Culture medium (LM 2) consisted of a modified LM-Hahn medium with only 2% chicken serum and no foetal bovine serum, plus 0.025 M-HEPES and 0.1% sodium bicarbonate. An optimal amount of Con A (25 μg/well) was added to one triplicate set, while the second set was left as a control. 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 (Titertek cell harvester, Flow Laboratories) on filter discs (Titertek, Flow Laboratories), and processed for liquid scintillation spectrophotometry. Results were expressed as the difference between c.p.m. of the means of the stimulated and control triplicates. Based on previous experience, a difference of 2000 c.p.m. was arbitrarily considered positive. A stimulation index (SI) was calculated as: SI = (c.p.m. in treated cultures − c.p.m. in untreated culture)/c.p.m. in untreated culture. An SI of 1.0 or greater was considered to indicate a response to Con A (Calnek et al., 1985). To be considered a responder, a donor chicken had to meet both criteria; i.e. there had to be a difference of at least 2000 c.p.m. and an SI of 1.0 or greater.

Examination of spleen cells for viral antigens. Direct fluorescent antibody (FA) tests for MDV-VIA were done before cultivation (0 h) and at 24 or 48 h after cultivation as described previously (Calnek et al., 1984).

Dual staining of spleen cells for detection of surface markers on VIA-positive cells. For detection of T cell or B cell surface markers, 1 × 10⁶ to 2 × 10⁶ cells were washed with MPBS and then sequentially treated (15 min at 4 °C followed by washing with MPBS) with 20 μl of the desired mouse monoclonal antibody, 20 μl of rabbit IgG anti-mouse Ig (heavy and light chain; Miles-Yeda, Rehovot, Israel) and 20 μl of goat IgG anti-rabbit Igs, conjugated with rhodamine (Cappel Laboratories). The monoclonal antibodies used for T cell markers and surface IgM (B cells) were those used previously (Calnek et al., 1984); i.e. MAC T and MAC IgM, respectively. After the final wash, 10 μl drops of cell suspensions were air-dried on glass slides, fixed in acetone and stained for MDV-VIA as above. The Leitz microscope used for examining the smears was equipped with filters suitable for exciting either conjugate, and by switching from one to the other it was possible to determine whether or not a given VIA-positive cell had a given surface marker. The fluorescein isothiocyanate-stained VIA was seen as granular intracytoplasmic and intranuclear greenish fluorescence and the rhodamine-stained surface markers were observed as strong patchy or solid-ring reddish fluorescence.

Examination of tissue sections for viral antigens. Tissue samples were collected from bursa of Fabricius, thymus, spleen, kidney and liver. They were sectioned on a cryostat, fixed in acetone and stained for MDV-VIA as described by Calnek & Hitchner (1969). For the present study, tissues with fluorescing cells were given a score of
Latency in Marek's disease

0.5 to 4.0 to denote the intensity of reaction which varied from rare, isolated, positive cells (0.5) to nearly all cells positive (4.0). Negative tissues received a score of 0.

**Immunosuppressive treatments.** Cyclosporin (Cs) was kindly provided by Sandoz Pharmaceuticals, E. Hanover, N.J., U.S.A. Cs was dissolved in a warm mixture of absolute ethanol and the semi-synthetic neutral oil, Mygliol 812 (Dynamit Nobel, Kay-Fries, Inc., Rockleigh, N.J., U.S.A.). Dosage was 50 mg/kg body weight injected intramuscularly every 3 days as previously described by Nowak et al. (1982) for depression of T cell responses in chickens. The treatment began at 1 day of age for chicks when the induction of latency was examined, or after latency had been already established when the effect on maintenance of latency was to be tested.

One part of betamethasone (BM; Sigma) was dissolved in 75 parts of warm absolute ethanol. It was administered as a single intramuscular injection of 4 mg/kg body weight, or as five daily doses of 2.2 mg/kg body weight (Powell & Davison, 1986). BM was used only in attempts to break latency.

Neonatal thymectomy (Tx) was performed on the first day after hatching (Herbert et al., 1973) in conjunction with cyclophosphamide (Cy) treatment. Cy treatment was carried out by intra-abdominal injection of 4 mg of Cytoxan (Mead Johnson & Co., Evansville, Ind., U.S.A.) per day per chick, given on days 1 to 4 after hatching (Linna et al., 1972). The combined treatment (Tx/Cy) had been shown in other studies to impair cellular immunity, whereas Tx alone did not (Calnek et al., 1978a). At necropsy, all Tx/Cy-treated chicks were examined for the presence of thymic tissues, and only those without remnants were included in the trial.

**Statistical analysis.** Where appropriate, data were compared for statistical significance by using Student’s t-test or the two-sample rank sum test performed by the Minitab computer program (Ryan et al., 1985). Statements of significance are based on a probability level of at least 95% (Snedecor & Cochran, 1980).

**Experimental design**

Three experiments were conducted to study the relationship between immunocompetence and the induction of latency (experiments 1, 2, 3). In addition one experiment was conducted to examine the effect of chemical immunosuppression on the maintenance of latency (experiment 4).

**Experiment 1.** Three trials were done in which MDV-inoculated and uninoculated groups of 3-week-old chickens were subdivided into three subgroups respectively treated with Cs, untreated, or mock-treated with carrier (Mygliol and ethanol). In the first trial, P-2a chickens were inoculated with CU-2 MDV, and in the second and third trials N-2a and P-2a birds were inoculated with JM-16 MDV. Samples in trials 1 and 2 were collected at 5, 8, 12 and 15 days p.i., and in trial 3 at 8 and 15 days p.i. FA tests for MDV-VIA were done on frozen tissue sections of bursa of Fabricius, thymus, spleen, kidney and liver. Spleen cell suspensions were examined for VIA-positive cells before (0 h) and after 48 h of cultivation.

**Experiment 2.** Intact and Tx/Cy P-2a chickens were inoculated with JM-16 MDV at 2 weeks of age and sampled at 8 and 15 days p.i. Intact birds in an uninoculated control group were sampled at 8 days p.i. only. FA tests for MDV-VIA were performed on spleen cells before (0 h) and at 48 h after cultivation as well as on frozen sections of bursa of Fabricius, thymus, spleen, kidney and liver.

**Experiment 3.** One-day-old, 2-week-old and 7-week-old P-2a chickens were inoculated with JM-16 MDV. Samples were collected at 0, 5, 6, 7, 8, 11 and 14 days p.i. Samples at 0 days p.i. were obtained before inoculation. Five 1-day-old chicks were left uninoculated as controls and sampled at 5 days p.i. FA tests for MDV-VIA were done on spleen cells at 0 h and 48 h after cultivation as well as on frozen tissue sections of bursa of Fabricius, thymus and spleen. Spleens obtained from the group of birds inoculated at 1-day-old and sampled during the first week were examined without separation over Ficoll-Paque. Mitogenic stimulation tests with Con A were also performed on spleen cells collected at 0 days p.i.

**Experiment 4.** Three-day-old P-2a chickens were divided into two groups, one of which was inoculated with JM-16 MDV. At 11 days p.i., each group was subdivided into four subgroups. One subgroup was left untreated; the others were given Cs, BM (single dose) or BM (five daily doses). Samplings were done at 0, 8 and 14 days after the treatments were started. Frozen tissue sections of bursa of Fabricius, thymus and spleen, and spleen cell suspensions before and after 24 h of cultivation in vitro were examined for the presence of VIA-positive cells by FA. Smears were made at 0 h from spleen cells that were not separated over Ficoll-Paque so that dead cells would not be removed from the samples. Positive cells in the suspension cultures were examined by dual fluorescence to identify surface markers. Mitogenic stimulation tests were performed on spleen cell samplings done at 0 and 14 days post-treatment (p.t.).

**RESULTS**

**Effects of immunocompetence on the induction of latency**

The results from experiment 1 are presented in Table 1. Data from untreated and mock-treated chickens were pooled because no differences were found between them. Birds without detectable cytolytic infections were latently infected, on the basis of the absence of VIA before culture (0 h) and its presence after 48 h of cultivation in vitro of spleen cells. Although there was
Table 1. *Cytolytic and latent infection in Cs-treated and normal chickens*  

<table>
<thead>
<tr>
<th>Trial</th>
<th>Strain</th>
<th>MDV</th>
<th>Cs</th>
<th>Days p.i.</th>
<th>No. examined</th>
<th>VIA-positive</th>
<th>0 h</th>
<th>48 h</th>
<th>Spleen</th>
<th>Thymus</th>
<th>Bursa</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P-2a</td>
<td>CU-2</td>
<td>-</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>(2.0)</td>
<td>(3.5)</td>
<td>(2.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>6</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>(0.5)</td>
<td>(1.5)</td>
<td>(1.5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>N-2a</td>
<td>JM-16</td>
<td>-</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>(1.0)</td>
<td>(1.8)</td>
<td>(2.5)</td>
<td>4</td>
<td>(2.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>(0.0)</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>(2.8)</td>
<td>(3.0)</td>
<td>(2.5)</td>
<td>4</td>
<td>(2.5)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>(0.3)</td>
<td>(0.3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>2</td>
<td>(0.5)</td>
<td>(2.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>P-2a</td>
<td>JM-16</td>
<td>-</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>(0.0)</td>
<td>(0.0)</td>
<td>(1.0)</td>
<td>3</td>
<td>(1.0)</td>
</tr>
<tr>
<td>+</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>(1.0)</td>
<td>(1.0)</td>
<td>3</td>
<td>(1.0)</td>
<td>(1.5)</td>
<td>2</td>
<td>(2.0)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>(0.0)</td>
<td>(1.0)</td>
<td>3</td>
<td>(1.5)</td>
<td>(1.5)</td>
<td>0</td>
<td>0</td>
<td>(0.0)</td>
</tr>
</tbody>
</table>

* Spleen cell suspensions (0 h and 48 h cultures) and frozen tissue sections from all organs collected from uninoculated controls were negative in FA tests for MDV-VIA.  
† Figures in parentheses are median FA test scores, statistical significance (two-sample rank sum test) between CS-treated and untreated controls: a, P < 0.005; b, P < 0.05.
some variation, it could generally be said that treatment with Cs caused a prolonged period of early cytolytic infection characterized by a large amount of VIA in lymphoid organs, a more widespread infection in liver and kidney evidenced by the presence of VIA and an enhanced late (15 days p.i.) cytolytic infection, especially in trial 1. In contrast, birds not treated with Cs generally displayed the pattern of a lytic infection followed by a latent infection as described previously (Calnek, 1986). Statistical significance was shown between some of the scores given to the tissues with fluorescing cells belonging to Cs-treated and the untreated controls.

The effect of the Tx/Cy treatment (experiment 2) is shown in Table 2. Cytolytic infection in spleen at both 8 days p.i. and 15 days p.i. and in non-lymphoid organs (liver and kidney) at 15 days p.i. was present in the Tx/Cy-treated birds. In contrast, intact untreated controls had only latent infection of spleen cells and no VIA was observed in non-lymphoid organs. VIA was seen in bursas of chickens from both groups, but because of the depletion of lymphocytes by Cy, in the Tx/Cy group it was in epithelial cells. Again, most birds which were not cytolytically infected had latent infections.

The effect of age was tested in experiment 3 (Table 3). Cytolytic infection was prolonged in birds infected at 1 day of age compared to those infected later. It was the only group with VIA in thymus at 8 days p.i. and some bursas were positive at all sampling times. In contrast, 2-week-old birds were free of detectable VIA in spleen and thymus after 8 days p.i. and only two birds had bursal infection at that time. None had any evidence of cytolytic infection in any organs tested at 11 or 14 days p.i. The 7-week-old group also was essentially in complete latency after 7 days p.i. except for one bird each at the 11 and 14 days p.i. samplings which had VIA-positive cells in the bursa. The presence of VIA-positive cells in those two birds was not statistically significant when compared to the other groups. Latent infections were demonstrated in most birds not cytolytically infected after 7 days p.i. The immunocompetence of the birds was evaluated by the blastogenic response of spleen cells to Con A. Table 4 shows the values of the mitogen stimulation tests of the three different age groups prior to inoculation. Only three out of eight 1-day-old chickens were responders compared to all 16 birds of the other two groups. Also, the 1-day-old chickens which did respond had lower responses than 2- and 7-week-old birds.

### Effects of immunocompetence on maintenance of latency

Table 5 summarizes the data from experiment 4 in which four, five or 10 birds per group were sampled at 0, 8 and 14 days after treatment with Cs, five daily doses of BM or a single dose of BM.
Table 3. Cytolytic and latent infection in 1-day-, 2-week- and 7-week-old P-2a chickens infected with JM-16 MDV*

<table>
<thead>
<tr>
<th>Age</th>
<th>Days p.i.</th>
<th>No. examined</th>
<th>Spleen culture 0 h</th>
<th>FA test on frozen tissues†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. positive (median FA score)</td>
<td>Spleen</td>
</tr>
<tr>
<td>1 day</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>3 (0-5)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>5 (1-0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>5 (1-0)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>5 (2-5)</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>0</td>
<td>4</td>
<td>2 (0-0)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0</td>
<td>5</td>
<td>4 (1-0)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>2 (1-8)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>4 (2-0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>2 (0-5)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 weeks</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>3 (0-5)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5 (1-0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>3 (1-5)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Spleen cell suspensions (0 h and 48 h cultures) and frozen tissue sections from all organs collected from uninoculated controls were negative in FA tests for MDV-VIA.
† Figures having the same superscript letter in a certain tissue were significantly different (P < 0.05) in the two-sample rank sum test.

Table 4. Effect of age of P-2a chickens on the response of spleen cells to Con A

<table>
<thead>
<tr>
<th>Age</th>
<th>No. responders/ no. tested</th>
<th>C.p.m. of responders (Mean ± S.E.M.*)</th>
<th>SI of responders (Mean ± S.E.M.†)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>3/8</td>
<td>24422 ± 9189</td>
<td>7 ± 2n,b</td>
</tr>
<tr>
<td>2 weeks</td>
<td>8/8</td>
<td>56703 ± 9532</td>
<td>22 ± 6a</td>
</tr>
<tr>
<td>7 weeks</td>
<td>8/8</td>
<td>46114 ± 8453</td>
<td>32 ± 11b</td>
</tr>
</tbody>
</table>

* Values are the arithmetic mean ± standard error of the mean (S.E.M.) derived from counts on triplicate samples from each bird.
† Figures having the same superscript letter were significantly different (P < 0.05).

Spleen cells for the 0 h smears were examined without separation over Ficoll-Paque because previous experiments had shown that the cytolytically infected cells shown to be present in the frozen spleen sections were apparently lost after centrifugation over Ficoll-Paque, probably because they were dead. Untreated controls had no evidence of cytolytic infection in spleen, whereas spleens from Cs-treated birds were all positive for VIA.

A proportion of the untreated birds did have VIA-positive cells in the bursa and thymus, but this was significantly enhanced in the Cs-treated birds. BM-treated groups had some birds with VIA-positive spleens, but thymic and bursal infections were similar to those in the control group.

To determine which cells become cytolytically infected in vivo after Cs-induced immunosuppression, monoclonal antibodies that specifically react with B cells or T cells were used. Dual fluorescence tests to detect surface markers and VIA were conducted with MDV-infected spleen cells freshly collected from Cs-treated chickens or after in vitro cultivation of spleen cells from other groups that had few or no VIA-positive cells at 0 h. After immunosuppression with Cs,
Table 5. Cytolytic infections after Cs or BM treatment of P-2a chickens latently infected with JM-16 MDV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days p.i.</th>
<th>No. examined</th>
<th>VIA in cultured spleen cells</th>
<th>FA test on frozen tissues†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Thymus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 h</td>
<td>24 h</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5 (12)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10</td>
<td>0</td>
<td>9 (6)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>0</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Cs</td>
<td>8</td>
<td>10</td>
<td>10 (11)</td>
<td>ND§</td>
</tr>
<tr>
<td>BM (5 doses)</td>
<td>14</td>
<td>5</td>
<td>5 (9)</td>
<td>ND</td>
</tr>
<tr>
<td>BM (single dose)</td>
<td>8</td>
<td>10</td>
<td>2 (&lt;1)</td>
<td>1 (11)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4</td>
<td>1 (&lt;1)</td>
<td>4 (2)</td>
</tr>
</tbody>
</table>

* Spleen cell suspensions (0 h and 48 h cultures) and frozen tissue sections from all organs collected from uninfected controls were negative in FA tests for MDV VIA.
† Mean number VIA-positive/10⁵ cells or mean FA score.
‡ Figures having the same superscript letter were significantly different: a,b,c, P<0.0005; d, P<0.005; e,f,g,h,i,j,k, P<0.05 in the two sample rank sum test.
§ ND, Not done.

Table 6. Mitogen responsiveness of spleen cells from uninfected and MDV-infected P-2a chickens before and after immunosuppressive treatments

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>Day p.t.</th>
<th>MDV infection</th>
<th>Treatment</th>
<th>Responders*</th>
<th>C.p.m.of responders (mean ± S.E.M.)†</th>
<th>SI of responders (mean ± S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0</td>
<td>No</td>
<td>–</td>
<td>4/5</td>
<td>24462 ± 13632</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>–</td>
<td>3/5‡</td>
<td>32589 ± 16420</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>25</td>
<td>14</td>
<td>No</td>
<td>–</td>
<td>3/5</td>
<td>12249 ± 5101</td>
<td>18 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cs</td>
<td>1/5§</td>
<td>2328 ± 0</td>
<td>2 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM (5 x)</td>
<td>1/5§</td>
<td>2224 ± 0</td>
<td>12 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM (1 x)</td>
<td>1/5§</td>
<td>4573 ± 0</td>
<td>6 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>–</td>
<td>3/5</td>
<td>5253 ± 741</td>
<td>9 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cs</td>
<td>1/5</td>
<td>2247 ± 0</td>
<td>3 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM (5 x)</td>
<td>4/4</td>
<td>30529 ± 9600</td>
<td>22 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BM (1 x)</td>
<td>1/4</td>
<td>108605 ± 0</td>
<td>33 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

* Number positive/total number.
† Values are the arithmetic mean ± standard error of the mean derived from counts on triplicate samples from each bird.
‡ One of the birds had a mean difference of 15308, but an SI of 0.6; for this reason it was not considered a responder. It had a high background, probably due to the presence of tumour cells.
§ Three birds from each of the three groups had an SI greater than 1.0, but a mean difference smaller than 2000 c.p.m. because all values were very low.

Cytolytic infection was found mostly in T cells, although many of the VIA-positive cells did not show markers for either T cells or B cells.

Mitogen stimulation tests were conducted in experiment 5 on uninoculated and inoculated chickens before and after administration of the different treatments. Prior to the immunosuppressive treatments no differences were found between infected and control groups and both showed a majority of responder birds (Table 6). Fourteen days p.t., the numbers of responders found among all treated subgroups in the uninfected group and all but the five dose BM treatment group in the infected group were lower than in the respective non-treated groups.
Birds treated with Cs were the lowest responders in the treated, infected group. Only one bird of the single dose BM-treated subgroup was a responder.

DISCUSSION

This is the first time that immunocompetence has been shown to influence the establishment and maintenance of latency in MDV infection. The results presented here suggest that latent MDV infection, like that with other herpesviruses, can be activated by immunosuppressive treatments. The results also suggest that treatments known to cause immunosuppression affect the establishment of latency in MD.

In MDV infection, two types of virus–cell interactions have been described: productive, where there is active synthesis of virus DNA, virus proteins and virions, and non-productive, in which latent infection and transformation occur (Calnek, 1980). We chose as our working definition of latency in MD, the condition in which the presence of the viral genome could be demonstrated by the absence of viral antigens detected by immunofluorescence (Calnek, 1986). Thus, latently infected cells were those which expressed VIA only after cultivation in vitro (Calnek et al., 1981, 1984). There are no experimental data to show whether or not cells latently infected with MDV transcribe parts of the viral DNA. However, with other herpesvirus infections, it appears that latency is relative and that some DNA transcription may take place (Stevens, 1980; Kieff et al., 1982) but with incomplete or complete blocks at the transcriptional and translational levels. The mechanisms responsible are not understood (Openshaw, 1984), although Stevens et al. (1987) suggested that RNA transcripts complementary to a herpesvirus α gene mRNA could be involved in maintaining the latent infection of herpes simplex virus (HSV).

Some direct evidence for immune responses being linked to herpesvirus latency comes from work by Stevens & Cook (1974) who showed that anti-HSV IgG bound to cell receptors was involved in modulating latency. However, work by others (Rotula et al., 1984) indicated that antibodies are not necessary to induce HSV latency and suggested that the transition from acute to latent HSV infection might be mediated mainly both by unknown virus factors and by host factors such as cellular immunity and interferon. Also, Schat et al. (1980) showed that chickens which had been bursectomized as embryos and hence lacking B cells, and hence Iggs, can become latently infected with MDV. Consequently, the immunosuppressive treatments chosen for these studies were those which are supposed to impair CMI. Cs treatment was selected because responsiveness to Con A of peripheral blood lymphocytes (PBL) from Cs-treated birds had been reported to be strongly depressed after 7 and 22 days of Cs administration (Nowak et al., 1982).

Corticosteroids are known to reduce CMI responses and BM was chosen because of a previous report on its use in the MD system (Powell & Davison, 1986) where it increased the incidence of MD in infected birds.

The various treatments were either shown, or could be inferred from other studies, to be effective. Uninfected or infected birds treated with either Cs or BM in experiment 4 (Table 6) clearly had reduced responses in terms of both number of responder birds and stimulation indices. An exception was the infected group given five daily doses of BM; this could have been because acute stress has been found to inhibit responsiveness while prolonged or chronic stress can be stimulatory (Monjan & Collector, 1977). In experiment 1, although CMI responsiveness was not tested, chickens were treated with Cs for at least 14 days before the first sampling.

Mitogen stimulation tests were not performed in experiment 2, but Tx/Cy had been shown previously to depress markedly Con A responsiveness and to delay or prevent skin graft rejection (Calnek et al., 1978a). Cy reportedly eliminates, or seriously impairs, B cell functions but it also has a transient depressive effect on the thymus (Linna et al., 1972).

The use of 1-day-old chickens, which are immunologically immature, was another approach to test the effect of the immune response. Con A stimulation tests (Table 4) showed only three of eight 1-day-old chicks to be responsive compared with eight of eight for each of the older groups. Furthermore, the mean stimulation index for the three 1-day-old responders was low compared to that of the others.

Immunocompetence seemed to be crucial in the establishment of latency with MDV. There
was at least a delay in latency induction with all treatments. This was shown in the age effect
experiment where MDV infection in 1-day-old chickens failed to enter latency in all lymphoid
organs whereas it did in more competent older birds. The 7-week-old group was essentially in
complete latency after 7 days p.i. except for one bird each at the 11 and 14 days p.i. samplings.
These two exceptions may have represented the beginning of the second cytolytic phase which
can accompany permanent immunosuppression from MDV infection. The role of immunocom-
petence in the induction of latency is also suggested from the results with the Cs and the Tx/Cy
treatments. Cs-treated birds had prolonged and more widespread cytolytic infection than
untreated controls in trials 1, 2 and 3 (Table 1). Tx/Cy treatment similarly prolonged cytolytic
infection in spleen and resulted in more widespread infection (liver and kidney) at 15 days p.i. It
would have been interesting to determine which cells were cytologically infected in the spleens of
Tx/Cy-treated birds at 8 and 15 days p.i., although it might be presumed that they were T cells.

In spite of immunosuppression, latency developed in many cases. Also, latency in intact birds
was not always complete in all organs. The bursa was often the last organ to show cytolytic
infection before latency was 'complete' and was also generally the only one positive at late
sampling times. Perhaps latency can be expressed to a variable degree in various organs with
MDV. Many birds have a persistent, low-level productive infection in the feather follicle
epithelium but apparently only latent infections in other tissues. Latent infection of splenic
lymphocytes and PBL persists, probably for the lifetime of the birds, but virus may be shed from
the skin during the same period (Witter et al., 1971; B. W. Calnek, unpublished data). Certain
changes in internal environment and certain apparent non-specific stimuli may trigger
induction of virus production more easily in some tissues than in others. It seems that at the host
level complete latency may not occur with MDV.

Because latency developed in spite of immunosuppression, albeit in a delayed fashion, and
because we do not have a method for determining whether latently infected cells are present
intermixed with cytolytically infected cells, one cannot rule out the possibility that an alternative
explanation for our findings is that immunocompetence is required for terminating cytolytic
infection but is irrelevant for the induction of latency. However, this explanation seems unlikely
because once latency was induced, immunosuppressive treatment clearly caused reactivation of
cytolytic infection (Table 5). The effects of induced immunosuppression might be clouded by the
fact that oncogenic MDV infection is in itself immunosuppressive and can cause permanent
suppression at 2 to 3 weeks p.i. Re-emergence of cytolytic infection coincides with this. Even so,
in experiment 5 (Table 5) substantial differences between untreated and treated groups were
observed wherein only treated groups had cytolytic infection in the spleen. Cs was more
effective than BM, but BM did have an effect, judging by the appearance of splenic infection in
at least a proportion of the birds in most of the sampling periods. The less striking effect of BM
could be because corticosteroids elicit their maximum immunosuppressive activity when
administered just prior to the antigen rather than after immunological challenge (Webb &
Winkelstein, 1982). Unfortunately, we only tested BM after latency was established. Although
we favour the conclusion that Cs or BM treatments had their effect indirectly by causing
immunosuppression we cannot exclude the possibility that these drugs had a direct effect by
activating the MDV genome. Additional studies are required to resolve this point.

The dual fluorescence tests on cells which were activated in vivo after Cs treatment showed
that the majority were T cells although some B cells were also observed. These results are in
agreement with those reported for latently infected cells by Calnek et al. (1984) and it seems
probable that the cells with cytolytic infection after immunosuppression represent the same
populations that were latently infected. Also, there were VIA-positive cells that did not react with
either the T cell or the B cell reagent. This could have been due to weaker than normal expression
of cell surface markers on the infected cells. Weak expression of T cell markers on MDV-
transformed lymphoblastoid cell lines has been reported (Ross et al., 1977; Matsuda et al., 1976;
Calnek et al., 1978b).

Our observations on the effect of immunosuppression on the induction or maintenance of
latency are in keeping with findings in other systems. There are data suggesting that the host
immune response which limits productive HSV infection may also play a role in the
establishment of latency protecting against it. CMI appears to be important in the control of initial HSV disease as well as other animal herpesvirus infections (Stanberry, 1986; Harris et al., 1984; Miller, 1985). In the guinea-pig model, Cs administration had adverse effects on the pathogenesis of primary cytomegalovirus infection (Bia et al., 1985), as it did on the pathogenesis of MDV infection in chickens (experiment 1, Table 1).

These results support the conclusion that immune responses are indeed involved in the maintenance and probably also in the establishment of latency although the mechanism(s) is not yet clear. Perhaps soluble factors such as interleukin 2 or gamma interferon, reported to be affected by the immunosuppressive treatments chosen in this study (Hess et al., 1986; Nelson, 1984; Cohen et al., 1984; Hall & Goldstein, 1982; Besedovsky et al., 1986), play a role. HSV-stimulated PBL were shown to produce a factor(s) that inhibits lymphokine activity (Sheridan et al., 1985). The absence of lymphokines may interfere in some way at the molecular level by derepressing transcription and translation. Alternatively, immunosuppression might provoke new factors which could directly contribute either to prolongation or reactivation of cytolytic infection. A combination of intrinsic and extrinsic influences may be involved in governing latency.

Although the cell-mediated responses seem to be important, the question is unresolved and additional studies will need to focus on identifying the specific components of the immune system that contribute to the control of latency.

We thank Mr Raymond Harris for excellent technical assistance and Mrs Gwen Troise for helping in the typing of the manuscript. This research was supported in part by Public Health Grant R01 CA06709-24 from the National Cancer Institute. It was presented in part at the section on Avian Medicine, 123rd Annual Meeting of the American Veterinary and Medical Association, Atlanta, Ga., U.S.A., 20 to 24 July 1986 and at the 10th Latin American Poultry Congress, Buenos Aires, Argentina, 29 September to 2 October 1987.

REFERENCES


(Received 20 July 1987)