Immunomodulation of peripheral T cells in chickens infected with Marek's disease virus: involvement in immunosuppression

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Marek's disease virus (MDV) causes T cell immunosuppression in chickens during latent infection. Morphological changes specific to apoptosis were demonstrated in peripheral blood mononuclear cells (PBMC) of MDV-infected chickens at 2–3 weeks post-inoculation (p.i.). Analysis of DNA fragmentation in T cell subsets in the peripheral blood revealed that CD4+ T cells but not CD8+ T cells underwent apoptosis after MDV infection. The proportion of CD4+ T cells, but not that of CD8+ T cells, in the peripheral blood expanded transiently at 16 days p.i., and rapidly decreased 1 week later. The decrease in CD4+ T cells might be mediated by apoptosis, because a rapid reduction in CD4+ T cells was observed when these cells underwent apoptosis.

Analysis of the T cell-receptor (TCR) repertoire of the peripheral blood showed that Vβ1 but not Vβ2-αβ TCR-bearing cells expanded at 16 days p.i., when the transient expansion of the CD4+ T cell population was observed in these chickens. Flow cytometric profiles also showed that the expression of CD8 was down-regulated after infection with MDV, but there was no difference in the expression level of CD4 molecules between normal and infected chickens. Northern blot analysis indicated that the down-regulation of CD8 occurred at the transcriptional level. These results suggest that both apoptosis of CD4+ T cells and down-regulation of CD8 molecules could contribute to the immunosuppression caused by MDV.

Introduction

Marek's disease (MD), caused by Marek's disease virus (MDV), is a lymphoproliferative disease of chickens, characterized by CD4+ T cell lymphoma formation (Schat et al., 1991). The pathogenesis of MD can be divided into three phases: early cytolytic infection with transient immunosuppression, latent infection combined with secondary cytolytic infection and permanent immunosuppression, and tumour development (Schat, 1987). Primarily, MDV causes lytic infection of B cells and a few T cells (early cytolytic phase). Following the lytic infection, T cells are activated to respond against the lytically infected cells. The activation of T cells during the early cytolytic infection is important for establishing the latent infection since the latent infection of MDV is maintained in activated T cells (latent phase). The secondary cytolytic infection starts in the feather follicle epithelium, which is the only place where infectious cell-free virus is produced (Shek et al., 1983; Calnek et al., 1984; Schat, 1987; Fynan et al., 1992). At the same time, MDV causes secondary immunosuppression, which is characterized by unresponsiveness to mitogen stimulation (Theis et al., 1975), delayed rejection of Rous sarcomas or MD transplantable tumour cells (Calnek et al., 1975), and increased susceptibility to coccidiosis (Biggs et al., 1968). Interestingly, cytotoxic T lymphocytes (CTL) against syngenic MD tumour cell lines, which had been thought to be important for tumour inhibition, were not detected in chickens infected with MDV (Schat et al., 1982). Subsequently, MDV can cause the development of CD4+ T cell lymphoma in chickens (Schat et al., 1991). However, the importance of secondary immunosuppression for tumour development is not known.

Apoptosis has long been recognized as an important feature in embryonic development, especially of the immune system (Cohen et al., 1992). Recently, it has been reported that several viruses can induce apoptosis of lymphoid cells, which are involved in viral immunosuppression. For example, human immunodeficiency virus (HIV) can cause depletion of peripheral CD4+ helper T cells via apoptosis, which may have a central
role in the pathogenesis of AIDS (Ameisen et al., 1994). Although the mechanism by which HIV causes apoptosis of CD4+ T cells has not been completely clarified, some models, including intra- and extracellular events, have been proposed (Groux et al., 1992; Laurence et al., 1992; Meyaar et al., 1992; Ameisen et al., 1994; Lu et al., 1994). In contrast, there is little knowledge about how MDV causes T cell immunosuppression in chickens.

In this paper, we examined the properties of peripheral T cell subsets in chickens latently infected with MDV. We report novel biological properties of MDV which might be involved in secondary immunosuppression.

Methods

Experimental chickens. Fertile eggs of Shaver 288 White Leghorn chickens were purchased from Hokuren Co. Ltd (Sapporo, Japan). These eggs were hatched, and birds were raised in our laboratory.

Virus and inoculation procedure. Chickens were challenged intra-muscularly with 8000 p.f.u. of strain Md5, a highly virulent MDV, at 5 days after hatching. This strain was propagated in chicken embryo fibroblasts (CEF), and virus titres were determined by plaque assays as described by Witter & Burmester (1979).

Monoclonal antibodies. The following monoclonal antibodies (MAbs) were used for flow cytometric analysis: CT4, which can recognize chicken CD4 molecules (Chan et al., 1988), and TCR2, which can recognize chicken Vβ1-β2 T cell receptor (TCR) (Chen et al., 1988). These MAbs were a gift from C-L. H. Chen (University of Alabama, Birmingham, Ala., USA); 11-39, which can recognize chicken CD8 molecules (Luhtala et al., 1993), was a gift from Y. Hirota (National Institute of Animal Health, Tsukuba, Japan); TCR3, which can recognize chicken Vβ2-β5 TCR (Chat et al., 1990), was purchased from Southern Biotechnology Associates, Inc.

Isolation of lymphocytes and flow cytometry. Peripheral blood lymphocytes (PBL) were isolated from heparinized blood samples after low speed centrifugation (60 g, 20 min) as described by Chen et al. (1984). Single cell suspensions of the spleen were obtained by disruption followed by Ficoll-Conray density gradient centrifugation to remove dead cells and red blood cells. Cells (1 x 10⁶) were incubated with MAbs for 30 min at 4 °C. After washing with PBS, cells were incubated with FITC-conjugated goat anti-mouse Ig (Cappel) for 30 min at 4 °C. Relative immunofluorescence of cells was analysed by a flow cytometer (EPICS-752; Coulter).

Isolation of CD4⁺ or CD8⁺ T cells. Peripheral blood mononuclear cells (PBMC) and spleen cells were isolated by Ficoll-Conray density gradient centrifugation. These cells (5 x 10⁸) were incubated with 1 ml of CT4 or 11-39 culture supernatant for 30 min at 4 °C. After incubation, cells were washed twice with DMEM/F-12 (GIBCO BRL) containing 10% heat-inactivated fetal calf serum (FCS; Filtron), 5 x 10⁻² M-2-mercaptoethanol (2-ME), 100 U/ml penicillin and 100 µg/ml streptomycin (complete DMEM/F-12). The cells were then resuspended in 500 µl of complete DMEM/F-12 containing 2 x 10⁷ sheep anti-mouse IgG magnetic beads (Dynabeads M-450, Dynal), and incubated at 4 °C for 45 min with gentle rotation. After incubation, cells attached to the beads were trapped by a magnet (MPC-E; Dynal). To separate the cells from the beads and to examine the fluorescence intensity of each molecule, the cells binding to the beads were cultured in RPMI 1640 (GIBCO BRL) containing 10 % heat-inactivated FCS, 5 x 10⁻² M-2-ME, 100 U/ml penicillin and 100 µg/ml streptomycin, at 40 °C for 3-5 h in a humidified atmosphere containing 5% CO₂. Flow cytometric analysis revealed that the purity of each T cell subset separated by this procedure ranged from 92-98%.

DNA fragmentation assay. DNA fragmentation was determined by the methods of Wyllie & Morris (1982) and Newell et al. (1990) with slight modifications. In brief, 5 x 10⁶ cells were lysed in 600 µl of hypotonic lysis buffer (10 mM-Tris (pH 7.4), 10 mM-EDTA, 0.2% Triton X-100) for 10 min on ice. The lysate was centrifuged at 13000 r.p.m. for 10 min at 4 °C to remove large DNA molecules. Supernatants were deproteinized by extracting once with phenol and once with phenol–chloroform–isoamyl alcohol (25:24:1). To the resulting supernatant (460 µl), 980 µl of ethanol and 29 µl 5 M-NaCl were added, and DNA was precipitated at -20 °C for 2 h. DNA samples were analysed on a 2% agarose gel, and stained with ethidium bromide.

Morphological study of cells. Apoptotic cells were identified by use of a light microscope. For light microscopic observation, cytocoentrifuged smears of PBMC isolated by Ficoll–Conray density centrifugation were stained with May–Grünewald–Giemsa solution, and examined with an oil immersion objective. With this staining procedure, apoptotic cells were easily identified by apoptosis-specific signs, such as apoptotic bodies, chromatin cleavage and chromatin condensation.

Preparation of cDNAs of chicken CD8α and CD8β chains. cDNAs encoding the CD8α and CD8β chains were amplified from a cDNA library of the chicken thymus by PCR using the primer sets 5' ACGATGGCAGCTCTCCTGC 3' and 5' CTCGACGTCGTC-ATAATGTGT 3', and 5' ATGATGTTGGACATGGCCAC 3' and 5' GCAGAGCAAGCTTCTGTGCT 3', respectively. These primers were designed to amplify entire coding regions of the CD8α and CD8β genes based on the nucleotide sequences reported by Tregaskes et al. (1995). The PCR products were cloned by using the pGEM-T vector system (Promega) (pGEM-Tα for CD8α, pGEM-Tβ for CD8β, respectively).

Northern blot hybridization. Transcripts for the CD8α and β chain genes were evaluated by Northern blot hybridization using appropriate 32P-labelled cDNA probes as follows: a Neol–Ndel fragment of pGEM-Tα as CD8α chain probe, and a SacII–NotI fragment of pGEM-β as CD8β chain probe. Total cellular RNAs were extracted from CD8⁺ T cells isolated from the peripheral blood and spleen with Trizol (GIBCO BRL) and chloroform. Total cellular RNA samples (2.5 µg each) were treated with 1% formaldehyde, electrophoresed on a 1% agarose gel in 10 mM-sodium phosphate buffer, pH 7.0, and transferred to a nylon membrane filter Hybond-N (Amersham). The filter was hybridized with 32P-labelled probes, washed three times with 2 x SSC plus 0.1% SDS at room temperature for 15 min, and washed once with 0.1 x SSC plus 0.1% SDS at 50 °C for 30 min. The filter was then subjected to autoradiography.

Statistical analysis. The statistical significance of the difference between the means of the samples was determined by Student's t-test.

Results

CD4⁺ T cells undergo apoptosis in chickens infected with MDV

It is generally accepted that MDV causes T cell immunosuppression during latent infection (Schat,
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1987), but the mechanism is still unclear. To study the mechanism of immunosuppression, we examined apoptotic changes in PBMC from chickens inoculated with strain Md5, a highly virulent MDV, 5 days after hatching. Apoptosis-specific signs, such as chromatin condensation, were observed in PBMC from MDV-infected chickens 2–3 weeks p.i., but not in those from age-matched normal chickens (Fig. 1a, b). To determine which T cell subpopulation, CD4+ or CD8+, underwent apoptosis, we studied DNA fragmentation in each T cell subset isolated from MDV-infected and uninfected chickens. As shown in Fig. 1(c), typical apoptotic ladders of nucleosomal DNA were observed in the CD4+ population (lane 5) but not in CD8+ T cells (lane 6) in the peripheral blood of MDV-infected chickens. CD4+ T cells in the spleen also underwent apoptosis in MDV-chickens, while CD8+ T cells showed marginal laddering of nucleosomal DNA (lanes 7 and 8). Both CD4+ and CD8+ T cell populations in the peripheral blood and spleen of normal chickens did not show these changes (lanes 1–4). These results indicate that MDV induces apoptosis in CD4+ T cells.

Flow cytometric analysis of T cell subsets after inoculation of MDV

Kinetic changes of T cell subpopulations in PBL were examined in chickens latently infected with MDV, during the period 9–30 days p.i. The CD4+ T cell population expanded transiently (58–65%), and rapidly declined 1 week later (about 50%) (Fig. 2a). In age-matched normal chickens, the percentage of CD4+ T cells was 45–55% during the experimental period. CD8+ T cells in normal chickens decreased gradually during the period of the experiment (Fig. 2b). In contrast, the CD8+ T cell population gradually expanded and reached about 26% at 30 days p.i. in MDV-infected chickens. Flow cytometric analysis showed that the level of expression of CD8 on CD8+ T cells fell after MDV infection, because the peak of relative fluorescence intensity of CD8 in MDV-infected chickens moved to the left compared with that in normal chickens (Fig. 3b). In contrast, the level of expression of CD4 in MDV-infected chickens was no different from that in normal chickens (Fig. 3a). The same pattern of flow cytometric profiles was also observed in spleen cells from MDV-infected chickens (Fig. 3c, d). Next, CD4+ and CD8+ T cell populations were isolated from the peripheral blood with magnetic beads, and the levels of expression of CD4 and CD8 on each subset in MDV-infected and normal chickens were analysed by flow cytometry. Compared to normal chickens, CD8+ T cells bearing a lower level of the CD8 molecules increased in MDV-infected chickens as
expected (Fig. 3f). The CD4 molecules on T cells from MDV-infected chickens were expressed at the same level as those from normal chickens (Fig. 3e). These observations suggest that MDV induces down-regulation of CD8.

**Down-regulation of CD8 transcripts in MDV-infected chickens**

To examine the mechanism of down-regulation of CD8 molecules, we analysed the transcription of the CD8 α and β chain genes in the CD8+ T cell population in normal and MDV-infected chickens (2 weeks p.i.). As shown in Fig. 4, in CD8+ T cells isolated from both the peripheral blood (a) and spleen (b), the transcriptional levels of the CD8 α and β chain genes were lower in MDV-infected chickens than in normal chickens, indicating that the down-regulation of CD8 occurred at the transcriptional level.

**Analysis of peripheral T cell-receptor repertoires in chickens inoculated with MDV**

Two types of αβ T cell-receptor (TCR) in chickens, the Vβ1 and Vβ2 gene products, have been reported (Chen et al., 1988; Char et al., 1990; Tjoelker et al., 1990). To further characterize T cells in MDV-infected chickens, we analysed TCR repertoires in PBL at 16 days p.i., when the CD4+ T cell population increased in the peripheral blood. As summarized in Table 1, the percentage of the T cell population that was Vβ1+ in MDV-infected chickens was significantly higher than that in uninfected birds (65.61% vs 57.24%; P < 0.01), whereas no significant difference was demonstrated in the percentage of Vβ2+ T cells when comparing normal and MDV-infected chickens (10.33% vs 11.77%). These results suggest that the transient increase in the CD4+ T cell population was mainly due to expansion of the Vβ1+ CD4+ T cell population.
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Fig. 4. Northern blot analysis of CD8 α and β chain gene expression. Total cellular RNA samples (2.5 μg) extracted from CD8+ T cells from peripheral blood (a) and spleen (b) were subjected to Northern blot hybridization with 32P-labelled probes to the CD8 α and β chain genes. Lanes 1 and 3, normal chicken CD8+ T cells; lanes 2 and 4, MDV-infected chicken CD8+ T cells. Ribosomal RNA on the agarose gel was stained with ethidium bromide (lanes 5 and 6) to ensure equal amount of loading in lanes 1 and 2, and lanes 3 and 4, respectively. Arrows show the position of CD8 α and β chain gene transcripts. Arrowheads show the positions of 18S and 28S ribosomal RNAs.

Table 1. Analysis of TCR repertoires in peripheral blood from chickens infected with MDV (strain Md5)*

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<th>TCR Vβ1+ T cells (%)</th>
<th>TCR Vβ2+ T cells (%)</th>
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<tr>
<td>Normal chickens</td>
<td>57 ± 2.66</td>
<td>10.3 ± 0.69</td>
</tr>
<tr>
<td>MDV-infected chickens</td>
<td>65 ± 0.91</td>
<td>11.7 ± 1.15</td>
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* Normal and MDV-infected chickens were studied at 16 days p.i. PBL from two chickens were pooled as one set and analysed by flow cytometry. Each result represents the mean ± SE of five to seven sets in each group. Significant difference: #, P < 0.01.

Discussion

MDV can induce two phases of immunosuppression. Primary immunosuppression occurs temporarily, immediately after systemic infection with MDV, due to lytic infection of B cells. In contrast, the secondary immunosuppression, which is characterized by functional deficiencies of T cells, starts about 2 weeks after infection, and continues for a long period (Schat, 1987). Although the secondary immunosuppression is a well-documented consequence of MDV infection, its mechanism is still unknown. Recently, it has been reported that several viruses can induce apoptosis of lymphoid cells and that this may be one of the causes of immunosuppression (Groux et al., 1992; Razvi & Welsh, 1993; Ameisen et al., 1994; Koga et al., 1994; Vasconcelos & Lam, 1994). To study the mechanism of T cell immunosuppression in MDV infection, we first studied apoptotic changes of peripheral lymphocytes morphologically and biochemically, and monitored apoptotic changes in CD4+ T cells from the peripheral blood and spleen (Fig. 1). Furthermore, flow cytometric analysis showed that the CD4+ but not the CD8+ T cell population in the peripheral blood rapidly decreased following a transient expansion in MDV-infected chickens (Fig. 2). These results indicate that MDV can cause a decrease in CD4+ T cells via apoptosis after a transient expansion, and suggest that the apoptosis observed with CD4+ T cells of MDV-infected chickens might play an important role in immunosuppression by MDV.
Tumour formation by MDV can be prevented by neonatal vaccination with an apathogenic MDV (Schat, 1987), although the mechanism of the anti-tumour effect is not clear. We postulated that the vaccination could rescue CD4+ T cells from apoptosis induced by a virulent MDV. We therefore examined DNA fragmentation in each T cell subset from chickens infected with strain Md5 after vaccination. This analysis, however, showed that the vaccination did not prevent apoptosis of CD4+ T cells (data not shown), suggesting that the progression of tumour formation in MD is independent of apoptosis induced by MDV infection.

The mechanisms of apoptosis induced by viral infections are divided into two types. For example, chicken anaemia virus can cause apoptosis in thymocytes and in a cell line by infecting the cells (Jeuring et al., 1992). Influenza A and B viruses also induce apoptosis of infected cells (Hinshaw et al., 1994). On the other hand, HIV can deplete the CD4+ T cell population via apoptosis not only in infected cells but also in uninfected cells (Groux et al., 1992; Laurence et al., 1992; Meyar et al., 1992; Ameisen et al., 1994; Lu et al., 1994). We are investigating whether infection of the target cells is essential for apoptosis induced by MDV. MDV can induce apoptosis in CD4+ T cells but not, or only marginally, in CD8+ T cells (Fig. 1), although the virus can infect both CD4+ and CD8+ T cells. We therefore postulate that MDV might induce apoptosis in CD4+ T cells without infecting the cells.

We found clonal expansion of Vβ1+ T cells in the peripheral blood in MDV-infected chickens at 16 days p.i., when the CD4+ T cell population was transiently increased in these chickens (Table 1). It is well known that superantigens can stimulate a large population of T cells bearing particular Vβ segments by interacting with the Vβ gene products and class II major histocompatibility (MHC) antigens without the need for antigen processing (White et al., 1989; Takimoto et al., 1990; Kawabe & Ochi, 1991). Therefore, it is possible that MDV might encode an antigen with a superantigen activity which stimulates Vβ1+ CD4+ T cells. However, it should also be noted that the increase in Vβ1+ T cells shown in our experiment was very small, although statistically significant when compared with control chickens. We are investigating the proportion of Vβ1 and Vβ2+ T cells in MDV-infected chickens.

Cytotoxic T lymphocytes (CTL), which mainly express CD8 and are restricted by class I MHC antigens, play an important role in the clearance of pathogenic agents (Byrne & Oldstone, 1984; Borrow et al., 1994). Recently, it was reported that down-regulation of CD8 in a CTL line specific to an influenza B virus nucleoprotein peptide might be important for clonal anergy (Robbins & McMichael, 1991). Furthermore, binding of CD8 to class I MHC triggers phosphorylation of the CD3 ζ chain by p56lck (Strauss & Weiss, 1992). These reports demonstrate the importance of CD8 in recognition of foreign antigens binding to class I MHC molecules. We showed that CD8 was down-regulated at the transcriptional level during MDV infection (Figs 3 and 4). It is well known that MDV can cause a functional deficiency of CTL, for example delayed rejection of Rous sarcomas or MD transplantable tumour cells (Calnek et al., 1975), increased susceptibility to coccidiosis (Biggs et al., 1968) and absence of specific cytotoxic activities against MD tumour cells (Schat et al., 1982; Powell et al., 1983). Down-regulation of CD8, therefore, may be important for the immunosuppression of T cells.

In this paper, we report that MDV can induce apoptosis of peripheral CD4+ T cells and down-regulate the expression of CD8 in infected chickens. These immunomodulations may be central to the immunosuppression of T cells induced by MDV, and it is important for understanding the pathogenesis of MD to clarify the mechanism of these immunomodulations.

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