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Priming of Immature Thymocytes to CD3-Mediated Apoptosis by Infection with Murine Cytomegalovirus

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Cytomegalovirus (CMV) causes severe clinical manifestations in immunocompromised hosts; however, it remains unclear whether the virus itself is a cause of immunosuppression or whether it is involved as an opportunistic bystander pathogen. This study was performed to elucidate the effect of CMV infection on the host's immune system. The double-positive thymocytes of BALB/c mice inoculated with a sublethal dose of murine CMV (MCMV) were extensively depleted by a 10-μg amount of anti-CD3 monoclonal antibody, while such an amount was unable to induce any apparent elimination of thymocytes in noninfected mice. In immature thymocytes of infected hosts, a markedly high level of susceptibility to apoptosis induction was found on treatment with anti-CD3 monoclonal antibody. Analysis of the signal transduction pathway of such double-positive thymocytes demonstrated a profound elevation of the intracellular Ca^{2+} level after anti-CD3 stimulation, implying that this aberrant mobilization of Ca^{2+} plays a crucial role in the signaling pathway leading these cells to an extensive apoptosis. Examination of the thymus by PCR was able to detect a low copy number of MCMV DNAs in thymic stromal cells but none at all in thymocytes. Therefore, it is suggested that a mechanism which is not associated with virus replication within the cells exerts a critical effect on rendering the thymocytes highly apoptosis sensitive in hosts infected with MCMV.

Virus can disturb the immune system directly by the consequence of virus replication in immunocompetent cells themselves or indirectly through the release of various cytokines and other soluble factors. Cytolysis, depressed expression of major histocompatibility complex class I and II gene products, and polyclonal activation of lymphocytes are the representatives of manifestations provoked in the immune system by those virus-induced mechanisms (14, 27).

Cytomegaloviruses (CMVs) classified as betaherpesviruses are ubiquitous microbes that commonly infect many animals, including humans and mice. These viruses are highly species specific. Like other herpesviruses, CMV persists in the host in a latent state after primary infection. CMV causes no apparent clinical manifestations in immunocompetent hosts, whereas it causes severe clinical symptoms in immunocompromised hosts such as fetuses, transplant recipients, and human immunodeficiency virus-infected individuals (1). In these situations, it is not clear whether the virus itself is a cause of immunosuppression and mortality or whether it is involved as an opportunistic bystander pathogen that is permitted to replicate in immunosuppressed hosts.

Peripheral blood mononuclear cells were found to be one of the sites of infection with CMV. The immediate-early (IE) and early antigens of CMV are expressed, but the late antigens (i.e., the CMV structure protein) are not detected in peripheral blood mononuclear cells; that is, no virions are released from such infected cells (20, 21). So far, it has been reported that infection with CMV resulted in depression of the following lymphocyte functions: T-cell proliferation to antigens or mitogens (23), NK activity (23), antibody production (14), and generation of CMV-specific cytotoxic T lymphocytes (19, 22). To elucidate further the mechanism of the immunodepression occurring in CMV-infected hosts, we prepared murine CMV (MCMV)-infected mice in the present study and found that their immature thymocytes are exceedingly sensitive to apoptosis induction by anti-CD3 monoclonal antibody (MAb). The details of the signal transduction pathway leading to such drastic apoptosis and the relevance of the virus to the apoptosis induction are investigated in this study.

MATERIALS AND METHODS

Mice. Female mice of an inbred BALB/c strain were obtained from Kyushu University Animal Center (Fukuoka, Japan) and maintained in a specific-pathogen-free condition. In this colony, contamination of mice with lymphocytic choriomeningitis virus, Sendai virus, and mouse hepatitis virus is checked every month by a serological examination. Mice were inoculated intraperitoneally (i.p.) with 10^5 PFU (0.02 50% lethal dose [LD_{50}]) of MCMV at 6 weeks of age and used for experiments at 4 weeks after the inoculation unless stated otherwise. The stock of MCMV (Smith strain; ATCC VR-194) used for inoculation was harvested from salivary gland tissue as a homogenate after being passaged twice in BALB/c mice. Infectious MCMV in various organs of infected mice was quantitated by a plaque assay using BALB-3T3 monolayer cell culture as described before (7). The detection limit of this assay was 10 PFU/organ.

Antibodies for injection. A hamster anti-mouse CD3 hybridoma, 145-2C11 (12), and a hamster anti-mouse TCR hybridoma, H57-597 (11), were cultured in a serum-free medium (Nissui Pharmaceutical Co., Tokyo, Japan) to prepare anti-CD3 and anti-TCR MAbs, respectively. Culture supernatants were purified by precipitation with saturated ammonium sulfate and dialysis in phosphate-buffered saline (PBS). About

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50% of the total protein of these MAb products was immunoglobulin. Usually, 20 μg of MAb was injected once i.p. into mice.

Fluorescence-activated cell sorter (FACS) analysis. The antigens expressed on the cells were examined by flow microfluorometry using a FACscan cell sorter as described elsewhere (10). Fluorescein isothiocyanate-conjugated 145-2C11 was used for the detection of CD3. Two-color analysis of CD4 and CD8 was performed in order to examine double-positive (DP), single-positive (SP), and double-negative (DN) thymocytes by using phycoerythrin (PE)-conjugated GK 1.5 and fluorescein isothiocyanate-conjugated anti-Lyt2 MAbs (both from Becton Dickinson, Sunnyvale, Calif.).

DNA fragmentation assay. DNA fragmentation was examined by the method of Newell et al. (17). Briefly, a single-cell suspension was prepared from thymus by disrupting it with frosted slide glasses in the medium. Cells (10^7) from the suspension were washed once with PBS and lysed in 0.5 ml of hypotonic lysing buffer (5 mM Tris-HCl, 5 mM EDTA, 0.5% Triton X-100, pH 7.4). The lysate was centrifuged at 13,000 × *g* for 15 min. The supernatant was incubated with RNase at 50 μg/ml for 2 h and deproteinized by extraction once in phenol-chloroform and once in chloroform-isoamyl alcohol (24:1). It was then precipitated at 10,000 × *g* after being incubated overnight at −20°C in 50% isopropanol–130 mM NaCl. After electrophoresis on a 2% agarose slab gel, DNA was stained with ethidium bromide. Quantitation of such low-molecular-weight DNA was done by the diphenylamine assay (2).

Measurement of intracellular Ca^{2+}. Thymocytes at 2 × 10^7/ml were loaded with 2 μM Fura acetoxymethyl ester in Ca^{2+} staining buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM glucose, 1 mg of bovine serum albumin per ml, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.4) for 20 min at 37°C, and then a 4× volume of staining buffer was added for a further 40-min incubation. Samples were washed twice, and 2 × 10^6 cells were resuspended in 2 ml of Ca^{2+} staining buffer and transferred to a quartz cuvette in which they were continuously stirred and maintained at 37°C during analysis. A spectrofluorometer, F-2000 (Hitachi, Tokyo, Japan), was used to measure fluorescence levels before and after stimulation with 145-2C11 or H57-597 at a concentration of 20 μg/ml. Ca^{2+} concentration was calculated by means of the program for intracellular calcium measurement (16).

Detection of MCMV DNA by PCR. A pair of 24-bp oligonucleotides, selected from exon 4 of the MCMV IE gene 1 from published sequence data (9), was used as primers. The base sequences are as follows: #1 (bp 1701 to 1724), 5′-ATC-AAT-CAG-CCA-TCA-ACT-CTG-CTA-3′; and #2 (bp 2400 to 2377, antisense), 5′-ATG-GTG-AAG-CTA-TCA-AAG-ATG-TGC-3′. This primer pair amplifies a 700-bp segment in the reaction. Sample DNA was prepared from thymus or salivary gland by a series of phenol-chloroform extractions and ethanol purifications. Gene amplification was performed according to the method reported by Pomeroy et al. (18). Amplified DNA samples were electrophoresed on a 1.2% agarose gel containing 0.5 μg of ethidium bromide per ml and photographed. DNA in the gel was then blotted to GeneScreen Plus (NEN, Boston, Mass.). A 24-bp oligonucleotide, #3, complementary to a sequence lying between the primers (bp 1801 to 1824) was used as the probe for Southern blot analysis and labeled by using the ECL 3′-oligolabeling and Detection System (RPN 2130; Amersham). The base sequence of oligonucleotide #3 is 5′-CAG-ATC-TGA-GTT-TGA-TAA-TGT-CCA-3′. pAMB25 (8) is a plasmid integrating the whole MCMV IE segment and was used as the standard for estimation of the copy number of the IE gene.

Separation of thymic cells. Thymic cells were separated into Thy-1^+ and Thy-1^- cells by the panning method using anti-Thy-1 antiserum as described elsewhere (13). Briefly, a polystyrene culture dish (diameter, 8.5 cm) was coated with anti-Thy-1 antiserum (Cedarlane, Hornby, Canada) diluted to 40 times the original concentration with RPMI 1640 medium by incubation at 4°C overnight. Next, 5 ml of thymic cell suspension (10^7 cells per ml in RPMI 1640 medium) was added to the dish and the mixture was incubated for 1 h at room temperature. The Thy-1^- cells were recovered by gently removing the nonadherent cell population, while the Thy-1^+ cells were harvested by detaching the adherent cell population by vigorous agitation. Almost complete detachment of adherent cells from the dish was confirmed by an inverted microscope. Thy-1^- and Thy-1^+ cell populations contained less than 10% Thy-1^+ and Thy-1^- cells by FACS analysis, respectively.

RESULTS

Depletion of DP thymocytes. In order to induce persistent infection for more than several weeks, female BALB/c mice were inoculated i.p. with 10^5 PFU of MCMV (0.02 LD_{50}) at 6 weeks of age, and at 10 weeks of age they were used for examination. As shown in Fig. 6, the mice at 4 weeks after the inoculation still have a high virus titer in the salivary glands. The body weight of infected mice was about 10% less on average than the weight of the age-matched noninfected mice at 10 weeks. Without the injection of antibodies, the total number of thymocytes (data not shown) and the ratios of DP/SP/DN thymocytes (Fig. 1A, uppermost panels) and $CD3^{high}/CD3^{low}/CD3^{negative}$ thymocytes (Fig. 1B, uppermost panels) showed no significant difference between infected and noninfected mice at 10 weeks. When 20 μg of anti-CD3 MAb was administered to mice at 0 h, a marked depletion of DP thymocytes occurred in infected mice at 48 h after injection (Fig. 1A; 75.7 to 3.3%), whereas only a slight reduction at 24 h (78.6 to 69.1%) followed by a recovery at 48 h (to 73.4%) of DP thymocytes happened in noninfected mice. In the infected mice at 48 h after the injection, analysis of surface CD3 on thymocytes by FACS also demonstrated the selective disappearance of $CD3^{negative}$ and $CD3^{low}$ cells, which are the thymocyte subsets compatible with DP thymocytes (Fig. 1B). Such selective depletion of DP thymocytes was able to be induced by the injection of as small an amount as 10 μg of anti-CD3 MAb in infected mice, while the decrease in DP thymocytes in noninfected mice was still moderate, even with the injection of 100 μg of this MAb (Fig. 2). These experimental results were always reproduced without exception in triplicate experiments (data not shown). Thus, it was indicated that DP thymocytes of MCMV-infected mice were highly susceptible to the cytolytic effect caused by anti-CD3 antibody. Mature T cells residing in spleen and lymph nodes of the infected mice were spared such elimination by 50 μg of anti-CD3 MAb (data not shown). Administration of up to 100 μg of anti-TCR MAb hardly provoked the depletion of DP thymocytes in the infected mice (data not shown).

Drastic apoptosis of thymocytes. It is already known that in vivo administration of anti-CD3 antibody to mice induces apoptosis in their immature thymocytes (24). To know whether the extensive cytolysis of DP thymocytes by anti-CD3 MAb in infected mice is associated with the induction of apoptosis, changes in the thymus were examined by electron microscopy and DNA fragmentation assay. In the MCMV-infected mice at 48 h after injection with 20 μg of anti-CD3 MAb, many

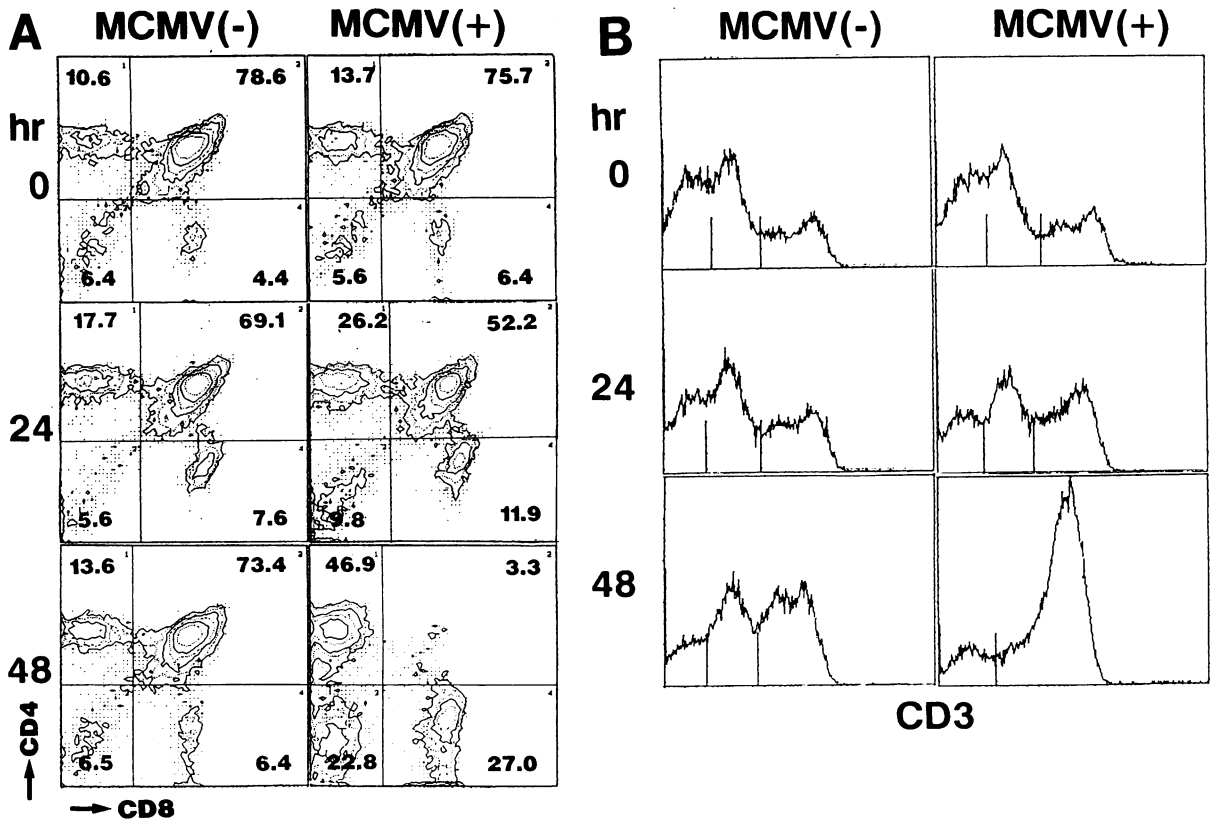

FIG. 1. FACS analysis of thymocytes. BALB/c mice which had been inoculated with 10^5 PFU of MCMV [MCMV(+)] 4 weeks before or age-matched uninoculated control BALB/c mice [MCMV(−)] were injected i.p. with 20 μg of 145-2C11 at 0 h, and their thymi were removed for FACS analysis at 0, 24, and 48 h. For two-color analysis of CD4 and CD8 (A), phycoerythrin-conjugated GK 1.5 and fluorescein isothiocyanate-conjugated anti-Lyt2 MAbs were used. The numbers in the subsquares indicate the percent ratio of the cell number belonging to its subpopulation. For single-color analysis of CD3 (B), fluorescein isothiocyanate-conjugated 145-2C11 was used.

thymocytes in the cortical area, where almost all the thymocytes bear the DP phenotype, exhibited the morphology typical of apoptosis such that cells shrank and their nuclei collapsed to condensed chromatin (Fig. 3B, arrowheads). In the cortical area of noninfected mice after the injection, however, cells showing the morphology of apoptosis were scarce (Fig. 3A). It

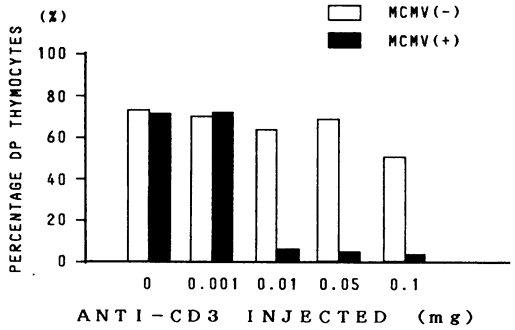

FIG. 2. Dose effect of anti-CD3 MAb on elimination of DP thymocytes. Various amounts of 145-2C11, as indicated at the bottom of each set of bars, were injected i.p. into the mice which had been inoculated with MCMV 4 weeks before [MCMV(+)] (shaded bars) or into the age-matched noninfected control mice [MCMV(−)] (open bars), and percentages of DP thymocytes were examined by FACS 48 h after the injection.

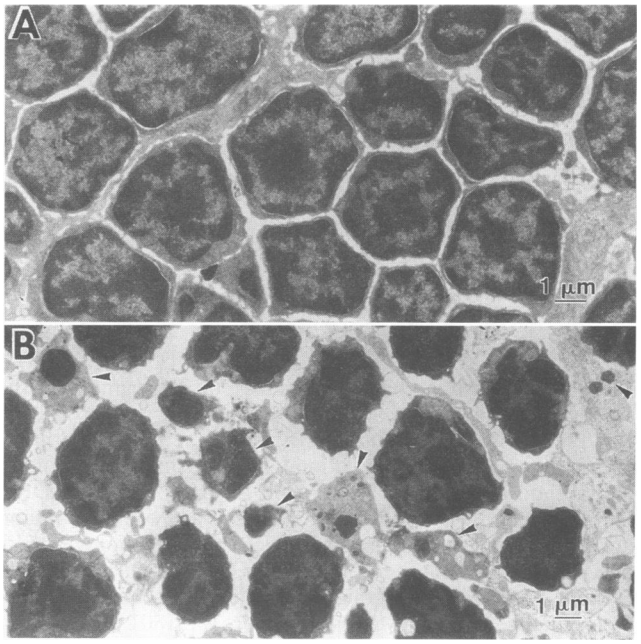

FIG. 3. Electron microscopy of thymus. Noninfected control mice (A) or BALB/c mice which had been inoculated with MCMV 4 weeks before (B) were injected i.p. with 20 μg of 145-2C11, and their thymi were removed 48 h later for examination by transmission electron microscopy.

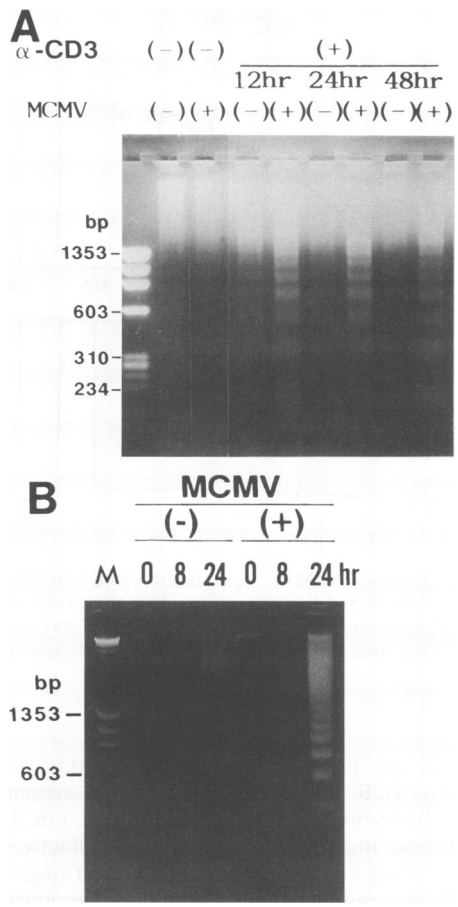

FIG. 4. DNA fragmentation assay. (A) Mice which had been inoculated with MCMV 4 weeks before [MCMV (+)] or noninfected mice [MCMV (−)] were injected with 20 μg of 145-2C11 [α-CD3 (+)] at 0 h, and their thymi were removed at 12, 24, and 48 h for DNA fragmentation assay. As a control, thymi were also removed from noninfected and infected mice which were not injected with 145-2C11 [α-CD3 (−)] and were examined. (B) A single-cell suspension of thymocytes at the concentration of 10^7/ml in RPMI 1640 medium was prepared from the thymi of infected mice [MCMV (+)] or noninfected mice [MCMV (−)] and was cultured for various times, as indicated in hours above each lane, at 37°C in a 24-well plate which had been coated with 145-2C11. Next, low-molecular-weight DNAs were extracted from the samples for analysis. For the coating, the plate was incubated with 20 μg of 145-2C11 per ml at 4°C for 24 h before culture. Lane M is loaded with φX174-*Hae*III marker.

was difficult to find apoptotic cells in the medullary area of the thymus not only in noninfected mice but also in infected ones. DNA fragmentation assay of the thymocytes of infected mice also demonstrated a remarkable increase in fragmented DNAs in multiples of about 200-bp units around 24 h, peaking after the injection of anti-CD3 MAb, but fragmentation was not so significant in the thymocytes of noninfected mice (Fig. 4A). Quantitation of such low-molecular-weight DNAs by the diphenylamine method revealed 3.9- and 1.6-fold increases at 24 h in infected and noninfected samples, respectively, in comparison with the DNA content of their control samples without anti-CD3 MAb treatment. These results indicated that the extensive depletion of DP thymocytes by anti-CD3 MAb is associated with a drastic induction of apoptosis in these cells. When thymocytes were stimulated with anti-CD3 MAb in vitro, a profound DNA fragmentation was also found in the thymocytes of infected mice at 24 h after stimulation but not in the thymocytes of noninfected mice (Fig. 4B). This means that some change(s) induced in the thymocytes themselves during infection renders these cells highly sensitive to the apoptosis induction triggered by the ligation of surface CD3 cells with antibody.

Extensive mobilization of intracellular Ca^{2+}. Thymocytes were next monitored for their intracellular Ca^{2+} concentration after stimulation with anti-CD3 MAb (Fig. 5). A marked increase in intracellular Ca^{2+} concentration, with a peak level more than 100 times the level in the noninfected group, was found in the thymocytes of infected mice. This remarkable Ca^{2+} mobilization by anti-CD3 MAb was able to be observed in the thymocytes of mice at 2 weeks and at 5 months after infection (data not shown), compatible with a period when extensive cytolysis due to apoptosis can be inducible. By contrast, the stimulation with anti-TCR MAb, which is able to induce neither cytolysis nor apoptosis, did not provoke any accentuated Ca^{2+} mobilization in the thymocytes of infected mice (Fig. 5). Thus, it is suggested that an aberrant increase in intracellular Ca^{2+} concentration plays an important role in the induction of drastic apoptosis and resultant cell depletion in the thymocytes of infected mice when they are injected with anti-CD3 MAb. Next, the whole thymocyte population was separated into its CD4 and CD8 subpopulations to measure their Ca^{2+} concentrations. Only the subpopulation containing DP thymocytes, which are the target cells for apoptosis-mediated cytolysis by anti-CD3 MAb, exhibited a high level of intracellular Ca^{2+} after stimulation (data not shown), indicating a close correlation between the apoptosis sensitivity and the level of intracellular Ca^{2+} mobilization after stimulation.

Detection of MCMV in the host. To elucidate the role of MCMV virions per se in the generation of apoptosis-sensitive thymocytes in MCMV-infected hosts, the number of MCMV virions capable of replication in various organs was counted serially after inoculation of 10^5 PFU of MCMV on day 0 (Fig. 6). High to moderate titers of MCMV were noted in the salivary gland, spleen, and lung up to 1 month after infection. In the thymus, on the other hand, no virus was detectable by plaque assay at any day after inoculation. To examine the possible existence of MCMV at levels below the level detectable by the plaque assay, PCR was employed to detect a low copy number of viral DNA in the thymus. As shown in Fig. 7A, a 700-bp DNA corresponding to the MCMV IE gene was detectable in the thymus of mice at 1 week after inoculation, whereas it was undetectable at 2 or 4 weeks. Densitometry analysis using pAMB25 as the standard assessed that one thymus contains 10^7 to 10^8 copies of the MCMV genome, although only the IE region of the MCMV genome was amplified by PCR for assessment. Next, the thymus at 1 week after infection was disrupted and separated by the panning method using anti-Thy-1 antibody with thymic T cells (Thy-1^+) and thymic stromal cells (Thy-1^-) in order to extract DNA for PCR assay from each of the thymic cell populations (Fig. 7B). A small amount of MCMV DNA, whose level was less than 10% of that in whole thymus (Fig. 7B, lane 2), was detectable in thymic stromal cells (lane 3), whereas it was not detectable at all in thymic T cells (lane 1). This indicates that most of the MCMV DNA detected in whole thymus is derived from the passenger viruses existing in the extracellular environment of the thymus.

DISCUSSION

Apoptosis has been demonstrated to occur in tissues undergoing processes as diverse as embryogenesis, metamorphosis,

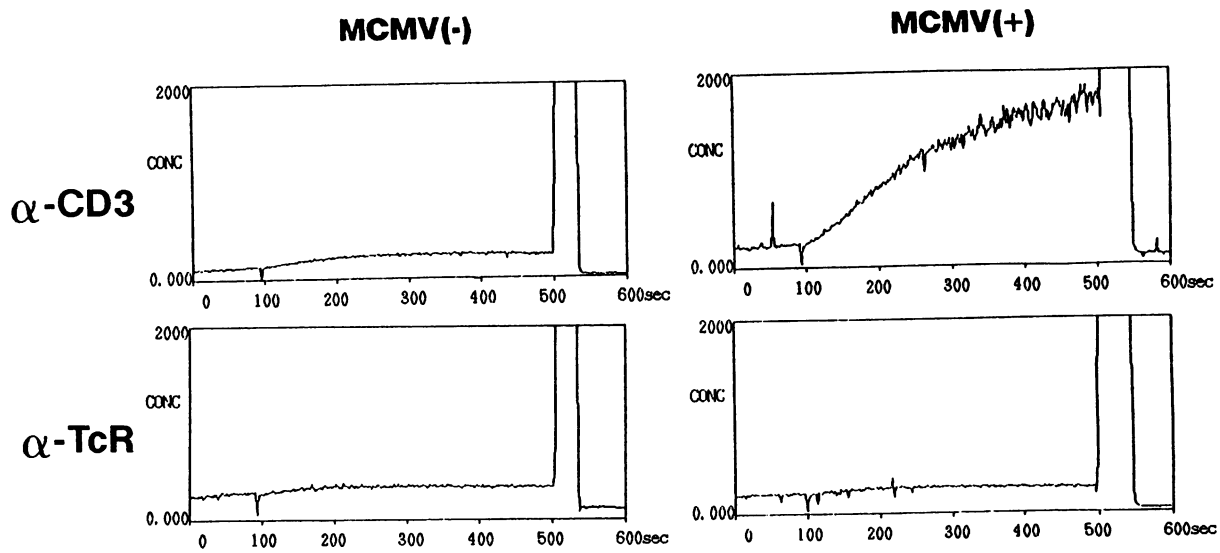

FIG. 5. CD3-mediated intracellular Ca^{2+} mobilization. Thymocytes obtained from infected [MCMV(+)] or noninfected [MCMV(−)] mice were labeled with Fura acetoxymethyl ester, suspended in Ca^{2+} staining buffer, and then transferred to a cuvette in which they were continuously stirred and maintained at 37°C during analysis. At 100 s after monitoring by a spectrofluorometer, 145-2C11 (α-CD3) or H57-597 (α-TCR) was added to the cells at the concentration of 20 μg/ml for stimulation. Horizontal and vertical axes indicate the mean time (in seconds) and Ca^{2+} concentration (in nanomolars), respectively.

and immunological development, where selective depletion of certain cells without concomitant inflammation is beneficial (4). Cells which had suffered from apoptosis were identified microscopically by the characteristic formation of collapsed nuclei. Later it was recognized that a major hallmark of this death process was the fragmentation of the genomic DNA into oligomers of 180- to 200-bp multiples, corresponding to the unit length of DNA coiled around a histone complex (3).

It was shown that exposure of thymocytes in organ culture to anti-CD3 antibody stimulates apoptosis, predominantly in the DP subset (26). This selective depletion of DP thymocytes has also been observed in mice following treatment with anti-CD3 MAb in vivo (24, 25). During intrathymic development, those DP thymocytes that bear TCR and CD3 capable of recognizing self antigens are negatively selected, and this selection is mediated by TCR and CD3 engagement, presumably via TCR-major histocompatibility complex interaction. Therefore, the observation that anti-CD3 antibodies effectively induce apoptosis in these cells presents the idea that negative selection may be a result of activation-induced apoptosis in this immature thymocyte population.

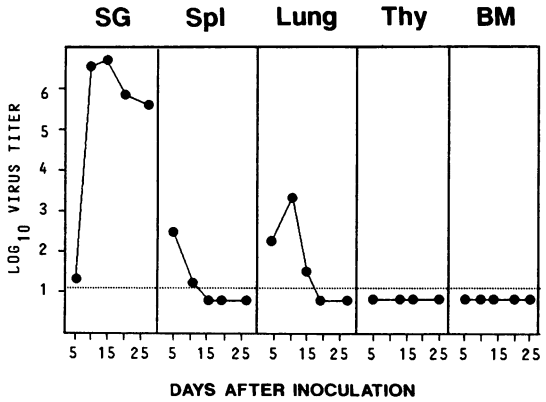

FIG. 6. Titration of MCMV by plaque assay. BALB/c mice were inoculated i.p. with 10^5 PFU of MCMV at 6 weeks of age. At 5, 7, 14, 21, and 28 days after the inoculation, various organs, including the salivary gland (SG), spleen (Spl), lung, and thymus (Thy), and bone marrow (BM) were removed in order to titrate the MCMV contained within them by plaque assay using BALB-3T3 monolayer cell culture.

In the present study, the DP thymocytes in MCMV-infected mice were drastically eliminated by an amount of anti-CD3 MAb so small that it is unable to induce such extensive depletion of DP thymocytes in noninfected mice. Also, a high level of susceptibility to apoptosis induction by anti-CD3 was found in immature thymocytes of infected hosts, as revealed by microscopical analysis and DNA fragmentation assay, indicating that the drastic elimination of DP thymocytes by anti-CD3 treatment in infected mice was due to the elevated sensitivity of these thymocytes to apoptosis induction. However, it still remains unclear how the elevation of apoptosis sensitivity of these immature thymocytes affects the further development of these cells in infected hosts. Up to 5 months after infection, no significant difference in either the total number of thymocytes or the ratio of DP thymocytes was found between MCMV-infected and noninfected hosts that had been kept in an environment free of specific pathogens (except MCMV) (data not shown). Hence, it seems unlikely that the negative selection of DP thymocytes is accelerated in MCMV-infected hosts maintained in a condition such that no further aggressive invasion to the host's immune system is occurring. On the other hand, if such infected hosts suffered from severe immunological assaults such as superinfection, tumor burden, and transplantation, the DP thymocytes might become exhausted, as shown by the administration of anti-CD3 antibody.

Neither infectious virus nor viral DNA could be detected in the thymus at 4 weeks after infection, when these thymocytes become highly sensitive to apoptosis. Therefore, it is unlikely that infectious virions or viral DNA residing in the thymus is the direct cause of apoptosis susceptibility of these thymocytes.

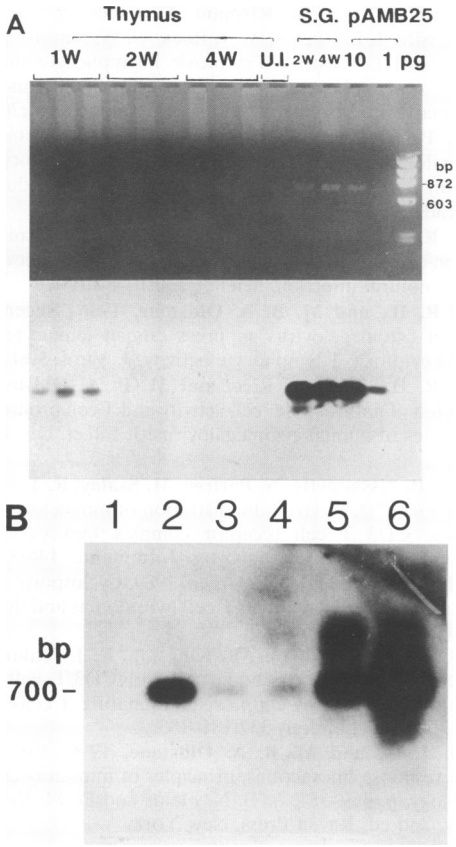

FIG. 7. Detection of the MCMV IE genome by PCR. (A) BALB/c mice were inoculated with 10^5 PFU of MCMV at 0 weeks, and their thymi and salivary glands (S.G.) were removed at 1, 2, and 4 weeks (1W, 2W, and 4W). DNAs were extracted from these organs, and 1 μg of DNA from each sample was used in the PCR assay for detection of the MCMV IE genome. A 1- or 10-pg amount of pAMB25 DNA mixed with 1 μg of genomic thymic DNA of noninfected mice was amplified in the PCR as the standard. Amplified samples were electrophoresed on a 1.2% agarose gel containing ethidium bromide (upper panel). DNA in the gel was then blotted onto the membrane and hybridized with #3 primer and developed by using the ECL system (lower panel). (B) DNAs were extracted from the whole thymus (lane 2), Thy-1^+ thymic cells (lane 1), and Thy-1^- thymic cells (lane 3) and were amplified by PCR to detect the MCMV IE genome as described in the legend to panel A. A 0.1-pg amount (lane 4), a 1-pg amount (lane 5), and a 10-pg amount (lane 6) of pAMB25 were also amplified as standards.

Some humoral factor(s) generated at the site of infection may affect the priming of such thymocytes, because at that time the salivary gland of the host is still infected with a high dose of MCMV. It is likely that some change(s) which occurred in the thymocytes themselves renders these cells apoptosis sensitive, because an extensive apoptosis was able to be induced in vitro by anti-CD3 stimulation. An important aspect of this finding which should be further investigated is the fate of those apoptosis-sensitive thymocytes when they maturate in the thymus and emigrate to the peripheral lymphoid organs. In our preliminary study, the mature T cells of MCMV-infected mice generated a severalfold greater amount of cytokines than the T cells of noninfected mice in the culture stimulated with anti-CD3 antibody. These highly cytokine-releasing T cells might be the progeny of the apoptosis-susceptible thymocytes.

Accumulation of intracellular Ca^{2+} is rapidly provoked when T cells are stimulated through TCR or CD3 engagement and is considered one of the essential phenomena for T-cell activation. Involvement of Ca^{2+} in the induction of apoptosis has been suggested because endonuclease requires a sustained increase of intracellular Ca^{2+} for cleavage of DNA in the nucleus. Furthermore, McConkey et al. (15) demonstrated that the apoptosis of immature thymocytes by stimulation via TCR or CD3 is dependent on elevation of the intracellular Ca^{2+} level. The DP thymocytes of MCMV-infected mice exhibited a marked, long-sustained elevation of intracellular Ca^{2+} induced by anti-CD3 stimulation in the present study. No such remarkable change was found in the activity of either protein tyrosine kinase or protein kinase C of those thymocytes. Therefore, it is thought that an aberrant increase of intracellular Ca^{2+} plays a crucial role in the signaling pathway leading the DP thymocytes of infected mice to such extensive apoptosis. Finkel et al. (5) reported that DP thymocytes exhibit far less response of intracellular Ca^{2+} level than mature T cells when TCRαβ is ligated with antibody. On the other hand, CD3 engagement on DP thymocytes has the same effect on the Ca^{2+} level as does CD3 engagement on mature T cells, suggesting that the signal transfer from TCRαβ to CD3 may be inefficient in DP thymocytes. Likewise, in the present study, the engagement of TCR with anti-TCR MAb did not induce any marked elevation of the Ca^{2+} level or apoptosis, even in the thymocytes of infected mice. This therefore suggests that the change, which occurs in DP thymocytes by infection and renders them hyperreactive to the Ca^{2+} influx response after the CD3 engagement but not the TCR engagement, may be located in the Ca^{2+} mobilization pathway connected directly with CD3-mediated signaling but not connected with TCRαβ-mediated signaling.

AIDS is a representative case of infectious diseases of viral origin in which an apoptosis pathway plays a critical role in the course of $CD4^+$ T-cell depletion. These apoptosis pathways may either be mediated directly by virus replication in the cells or indirectly through the priming of uninfected cells to apoptosis when triggered by different agents (6). Here, in the present study, we report a novel case of apoptosis induction associated with MCMV infection in which uninfected thymocytes are primed for apoptosis.

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