

Characterization of T Cells Expanded *in vivo* during Primary Mouse Hepatitis Virus Infection in Mice

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ABSTRACT. After intraperitoneal infection with mouse hepatitis virus, strain JHM (JHMV), JHMV replicated in the spleen of C57BL/6 mice for a few days but cleared within a week. The acute viral clearance coincided with moderate expansion of CD8⁺T cells and modest expansion of CD4⁺T cells, and was impaired moderately in mice depleted of CD8⁺T cells and completely in mice depleted of both CD4⁺ and CD8⁺T cell subsets. Flow cytometric analysis showed that expression of cell surface markers on the spleen T cells changed during JHMV infection. CD8⁺T cells expressing increased amounts of CD11a, CD43, CD44 and CD49d, and those expressing decreased levels of T cell receptor $\alpha\beta$, CD8, CD45RB and L-selectin were expanded in the spleen after JHMV infection. However, they did not express CD11b, CD25 or NK1.1. They used highly heterogeneous V β chains for their T cell receptors. In addition to CD11a^{high}CD8⁺T cells, CD11a^{high}CD4⁺T cells were detected transiently after JHMV infection. The virus-specific cytotoxic T lymphocyte (CTL) activity was observed in both CD4⁺ and CD8⁺ spleen T cells from mice 7 days post-infection. The present study shows the dynamics of CD8⁺ and CD4⁺T cells in the spleen during JHMV infection in mice and suggests that CD11a^{high}T cells may be involved in JHMV clearance *in vivo* because their appearance was temporally correlated with T cell-mediated viral clearance *in vivo* and antiviral CTL activity *in vitro*. — **KEY WORDS:** CD11a, CTL activity, flow cytometry, mouse hepatitis virus, T cell.

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It has been well documented that T cell-mediated immune responses play an important role in various virus infections in both animals and humans [6]. Recent studies in particular indicate the clinical importance of virus-specific cytotoxic T lymphocyte (CTL) activities in the control of both acquired immunodeficiency syndrome and viral hepatitis in humans [11, 25, 34]. It is also the case with mouse hepatitis virus infection in mice. After intraperitoneal (ip) infection with mouse hepatitis virus, strain JHM (JHMV), immunocompetent mice develop an acute but mild splenitis and hepatitis which subsequently resolves, although JHMV infection in the central nervous system induces fatal encephalitis in mice of most laboratory strains [13]. In contrast, athymic nude mice infected ip with JHMV develop a fatal disease with increasing viral growth. In nude mice the disease is cured by adoptive transfer of H-2-matched immune spleen T cells, suggesting the importance of T cell-mediated immunity [14]. Protective effects of T cells in JHMV infection are also demonstrated by adoptive transfer of JHMV-specific T cell clones [32, 33].

Although the several lines of evidence mentioned above suggest that T cell-mediated immune responses play an essential role in protection against JHMV infection, little has been known about the antiviral T cells *in vivo* during

infection. In the present study, numerical and phenotypic changes in T cells in C57BL/6 (B6) following ip infection with JHMV were examined by flow cytometry. The viral clearance and the virus-specific CTL activity were also examined and correlation among these parameters was analyzed.

MATERIALS AND METHODS

Mice: Specific pathogen-free B6 mice at 6 to 8 weeks of age were purchased from SLC, Hamamatsu, Japan. CB-17. SCID mice were obtained from CLEA Japan, Tokyo.

Virus and cells: The DL variant of JHMV was propagated and plaque assayed on DBT cells as described previously [12]. Mice were infected ip with 1×10^6 plaque-forming units (PFU) of JHMV. IC-21 cells, a macrophage-like tumor cell line of B6 mouse origin [18] and J774.1 cells [33] were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes and antibiotics.

Depletion of CD4⁺ and/or CD8⁺T cells *in vivo*: To deplete CD4⁺T cells and/or CD8⁺T cell subsets, B6 mice were injected with 200 μ g of anti-CD4 (GK1.5) [5] or anti-CD8 (2.43) [26] monoclonal antibodies (mAb) three times per week from 2 days prior to infection. Antibody was purified from ascitic fluid collected from CB-17.SCID mice injected with the hybridomas, by caprylic acid precipitation and then by ammonium sulfate precipitation [9]. Depletion of T cell subsets was confirmed by flow cytometry as described below, and approximately a 95% reduction in the desired T cell subset was obtained when mice were infected (data not shown).

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Flow cytometric analysis: Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and anti-CD8 mAb were obtained from Beckton Dickinson, Mountain View, CA. R-phycoerythrin-conjugated anti-T cell receptor (TCR) $\alpha\beta$, anti-L-selectin, anti-CD44 (Pgp-1), anti-NK1.1 mAb and biotin-conjugated anti-CD45RB, anti-V β 3 and anti-V β 6 mAb were obtained from Pharmingen, San Diego, CA. Biotin-conjugated anti-CD11b (Mac-1 α chain), anti-V β 10 and anti-CD25 (IL-2 receptor α chain) mAb were obtained from Caltag, South San Francisco, CA, Serotec, Kidlington, UK and GIBCO BRL, Gaithersburg, MD, respectively. Hybridomas secreting anti-CD11a (FD441.8) [26], anti-CD43 (S7) [8] and anti-CD49d (α 4 integrin) (PS/2) [20] antibodies were kindly provided by Drs. H. Nariuchi, Institute of Medical Science, University of Tokyo, J. D. Kemp, University of Iowa Hospitals and Clinics and K. Miyake, Saga Medical School, respectively. Hybridomas secreting anti-TCR V β 2 (B20.6) [7], anti-TCR V β 4 (KT4) [28], anti-TCR V β 5 (MR9-4) [31], anti-TCR V β 7 (TR310) [23], anti-TCR V β 8.1,8.2 (KJ-16) [10], anti-TCR V β 9 (MR10-2) [30] and anti-TCR V β 11 (RR3-15) [2] antibodies were generous gifts from Drs. Y. Yoshikai, Nagoya University, N. Shinohara, Mitsubishi Life Science Institute, P. Marrack, University of Colorado, CO and H. Nakauchi, Tsukuba University. Antibody was purified as described above and biotinylation was performed with a succinimide ester of biotin, GIBCO BRL, Gaithersburg, MD, by standard methods.

Spleens were removed from mice and single cell suspension was prepared in Hank's balanced salt solution supplemented with 5% FCS. A total of 1×10^6 cells were resuspended in chilled phosphate-buffered saline (pH 7.2) containing 2% FCS, 0.02% EDTA and 0.05% NaN₃, incubated with normal rat immunoglobulins at 4°C for 10 min and followed by incubation with optimal mAb concentration at 4°C for 30 min. Cells incubated with biotinyl antibody of interest at 4°C for 30 min were washed three times and further incubated with R-phycoerythrin-conjugated streptavidin, Biomed, Foster City, CA, at 4°C for 30 min. The samples were resuspended in chilled phosphate-buffered saline containing 2% FCS, 0.02% EDTA, 0.05% NaN₃ and 2 μ g/ml 7-aminoactinomycin D, and analyzed by a FACScan flow cytometer, Beckton Dickinson, Mountain View, CA. Data on viable cells only were acquired by judging from a combination of forward versus side scatter dot plot and fluorescent intensity of 7-aminoactinomycin D detected by the FL3 channel. All stainings were carried out on groups of three mice.

CTL assay: CTL activity of fresh spleen cells from JHMOV-infected B6 mice was measured by ⁵¹Cr release from labelled target cells [33], with a minor modification. Briefly, IC-21 cells (H-2^b) were infected with JHMOV at a multiplicity of infection of 3. Four hours later, 1×10^6 IC-21 cells were incubated with 0.1 mCi of sodium [⁵¹Cr]chromate for 1 hr at 37°C and then washed three times. JHMOV-infected J774.1 cells (H-2^d) were prepared as described previously [33]. A total of 1×10^4 target cells were mixed with B6 splenocytes to give the desired effector/target (E/T) ratio in

200 μ l of RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, 10 mM Hepes and antibiotics. Depletion of B cells from splenocytes was carried out by panning with goat anti-mouse immunoglobulins, Cappel, Durham, NC, as described elsewhere [16]. Depletion of CD4⁺ and/or CD8⁺ T cells *in vitro* was performed as previously described [15]. After incubation at 37°C for 5 hr, supernatants were collected and the radioactivities were counted by a γ -ray counter. The maximum release was determined by adding 1% Triton X-100. CTL activity is expressed as percent specific lysis calculated as previously described [31]. Spontaneous releases of both uninfected and JHMOV-infected IC-21 cells, and JHMOV-infected J774.1 cells were less than 15% of the maximum releases, respectively.

RESULTS

Viral growth in the spleen in B6 mice depleted of CD4⁺ and/or CD8⁺ T cells: Viral growth in the spleen in B6 mice depleted of CD4⁺ and/or CD8⁺ T cells was examined (Fig. 1). Female, 6- to 8-week-old B6 mice were injected with anti-CD4 and/or anti-CD8 mAb, infected ip with JHMOV as mentioned above, and spleen viral titer at 3, 5 and 7 days post-infection (pi) was determined by plaque assay. JHMOV replicated for a few days but was nearly cleared from the spleen within a week in control mice. There was no difference in viral titers among experimental groups at 3 days pi, although T cell subset(s) had depleted before infection. This suggests that viral growth is not swayed by T cells for the first 3 days but subsequent viral titers varied

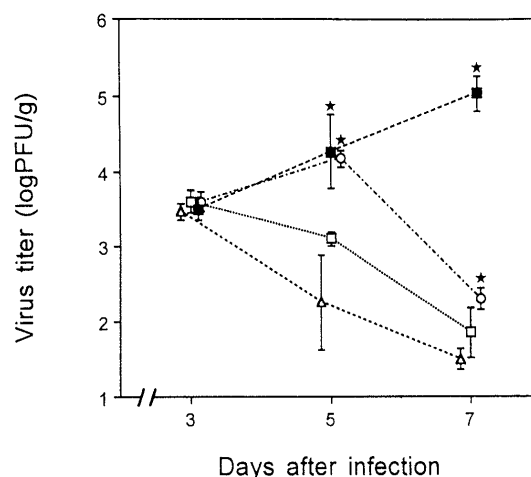


Fig. 1. Viral growth in mice injected with anti-CD4, anti-CD8 and both mAb. B6 mice received multiple injections with anti-CD4 (□), anti-CD8 (○) or both mAb (■) from 2 days prior to infection and were inoculated ip with 1×10^6 PFU of JHMOV. Control mice (△) received the same volume of PBS and infected in the same manner. Spleens were removed and viral titers were determined by plaque assay with DBT cells. Mean \pm standard deviation for three mice. Viral titers that are significantly different from those in control mice are indicated by asterisks (★, $p < 0.05$).

Table 1. The numbers of total, CD4⁺ and CD8⁺ cells in the spleen during primary JHMV infection in B6 mice

Days pi	Total Spleen cells ^{a)} ($\times 10^6$)	CD4 ⁺ T cells ^{b)}		CD8 ⁺ T cells ^{c)}	
		($\times 10^6$)	%	($\times 10^6$)	%
0	105 \pm 12	26 \pm 4	25 \pm 1	14 \pm 2	13 \pm 0
3	88 \pm 20	ND ^{c)}	ND	10 \pm 3	11 \pm 1
5	117 \pm 19	ND	ND	13 \pm 3	11 \pm 1
7	226 \pm 48	56 \pm 10	25 \pm 1	40 \pm 11	18 \pm 1
10	102 \pm 2	ND	ND	15 \pm 1	15 \pm 1
14	98 \pm 4	24 \pm 2	24 \pm 1	12 \pm 1	12 \pm 1

a) Total spleen cells were counted microscopically. Mean \pm standard deviation for three mice.

b) CD4⁺ and CD8⁺T cells were determined by flow cytometry. Mean \pm standard deviation for three mice.

c) Not determined.

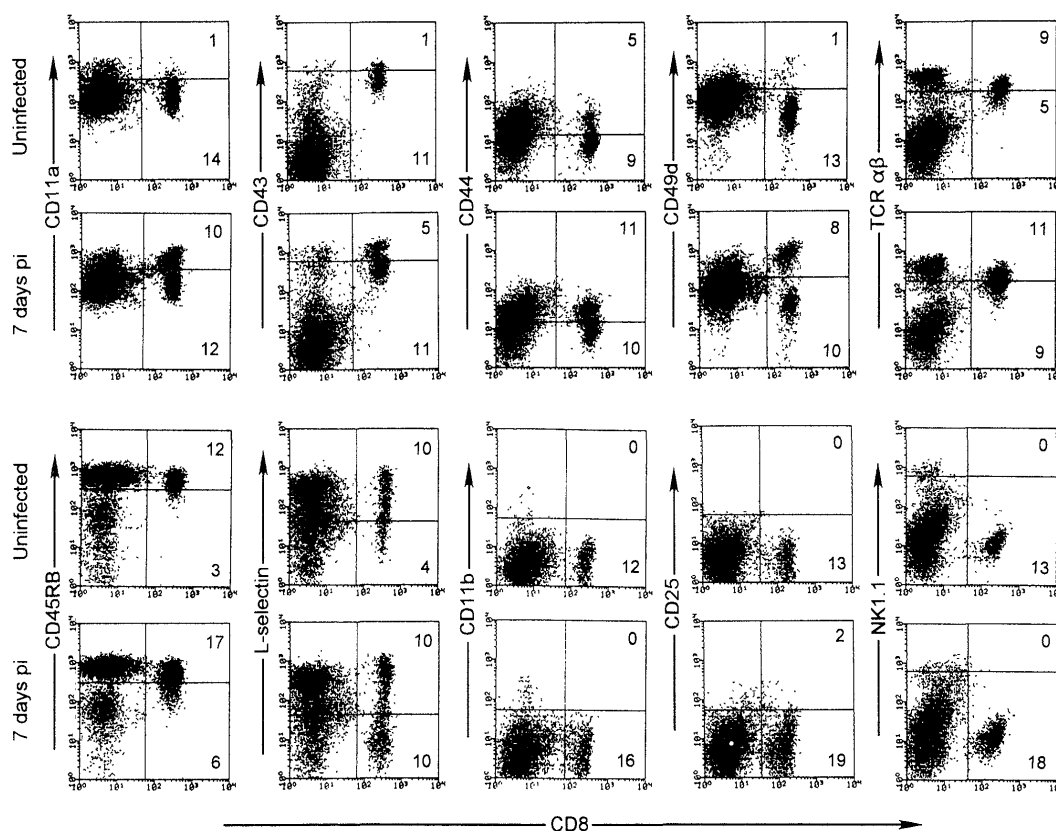


Fig. 2. Expression of various cell surface markers on CD8⁺T cells from uninfected and JHMV-infected B6 mice. Spleen cells from B6 mice infected with JHMV 7 days before and those from uninfected mice were incubated with FITC-conjugated anti-CD8 mAb and either with R-phycoerythrin-conjugated anti-TCR $\alpha\beta$, anti-CD45RB, anti-CD44, anti-L-selectin or anti-NK1.1 mAb, or biotin-conjugated anti-CD11a, anti-CD43, anti-CD49d, anti-CD25 or anti-CD11b mAb followed by R-phycoerythrin-conjugated streptavidin, and then analyzed by flow cytometry. Representative dot plots of three mice are shown with the percentages for the second and fourth quadrants. Similar results were obtained in two separate experiments.

between experimental groups. In CD4⁺T cell-depleted mice, virus clearance rate was almost the same as that in control mice. On the other hand, viral clearance was significantly inhibited in mice depleted of CD8⁺T cells and both T cell subsets; viral titers at 5 days pi were approximately 100 times those of control mice. These results suggest that

CD8⁺T cells play a key role in viral clearance from the spleen after JHMV infection in B6 mice. Although spleen viral titer subsequently decreased in CD8⁺T cell-depleted mice still significantly higher than control mice, JHMV continued to replicate in the spleen for another two days in B6 mice depleted of both T cell subsets.

Table 2. The levels of cell surface antigen expression of CD8⁺T cells from uninfected and JHMV-infected B6 mice

Antigen	Fluorescence intensity ^{a)}		Index ^{b)}
	Uninfected	7 days pi	
CD8	306 ± 3	226 ± 16	0.74
CD11a	176 ± 18	399 ± 24	2.27
CD43	307 ± 29	527 ± 1	1.72
CD44	19 ± 0.5	22 ± 1	1.16
CD49d	92 ± 27	298 ± 57	3.24
TCR $\alpha\beta$	75 ± 1	69 ± 1	0.92
CD45RB	508 ± 18	445 ± 24	0.88
L-selectin	243 ± 18	213 ± 19	0.88

a) Mean channel \pm standard deviation for three mice.

b) Mean fluorescence intensity at 7 days pi/that from uninfected mice.

Expansion of CD4⁺ and CD8⁺T cells during primary JHMV infection in B6 mice: B6 mice were infected ip with JHMV and the numbers of CD4⁺, CD8⁺ and total spleen T cells were examined during primary JHMV infection (Table 1). After JHMV infection, the total number of spleen cells approximately doubled by 7 days pi and decreased again to the levels before infection by 10 days pi. The percentage of CD8⁺T cells in the spleen had also moderately increased at 7 days pi, and the number of CD8⁺T cells in the spleen was therefore approximately three times as great as before infection. The number of CD4⁺T cells in the spleen at 7 days pi also was double before infection, although the percentage remained unchanged during infection. In summary, both T cell subsets expanded, CD8⁺T cells preferentially.

Phenotypic changes in T cells and transient appearance of CD11a^{high}T cells during JHMV infection: The expression of various cell surface markers on T cells expanded in B6 mice at 7 days pi was examined (Fig. 2, Table 2). Flow cytometric analysis showed that CD8⁺T cells expressing higher amounts of CD11a, CD43, CD44 and CD49d, and lower amounts of CD8, TCR $\alpha\beta$, CD45RB and L-selectin were expanded in the spleen at 7 days pi. Few CD8⁺T cells expressed CD11b, CD25 or NK1.1. To see whether CD8⁺T cells expanded after JHMV infection are oligoclonal or polyclonal, we examined TCR V β chain expression on CD8⁺T cells. TCR V β chains expressed on CD8⁺T cells at 7 days pi were highly heterogenous, the same as those of uninfected mice (Fig. 3). Since the number of CD8⁺T cells approximately tripled by 7 days pi (Table 1), the results indicate that all the V β population examined increased twice or more. Therefore, it is unlikely that monoclonal or oligoclonal CD8⁺T cells expanded in the spleen during JHMV infection.

Forward and side scatter profiles indicated that the CD11a^{high}CD8⁺T cells at 7 days pi were larger and more complex than the CD11a^{low}CD8⁺T cells (data not shown), suggesting that these T cells were activated. The number of CD11a^{high}CD8⁺T cells began to increase 5 days pi and peaked at 7 days pi (Fig. 4) but they gradually decreased thereafter to

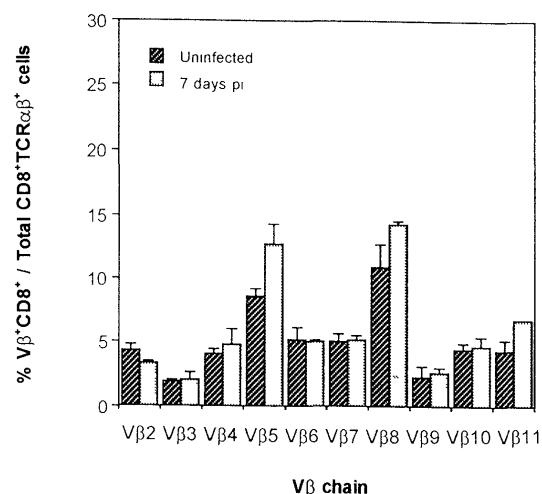


Fig. 3. Frequency of V β chains on CD8⁺T cells in the spleen from uninfected and JHMV-infected B6 mice. Spleen cells from B6 mice infected with JHMV 7 days before and those from uninfected mice were incubated with FITC-conjugated anti-CD8 mAb and biotin-conjugated V β -specific mAb followed with R-phycoerythrin-conjugated streptavidin, and then analyzed by flow cytometry. Percentage of V β CD8⁺ cells/Total CD8⁺TCR $\alpha\beta$ ⁺ cells in each mouse was calculated. Mean values \pm standard deviations for three mice are shown. Similar results were obtained in two separate experiments.

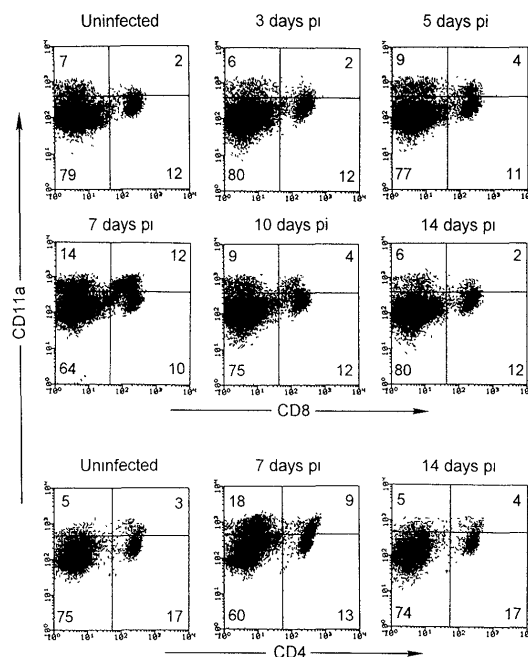


Fig. 4. Appearance of CD11a^{high}T cells during primary JHMV infection in B6 mice. Spleen cells from B6 mice infected with JHMV 3, 5, 7, 10 or 14 days before and those from uninfected mice were incubated with either FITC-conjugated anti-CD8 or anti-CD4 mAb and biotin-conjugated anti-CD11a mAb followed with R-phycoerythrin-conjugated streptavidin, and then analyzed by flow cytometry. Representative dot plots of three mice are shown with the percentage for each quadrant. Similar results were obtained in separate experiments.

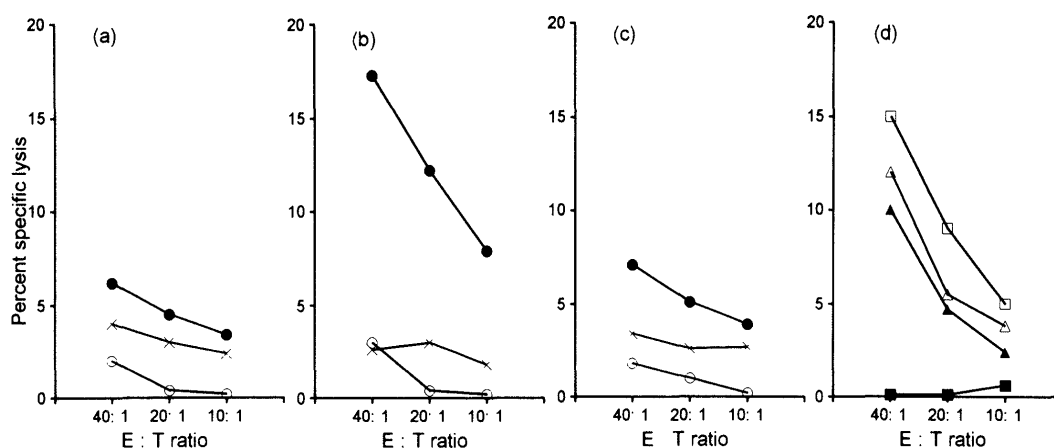


Fig. 5. Virus-specific CTL activity of spleen cells from B6 mice infected with JHMV. Spleen cells from uninfected B6 mice (a) and those from mice infected with JHMV 7 (b) or 14 (c) days before were incubated with uninfected (○) or JHMV-infected (●) IC-21 cells, or JHMV-infected J774.1 cells (×) to determine virus-specific CTL activity. Spleen cells from B6 mice infected with JHMV 7 days before were treated with either complement alone (□), anti-CD4 mAb plus complement (△), anti-CD8 mAb plus complement (▲), or a cocktail of anti-CD4 and anti-CD8 mAb plus complement (■), adjusted cell numbers to the desired E/T ratio, and then examined for antiviral CTL activity with JHMV-infected IC-21 cells (d). Similar results were obtained in two or three separate experiments.

the levels before infection at 14 days pi. Similarly, CD11a^{high}CD4⁺T cells were observed at 7 days pi but not 14 days pi. Concurrent analysis indicated that expression of TCR $\alpha\beta$ and CD8 was inversely regulated during primary JHMV infection: their expression was lowest at 7 days pi and they began to increase thereafter (date not shown).

Virus-specific CTL activity during primary JHMV infection in B6 mice: Primary virus-specific CTL activity of spleen cells from B6 mice at 7 days and 14 days pi, and of those from uninfected mice was measured (Fig. 5). A weak, but significant, cytotoxic activity against JHMV-infected IC-21 cells (H-2^b), but neither uninfected IC-21 cells nor JHMV-infected J774.1 cells (H-2^d), was observed in fresh spleen cells from B6 mice infected with JHMV 7 days before. But, spleen cells from mice infected 14 days before as well as those from naive mice, which did not contain CD11a^{high}T cells did not show primary virus-specific CTL activity. To characterize effector cells in the spleen cells 7 days pi, cells were treated with anti-CD4 mAb plus complement, anti-CD8 mAb plus complement and both mAb plus complement *in vitro*, and then the CTL activity of each sample was measured (Fig 5d). Although spleen cells 7 days pi, which had been treated *in vitro* with either anti-CD4 mAb plus complement or anti-CD8 mAb plus complement prior to CTL assay, showed virus-specific CTL activity, the spleen cells treated with both anti-CD4 and anti-CD8 mAb plus complement did not show antiviral CTL activity. These results suggest that both CD4⁺ and CD8⁺T cells at 7 days pi, which contain CD11a^{high}T cells, mediate virus-specific CTL activity.

DISCUSSION

We have shown that CD8⁺T cells play a key role in acute viral clearance from the spleen during JHMV infection.

However, CD4⁺T cells may be involved in the viral clearance in late phase of JHMV infection, because spleen viral titer in CD8⁺T cell-depleted mice decreased at 7 days pi although JHMV continued to replicate in B6 mice depleted of both T cell subsets. CD4⁺T cells may require a longer period to acquire antiviral activity than CD8⁺T cells, although the detailed mechanism remains to be elucidated. In JHMV infection in mice both CD8⁺ and CD4⁺T cells changed in number and in the expression of cell surface markers *in vivo*. In addition, fresh spleen cells of both T cell subsets showed the virus-specific CTL activity transiently during infection. In lymphocyte choriomeningitis virus (LCMV) infection in mice, CD8⁺ CTL are considered as the major effector cells against infection and they change in number and in the expression of cell surface markers following infection [1, 3, 19]. It is likely that numerical and phenotypic changes in T cells may decisively correlated with the functional activity.

During primary JHMV infection in mice, expression of several cell surface molecules changed. CD8⁺T cells 7 days pi expressed most of the activation or memory markers, and were characterized by decreased expression of CD8, TCR $\alpha\beta$, L-selectin and CD45RB, and increased expression of CD11a, CD43, CD44 and CD49d. Although a considerable number of CD25 (IL-2R α chain)⁺CD8⁺T cells are observed after LCMV infection [1, 27], such cells were only marginally detected in JHMV-infected mice. In addition, expression of CD11b (Mac-1) is described as a characteristic marker of CD8⁺T cells induced following LCMV infection [1, 19] and may facilitate homing to inflammatory sites [22], but no CD11b⁺CD8⁺T cells were induced after JHMV infection. The basis of the difference between infections remains unknown.

It may be important that a transient reduction in CD8 and TCR expression on T cells follows JHMV infection. A

recent paper has shown a similar phenomenon in mice transgenic for a TCR specific for a peptide of the nucleoprotein of the influenza virus [17]. Treatment of these mice with the antigenic peptide for 2, 3 or 4 days leads to a gradual reduction in CD8 and TCR to levels below those of non-treated transgenic mice. It is therefore tempting to speculate that repeated interaction between CD8⁺T cells and cells expressing MHC class I and peptides from JHMV might result in the decreased expression of CD8 and TCR on CD8⁺T cells in JHMV-infected mice. It is noteworthy, however, that CD8⁺T cells which were expanded at 7 days pi appeared to be highly heterogeneous, similar to those in the acute phase of LCMV infection in mice [21]. This may be explained by the fact that not only highly virus-specific T cells but also T cells with low affinity with viral antigens are activated by IL-2 secreted during acute viral infections. Moreover, the changes may be advantageous to the protection, recruiting more T cells to fight viral infections. On the other hand, such a consequence may provide the reason why viral infections sometimes trigger autoimmune-like responses [15].

We have paid special attention to the CD11a^{high}T cells, because CD11a is a component of lymphocyte function-associated antigen-1 (LFA-1) which is an important adhesion molecule involved in immune recognition of T cells [26], and induction of CD11a^{high}T cells is observed in mice and humans following experimental or natural virus infections [1, 4, 24]. We found a temporal correlation between the induction of CD11a^{high}T cells, T cell-mediated viral clearance *in vivo* and virus-specific CTL activity *in vitro* after JHMV infection. Although a recent study has suggested that protein tyrosine kinase p56-Lck, which has been found in physical association with the cytosolic domains of T cell surface receptors including CD2, CD4, CD8 and IL-2 β , can regulate LFA-1 expression and cytolytic effector function in a cloned T cell [29], and the temporal correlation between the induction of CD11a^{high}T cells and antiviral functions observed during JHMV infection suggests that the consequence can be induced not only in an *in vitro* system but also in response to viral infection *in vivo*.

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