JOURNAL OF VIROLOGY, June 1988, p. 2102–2106
0022-538X/88/062102-05$02.00/0

Vol. 62, No. 6

# $T4^+$ T Helper Cell Function In Vivo: Differential Requirement for Induction of Antiviral Cytotoxic T-Cell and Antibody Responses

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Received 2 November 1987/Accepted 9 February 1988

**This study documents the differential requirements of $T4^+$ T helper cells in the induction of virus-specific cytotoxic T-lymphocyte (CTL) and antibody responses during acute lymphocytic choriomeningitis virus infection. Two monoclonal antibodies (GK1.5 and RL172.4) directed against the L3T4 (T4) molecule were used for depleting T helper cells from mice. Depletion of $T4^+$ cells caused a pronounced suppression of antiviral antibody response (20-fold decrease) but had minimal effect on virus-specific CTL response (<2-fold reduction). Despite the elimination of >90% of T helper cells, anti-L3T4-treated mice were able to generate a CTL response of sufficient magnitude to control the viral infection. In contrast, depletion of $Lyt2^+$ T cells abrogated the CTL response and the ability to eliminate virus. Thus, our results underscore the importance of the $Lyt2^+$ T-cell subset in controlling infection with this virus and show that a deficiency of $T4^+$ T cells is likely to have a more severe effect on antibody production than on CTL responses.**

T helper cells, characterized by surface expression of the T4 molecule, play a central role in the generation of immune responses (4, 8, 10, 12, 13, 29, 30). It is generally accepted that $T4^+$ T cells provide help for antibody production as well as for the induction of $Lyt2^+$ cytotoxic T lymphocytes (CTL). The relative requirements of $T4^+$ T helper cells in the generation of humoral responses versus the induction of CTL effector functions are not fully understood. In this report we examined the role of $T4^+$ cells in the development of antiviral antibody and CTL responses following infection of mice with lymphocytic choriomeningitis virus (LCMV). Two monoclonal antibodies (MAbs), GK1.5 and RL172.4, against the cell surface molecule L3T4 were used to deplete T helper cells. We found that elimination of >90% of T helper cells caused a marked suppression of antiviral antibody response but had minimal effect on virus-specific CTL response. All anti-L3T4-treated mice generated an LCMV-specific CTL response that was of sufficient magnitude to control the viral infection. These results suggest that a deficiency of $T4^+$ T helper cells is more likely to affect antiviral antibody production than the induction of virus-specific CTL.

## MATERIALS AND METHODS

**Mice.** BALB/cByJ ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice (6 to 8 weeks old) were purchased from the Jackson Laboratory, Bar Harbor, Maine.

**Virus.** The Armstrong CA1371 strain of LCMV (Arm-7) was used in this study (3).

**MAbs.** The rat anti-mouse hybridoma MAb GK1.5 was obtained from the American Type Culture Collection, Rockville, Md. This antibody reacts with the L3T4 (T4) molecule and is of the immunoglobulin G2b (IgG2b) subclass (11). GK1.5 antibody was partially purified from ascitic fluids by precipitation with a 50% saturated solution of ammonium sulfate. The antibody concentration was determined by an immunodiffusion test with rat IgG standards. The rat anti-mouse hybridoma MAb RL172.4 was kindly provided by M. Bevan, Scripps Clinic and Research Foundation. This antibody (IgM) also reacts with the L3T4 molecule and is effective in complement-mediated in vitro depletion of $T4^+$ cells (9). The AD4(15) MAb was purchased in the form of ascites fluid from Cedarlane and used at concentrations specified by the supplier to deplete $Lyt2^+$ T cells.

**T-cell subset depletion.** Two protocols were used for depleting $L3T4^+$ cells. (i) For in vivo treatment with GK1.5, mice were injected intraperitoneally (i.p.) with a total of 1.5 mg of GK1.5 antibody. This was given in five doses of 300 μg each on days −3, −1, +1, +3, and +5 (mice were challenged with virus on day 0). (ii) The second protocol used two MAbs against L3T4: RL172.4 for complement-mediated depletion in vitro followed by in vivo treatment with GK1.5. The in vitro treatment with RL172.4 was done as described (2). Briefly, $3 \times 10^8$ spleen cells at a concentration of $10^8$/ml were treated with RL172.4 and rabbit complement (low tox M; Cedarlane). To ensure effective depletion of $L3T4^+$ cells, the cytotoxicity procedure was done twice. The cytotoxic index (CI) was calculated as follows: CI = 100 × [(% cytotoxicity of antibody and complement − % cytotoxicity of complement alone)/(100 − % cytotoxicity of complement alone)]. The RL172.4- and complement-treated spleen cells were then transferred intravenously (i.v.) into irradiated (650 rads) mice ($5 \times 10^7$ cells per mouse). These reconstituted mice were injected i.p. with 900 μg of GK1.5 antibody (three injections of 300 μg each on days −1, 0, and +1). The time of LCMV challenge was considered day 0.

For depletion of $Lyt2^+$ T cells, spleen cells were treated in vitro with MAb AD4(15) plus complement as described (2) and transferred i.v. into irradiated (650 rads) mice ($5 \times 10^7$ cells per mouse).

**Flow cytometry.** T-cell subsets in the spleens of anti-L3T4-treated and control mice were quantitated by flow cytometry. Phycoerythrin-conjugated anti-mouse L3T4 MAb (clone GK1.5), biotin-conjugated anti-mouse Lyt2 MAb (clone 53-6), and streptavidin-phycoerythrin were purchased from Becton Dickinson, Mountain View, Calif. Fluorescein isothiocyanate-conjugated goat anti-rat IgG was from Cooper Biomedical, Malvern, Pa. Spleen cells were stained with these reagents and analyzed in a Becton Dickinson FACS II fluorescence-activated cell sorter.

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TABLE 1. Effect of anti-L3T4 treatment on LCMV-specific CTL and antibody responses and ability to control infection

| Expt no.[a] | Group (treatment) | LCMV-specific CTL in spleen: % Specific $^{51}$Cr release at indicated E:T ratio[b] | | | | | | LU/$10^7$ spleen cells[c] | LCMV-specific antibody in serum (ELISA titer) | LCMV titer ($log_{10}$ PFU/ml or organ) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LCMV infected | | | | Uninfected | | | | | | |
| | | 1.8:1 | 5.5:1 | 16.6:1 | 50:1 | 16.6:1 | 50:1 | | | Serum | Spleen | Liver |
| 1 | Control (PBS) | 30 | 63 | 84 | 80 | 2 | 2 | 277 | 40,342 | <1.6 | <1.6 | <1.6 |
| | | 28 | 60 | 89 | 90 | 1 | 5 | 250 | 30,573 | <1.6 | <1.6 | <1.6 |
| | | 27 | 57 | 86 | 84 | 3 | 7 | 247 | 26,615 | <1.6 | <1.6 | <1.6 |
| | Anti-L3T4 (GK1.5) | 21 | 52 | 73 | 73 | 1 | 0 | 203 | 274 | <1.6 | <1.6 | <1.6 |
| | | 2 | 35 | 71 | 85 | 0 | 0 | 108 | 588 | <1.6 | <1.6 | <1.6 |
| | | 17 | 43 | 69 | 87 | 0 | 0 | 156 | 724 | <1.6 | <1.6 | <1.6 |
| 2 | Control (complement + PBS) | | 12 | 35 | 55 | 1 | 3 | 39 | 6,208 | <1.6 | <1.6 | <1.6 |
| | | | 18 | 48 | 70 | 0 | 2 | 58 | 7,131 | <1.6 | <1.6 | <1.6 |
| | | | 12 | 37 | 57 | 0 | 2 | 41 | 2,048 | <1.6 | <1.6 | <1.6 |
| | Anti-L3T4 (RL172.4 + complement + GK1.5) | | 18 | 49 | 65 | 0 | 0 | 59 | 150 | <1.6 | <1.6 | <1.6 |
| | | | 15 | 38 | 54 | 0 | 0 | 44 | 58 | <1.6 | <1.6 | <1.6 |
| | | | 12 | 33 | 40 | 0 | 0 | 34 | 46 | <1.6 | <1.6 | <1.6 |
| | Irradiated | | 0 | 0 | | 0 | | | <25 | 3.4 | 4.0 | 4.6 |
| | | | 0 | 0 | | 0 | | | <25 | 3.6 | 3.9 | 4.4 |

[a] In experiment 1, BALB/cByJ mice were injected i.p. with 300 μg of anti-L3T4 MAb (GK1.5) on days −3, −1, +1, +3, and +5 (total, 1.5 mg). The control group received five injections of PBS. All mice were infected i.p. with $10^5$ PFU of LCMV on day 0. LCMV-specific CTL, antibody, and virus titers were checked eight days postinfection. In experiment 2, spleen cells from BALB/cByJ mice were first treated in vitro with RL172.4 plus complement. These anti-L3T4-treated cells were transferred i.v. into irradiated (650 rads) mice ($5 \times 10^7$ cells per mouse) and then treated in vivo with GK1.5 (300 μg each on days −1, 0, and +1). For the control group, spleen cells were treated in vitro with complement only and transferred into irradiated mice ($5 \times 10^7$ cells per mouse). These mice were then injected with PBS instead of GK1.5. All mice were challenged with virus on day 0. LCMV-specific CTL and antibody responses and virus titer were checked 8 days postinfection.

[b] E:T ratio, Effector/target cell ratio. Not all ratios were tested with all groups.

[c] The LCMV-specific CTL response was calculated as lytic units (LU) per $10^7$ spleen cells. One lytic unit is defined as the number of lymphocytes needed to kill 30% of infected targets in a 6-h assay.

**Antibody titrations.** LCMV-specific antibody was measured as described previously (3) by a solid-phase enzyme-linked immunosorbent assay (ELISA) with purified virus as the antigen.

**Cytotoxicity assay.** LCMV-specific CTL activity in the spleen was determined by a 6-h $^{51}$Cr release assay (3). All samples were assayed in triplicate, and the standard error was <2%.

**Virus titrations.** Infectious LCMV in serum and tissues was quantitated by plaque assay on Vero cell monolayers as described previously (3).

## RESULTS

**Quantitation of T-cell subsets after treatment with anti-L3T4 MAbs.** Two injections of GK1.5 (300 μg each on days −3 and −1) caused >90% depletion of L3T4$^+$ cells. The percentages of L3T4$^+$ and Lyt2$^+$ T cells in the spleens of mock (phosphate-buffered saline [PBS])-treated and GK1.5-treated mice were determined by flow cytometry. Spleens of mock-treated mice contained 21 to 25% L3T4$^+$ cells and 10 to 12% Lyt2$^+$ cells, whereas GK1.5-treated mice contained 1 to 3% L3T4$^+$ cells and 15 to 18% Lyt2$^+$ cells (average of six mice per group). In addition, mice receiving GK1.5 also contained 1 to 2% cells that were rat IgG positive, suggesting the presence of a small percentage of L3T4$^+$ cells with bound GK1.5 antibody. These results, showing elimination of ~90% of L3T4$^+$ cells after two injections of GK1.5, are in agreement with the results obtained by others with this MAb (16). In our experiments, three additional injections of GK1.5 were given after challenge with LCMV (300 μg each on days +1, +3, and +5) to prevent the possible reemergence of L3T4$^+$ cells during the viral infection. Quantitation of T helper cells on the day that virus-specific responses were measured (usually day +8) showed that there were ≤5% L3T4$^+$ cells. Thus, in vivo treatment with GK1.5 was effective in eliminating the majority (>90%) of L3T4$^+$ T cells from the spleen. The spleens of anti-L3T4-treated mice were also smaller than those of the control group. At 8 days postinfection, the spleens of control (PBS-treated) mice contained $120 \times 10^6$ to $150 \times 10^6$ cells, whereas GK1.5 treated mice contained $50 \times 10^6$ to $90 \times 10^6$ cells. Similar results were obtained with both BALB/cByJ and C57BL/6 strains of mice.

In the depletion protocol with two MAbs against L3T4, spleen cells were first treated in vitro with RL172.4 plus complement. The CI after this treatment was 30 to 35%, and there were no more than 1 to 2% L3T4$^+$ cells remaining, as determined by immunofluorescence. This cell population was then transferred into irradiated mice and subjected to in vivo treatment with GK1.5 (three injections of 300 μg each on days −1, 0, and +1). This protocol (RL172.4 and GK1.5) was effective in eliminating 90 to 95% of L3T4$^+$ T cells.

**Effect of anti-L3T4 treatment on LCMV-specific immune response and ability to clear viral infection.** Depletion of T4$^+$ T helper cells resulted in pronounced suppression of LCMV-specific antibody response but had only a marginal effect on virus-specific CTL response. The results of two experiments, one with GK1.5 and the second with both RL172.4

and GK1.5 used to deplete $T4^+$ cells, are shown in Table 1. All anti-L3T4-treated mice generated a potent LCMV-specific CTL response, although the number of lytic units per $10^7$ spleen cells was lower in some of the treated mice than in the control group. In contrast to the variable and low suppression of the CTL response, there was severe depression of the antibody response (97.1 to 99.1% suppression). Even mice that showed minimal to no effect on the CTL response (0 to 20% suppression) exhibited 97 to 99% suppression of LCMV-specific antibody response. The anti-L3T4 treatment had no effect on the ability of mice to clear the viral infection. There was no detectable infectious virus in the serum, spleen, or liver of these mice at 8 days postinfection. In contrast, mice made immunodeficient by whole-body irradiation (650 rads) contained high levels of virus.

Figure 1 summarizes data from six experiments with two strains of mice (BALB/cByJ and C57BL/6). Depletion of ≥90% of $T4^+$ helper cells caused on average a less than 2-fold decrease (ranging from no reduction to 3-fold reduction) in LCMV-specific CTL response and a 20-fold decrease (5-fold to 270-fold decrease) in LCMV-specific antibody response. Thus, there is clearly a differential requirement for $T4^+$ helper cells in the induction of antiviral CTL and antibody responses.

**Effect of anti-Lyt2 treatment on LCMV-specific immune response and ability to eliminate virus.** As shown in Table 2, depletion of $Lyt2^+$ T cells completely abrogated the CTL response and had no effect on the antibody response. Anti-Lyt2-treated mice were unable to resolve the infection and contained high levels of infectious virus in the serum and tissues at 8 days postinfection.

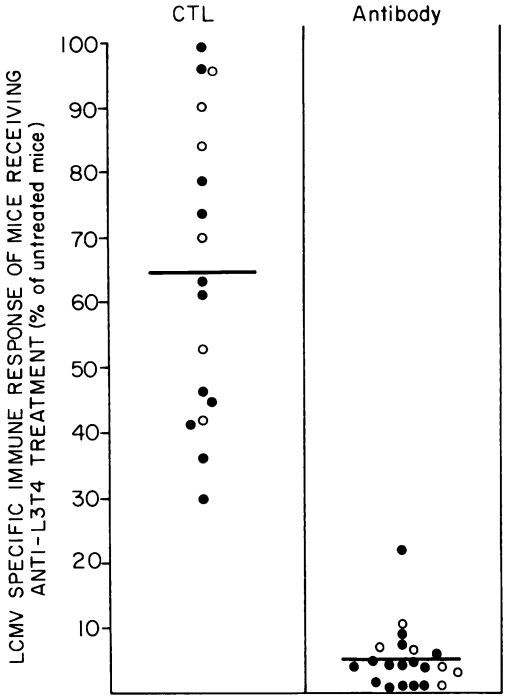


FIG. 1. Depletion of $T4^+$ T helper cells has a differential effect on LCMV-specific CTL and antibody responses. The response of anti-L3T4-treated mice is shown as percent response of untreated control mice. The data presented are from six experiments; each dot represents an individual anti-L3T4-treated mouse, and the horizontal bar is the average response (●, BALB/cByJ mice; ○, C57BL/6 mice). Two to four untreated control mice were included in each experiment, and their average response was considered 100%. The CTL response was calculated as lytic units per $10^7$ spleen cells, and antibody response was calculated as ELISA titer per milliliter of serum.

## DISCUSSION

This study documents the differential requirements of $T4^+$ T helper cells in the induction of antiviral antibody and CTL responses. Mice treated with anti-L3T4 MAbs exhibited suppression of LCMV-specific antibody response but no effect on virus-specific CTL responses. Even after depletion of >90% of $T4^+$ T helper cells, anti-L3T4-treated mice were able to generate a CTL response that was potent enough to control the viral infection. An important implication of these findings is that a deficiency of $T4^+$ helper cells is likely to have a more severe effect on humoral responses than on the induction of CTL responses.

CTL play an important role in controlling viral infections. Our studies, as well as those of other investigators, have shown that $Lyt2^+$ T cells are required for clearing LCMV from both acutely and persistently infected mice (1, 7, 19, 24, 25, 31). However, the mechanism(s) of induction of virus-specific CTL in vivo is not fully understood. Our results suggest that $T4^+$ T helper cells may not be required for the generation of LCMV-specific CTL and that the necessary lymphokines (such as interleukin-2 [IL-2]) are produced by the CTL themselves or by other $Lyt2^+$ T cells. The existence of $Lyt2^+$ "helper" T cells and the ability of $Lyt2^+$ CTL clones to produce IL-2 have been shown in other systems (23). Our studies do not completely rule out a requirement

TABLE 2. $Lyt2^+$ T cells are required for viral clearance

| Group (treatment)[a] | LCMV-specific CTL in spleen (% specific $^{51}Cr$ release) at indicated E:T ratio | | | | | | LCMV-specific antibody in serum (ELISA titer) | LCMV titer ($log_{10}$ PFU/ml or organ) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | LCMV infected | | | Uninfected | | | | | | |
| | 5.5:1 | 16.6:1 | 50:1 | 5.5:1 | 16.6:1 | 50:1 | | Serum | Spleen | Liver |
| Control (complement only) | 46 | 64 | 84 | 0 | 0 | 2 | 5,042 | <1.6 | 2.0 | <1.6 |
| | 62 | 80 | 80 | 0 | 0 | 3 | 2,352 | <1.6 | <1.6 | <1.6 |
| | 58 | 70 | 82 | 1 | 0 | 3 | 6,653 | <1.6 | <1.6 | <1.6 |
| Anti-Lyt2 [AD4(15) + complement] | 0 | 2 | 2 | 0 | 3 | 4 | 2,352 | 3.0 | 4.2 | 4.1 |
| | 0 | 2 | 3 | 0 | 0 | 2 | 4,096 | 3.1 | 5.2 | 4.3 |
| | 0 | 3 | 5 | 0 | 1 | 3 | 5,792 | 3.7 | 5.0 | 5.2 |

[a] To deplete $Lyt2^+$ T cells, spleen cells from BALB/cByJ mice were treated in vitro with AD4(15) plus complement and transferred i.v. into irradiated mice ($5 \times 10^7$ cells per mouse). For the control group, cells were treated with complement prior to transfer. All mice were infected with $10^5$ PFU of LCMV on the day of cell transfer. LCMV-specific CTL and antibody responses and virus titers were checked 8 days postinfection. E:T ratio, Effector/target cell ratio.

for T4+ T helper cells in the in vivo generation of LCMV-specific CTL responses. Although >90% of T4+ cells were eliminated by our depletion protocols, a residual population of T4+ cells was present, and these cells may have been sufficient to provide help for the CTL response. Nevertheless, it is clear from our studies that the relative numbers of T4+ T helper cells required for LCMV-specific antibody and CTL responses are vastly different.

Mice infected with LCMV at birth or in utero become lifelong carriers, with high levels of infectious virus in most of their tissues. Such persistently infected mice are deficient in generating LCMV-specific CTL responses and also make low levels of antibody against the virus (3, 5, 21). We have recently shown that T cells of the helper subset (T4+) are infected with LCMV during persistent infection in vivo and there is minimal or no infection of Lyt2+ T cells and mature B cells (2). This finding implied that infection of T4+ T helper cells may be the underlying cause of the suppression of LCMV-specific immune responses. The results of the present study suggest that lack of appropriate T4+ helper cells is unlikely to be the sole cause of the suppression of LCMV-specific CTL responses in carrier mice. However, it is possible that the development of memory CTL, in contrast to the generation of the primary CTL response, is more dependent on T4+ T cells. The CTL response to LCMV may progress from a T helper-independent to a more T helper-dependent phase. In this case, lack of functional LCMV-specific T helper cells could still explain the suppression of LCMV-specific CTL responses in carrier mice. Studies are in progress to address this issue.

A recent study by Leist et al. (22) also examined the role of T4+ T cells in generation of the LCMV-specific CTL response by treating thymectomized C57BL/6 and CBA/J mice with anti-L3T4 MAb (YTS 191.1). They found that anti-L3T4-treated mice made a readily detectable CTL response (~70% specific killing at an effector/target cell ratio of 50:1 on virus-infected syngeneic targets, as measured by the $^{51}Cr$ release assay), although the number of lytic units per spleen was about 10-fold lower than in control mice. In addition, this response was sufficient to cause the lethal choriomeningitis that is mediated by LCMV-specific Lyt2+ T cells (22). All anti-L3T4-treated mice died at the same time as untreated mice (7 days postinfection) following intracerebral inoculation of LCMV (22). Thus, the results of this study showing that LCMV-specific CTL responses are generated by anti-L3T4-treated mice are in agreement with our findings. Leist et al. (22) did not examine the requirement for T4+ T helper cells in the induction of LCMV-specific antibody response or in controlling viral infection.

It has been accepted as dogma that T helper cells are essential for the generation of CTL responses (4, 8, 12, 13, 29). However, this belief has been challenged by several reports showing that Lyt2+ T cells can generate allogeneic CTL responses both in vitro and in vivo in the absence of T4+ T helper cells (26–28). We have now shown that a potent LCMV-specific CTL response that is sufficient to control the viral infection is made by anti-L3T4-treated mice. Similar results have recently been reported for ectromelia virus (6). In this study, GK1.5-treated mice made an optimal CTL response against ectromelia virus and recovered from the mouse pox. Thus, taken together, these studies show that antiviral CTL responses can be generated even when there is a deficiency of T4+ T helper cells. It is possible that CTL responses to some viruses may be T helper cell independent and to other viruses may be T helper cell dependent. Studies addressing this issue will better define the role of T4+ T cells in antiviral host defense mechanisms and may provide a clearer understanding of the pathogenesis of acquired immunodeficiency syndrome, a disease characterized by a deficiency of T4+ T cells and susceptibility to opportunistic infections (14, 15, 17, 18, 20).

## ACKNOWLEDGMENTS

We thank Rita J. Concepcion for excellent technical assistance and Jeannie Martinez and Lori Kenton for help in preparing the manuscript.

This work was supported by Public Health Service grant NS-21496 and an award (H860623) from the UC Systemwide Task Force on AIDS to R. Ahmed. R.A. is a Harry Weaver Neuroscience Scholar of the National Multiple Sclerosis Society. Laveeza Bhatti is supported by a scholarship from the Ministry of Science and Technology, Government of Pakistan.

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