JOURNAL OF VIROLOGY, July 1988, p. 2505–2507
0022-538X/88/072505-03$02.00/0

Vol. 62, No. 7

Establishment of Cytotoxic T-Cell Clones Specific for Cells Infected with Mouse Hepatitis Virus

KENJIRO YAMAGUCHI,* SHIGERU KYUWA, KAZUE NAKANAGA, AND MASANORI HAYAMI

Department of Animal Pathology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Received 26 October 1987/Accepted 22 March 1988

Mouse hepatitis virus (MHV)-specific T-lymphocyte clones were established from MHV-infected BALB/c mice. They expressed Thy1 and Lyt2 antigens but lacked L3T4 and NK1 antigens. The clones killed MHV-infected but not uninfected or influenza virus-infected J774.1 cells. The specificity was further defined by a cold-target competition test.

In vitro assay systems for measuring cell-mediated cytotoxicity against virus-infected cells have been established for various viruses, such as influenza virus, lymphocytic choriomeningitis virus, Sendai virus, and cytomegalovirus (1, 4, 6, 8, 14), and have enabled investigators to prove the correlation between in vitro cytotoxicity against virus-infected target cells and in vivo effects of cytotoxic T lymphocytes (CTLs). In fact, the importance of CTLs in virus elimination from infected animals was indicated by adoptive-transfer experiments using a polyclonal or monoclonal CTL population (7, 10, 12, 15). However, no assay system for cytotoxicity has been established for mouse hepatitis virus (MHV) and other coronavirus infections, and the existence of CTLs against MHV has not been demonstrated. We tried to establish an assay system for CTL activity in MHV infection, using MHV-infected J774.1 cells as target cells and cloned CTL cell lines derived from MHV-infected BALB/c mice as effector cells.

We established five CTL clones, P11B, P11D, P1C5, P4A3, and P8B5, from BALB/c mice infected with MHV strain JHV/MHV type 4 (MHV-4) as described previously (11). These clones have been maintained in the presence of interleukin-2 (IL-2) and MHV-infected BALB/c spleen cells. Flow cytometric analysis revealed that all the clones expressed Thy1 and Lyt2 antigens but not Lyt1, L3T4 (11), and NK1 (data not shown), the last of which is thought to be a marker of natural killer cells (5). To determine whether the clones were reactive for MHV antigens, cloned T cells which had been cultured for a week after the last antigen pulse were cocultured with uninfected syngeneic spleen cells, MHV-infected spleen cells, or MHV-infected spleen cells plus IL-2. The proliferative response of the cells was estimated by the uptake of [^{3}H]thymidine, as described previously (11). Representative results of two separate experiments are shown in Table 1. Clones P11D, P1C5, P4A3, and P8B5 showed a weak response to MHV-infected spleen cells only, and this response was enhanced by the addition of exogenous IL-2. On the other hand, clones D4C, P9A, and P12B, expressing L3T4 but not Lyt2 antigen (11), proliferated markedly in the presence of MHV-infected spleen cells without exogenous IL-2. These data indicate that Lyt2-positive clones require exogenous IL-2 for proliferation (2, 3). However, some clones, P1C5, P4A3, and D4C, markedly proliferated in the presence of exogenous IL-2.

TABLE 1. MHV specificity and requirement for IL-2 for proliferation of CTL clones

Clone[a]	[^{3}H]thymidine incorporation (cpm)[b]				
	Medium	Uninfected SPC[c]	MHV-infected SPC[d]	MHV-infected SPC + IL-2[e]	IL-2
P11D	567 ± 229	322 ± 182	7,463 ± 569	35,079 ± 1,783	6,114 ± 1,531
P1C5	731 ± 533	1,387 ± 376	2,097 ± 192	26,045 ± 1,786	17,604 ± 1,531
P4A3	684 ± 661	343 ± 136	5,508 ± 473	27,981 ± 2,285	21,369 ± 1,088
P8B5	2,146 ± 792	1,279 ± 299	2,833 ± 95	18,510 ± 691	8,821 ± 264
D4C	173 ± 81	650 ± 206	28,982 ± 2,003	29,815 ± 495	27,465 ± 1,589
P9A	567 ± 664	664 ± 271	25,668 ± 258	27,962 ± 2,478	9,143 ± 1,178
P12B	582 ± 90	458 ± 201	19,051 ± 706	16,910 ± 1,035	1,708 ± 357

[a] Cloned CTL lines were tested 7 days after cultivation with MHV-infected, irradiated spleen cells and 10% concanavalin A conditioned medium.
[b] Mean cpm ± standard deviation from triplicate cultures.
[c] Gamma-irradiated, uninfected BALB/c spleen cells.
[d] Gamma-irradiated, MHV-infected BALB/c spleen cells.
[e] Ten percent rat concanavalin A conditioned medium.

We examined the cytotoxic activity of the clones by using MHV-infected J774.1 cells as target cells. J774.1 cells derived from DBA/2 mice were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. The major histocompatibility complex products of J774.1 cells are compatible with those of clones derived from

* Corresponding author.

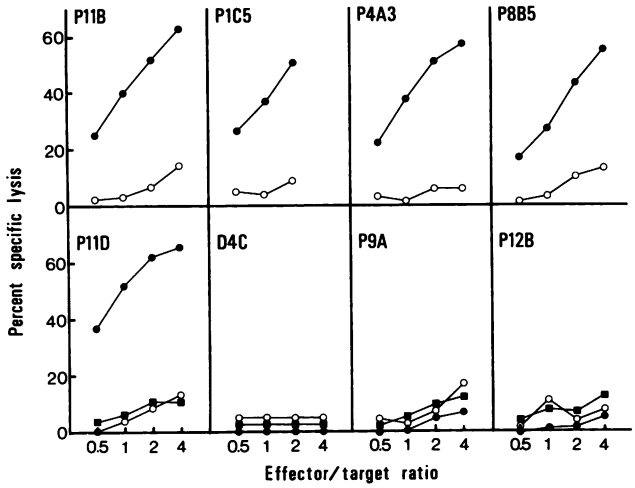

FIG. 1. Cytotoxic activity of MHV-specific CTL clones against MHV-infected J774.1 cells. Symbols: ●, MHV-infected J774.1 cells; ○, uninfected J774.1 cells; ■, A/PR/8-infected J774.1 cells. The percent specific lysis was calculated by the following formula: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release and maximum release were determined by incubation of 10^4 cells with medium alone and with a 1% solution of Triton X-100, respectively. All tests were done in triplicate, and standard deviations were less than 10%.

BALB/c mice. J774.1 cells were infected with MHV-4 at a multiplicity of infection of 5 or with influenza virus strain A/PR/8 (multiplicity of infection, 6), and the percentage of infected cells was 65 to 75% and 80 to 90%, respectively. Four hours later, target cells were labeled by incubating 10^6 cells with sodium [^{51}Cr]chromate for 1 h at 37°C. The cells were washed and plated at 10^4 cells per well in 96-well flat-bottom plates. T-cell clones were added to ^{51}Cr-labeled target cells at various effector-to-target-cell ratios. After incubation for 6 h at 37°C, supernatants were collected and the radioactivity was counted. Lyt2-positive clones P11B, P11D, P1C5, P4A3, and P8B5 showed cytotoxic activity against more than 50% of the MHV-infected J774.1 cells at the highest effector-to-target-cell ratio (Fig. 1). However, these clones did not lyse uninfected J774.1 cells. Similarly, clone P11D lysed MHV-infected but not uninfected P388D1 cells, a macrophagelike cell line established from a DBA/2 mouse (data not shown). No difference was observed in cytolyses of uninfected and influenza virus-infected J774.1 cells by clone P11D. L3T4-positive clones D4C, P9A, and P12B did not show significant cytotoxicity against MHV-infected, uninfected, or influenza virus-infected J774.1 cells.

Moreover, we carried out a cold-target competition test to determine whether these CTL clones specifically recognized and lysed MHV-infected target cells. Unlabeled MHV-infected (4 h postinfection) or uninfected J774.1 cells were used as cold target cells. At an effector-to-hot-target-cell ratio of 2:1, cold target cells were mixed at various ratios of cold to hot target cells. Lysis of MHV-infected J774.1 and P388D1 cells was inhibited by cold MHV-infected J774.1 cells (Fig. 2). However, uninfected J774.1 cells did not inhibit the lysis of either of these target cell lines. These results suggest that clone P11D recognizes a molecule present on MHV-infected but not uninfected P388D1 and J774.1 cells. Moreover, these CTL clones lacked natural killer activity against YAC-1 cells (data not shown). Taken together, these T-cell clones were considered to be MHV-specific CTLs.

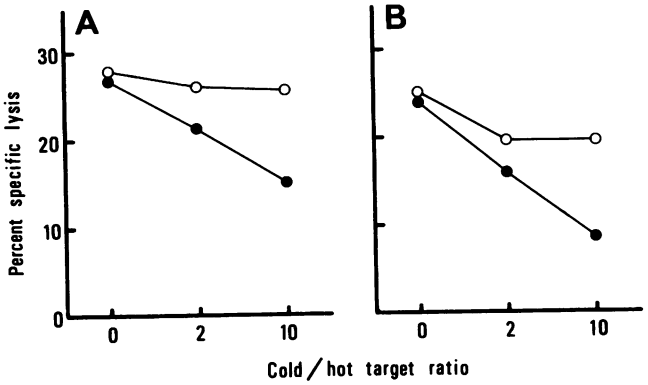

FIG. 2. Inhibition of cytotoxic activity of clone P11D against MHV-infected target cells by unlabeled J774.1 cells. MHV-infected J774.1 (A) and P388D1 (B) cells were used as hot target cells. MHV-infected (●) and uninfected (○) J774.1 cells were used as cold target cells. The percent specific lysis was calculated by the formula given in the legend to Fig. 1. Points are means for three replicate wells. Standard deviations were less than 10%.

No cytotoxicity assay system has been established for MHV infection, and the existence of CTLs against MHV has not previously been demonstrated. One reason for this may be that there are few, if any, CTL cells in MHV-infected mice (13). Another reason is that target cells are difficult to prepare because MHV induces acute cytolysis of almost all MHV-susceptible cell lines in vitro. Moreover, the fact that normal spleen cells, probably B lymphocytes, also lyse MHV-infected cells (9, 18) complicates the demonstration of specific CTLs in MHV infections. In the present study we demonstrated the existence of MHV-specific CTLs in MHV-infected mice by establishment of CTL clones. The use of cloned T cells as effector cells eliminated the effects of B lymphocytes and other nonspecific cytotoxic cells in the cytotoxicity assay system.

The role of the CTL population in MHV-infected mice has not been elucidated. There are reports of inhibition of virus replication in MHV-infected cells by macrophages (16) and of the lytic activity of spleen cells from uninfected mice against MHV-infected cells (9). In addition to these antiviral activities, the MHV-specific CTL population may be important in eliminating virus in vivo, as suggested by adoptive transfer of immunized T cells (17). The CTL clones and cytotoxicity assay system established in this study will be useful for studying the pathogenesis of MHV-4 infection in mice.

We thank H. Nariuchi for a gift of J774.1 and P388D1 cells and K. Nakajima for a gift of influenza virus.

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

1. **Ada, G. L., K. N. Leung, and H. Ertl.** 1981. An analysis of effector T cell generation and function in mice exposed to influenza A or Sendai virus. Immunol. Rev. **58**:5–24.
2. **Andrew, M. E., V. L. Braciale, and T. J. Braciale.** 1984. Regulation of interleukin 2 receptor expression on murine cytotoxic T lymphocyte clones. J. Immunol. **132**:839–844.
3. **Andrew, M. E., A. M. Churilla, T. R. Malek, V. L. Braciale, and T. J. Braciale.** 1985. Activation of virus specific CTL clones: antigen-dependent regulation of interleukin 2 receptor expression. J. Immunol. **134**:920–925.
4. **Braciale, T. J., M. E. Andrew, and V. L. Braciale.** 1981.

Heterogeneity and specificity of cloned lines of influenza virus-specific cytotoxic T lymphocytes. J. Exp. Med. **153**:910–923.
5. **Burton, R. C., and H. J. Winn.** 1981. Studies on natural killer (NK) cells. I. NK cell specific antibodies in CE anti-CBA serum. J. Immunol. **126**:1985–1989.
6. **Byrne, J. A., R. Ahmed, and M. B. A. Oldstone.** 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus. I. Generation and recognition of virus strain and H-2 mutants. J. Immunol. **133**:433–439.
7. **Byrne, J. A., and M. B. A. Oldstone.** 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. J. Virol. **51**:682–686.
8. **Finberg, R., and B. Benacerraf.** 1981. Induction, control and consequences of virus specific cytotoxic T cells. Immunol. Rev. **58**:157–180.
9. **Holmes, K. V., R. M. Welsh, and M. V. Haspel.** 1986. Natural cytotoxicity against mouse hepatitis virus-infected target cells. I. Correlation of cytotoxicity with virus binding to leukocytes. J. Immunol. **136**:1446–1453.
10. **Kast, W. M., A. M. Bronkhorst, L. P. De Waal, and C. J. M. Melief.** 1986. Cooperation between cytotoxic and helper T lymphocytes in protection against lethal Sendai virus infection: protection by T cells is MHC-restricted and MHC-regulated; a model for MHC-disease associations. J. Exp. Med. **164**:723–738.
11. **Kyuwa, S., K. Yamaguchi, M. Hayami, and K. Fujiwara.** 1987. Characterization of mouse hepatitis virus-reactive T cell clones, p. 391–398. *In* M. C. Lai and S. A. Stohlman (ed.), Coronaviruses. Plenum Publishing Corp., New York.
12. **Lin, Y. L., and B. A. Askonas.** 1981. Biological properties of an influenza A virus-specific killer T cell clone: inhibition of virus replication *in vivo* and induction of delayed-type hypersensitivity reactions. J. Exp. Med. **154**:225–234.
13. **Natuk, R. J., and R. M. Welsh.** 1987. Accumulation and chemotaxis of natural killer/large granular lymphocytes at sites of virus replication. J. Immunol. **138**:877–883.
14. **Reddehase, M. J., H.-J. Buhring, and U. H. Koszinowski.** 1986. Cloned long-term cytolytic T-lymphocyte line with specificity for an immediate-early membrane antigen of murine cytomegalovirus. J. Virol. **57**:408–412.
15. **Sethi, K. K., Y. Omata, and K. E. Schneweis.** 1983. Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. J. Gen. Virol. **64**:443–447.
16. **Stohlman, S. A., J. G. Woodward, and J. A. Frelinger.** 1982. Macrophage antiviral activity: extrinsic versus intrinsic activity. Infect. Immun. **36**:672–677.
17. **Sussman, M. A., J. O. Fleming, H. Allen, and S. A. Stohlman.** 1987. Immune mediated clearance of JHM virus from the central nervous system, p. 399–410. *In* M. C. Lai and S. A. Stohlman (ed.), Coronaviruses. Plenum Publishing Corp., New York.
18. **Welsh, R. M., M. V. Haspel, D. C. Parker, and K. V. Holmes.** 1986. Natural cytotoxicity against mouse hepatitis virus-induced cells. II. A cytotoxic effector cell with a B lymphocyte phenotype. J. Immunol. **136**:1454–1460.