Jpn. J. Cancer Res. **80**, 649–654, July 1989

The Roles of $CD8^+$ and $CD4^+$ Cells in Tumor Rejection

Heiichiro Udono, Masahiro Mieno, Hiroshi Shiku and Eiichi Nakayama

Department of Oncology, Nagasaki University School of Medicine, 12-4 Sakamoto-cho, Nagasaki 852

***In vivo* administrations of anti-Lyt-2.2 (CD8) mAb and anti-L3T4 (CD4) mAb selectively eliminated $CD8^+$ cells amd $CD4^+$ cells, respectively. The relative potencies of $CD8^+$ cells and $CD4^+$ cells and their roles in primary tumor rejections were studied by investigating the effects of these mAbs on tumor growth. $CD8^+$ cells were themselves fully capable of mediating rejection in 5 different tumor rejection systems: two radiation leukemia virus (RadLV)-induced leukemias, B6RV2 and BALBRVD, a radiation-induced leukemia BALBRL♂1, and a plasmacytoma BALBMOPC-70A in $CB6F_1$ mice, and a Friend virus-induced leukemia B6FBL-3 in B6 mice. On the other hand, $CD4^+$ cells were capable of resisting tumor growth of B6FBL-3, but not of the other four tumors. Furthermore, for efficient rejection of $CB6F_1$UV♀1 sarcoma by $CB6F_1$ mice, synergy of $CD8^+$ and $CD4^+$ cells was necessary. Blocking of UV♀1 rejection was abrogated by delayed administration of anti-L3T4 (CD4) mAb but not anti-Lyt-2.2 (CD8) mAb, indicating the involvement of $CD4^+$ cells in only the initial phase of rejection.**

Key words: T-cell subsets — Tumor rejection

The effector cell mechanisms for tumor rejection are still poorly understood. In previous studies on adoptive transfer into adult, thymectomized, bone marrow-reconstituted (ATXBM) or athymic mice, either $CD8^+$ cells[1-6] or $CD4^+$ cells[7,8] were found to be capable of mediating rejection of tumors which were negative for class II antigen. Each T-cell subset appeared to be differentially activated, possibly by a factor(s) such as the type of tumor, the number of tumor cells inoculated, or the type of rejection response (primary or secondary) involved. In this study, we investigated the relative potencies of $CD8^+$ and $CD4^+$ cells and their roles in mediating primary rejection of 6 different tumors: 2 radiation leukemia virus (RadLV)-induced leukemias, a radiation-induced leukemia, a plasmacytoma, a Friend virus-induced leukemia and a fibrosarcoma induced by irradiation with ultraviolet light (UV). For this purpose we examined the effects on tumor rejection of *in vivo* administration of anti-Lyt-2.2 (CD8) mAb, anti-L3T4 (CD4) mAb, or both.

MATERIALS AND METHODS

Mice C57BL/6 (B6), BALB/c, and (BALB/c×C57BL/6)F_1 ($CB6F_1$) mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka). Breeding pairs of B6.C-H-2^{bm1} (bm1) and B6.C-H-2^{bm12} (bm12) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were bred in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine.

Tumors B6RV2 and BALBRVD are leukemias induced by injection of radiation leukemia virus into neonates.[9] RL♂1 is a radiation-induced leukemia of BALB/c origin.[10] FBL-3 is a Friend virus-induced leukemia of B6 origin.[11] UV♀1 is an ultraviolet light (UV)-induced fibrosarcoma of $CB6F_1$ origin[12] B6RV2, BALBRVD and RL♂1 were maintained in ascites form in the strain of origin. FBL-3 and UV♀1 were maintained in ascites form in athymic (*nu/nu*) B6 and $CB6F_1$ mice, respectively.

Monoclonal antibodies Anti-Lyt-2.2 (CD8) mAb has been described previously.[13] Anti-L3T4 (CD4) mAb, a rat antibody of the IgG2b immunoglobulin class, produced by hybridoma GK1.5,[14] was kindly provided by Dr. F. Fitch, University of Chicago (Chicago, IL). The titers of both anti-Lyt-2.2 (CD8) mAb and anti-L3T4 (CD4) mAb determined by antibody-mediated complement-dependent cytotoxicity assay were 1:20,000. These antibodies were used in the form of ascites from hybridoma-bearing mice. The concentrations of anti-Lyt-2.2 (CD8) and anti-L3T4 (CD4) mAb in pooled ascites were 7.1 and 2.8 mg/ml, respectively, as quantified by protein assay (Bio-Rad Laboratories, Richmond, CA), and by quantitative cellulose acetate electrophoresis.

Skin grafting The method used was described previously.[15] The arithmetic mean time of graft survival in days (MGS) was calculated for each group of mice.

Tumor assay Tumors were harvested in Eagle's minimum essential medium (MEM) and washed twice with the same medium. Then the desired number of tumor

Abbreviations: RadLV, radiation leukemia virus; UV, ultraviolet light; B6, C57BL/6; $CB6F_1$, (BALB/c×C57BL/6)F_1; bm1, B6.C-H-2^{bm1}; bm12, B6. C-H-2^{bm12}; MGS, mean graft survival; MEM, minimum essential medium; MSV, Moloney sarcoma virus; CTL, cytotoxic T-lymphocytes; Th, helper T-cells.

cells (in 0.2 ml) was injected intradermally into the backs of mice through a 27-gauge needle. Before inoculation of tumor cells, the hair of the back was shaved with clippers. The diameters of tumors at right angles were measured with vernier calipers to calculate the mean diameter.

Antibody administration Mice were anesthetized with ether and a volume of 0.2 ml of antibodies (ascites), diluted 1:8 with MEM, was injected through the retrobulbar venous plexus.

RESULTS

The effects on tumor rejection of *in vivo* administration of anti-Lyt-2.2 (CD8) mAb, anti-L3T4 (CD4) mAb or both were investigated. Tumor cells were inoculated into the back of mice and tumor growth was measured 2–3 times a week. Mice were treated iv with mAb on days 0 and 4 after tumor inoculation. We showed previously that on *in vivo* administration, anti-Lyt-2.2 (CD8) mAb selectively eliminated $CD8^+$ cells for more than 50 days, whereas anti-L3T4 (CD4) mAb selectively eliminated $CD4^+$ cells for more than 30 days.[15] The functional blocking of $CD8^+$ cells and $CD4^+$ cells by *in vivo* administrations of these mAbs was examined by studies on rejection of bm1 and bm12 skin grafts on B6 mice with H-2 class I and class II antigen differences, respectively. Administration of anti-Lyt-2.2 (CD8) mAb prolonged survival of bm1 (MGS, 29.6±2.4 days; MGS of MEM-injected controls, 14.2±3.7 days) but not bm12 (MGS, 11.6±0.8 days; MGS of MEM-treated controls, 11.5±0.5 days) skin grafted onto B6 mice. On the other hand, administration of anti-L3T4 (CD4) mAb prolonged survival of bm12 (MGS, 29.6±6.1 days) but not bm1 (MGS, 10.3±1.1 days) skin grafted onto B6 mice. These findings were consistent with previous results.[15, 16]

The rejections of two RadLV-induced leukemias B6RV2, BALBRVD, a radiation-induced leukemia BALBRL♂1, and a plasmacytoma BALBMOPC-70A by $CB6F_1$ recipient mice were first studied. Figure 1 shows results with BALBRL♂1 and BALBMOPC-70A. The rejection was blocked by *in vivo* administration of anti-Lyt-2.2 (CD8) mAb. Administration of anti-L3T4 (CD4) mAb with anti-Lyt-2.2 (CD8) mAb did not alter the results. The tumor growth and survival of mice after these treatments were not significantly different from those of BALB/c mice inoculated with these tumors without further treatment. Rejection was not affected by administration of anti-L3T4 (CD4) mAb alone. The results were essentially similar with inocula of 2×10^5–1×10^6 tumor cells and with two RadLV-induced leukemias. These results indicated that $CD8^+$ cells were fully capable of mediating rejection of these tumors and that $CD4^+$ cells were not involved in the rejection.

Next we examined the rejection of FBL-3 leukemia by B6 mice (Fig. 2). On administration of anti-Lyt-2.2 (CD8) mAb, rejection of local tumors induced by inocula of 2×10^5 cells was delayed, but mice died in 45–55 days from systemic lymph node metastases. The same treatment blocked rejection of tumors induced by inocula of 1×10^6 FBL-3 cells but prolonged survival. In both cases rejection was blocked completely by anti-Lyt-2.2 (CD8) mAb plus anti-L3T4 (CD4) mAb, but was not

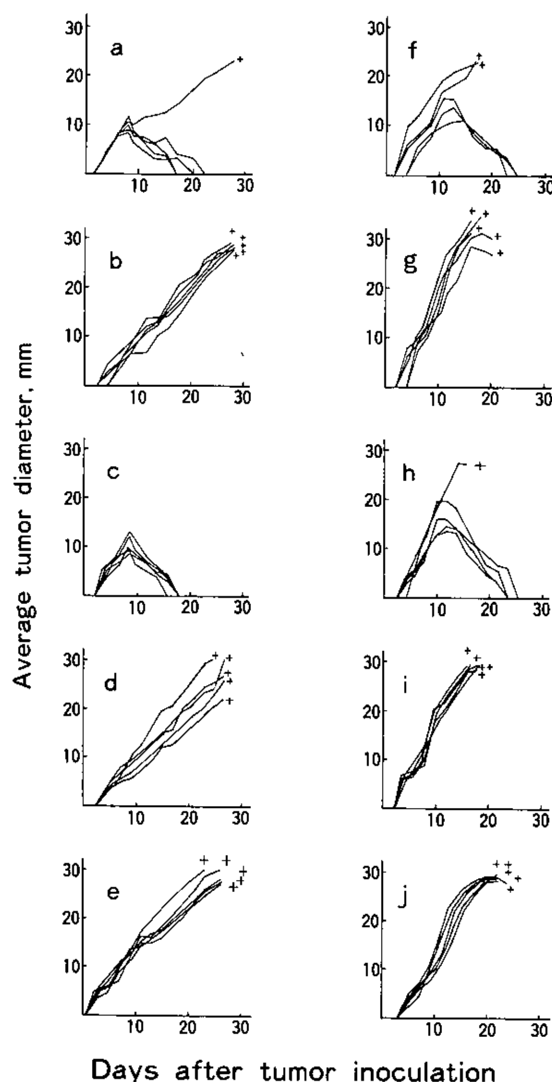

Fig. 1. Effects of *in vivo* administration of anti-Lyt-2.2 (CD8) mAb (b, g), anti-L3T4 (CD4) mAb (c, h), both (d, i) or MEM (control) (a, f) on rejection of BALBRL♂1 (a–e) and BALBMOPC-70A (f–j) by $CB6F_1$ mice. 1×10^6 RL♂1 and 5×10^5 MOPC-70A cells were inoculated into recipient mice. mAbs were injected on days 0 and 4. e, RL♂1 growth in BALB/c male mice. j, MOPC-70A growth in BALB/c female mice.

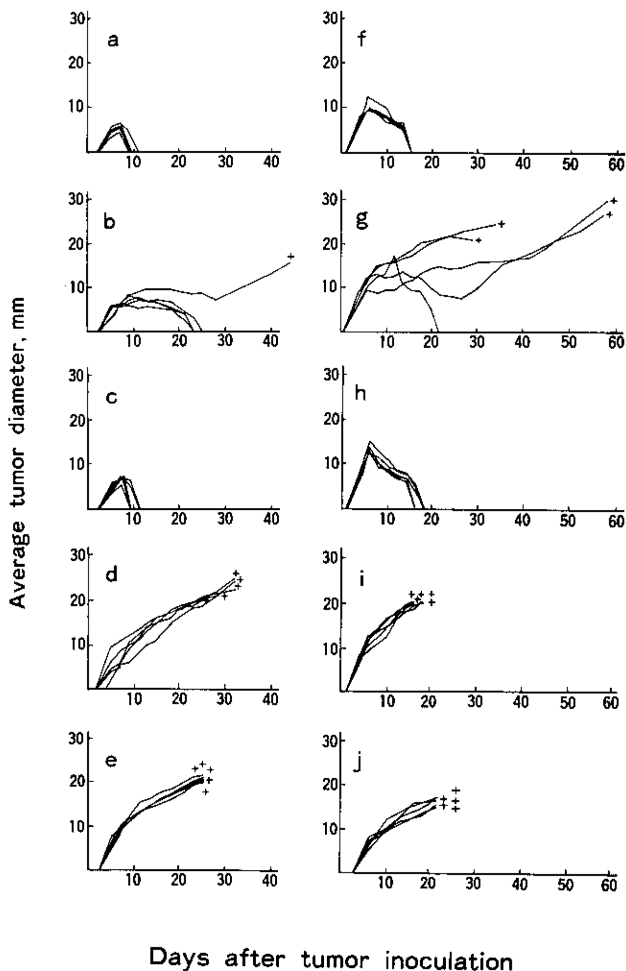

Fig. 2. Effect of *in vivo* administration of anti-Lyt-2.2 (CD8) mAb (b, g), anti-L3T4 (CD4) mAb (c, h), or both (d, i) or MEM (control) (a, f) on rejection of B6FBL-3 leukemia by B6 mice inoculated with 2×10^5 (a–e) or 1×10^6 (f–j) cells. e and j, FBL-3 growth in B6 *nu/nu* mice after inoculation of 2×10^5 and 1×10^6 cells, respectively. In b, 3 of 4 mice in which local tumors regressed died of systemic lymph node metastases on days 46, 51, and 55, but the fourth remained tumor-free for more than 120 days.

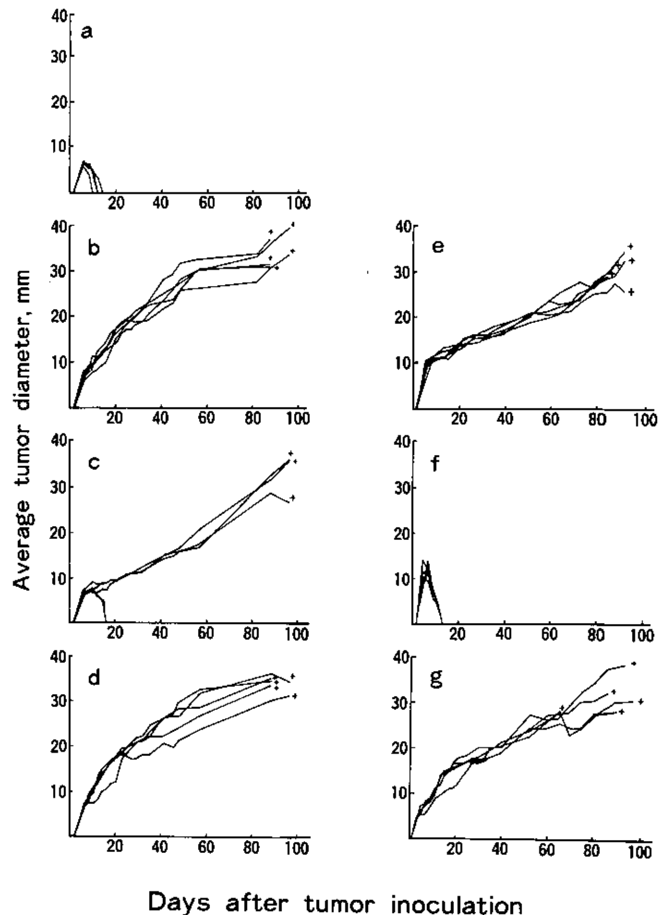

Fig. 3. Effect of *in vivo* administration of anti-Lyt-2.2 (CD8) mAb (b, e), anti-L3T4 (CD4) mAb (c, f) or both (d), or MEM (control) (a) on rejection of UV♀1 sarcoma. mAbs or MEM were injected on days 0 and 4 (a–d) and days 5 and 9 (e, f). g, UV♀1 growth in $CB6F_1$ *nu/nu* mice.

affected by anti-L3T4 (CD4) mAb alone. These results suggest that in rejection of FBL-3 tumors by B6 mice, CD8$^+$ cells were fully capable of mediating rejection and CD4$^+$ cells were weakly capable of resisting tumor growth.

The rejection of UV♀1 sarcoma by syngeneic $CB6F_1$ mice was also studied. The rejection was totally blocked by *in vivo* administration of anti-Lyt-2.2 (CD8) mAb and partially blocked by administration of anti-L3T4 (CD4) mAb (Fig. 3). The rejection was also blocked by the delayed administration of anti-Lyt-2.2 (CD8) mAb on days 5 and 9, but not by delayed administration of anti-L3T4 (CD4) mAb. These results suggested that for efficient rejection of UV♀1 sarcoma, both CD8$^+$ cells and CD4$^+$ cells were necessary, and that CD4$^+$ cells were involved in the initial stage and CD8$^+$ cells were involved in the initial and later stages of rejection.

DISCUSSION

The tumors used in this study were immunogenic to syngeneic or semisyngeneic recipient mice. The regression of B6RV2 and BALBRVD tumors after initial growth in semisyngeneic female mice but not male mice indicated the involvement of H-Y antigen in their rejec-

Table I. Capabilities of $CD8^+$ and $CD4^+$ Cells for Mediating Tumor Rejections and Requirement of Their Synergy

Tumor	Recipient	Capability for tumor rejection		Synergy requirement
		$CD4^+$ cells	$CD8^+$ cells	
B6RV2	$CB6F_1$♀	−	++	−
BALBRVD	$CB6F_1$♀	−	++	−
BALBRL♂1	$CB6F_1$♀	−	++	−
BALBMOPC-70A	$CB6F_1$♀	−	++	−
B6FBL-3	B6♀	+	++	−
$CB6F_1$UV♀1	$CB6F_1$♀	−	++	+

tion, probably as a helper antigen for recognition of tumor antigen by effector cells of recipient mice.[9,17] A radiation-induced leukemia BALBRL♂1 was rejected by either male or female hybrid mice obtained by crossing BALB/c with certain other strains of mice that have the immune response gene to the rejection antigen on this tumor.[10] Likewise, a plasmacytoma BALBMOPC-70A was rejected by hybrid mice obtained by crossing BALB/c with certain strains.[18] A Friend virus-induced leukemia FBL-3[11] and UV-induced fibrosarcoma UV♀1[12] were highly immunogenic and were rejected by syngeneic mice. With all these tumors, cytotoxic T-cells were induced in spleen cells from mice that rejected tumors followed by *in vitro* sensitization with the corresponding tumor cells. The cytotoxic T-cells thus induced mainly recognized the individually distinct (unique) antigen of B6RV2,[9] BALBRVD,[17] RL♂1[19] and UV♀1,[12] and the common antigen of MOPC-70A (unpublished) and FBL-3.[11]

In this study, with these 6 systems of tumor rejection, we investigated the relative potencies and the roles of $CD8^+$ cells and $CD4^+$ cells for mediating rejection, and showed that different T-cell mechanisms were involved. We found that synergy between $CD8^+$ cells and $CD4^+$ cells was necessary for efficient rejection of UV♀1 sarcoma by $CB6F_1$ mice. The findings that $CD4^+$ cells were involved in the initial stage, and $CD8^+$ cells were involved in the initial and later stages suggested that the effector and helper cells in collaboration were $CD8^+$ cells and $CD4^+$ cells, respectively (Table I). The finding that UV♀1 was not rejected even after recovery of $CD8^+$ and $CD4^+$ cells in 50–90 days after tumor inoculation could simply be due to too much tumor burden or other mechanisms. This problem requires investigation. In five other systems of tumor rejection, $CD8^+$ cells were themselves fully capable of mediating rejection. On the other hand, $CD4^+$cells were shown to be capable of resisting tumor growth only in FBL-3 leukemia, though their effect was weak and so after prolonged survival the tumor eventually killed the mice. In other tumor rejection systems, $CD4^+$ cells were not capable of mediating rejection. Activation of $CD4^+$ cells in rejection of FBL-3 leukemia by B6 mice was not attributable to the high antigenicity of this tumor. No activation of $CD4^+$ cells in mediating rejection was observed in UV♀1, which is also highly immunogenic. Furthermore, the finding that involvement of $CD4^+$ cells in rejection was observed with a range of cell doses of 2×10^5–1×10^6 suggested that it was not dependent on the quantity of antigen, but rather on the characteristics of FBL-3 antigen. These findings were consistent with previous findings that $Lyt\text{-}2^-$ cells were effective for eradication of this tumor in adoptive chemoimmunotherapy.[8] Thus, in this study, it was shown that the contribution and the role of $CD4^+$ cells for rejection responses were variable depending on the tumor.

The antigens involved in rejection of UV-induced tumors were shown to belong to a class of individually distinct antigens.[20] The rejection antigen on one UV-induced sarcoma was found to be MHC class I molecule related to the L^d antigen, [21] although no other example of L^d involvement was found. The antigen for rejection of FBL-3 lekemia appears to be MuLV-related.[11] Recently, Klarnet *et al.*[22] showed that immunization of B6 mice with a Friend MuLV generated *gag*-specific $CD8^+$ CTL and *env*-specific $CD4^+$ Th-cell responses. Activation of $CD4^+$ cells in resisting FBL-3 growth could thus be directed against *env* gene product. No information is available on the nature of the antigens responsible for rejection of the RadLV and radiation-induced leukemias used in this study. Individually distinct antigens on these tumors were recognized by cytotoxic T-cells, but their relations to MuLV related antigen and tumor rejection antigens remain to be elucidated.

We did not find a tumor rejection system in which only $CD4^+$ cells were involved. The tumors used in this study were all H-2 class II antigen-negative (data not shown), and the rejection response of class II antigen-positive

tumors has yet to be investigated. Bateman *et al.*[23] observed that in the primary reaction against MSV-induced sarcoma growth, L3T4+ cells, but not Lyt-2+ cells, exerted a protective effect. But a humoral response to MSV may also have been involved in their system.

ACKNOWLEDGMENTS

We thank Ms. Yamashita for expert technical assistance and members of the Laboratory Animal Center of Nagasaki University for care of mice.

(Received March 9, 1989/Accepted May 27, 1989)

REFERENCES

1) Mills, C. D. and North, R. J. Expression of passively transferred immunity against an established tumor depends on generation of cytolytic T cells in recipients. Inhibition by suppressor T cells. *J. Exp. Med.*, **157**, 1448–1460 (1983).
2) Rosenstein, M., Eberlein, T. J. and Rosenberg, S. A. Adoptive immunotherapy of established syngeneic solid tumors: role of T lymphoid subpopulations. *J. Immunol.*, **132**, 2117–2122 (1984).
3) Dailey, M. O., Pillemer, E. and Weissman, I. L. Protection against syngeneic lymphoma by a long-lived cytotoxic T-cell clone. *Proc. Natl. Acad. Sci. USA*, **79**, 5384–5387 (1984).
4) Rosenstein, M. and Rosenberg, S. A. Generation of lytic and proliferative lymphoid clones to syngeneic tumor: *in vitro* and *in vivo* studies. *J. Natl. Cancer Inst.*, **72**, 1161–1165 (1984).
5) Eberlein, T. J., Rosenstein, M. and Rosenberg, S. A. Regression of a disseminated syngeneic solid tumor by systemic transfer of lymphoid cells expanded in interleukin 2. *J. Exp. Med.*, **156**, 385–397 (1982).
6) Engers, H. D., Lahaye, T., Sorenson, G. D., Glasebrook, A. L., Horvath, C. and Brunner, K. T. Functional activity *in vivo* of effector T cell populations. **II.** Anti-tumor activity exhibited by syngeneic anti-MoMuLV-specific cytolytic T cell clones. *J. Immunol.*, **133**, 1664–1670 (1984).
7) Fujiwara, H., Fukuzawa, M., Yoshioka, T., Nakajima, H. and Hamaoka, T. The role of tumor-specific Lyt-1^+2^- T cells in eradicating tumor cells *in vivo*. I. Lyt-1^+2^- T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of *in vivo* immunity. *J. Immunol.*, **133**, 1671–1676 (1984).
8) Greenberg, P. D., Cheever, M. A. and Fefer, A. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1^+2^- lymphocytes. *J. Exp. Med.*, **154**, 952–963 (1982).
9) Nakayama, E., Uenaka, A., Stockert, E. and Obata, Y. Detection of a unique antigen on radiation leukemia virus-induced leukemia B6RV2. *Cancer Res.*, **44**, 5138–5144 (1984).
10) Sato, H., Boyse, E. A., Aoki, T., Iritani, C. and Old, L. J. Leukemia-associated transplantation antigens related to murine leukemia virus. The X.1 system: immune response controlled by a locus linked to H-2. *J. Exp. Med.*, **138**, 593–606 (1973).
11) Matis, L. A., Ruscetti, S. K., Longo, D. L., Jacobson, S., Brown, E. J., Zinn, S. and Kruisbeek, A. M. Distinct proliferative T cell clonotypes are generated in response to a murine retrovirus-induced syngeneic T cell leukemia: viral gp70 antigen-specific MT4+ clones and Lyt-2+ cytolytic clones which recognize a tumor-specific cell surface antigen. *J. Immunol.*, **135**, 703–713 (1985).
12) Kuribayashi, K., Tanaka, C., Matsubayashi, Y., Masuda, T., Udono, H., Abe, M., Nakayama, E. and Shiku, H. Anti-idiotypic antibodies against UV-induced tumor-specific CTL clones: preparation in syngeneic combination. *J. Immunol.*, **141**, 4047–4080 (1988).
13) Nakayama, E. and Uenaka, A. Effect of *in vivo* administration of Lyt antibodies. Lyt phenotype of T cells in lymphoid tissues and blocking of tumor rejection. *J. Exp. Med.*, **161**, 345–355 (1985).
14) Dialynas, D. P., Quan, Z. S., Wall, K. A., Pierres, A., Quintans, J., Loken, M. R., Pierres, M. and Fitch, F. W. Characterization of the murine T cell surface molecule, designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.*, **131**, 2445–2451 (1983).
15) Ichikawa, T., Nakayama, E., Uenaka, A., Monden, M. and Mori, T. Effector cells in allelic H-2 class I-incompatible skin graft rejection. *J. Exp. Med.*, **166**, 982–990 (1987).
16) Rosenberg, A. S., Mizuochi, T., Sharrow, S. and Singer, A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. *J. Exp. Med.*, **165**, 1296–1315 (1987).
17) Morishita, H., Shiku, H., Horibe, K., Obata, Y., Stockert, E., Oettgen, H.F., Old, L. J. and Yamada, K. Cell surface antigens of murine leukemias induced by radiation leukemia virus. Recognition of individually distinct cell surface antigens by cytotoxic T cells on leukemias expressing crossreactive transplantation antigens. *J. Exp. Med.*, **163**, 452–457 (1986).
18) Kaneko, Y., Natsuume-Sakai, S., Moriwaki, K. and Migita, S. F_1 hybrid resistance to murine plasmacytoma MOPC-70A: hybrid resistance to MOPC-70A controlled by a locus linked to H-2. *J. Immunol.*, **125**, 2031–2038 (1980).
19) Nakayama, E., Shiku, H., Takahashi, T., Oettgen, H. F. and Old, L. J. Definition of a unique cell surface antigen of mouse leukemia RL♂1 by cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA*, **76**, 3486–3490 (1979).
20) Kripke, M. L. Antigenicity of murine skin tumors induced by ultraviolet light. *J. Natl. Cancer Inst.*, **53**, 1333–1336

(1974).
21) Stauss, H. J., van Waes, C., Fink, M. A., Starr, B. and Schreiber, H. Indentification of a unique tumor antigen as rejection antigen by molecular cloning and gene transfer. *J. Exp. Med.*, **164**, 1516–1530 (1983).
22) Klarnet, J. P., Kern, D. E., Okuno, K., Holt, C. Lilly, F. and Greenberg, P. D. FBL-reactive $CD8^+$ cytotoxic and $CD4^+$ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigens. *J. Exp. Med.*, **169**, 457–467 (1989).
23) Bateman, W. J., Jenkinson, E. J. and Owen, J. J. T. T-Cell immunity to murine Moloney sarcoma virus-induced tumours: $L3T4^+$ T cells are necessary for resistance to primary sarcoma growth, but Lyt-2^+ T cells are required for resistance to secondary tumour cell challenge. *Immunology*, **61**, 317–320 (1987).