EFFECT OF IN VIVO ADMINISTRATION OF Lyt ANTIBODIES

Lyt Phenotype of T Cells in Lymphoid Tissues and Blocking of Tumor Rejection

BY EIICHI NAKAYAMA AND AKIKO UENAKA

From the Department of Tumor Immunology, The Center for Adult Diseases, Osaka 537, Japan

Cytotoxic T cells are generated in vivo during allograft rejection and can be recovered from mice bearing an allogeneic tumor. From this circumstantial evidence, graft rejection is generally considered to be mediated by cytotoxic T lymphocytes. However, Loveland and his colleagues (1-3) challenged this notion by demonstrating that immunity to an allograft could be adoptively transferred to adult, thymectomized, irradiated, and bone marrow-reconstituted (ATXBM) mice by Lyt-2+ -depleted cells, but not by Lyt-1+ -depleted cells. They interpreted this finding as indicating that Lyt-1+2−3− cells caused graft rejection, whereas Lyt-2+ cells did not. But, there is no direct evidence that Lyt-2+ cells of either the host or donor are not involved in graft rejection. Moreover, LeFrancois and Bevan (4) recently raised doubt about the validity of using ATXBM mice as a T cell-deficient model and of eliminating cells by α-Lyt and complement to define T cell subsets mediating certain immunological functions. They found that host-derived cytotoxic T cells were activated long after adoptive transfer of Lyt-2+ -depleted cells and that donor-derived cytotoxic T cells were activated soon after transfer of a Lyt-1+ - or Lyt-2+ -depleted population. Their results indicate that host-derived Lyt-2+ cells are present in ATXBM mice and that small numbers of viable cells remaining after treatment with antisera and complement can proliferate in vivo in response to continual antigenic stimulation. In fact, ATXBM mice do not seem to be truly T cell-deficient (5). There are many technical problems in quantifying complex Lyt reagents, and these must be considered when evaluating findings obtained by their use, under conditions leading to incomplete elimination of a particular Lyt population (6, 7). It is still unknown whether host or donor Lyt-2+ cells are involved or have any functional role in rejection.

In this study, to circumvent these problems, we directly evaluated the effect of Lyt monoclonal antibodies (mAb)1 on syngeneic and semisyngeneic tumor rejection by administering them to recipient mice without exogenous complement. The results indicate the apparent involvement of Lyt-2+3+ cells in tumor rejection.

1 Abbreviations used in this paper: mAb, monoclonal antibody; MEM, minimum essential medium.
Materials and Methods

**Mice.** C57BL/6 (B6), BALB/c, and (BALB/c × B6)F₁ (CB6F₁) mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). B6-Lyt-1.1 and B6-Lyt-2.1,3.1 mice were originally provided by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, New York and bred in our laboratory. (BALB/c × B6-Lyt-2.1,3.1)F₁ (CB6-Lyt-2.1,3.1F₁) mice were obtained from our breeding colonies. The Lyt phenotypes of B6, B6 Lyt congeneric, and BALB/c mice are as follows: B6, Lyt-1.2,2.2,3.2; B6-Lyt-1.1, Lyt-1.1,2,2,3.2; B6-Lyt-2.1,3.1, Lyt-1.2,2,1,3.1; BALB/c, Lyt-1.2,2,3.2.

**Tumors.** B6RV2 is a B6 leukemia induced by injection of neonatal B6 mice with radiation leukemia virus (8). RLd1 is a radiation-induced leukemia of BALB/c origin (9, 10). These tumors were maintained in ascites form in the strain of origin.

**Antibodies.** The Lyt and Thy-1 mAb used in this study are listed in Table I. The Ig class of concentrated culture supernatants was determined by immunodiffusion. These were used in the form of ascites from hybridoma-bearing nu/nu (BALB/c) mice. The concentration of antibodies in pooled ascites was determined by single radial immunodiffusion (11) using goat anti-mouse heavy chain antibodies (Meloy Laboratories, Inc., Springfield, VA).

**Tumor Assay.** Tumors were harvested in Eagles' minimum essential medium (MEM) and washed twice with medium. Then the desired number of tumor cells (in 0.2 ml) was injected intradermally into the backs of mice through a 30-gauge stainless steel needle. Before inoculation of tumor cells, the hair was shaved with clippers. The diameters of tumors were measured with vernier calipers, twice, at right angles, to calculate the mean diameter.

**Intravenous Injection of Antibody.** Mice were anesthetized with ether and injected through the retrobulbar venous plexus with 0.2 ml of antibodies (ascites), diluted 1:4 with MEM, through a 1-ml disposable tuberculin syringe fitted with a 26-gauge needle (Terumo, Inc., Tokyo).

**Antibody-mediated, Complement-dependent Cytotoxicity.** Tests were performed as described previously (12). Before tests on the Lyt phenotype of T cells from the spleen or lymph node, dead cells were removed by density gradient centrifugation in bovine serum albumin (13).

Results

**Effect of In Vivo Administration of Lyt mAb on Tumor Rejection Mediated by Syngeneic or Semisyngeneic Recipient Mice.** Fig. 1 illustrates the growth of B6RV2

| Antibody Specificity | Immunization* | Ig class | Antibody titer* | Antibody concentration (mg/ml) |
|----------------------|---------------|----------|-----------------|-------------------------------|
| Lyt-1.1              | B6 anti-DBA/2  | μκ        | 6,400–12,800    | 3.2                           |
| Lyt-1.2              | B6-Lyt-1.1 anti-BALB/c | γ2ακ     | 12,800–25,600 | 116.0                       |
| Lyt-2.1              | B6-H-2α anti-B6-H-2α CE-Lyt-2.1:DS | γ2κακ    | 6,400–12,800 | 3.0                          |
| Lyt-2.2 (C57H/An × B6-Lyt-2.1)F₁ anti-ERLD | γ2ακ  | 6,400–12,800 | 2.9                  |
| Lyt-3.2              | C58 anti-C58 CE-Lyt-3.2:DS | μκ | 204,800–409,600 | 2.5                           |
| Thy-1.2 (A-Thy-1.1 × AKR-H-2α)F₁ anti-ASL1 | μκ | 409,600–819,200 | 0.7                  |

* For production of hybridoma, spleen cells from immunized mice were fused with BALB/c myeloma NS-1 by the method of Köhler and Milstein (28).

* Reciprocal of antiseraum dilution giving 50% cell lysis in cytotoxic tests with thymocytes of the appropriate Lyt and Thy-1 phenotype.

* Provided by Drs. Shoji Kimura and Nobuhiko Tada.

* Derived stock.

* Provided by Dr. Ulrich Hämmerling.
in B6 mice after intradermal injection of $1 \times 10^6$ B6RV2 cells. In male B6 mice (Fig. 1A), tumors grew progressively until the mice generally died 2–4 wk after B6RV2 transplantation. In female B6 mice (Fig. 1B), initial B6RV2 growth was followed by tumor regression. These observations suggest that the H-Y antigen on B6RV2 was involved in B6RV2 rejection by female B6 mice, although the predominant antigen involved in humoral and cellular immunity of these mice against B6RV2 was a unique tumor antigen on B6RV2, and not the H-Y antigen, as shown previously (8). When BALB.RL31 leukemia cells were inoculated intradermally ($5 \times 10^5$) into CB6F1 mice, tumor regression after initial growth was observed in both males and females (9, 10). In BALB/c mice (male and female), RL31 grew progressively and generally killed the mice within 4–5 wk.

Using these two tumor regression systems, B6RV2 rejection by B6 and B6-Lyt-2.1,3.1 female mice, and RL31 rejection by CB6F1 and CB6-Lyt-2.1,3.1F1 mice, we investigated the effects of in vivo administration of Lyt and Thy-1.2 mAb. mAb were injected intravenously into recipient mice on days 0, 4, and 8 after tumor inoculation, and the growth of the tumors was observed. Figs. 2 and 3 illustrate the results of Lyt-2 mAb on rejection of B6RV2 and RL31, respectively. Figs. 4 and 5 show the results of Lyt-3 mAb on rejection of B6RV2 and RL31, respectively. Lyt-2.1 mAb blocked B6RV2 rejection by B6-Lyt-2.1,3.1 female mice, and RL31 rejection by CB6-Lyt-2.1,3.1F1 mice, but did not block B6RV2 rejection by B6 female mice or RL31 rejection by CB6F1 mice. On the other hand, Lyt-2.2 mAb blocked B6RV2 rejection by B6 female mice and RL31 rejection by both CB6-Lyt-2.1,3.1F1 and CB6F1 mice, but did not block B6RV2 rejection by B6-Lyt-2.1,3.1F1 mice. Lyt-3.2 mAb blocked B6RV2 rejection by B6 female mice and RL31 rejection by CB6F1 mice, but did not block B6RV2 rejection by B6-Lyt-2.1,3.1 female mice. In these experiments, mice were usually each injected three times. But we observed blocking of tumor rejection in mice given only a single injection (50 μl) of Lyt-2,3 mAb on day 0. Therefore, we investigated the effect of antibody injection at different stages during the course of tumor rejection. As shown in Fig. 6, a single injection of Lyt-2.2 mAb on day 0, 3, 6, or 9 after inoculation of B6RV2 was effective for blocking rejection. No blocking effect was observed with Thy-1.2 mAb on B6RV2 rejection by B6 mice.

**Figure 1.** Growth of B6RV2 leukemia in B6 male (A) and female (B) mice injected intradermally with $1 \times 10^6$ B6RV2. (•) Death from progressive B6RV2 growth. The ratio of the number of mice in which a tumor was recognized macroscopically to the number of mice injected with tumor cells is shown in A and B. Growth of B6RV2 in CB6F1 mice is described elsewhere (8).
Figure 2. Blocking of B6RV2 rejection by in vivo administration of Lyt-2 mAb. B6-Lyt-2.1,3.1 (A, B, C) and B6 (D, E, F) female mice were either not treated (A, D) or were injected intravenously with Lyt-2.1 (B, E) or Lyt-2.2 (C, F) mAb on days 0, 4, and 8 after transplantation of 1 × 10⁶ B6RV2 cells.

Figure 3. Blocking of BALB.RLd1 rejection by in vivo administration of Lyt-2 mAb. CB6-Lyt-2.1,3.1F₁ (A, B, C) and CB6F₁ (D, E, F) mice were either not treated (A, D) or were injected intravenously with Lyt-2.1 (B, E) or Lyt-2.2 (C, F) mAb on days 0, 4, and 8 after transplantation of 5 × 10⁵ RLd1 cells.

(Fig. 4) or on RLd1 rejection by CB6F₁ mice (Fig. 5), or with Lyt-1.1 mAb on B6RV2 rejection by B6-Lyt-1.1 mice (data not shown).

Direct Estimation of the Lyt Phenotype of T Cells Derived from Mice Treated with Lyt mAb. Mice were injected intravenously with Lyt or Thy-1.2 mAb on days 0, 4, and 8. The serum antibody levels of individual mice were assayed kinetically after the last injection. Antibody activities gradually fell and were generally not
FIGURE 4. Effects of Lyt-3.2 and Thy-1.2 mAb on rejection of B6RV2. B6-Lyt-2.1,3.1 (A, B) and B6 (C, D, E) mice were either not treated (A, C) or were injected intravenously with Lyt-3.2 (B, D) or Thy-1.2 (E) mAb on days 0, 4, and 8 after transplantation of $1 \times 10^6$ B6RV2 cells. Lyt-3.2 mAb blocked rejection by B6, but not by B6-Lyt-2.1,3.1 mice. Thy-1.2 mAb did not block rejection by B6 mice.

FIGURE 5. Effects of Lyt-3.2 and Thy-1.2 mAb on rejection of RLd1. CB6F1 mice were either not treated (A) or were injected intravenously with Lyt-3.2 (B) or Thy-1.2 (C) mAb on days 0, 4, and 8 after transplantation of $5 \times 10^5$ RLd1 cells. Lyt-3.2 mAb blocked rejection, whereas Thy-1.2 did not.

detectable after 2–3 wk. After the antibody activity became undetectable, thymocytes, lymph node, and spleen cells were typed for Lyt and Thy-1 in direct, antibody-mediated, complement-dependent cytotoxicity tests. Before tests on lymph node and spleen cells, dead cells were removed by density gradient centrifugation in bovine serum albumin. Normally, the thymocyte population was $\geq 90\%$ positive for Thy-1, Lyt-1, Lyt-2, and Lyt-3. The lymph node cell population contained $\sim 40$–$45\%$ Thy-1$^+$ and Lyt-1$^+$ cells, and $50\%$ Lyt-2$^+$ and Lyt-3$^+$ cells. The spleen cells were approximately $35$–$40\%$ Thy-1$^+$ and Lyt-1$^+$ cells, and $25$–$30\%$ Lyt-2$^+$ and Lyt-3$^+$ cells. These results were not significantly different in the different strains of mice. Fig. 7 illustrates tests with lymph node cells from B6-Lyt-2.1, 3.1 and B6 mice injected with Lyt-2 mAb. $\sim 40$–$45\%$ of the lymph node cells from untreated mice or mice injected with Lyt-2 mAb to
Lyt-2,3 BLOCKING OF TUMOR REJECTION

FIGURE 6. Effect of Lyt-2 mAb injected at different stages during the course of tumor regression. B6 mice were given a single injection of Lyt-2.2 mAb on day 0 (A), 3 (B), 6 (C), or 9 (D) after transplantation of $1 \times 10^6$ B6RV2 cells. (E) Untreated control.

FIGURE 7. Direct Lyt phenotyping of T cells from B6-Lyt-2.1,3.1 (A, B, C) and B6 (D, E, F) mice that were either not treated (A, D) or were injected intravenously with Lyt-2.1 (B, E) or Lyt-2.2 (C, F) mAb. Lymph node cells were typed for Thy-1.2 (○), Lyt-1.2 (●), Lyt-2.1 (△), Lyt-2.2 (▲), and Lyt-3.2 (■), in antibody-mediated, complement-dependent cytotoxicity tests, after elimination of dead cells on a density gradient of bovine serum albumin. (——), Background lysis.
the alternative allele were Thy-1+ and Lyt-1+ cells, and 30% were Lyt-2+ and Lyt-3+ cells. In contrast, lymph node cells from mice injected with Lyt-2 mAb to the appropriate allele contained ~40–45% Thy-1+ and Lyt-1+ cells, and ≤15% Lyt-2+ and Lyt-3+ cells. These results indicate that Lyt-2+ and -3+ cells were reduced to background level and that the T cell population phenotype in these mice was mainly Lyt-1+2−3−. Similar results were obtained with spleen cells. Blocking of the Lyt-2+3+ population was still observed on day 57 (8 wk), but had recovered partially on day 80 (11 wk), and completely on day 94 (14 wk), after the end of treatment with Lyt-2.1 or Lyt-2.2 mAb. Blocking was also observed in mice given only one or two injections of Lyt-2 and -3 mAb. In the thymocyte population, no blocking of the Lyt-2+3+ population was observed. The cell yields and levels of T cells (Thy-1+ cells) in the lymph node and spleen of B6 and CB6F1 mice after intravenous injection of Lyt-2.1, Lyt-2.2, or Lyt-3.2 mAb were not significantly different from those of untreated mice. Administration of Thy-1.2 mAb to B6 or CB6F1 mice, and of Lyt-1.1 mAb to B6-Lyt-1.1 mice, did not alter the Lyt population.

Discussion

There are several differences between the characteristics of regression of B6RV2 leukemia mediated by B6 or CB6F1 female mice and those of BALB.RL61 mediated by CB6F1 mice. First, as described in Results, H-Y antigen is apparently involved in the in vivo rejection of B6RV2, but not in that of RLd1. Second, an Ir gene, possibly identical to the Rgu-I gene, is responsible for RLd1 rejection. Thus, RLd1 leukemia is rejected by hybrids of BALB/c and certain other mouse strains that possess the responder allele, although BALB/c mice themselves exhibit no detectable resistance (9). Third, the tumor sizes during the course of regression of these two tumors are different. The peak diameter of B6RV2 reaches ~20 mm, whereas that of RLd1 is no more than 10 mm. These differences suggest that the mechanisms of regression of these two tumors in syngeneic and semisyngeneic recipient mice are different.

In the present study, we demonstrate that the rejection of these two tumors in syngeneic and semisyngeneic recipients was blocked by either Lyt-2 and -3 mAb administered in vivo without exogenous complement. The specificity of Lyt-2,3 blocking was shown by the fact that the blocking activities of Lyt-2 and -3 mAb in recipient mice derived from B6 Lyt congeneric stocks were consistent with the Lyt phenotype of these mice, and that no blocking was observed after in vivo administration of Thy-1 or Lyt-1 (data not shown) mAb. These effects cannot be ascribed to a certain class of Ig because the Ig of Lyt-2.1, Lyt-2.2, and Lyt-3.2 mAb are γ2b, γ2α, and μκ, respectively. Blocking of B6RV2 or BALB.RLd1 rejection by Lyt-2.1 mAb in appropriate Lyt-congeneric recipient mice provided further proof that Lyt-2 blocking was at the effector level, not at the target tumor cell level (Lyt phenotype of B6 and BALB/c, Lyt-1.2,2.2,3.2).

There are several reports on the effect of in vivo administration of mAb. Infusion of Thy-1.1 mAb with or without exogenous complement has a therapeutic effect on AKR/J mice with transplanted AKRSL2 leukemia (14). Since antigen-negative variants are not eliminated by the treatment, this effect appears to be at the target cell level. In the case of RBL-5 (B6) leukemia transplanted
into BALB/c mice, injection of Thy-1.2 mAb tends to prolong the rejection time, and Lyt-1 or Lyt-2 mAb have no effect (15). Michaelides et al. (16), however, observed blocking of allograft rejection after administration of Lyt-1.1 mAb to CBA/H mice. The reason for these discrepancies is unknown. Possible reasons are (a) differences in antigenic strength; allogeneic and syngeneic (or semisyngeneic) tumor rejection used for assay of the effect, (b) different tumor types, or (c) differences in activity of mAb.

It has been widely accepted that cytotoxic T cells are effector cells in graft rejection. Recently, however, some investigators have raised questions about the functional role of Lyt-2+ cells as effectors in rejection of allografts (1) or syngeneic tumors (17), by demonstrating that immunity is passively transferred to immunodeficient mice by spleen cells from which Lyt-2+ cells had been eliminated by treatment with α-Lyt-2 and complement. However, it was also shown that the adult thymectomized, irradiated, and bone marrow–reconstituted mice used in these studies were not truly T cell deficient (5) and that, in fact, host-derived cytotoxic T cells immune to the allograft were recovered from mice to which Lyt-2÷-depleted populations were adoptively transferred (4). Thus, it was still possible that Lyt-2+ cells are involved in graft rejection. Recently (18), it was reported that immunity to syngeneic fibrosarcoma can be adoptively transferred by Lyt-2+ cells. Consistent with these findings, immunity is also adoptively transferred by infusion of interleukin 2–dependent cytotoxic T cells to syngeneic tumors (19, 20) and allogeneic tumors (21) or tissue (22). Although our results support these findings and demonstrate directly that Lyt-23÷ cells (and/or Lyt-2,3 antigen) is essential for tumor rejection by syngeneic and semisyngeneic recipients, it is still unknown by which mechanism Lyt-2 and -3 mAb cause blocking, or at which step of the rejection process Lyt-2 and -3 mAb are operative. The finding that a single injection of Lyt-2 mAb on day 0–9 after B6RV2 transplantation effectively blocked tumor rejection suggests that effector cells were functionally blocked rather than that the generation of these cells was inhibited. Previously, we and others demonstrated that in vitro T cell cytotoxicity (6, 23–26) and proliferation (7, 27) in response to alloantigen stimulation, were blocked by α-Lyt-2,3 without added complement. We suggested that molecules bearing Lyt-2 and -3 determinants may be involved in T cell recognition. The finding of Lyt-2,3 blocking of tumor rejection is consistent with these in vitro effects of α-Lyt-2,3.

The alteration of the T cell population to result in Lyt-1+2-3÷ cell predominance in the lymph node and spleen of mice injected with Lyt-2,3 mAb is also consistent with findings that, in in vitro cultures of H-2 antigen–stimulated T cells, Lyt-1+2+3÷ cells predominate under normal circumstances and Lyt-1+2-3÷ cells predominate when Lyt-1+2+3÷ cells are blocked by Lyt-2 or -3 antiserum in the absence of complement (7). The decrease of Lyt-2+3÷ cells in mice injected with Lyt-2,3 mAb did not appear to be due to killing of Lyt-2+3÷ cells by Lyt mAb and complement from the recipient mice, because Thy-1 and Lyt-1 mAb (both with extremely high titers in cytotoxic tests) did not alter the T cell population. Our results do not indicate why there was no change in the Lyt population in the thymus. One possible reason is that insufficient antibodies reached the thymus, because of the low blood supply or a barrier to the organ.
Another reason could be that in the thymus, antibody-coated Lyt-2\(^{-}3^{+}\) cells were eliminated rapidly and the population of uncoated cells was continuously renewed. In contrast, in the lymph node and spleen, it is possible that the population of Lyt-2\(^{+}\) cells was not restored quickly after treatment with antibody and that Lyt-1\(^{+}\)2\(^{-}\)3\(^{-}\) cells proliferated instead.

**Summary**

After transplantation of B6RV2 leukemia, initial tumor growth was followed by tumor regression in B6 (CB6F\(_1\)) female, but not male, mice. This indicated that H-Y antigen is involved in B6RV2 rejection by syngeneic female recipient mice. In the case of another leukemia, BALB.RL\(_{61}\), an \(I_r\) gene, probably identical to the \(Rg_2-1\) gene, is responsible for RL\(_{61}\) rejection. Thus, F\(_1\) hybrids of BALB/c with certain other strains of mice can reject RL\(_{61}\). Using these two different systems of tumor rejection, we investigated the effects of in vivo administration of Lyt and Thy-1 monoclonal antibodies (mAb). Results showed that Lyt-2 and -3 mAb blocked both B6RV2 rejection by B6 female mice and BALB.RL\(_{61}\) rejection by CB6F\(_1\) mice. The specificity of blocking was confirmed by use of Lyt-2 and -3 mAb to reciprocal alleles and mice from B6 Lyt-congeneic stocks. No blocking was observed with Lyt-1 and Thy-1 mAb. The Lyt phenotype of T cells in lymphoid tissues from mice treated with mAb was then studied. Blocking of the Lyt-2\(^{-}3^{+}\) population was observed in the lymph node and spleen, but not in the thymus.

These results indicate the involvement of Lyt-2\(^{-}3^{+}\) cells (or Lyt-2,3 antigen) in tumor rejection. The precise mechanism of blocking is unknown, but it was observed after even a single injection of Lyt-2,3 mAb on day 9 after tumor transplantation, suggesting that effector cells were functionally blocked, rather than that the generation of these cells was inhibited.

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