# BLOCKING EFFECT OF Lyt-2 ANTIBODIES
# ON T CELL FUNCTIONS*

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Thymus-derived (T)[1] lymphocytes mediate a variety of immunological functions. The antigen specificity revealed in their responses postulates the existence of specific T cell receptors (1-3). Antigen-specific receptors of B cells consist of immunoglobulin (Ig) molecules and can be blocked by antibodies to constant region determinants of Ig molecules (4-6). The nature of the specific T cell receptor, however, is still controversial. Idiotype studies suggest the expression of Ig heavy chain variable gene products on T cell receptors, although conventional Ig constant determinants have not been demonstrated (7-9). Thus, if T cell receptors, like those of B cells, possess constant regions and if T cell functions could be blocked by antibodies to such constant determinants, studies on the nature of T cell receptors may be greatly facilitated.

Lymphocyte differentiation (Lyt) antigens are useful markers for T lymphocyte subpopulations. Although several systems of Lyt antigens are known, the Lyt-1 system and the Lyt-2,3 system have been most extensively characterized (10-12). T cells with different functions, particularly helper and killer activities, can be distinguished on the basis of their Lyt phenotype. Thus, with only few exceptions (13, 14), it has been widely accepted, that helper cells express the $Lyt-1^+2^-3^-$ phenotype, whereas killer cells have the $Lyt-1^-2^+3^+$ phenotype (15). The question with regard to Lyt antigens is whether they play a role in the function of T cells.

Previous attempts to block T cell killing of allogeneic target cells by a variety of antibodies including Lyt antisera (in the absence of added complement) were generally unsuccessful (16, 17), although successful blocking studies with Lyt 2 antibodies have been recently reported (18, 19). The availability of highly specific high titer monoclonal antibodies, reactive with T cell membrane components, prompted us to investigate the effect of these antibodies on T cell-mediated cytotoxicity and T cell-proliferative responses. In this paper we describe the inhibition of certain T cell functions by anti-Lyt-2 antibodies.

[1] *Abbreviations used in this paper:* α, anti; C, complement; CML, cell-mediated lymphocytotoxicity; Con A, concanavalin A; FACS, flourescence-activated cell sorter; FCS, fetal calf serum; [$^3$H]TdR, tritiated thymidine; MLC, mixed lymphocyte culture; M-MSV, Moloney murine sarcoma virus.

## Materials and Methods

*Mice.* 8- to 12-week-old BALB/c, CBA/J, AKR/J, DBA/2J, C3H/eJ, SWR/J, BALB/B, BALB/K, and C57BL/6J male mice were used.

*Target Cells.* LSTRA-Moloney virus-induced lymphoma cells of BALB/c origin, P815-mastocytoma cells of DBA/2 origin, EL-4 lymphoma cells of C57BL/6 origin, and MSV85-Moloney virus-induced sarcoma cells of BALB/c origin, were used as target cells for cell-mediated lymphocytotoxicity (CML) assays. They were maintained in culture as cell lines.

*Antibodies.* Monoclonal Lyt-1 (subclone 53 - 7.313) and Lyt-2 (subclone 53 - 6.72) antibody-producing hybridoma cell lines were a generous gift from J. Ledbetter of Stanford University. They secrete γ2A antibodies directed to constant determinants of mouse Lyt-1 and Lyt-2 (20). 31-11 is a monoclonal rat γ2A antibody directed to a constant determinant of mouse Thy-1 (21). 42-21 is a monoclonal rat IgM antibody directed to a constant determinant of mouse Thy-1 (22). 43-13 is a monoclonal rat γ2B antibody specific for an AKR thymic lymphoma antigen (21). All antibodies were harvested from cultures of the hybridoma cells. To be able to use high antibody concentrations in small volumes, hybridoma culture media supernates were precipitated with 50% ammonium sulfate, the precipitate was dissolved in a 10th of the initial volume, and dialyzed four times against RPMI-1640 medium.

*Pretreatment of Cells with Anti- (α) Lyt and Complement (C).* Because the monoclonal Lyt antibodies which we used for blocking studies were not cytotoxic with complement, (although they are efficient in sandwich cytotoxicity systems [J. Ledbetter. Personal communication.]), conventional Lyt antisera were used for this purpose. Pelleted spleen cells ($10 \times 10^6$) were suspended in 0.4 ml antiserum diluted 1:10 in medium and incubated for 30 min at 4°C. The cells were then washed once and resuspended in 1.5 ml of rabbit serum diluted 1:8 in medium. After incubation at 37°C for 30 min the cells were washed twice and resuspended in the culture medium.

*Generation of Effector Cells.* For generation of allogeneic killer cells $2 \times 10^7$ spleen cells were cultured with $2 \times 10^7$ mitomycin C-treated stimulator spleen cells (mitomycin C added at 40 μg/ml for 1 h) in 5 ml of RPMI 1640 medium supplemented with glutamine, antibiotics, 0.05 mM 2-mercaptoethanol, and 15% heat-inactivated fetal calf serum (FCS). Cells were harvested after 4 d in culture and tested for cytolytic activity. Purified T cells were prepared by passage of spleen cells over nylon wool columns (23). Nylon wool nonadherent spleen cells are usually 75–85% Thy-1 positive in our laboratory, as compared to 25–30% of untreated spleen cells. For generation of syngeneic killer cells, BALB/c mice were injected intramuscularly with Moloney sarcoma virus (M-MSV). Spleen cells were assayed for killing activity 14 d after immunization. Such spleen cells do not lyse Moloney-infected C57BL/6J cells, nor do C57BL/6J Moloney-immune spleen cells lyse LSTRA or MSV85 targets.

*Cell-mediated Cytotoxicity Assay.* Cytotoxic activity was assayed in microtiter plates containing $1 \times 10^4$ $^{51}$Cr-labeled target cells and $2 \times 10^5$ allogeneic effector cells or $1 \times 10^6$ syngeneic effector cells in 0.2 ml medium per well. The plates were incubated for 3 h (allogeneic killing) or for 6 and 14 h (syngeneic killing) at 37°C. Cultures were terminated by centrifugation at 400 g for 10 min at 4°C. Supernates were collected and counted in a Beckman gamma counter (Beckman Instruments, Fullerton, Calif.) Percentage of specific lysis was calculated as follows: (experimental $^{51}$Cr release − spontaneous release)/(maximum $^{51}$Cr release − spontaneous release) × 100.

*Mixed Lymphocyte Cultures (MLC).* Proliferative responses of allogeneic mixed cells were assayed in microtiter plates. $2 \times 10^5$ responder spleen cells were incubated with $2 \times 10^5$ mitomycin C-treated stimulator spleen cells in 0.2 ml medium per well. At 90 h, 1 μCi tritiated thymidine ([$^3$H]TdR) was added, cultures were terminated at 96 h and [$^3$H]TdR incorporation was determined.

*Activation of Cells with Concanavalin A (Con A).* $2 \times 10^5$ spleen cells were incubated in microplates in 0.2 ml medium containing 5 μg/ml Con A. At 48 h, 1 μCi [$^3$H]TdR was added, cultures were terminated at 54 h and [$^3$H]TdR incorporation was determined.

*Analysis of Cell Populations by the Fluorescence-activated Cell Sorter (FACS).* $2 \times 10^6$ cells were incubated with 0.1-ml monoclonal antibodies diluted 1:10 in medium +5% FCS. After a 20-min incubation on ice, the cells were washed through FCS. The cell pellet was resuspended in 0.05 ml of a fluorescein-conjugated rabbit anti-rat Ig second stage and incubated for 20 min on

ice. After an additional wash through FCS the cells were fixed in 1 ml of 1% formalin for FACS analysis.

## Results

*Blocking of Effector Cell Cytotoxicity by Lyt-2 Antibodies.*   C57BL/6J effector cells were generated by in vitro sensitization with mitomycin C-treated BALB/c spleen cells and tested for cytotoxicity against BALB/c LSTRA (H-2$^d$) and DBA/2 P815 (H-2$^d$) target cells. The effect of Lyt antibodies on killer cell activity in the absence of added complement is shown in Table I. P815 target cells, known as extremely fragile, and LSTRA cells, which are less sensitive targets, were used in parallel to exclude a possible influence of the overall efficiency of the killing system on the results obtained. With both target cells, blocking of cytotoxicity was mediated by αLyt-2 but not by αLyt-1. Blocking by αLyt-2 is at the level of the effector cells and not the target cells, because both LSTRA and P815 cells do not bind Lyt antibodies. 31-11 (αThy-1) antibodies, which bind to the effector cells to the same (or greater) extent as Lyt-2 antibodies (Table VI), did not block cytotoxicity, indicating that mere coating of killer cells with antibodies is insufficient to inhibit the cytolytic activity. 43-13 antibodies are specific for an AKR thymic lymphoma and do not bind to normal lymphoid cells (21). Because they were produced and processed by the same procedure as the other antibodies, they were used as negative controls, to exclude a nonspecific effect of concentrated Ig on cytotoxic reactions.

Lyt antibodies were further tested for their effect on killing of syngeneic tumor cells. BALB/c mice were injected with MSV and the cytotoxic activity of their spleen cells was assessed using as target cells both LSTRA-BALB/c Moloney virus-induced lymphoma cell line, and MSV85-BALB/c Moloney virus-induced sarcoma cell line. The two different target cells were employed because it had been suggested that T cells reactive in short-term killing of lymphoma cells are distinct with respect to their Lyt phenotype from those reactive against adherent cells (24). As shown in Table II, αLyt-2 blocked killing of both LSTRA and MSV85 target cells, whereas no blocking effect could be demonstrated with αLyt-1 or αThy-1.

*Blocking of Mixed Lymphocyte Reactions by Lyt-2 Antibodies.*   Proliferation of T cells was

### TABLE I
*Inhibitory Effect of αLyt-2 Antibodies on Allogeneic Killer Cells**

| Experi-ment | Target cells | Antibodies‡ | | | | |
|---|---|---|---|---|---|---|
| | | — | 43-13 | 31-11 | αLyt-1 | αLyt-2 |
| 1 | P815 | 66§ | 66 | 81 | 67 | 27‖ |
| | LSTRA | 30 | 37 | 35 | 35 | 7¶ |
| 2 | P815 | 50 | ND** | 42 | 46 | 15‖ |
| | LSTRA | 35 | ND | 24 | 36 | 5¶ |

\* C57BL/6J spleen cells were sensitized in vitro to BALB/c cells. For details on the cytotoxicity assay see Materials and Methods.
‡ Antibodies added at a final dilution of 1:8.
§ Percent specific $^{51}$Cr release in the presence of the indicated antibodies.
‖ Significantly lower than control (P = 0.001).
¶ Significantly lower than control (P < 0.01).
** Not determined.

TABLE II

Inhibitory Effect of αLyt-2 Antibodies on Syngeneic Killer Cells*

| Experi-ment | Target cells | Antibodies‡ | | | | |
|---|---|---|---|---|---|---|
| | | — | 43-13 | 31-11 | αLyt-1 | αLyt-2 |
| 1 | LSTRA | 23§ | 23 | 32 | 21 | 3‖ |
| | MSV85 | 19 | 26 | 27 | 29 | 7¶ |
| 2 | LSTRA | 32 | 34 | 58 | 39 | 13‖ |
| | MSV85 | 33 | 35 | 25 | 47 | 11¶ |

* BALB/c mice were injected intramuscularly with Moloney sarcoma virus. Spleen cells were assayed for cytotoxic activity 14 d after immunization.
‡ Antibodies added at a final dilution of 1:8.
§ Percent specific $^{51}$Cr release in the presence of the indicated antibodies.
‖ Significantly lower than control ($P < 0.001$).
¶ Significantly lower than control ($P < 0.02$).

TABLE III

Inhibitory Effect of αLyt-2 Antibodies on Cell Proliferation in MLC*

| Experi-ment | Antibodies‡ | | | | | Percent inhibition by αLyt-2 |
|---|---|---|---|---|---|---|
| | — | 43-13 | 31-11 | αLyt-1 | αLyt-2§ | |
| 1 | 82,777‖ | 81,435 | 83,150 | 91,156 | 32,278 | 60 |
| 2 | 118,052 | 83,210 | ND¶ | 94,994 | 36,050 | 71 |
| 3 | 128,692 | 90,188 | 88,553 | 101,981 | 41,408 | 68 |
| 4 | 54,067 | 65,363 | ND | 102,622 | 21,856 | 60 |

* C57BL/6J responder spleen cells were cultured with mitomycin C-treated BALB/c cells. Thymidine incorporation was determined at 96 h.
‡ Antibodies added at a final dilution of 1:20 at times 0, 24, 48, and 72 h.
§ Values for αLyt-2 are statistically different from control values ($P < 0.001$).
‖ Counts per minute in the presence of the indicated antibodies.
¶ Not determined.

induced by allogeneic stimulation in MLC. 0.01 ml of antibodies was successively added to each culture at time 0, 24, 48, and 72 h. No complement was added. [$^3$H]-TdR was added at 90 h and cultures were harvested at 96 h. The results of four independent experiments are presented in Table III. The pattern of antibody effects on MLC resembled that observed for cytolysis, namely: αLyt-2 inhibited cell proliferation in response to allogeneic stimulus, whereas αLyt-1 or αThy-1 exerted no effect on the response; in fact, in some experiments αLyt-1 enhanced the proliferative response. Cell proliferation was inhibited by anti-Lyt-1 antibodies as well when nylon wool-purified spleen lymphocytes were responding in MLC (Table IV). Thus, anti-Lyt-2 antibodies exert the same effect on whole spleen cell populations and on cell preparations enriched for T cells.

It is widely accepted that both Lyt-1$^+$ and Lyt-2$^+$ cells contribute to the MLC response to allogeneic antigens (15, 25). As shown in Table V, we ascertained that our cultures follow this rule. Thus, pretreatment of responder cells with either Lyt-1 or Lyt-2 antibodies and complement reduced subsequent proliferation in MLC; depletion of Lyt 1$^+$ cells resulted in 74% reduction, whereas depletion of Lyt 2$^+$ cells resulted in 46% reduction in MLC activity. The finding that Lyt-2 antibodies, but

TABLE IV

*Inhibitory Effect of αLyt-2 Antibodies on Proliferation of Nylon Wool-purified Spleen Cells\**

| Experiment | Antibodies‡ | | | | Percent inhibition |
|---|---|---|---|---|---|
| | — | 31-11 | αLyt-1 | αLyt-2 | |
| 1 | 25,582§ | ND‖ | 31,377 | 6,291 | 75 |
| 2 | 22,109 | 25,057 | 31,601 | 9,175 | 60 |

\* C57BL/6J responder spleen cells were passed over nylon wool columns and cultured with mitomycin C-treated BALB/c cells. Thymidine incorporation was determined at 96 h.
‡ Antibodies added at a final dilution of 1:20.
§ Counts per minute in the presence of the indicated antibodies.
‖ Not determined.

TABLE V

*The Effect of Pretreatment with Lyt Antiserum and C on the Capacity to Proliferate in MLC*

| Experiment | C57BL/6J spleen cells treated with:\* | cpm | Percent inhibition |
|---|---|---|---|
| 1 | C | 64,185 | |
| 2 | αLyt-1 + C | 16,680 | 74 |
| 3 | αLyt-2 + C | 35,009 | 46 |
| 4 | Cells of groups 2 and 3 mixed (1:1 ratio) before culture | 60,728 | 6 |

\* After treatment of C57BL/6J spleen cells with antibodies and C, cells in each group were adjusted to the same final concentration of viable cells, mixed with BALB/c stimulator cells and cultured for 4 d.

not Lyt-1 antibodies, blocked MLC when present in the cultures (Table III) in spite of a requirement for both Lyt-1$^+$ and Lyt-2$^+$ subclasses, indicates a functional or structural difference between Lyt-1 and Lyt-2.

It should be noted that the inhibition levels (60–70%) of cell proliferation, as assessed by thymidine incorporation, are in fact higher (75–88%) when considering T cell proliferation, since 20% of the lymphoblasts in the MLC were non-T cells (Table VI), thus resistant to antibodies against T cell antigens. Such high inhibition levels by αLyt-2 would be conceivable if the majority of T cells proliferating on the 5th d of MLC are of Lyt-2 phenotype. The cells harvested after 4 d in culture were therefore stained with Lyt antibodies followed by a fluorescein-conjugated rabbit anti-rat reagent, and analyzed by the FACS. As shown in Table VI, the harvested lympho-blasts expressed predominantly Lyt-2. Although 50% of MLC-induced blast cells were positive for Lyt-1, the intensity of their stain was very close to background. (The median fluorescense was only 9 units above background, compared to 56 units for Lyt-2.) This dull staining was not caused by technical problems, because unstimulated spleen cells and Con A-induced blast cells were brightly stained by the same Lyt-1 antibodies. It therefore appears that mixed lymphocyte reactions select for Lyt-2$^+$ cells. Whether the Lyt-2$^+$ MLC-induced blast cells also express low amounts of Lyt-1 or are Lyt-1$^-$ cannot be concluded from the FACS-analysis, because fluorescence is

TABLE VI

*Typing of Cells Proliferating in MLC and in Response to Con A*

| Test cells | Antibodies used for first stage pretreatment* | Percent positive | Median fluorescence‡ |
|---|---|---|---|
| Normal spleen cells | αLyt-1 | 22.3 | 57 |
| | αLyt-2 | 15.4 | 46 |
| | 31-11 | 28.8 | 91 |
| | 43-13 | 0.2 | — |
| | — | 0.7 | — |
| MLC-induced blast cells | αLyt-1 | 50.0 | 9 |
| | αLyt-2 | 71.8 | 56 |
| | 31-11 | 80.6 | 60 |
| | 43-13 | 2.6 | — |
| | — | 1.7 | — |
| Con A-induced blast cells | αLyt-1 | 66.6 | 45 |
| | αLyt-2 | 81.2 | 53 |
| | 31-11 | 86.6 | 88 |
| | 43-13 | 1.2 | — |
| | — | 1.2 | — |

* Treatment of cells with the indicated antibodies as first stage, was followed by treatment with fluorescein-conjugated rabbit anti-rat Ig antibodies as second stage. Cells were then analyzed by the FACS for percentage of positive cells and intensity of their stain. Only large blast cells were analyzed.
‡ FACS units expressing intensity of cell staining as compared to background fluorescence.

TABLE VII

*The Effect of Lyt Antiserum and C on the Capacity of MLC-Blasts to Proliferate* *

| Experiment | Treatment | cpm | Percent inhibition‡ |
|---|---|---|---|
| 1 | C | 44,596 | |
| 2 | αLyt-1 + C | 36,152 | 24 |
| 3 | αLyt-2 + C | 19,794 | 71 |
| 4 | αLyt-1 + αLyt-2 + C | 12,722 | 91 |
| 5 | 42-21 + C | 9,258 | |

* C57BL/6J anti-BALB/c lymphoblasts were harvested on 5th d of MLC and treated with Lyt antiserum and complement. Cells were labeled with [$^3$H]-TdR after treatment and thymidine uptake was determined.
‡ Because 42-21 antibodies kill 100% of T cells, cpm after treatment with 42-21 were used as background for calculation of percent inhibition by αLyt.

measured by arbitrary units. Borderline levels should therefore be considered on a quantitative, rather than exclusively qualitative, basis.

To study further the Lyt phenotype expressed by cells proliferating in MLC, cells harvested on the 5th d of MLC were assayed for thymidine uptake after treatment with Lyt antibodies and complement. As shown in Table VII treatment of MLC-stimulated cells with αLyt-2 and complement significantly reduced their proliferation. 42-21 is a monoclonal IgM antibody directed to mouse Thy-1. It is a highly efficient

cytolytic antibody which kills 100% of T cells, including MLC-induced blast cells. When compared to thymidine uptake after treatment with 42-21 plus complement, αLyt-2 plus complement eliminated 71% of MLC-induced T cell proliferation, whereas αLyt-1 plus complement eliminated only 24% of the proliferating activity. These results indicate, in agreement with the data obtained by staining, that the majority of T cells proliferating on the 5th d of MLC are of Lyt-2 phenotype.

*The Effect of T Cell Monoclonal Antibodies on the T Cell Response to Con A.* The effect of Lyt-1, Lyt-2, and Thy-1 antibodies on the polyclonal activation of T cells by Con A was studied. 0.01 ml of antibodies was added to each culture at time 0 and 24 h, [³H]TdR was added at 48 h and cultures were harvested at 54 h. The results, summarized in Table VIII, demonstrate that distinct effects are mediated by the antibodies on Con A activation, as compared to allogeneic activation. Thus, Con A cultures were inhibited by αThy-1, but not by αLyt-2, whereas MLC was blocked by αLyt-2, but not by αThy-1. αLyt-1 did not affect either Con A or allogeneic stimulation; in fact it occasionally enhanced the proliferative responses.

Although αThy-1 reproducibly inhibited Con A-mediated cell proliferation, levels of inhibition were variable, ranging from 40 to 80%, and dependent on high antibody concentrations. As shown in Table IX, αThy-1 had no effect when added at final dilution of 1:100. This is in contrast to the inhibition of MLC by αLyt-2, which was as effective at 1:100 as at 1:20 dilution, and in fact could be diluted to concentrations lower than 1:100 without losing activity (not shown). It therefore seems that inhibition of MLC by αLyt-2 and inhibition of Con A activation by αThy-1 are mediated by different blocking mechanisms.

*The Effect of Lyt-2 Antibodies on T Cells of Different Mouse Strains.* To ascertain whether the inhibition of Lyt-2⁺ T cell function by αLyt-2 antibodies is a general phenomenon, the experiments described for C57BL/6 anti BALB/c reactions were repeated with other mouse strain combinations. As shown in Table X, cell-mediated cytotoxicity was generally blocked by αLyt-2 in all strain combinations. Blocking by αLyt-2 is at the level of the effector cells, since both P815 and EL₄ target cells do not bind Lyt-2 antibodies.

However, when cell proliferation in MLC was tested, quantitative differences were observed between different strain combinations; spleen cells of certain mouse strains were more readily blocked by αLyt-2 than others (Table XI). In general, C57BL/6J

TABLE VIII

*Con A-induced Lymphocyte Proliferation in the Presence of αThy-1 and αLyt Antibodies**

| Experiment | Antibodies‡ | | | | | Percent inhibition by αThy-1 |
|---|---|---|---|---|---|---|
| | — | 43-13 | 31-11§ | αLyt-1 | αLyt-2 | |
| 1 | 189,502‖ | 179,840 | 99,496 | 440,257 | 177,470 | 47 |
| 2 | 120,001 | 78,965 | 19,212 | 267,465 | 88,249 | 84 |
| 3 | 146,426 | 107,287 | 76,991 | 156,508 | 130,086 | 48 |
| 4 | 111,897 | 74,910 | 43,735 | 131,835 | 102,719 | 62 |
| 5 | 107,891 | 79,042 | ND¶ | 116,773 | 97,228 | ND |

* C57BL/6J spleen cells activated by 5 μg Con A/ml. Thymidine incorporation was determined at 54 h.
‡ Antibodies at a final dilution of 1:20 were added at times 0 and 24 h.
§ Values for 31-11 (αThy-1) are statistically different from control values (*P* < 0.001).
‖ CPM in the presence of the indicated antibodies.
¶ Not determined.

Table IX

Comparison of αLyt-2 and αThy-1 Inhibitory Effect on Lymphocyte Proliferation

| Antibodies added | | Proliferation induced by: | |
|---|---|---|---|
| | | Con A | MLC |
| — | | 154,152* | 84,882 |
| 43-13 | 1:20 | 153,790 | 78,217 |
| | 1:100 | 287,457 | 92,437 |
| 31-11 | 1:20 | 94,114 | 68,139 |
| | 1:100 | 221,888 | 102,925 |
| αLyt-1 | 1:20 | 321,325 | 82,057 |
| | 1:100 | 463,307 | 106,845 |
| αLyt-2 | 1:20 | 314,101 | 35,344 |
| | 1:100 | 230,826 | 32,585 |

* Counts per minute in the presence of the indicated antibodies.

Table X

Inhibitory Effect of αLyt-2 Antibodies on Allogeneic Killer Cells of Different Strains*

| Killer cells | Target cells | Antibodies‡ | | |
|---|---|---|---|---|
| | | — | αLyt-1 | αLyt-2 |
| C57BL/6J anti-BALB/c | P815 | 83§ | 77 | 38 |
| DBA/2J anti-C57BL/6J | EL4 | 31 | 28 | 7 |
| CBA/J anti-C57BL/6J | EL4 | 42 | 39 | 9 |
| CBA/J anti-BALB/c | P815 | 77 | 87 | 30 |
| AKR/J anti-C57BL/6J | EL4 | 58 | 51 | 9 |
| AKR/J anti-BALB/c | P815 | 83 | 84 | 34 |

* Killer cells were induced for 4 d in MLC.
‡ Antibodies added at a final dilution of 1:8.
§ Percent specific $^{51}$Cr release in the presence of the indicated antibodies.

cells manifested the highest inhibition rate, BALB/c and CBA cells were significantly blocked, but with an inhibition percentage somewhat lower than the rates demonstrated by C57BL/6 cells, and AKR or DBA/2 cells manifested low inhibition rates. A possible explanation to account for such differences between various strains may be a quantitative difference in the fraction of Lyt-2$^+$ cells proliferating in MLC. Nylon wool- purified normal spleen cells of the various strains were therefore stained with Lyt antibodies followed by fluorescein-conjugated rabbit anti-rat reagent and the Lyt phenotype of the cells was analyzed. Striking differences were found in the Lyt phenotype of different mouse strains (Table XII). C57BL/6 normal spleen T cells contain a very large fraction of Lyt-2 cells, compared to other strains. MLC blast cells also exhibited differences in the cell fraction positive for Lyt-2 (Table XIII), although not as marked as the differences demonstrated for normal cells, possibly because MLC reactions select for Lyt-2$^+$ cells which proliferate and take over the culture. As

Table XI

*Inhibitory Effect of αLyt-2 Antibodies on MLC Carried Out with Different Strain Combinations*

| Experiment | Strain combination | Percent inhibition by αLyt-2 |
|---|---|---|
| 1 | C57BL/6J anti-BALB/c | 51 |
| | BALB/c anti-C57BL/6J | 41 |
| | CBA/J anti-BALB/c | 46 |
| | CBA/J anti-C57BL/6J | 9 |
| | AKR/J anti-SWR/J | 31 |
| | AKR/J anti-C57BL/6J | 24 |
| 2 | C57BL/6J anti-BALB/c | 51 |
| | C57BL/6J anti-C3H/eJ | 59 |
| | BALB/c anti-C3H/eJ | 33 |
| | DBA/2J anti-SWR/J | 36 |
| | CBA/J anti-SWR/J | 21 |
| | CBA/J anti-BALB/c | 33 |
| 3 | C57BL/J anti-BALB/c | 55 |
| | C57BL/J anti-CBA/J | 58 |
| | C57BL/J anti-AKR/J | 38 |
| | C57BL/J anti-DBA/2J | 60 |
| | BALB/c anti-C57BL/6J | 40 |
| | BALB/c anti-CBA/J | 47 |
| | BALB/c anti-AKR/J | 56 |
| | CBA/J anti-BALB/c | 54 |
| | AKR/J anti-BALB/c | 27 |

Table XII

*Lyt Phenotype of Normal Spleen Cells Derived from Different Strains*[*]

| Strain | Percent of T cells[‡] | |
|---|---|---|
| | Lyt-1 | Lyt-2 |
| C57BL/6J | 90 | 72 |
| BALB/c | 86 | 25 |
| CBA/J | 85 | 38 |
| DBA/2J | 92 | 28 |
| AKR/J | 92 | 42 |

[*] Nylon wool-purified spleen cells were typed.

[‡] Cells were stained with 31-11 (αThy-1), αLyt-1, and αLyt-2. The fraction of Lyt-1- and Lyt-2-positive cells was calculated as percentage of the Thy-1-positive cells.

mentioned above, since MLC-induced lymphoblasts express very low amounts of Lyt-1 with staining intensity close to background, no qualitative differences regarding Lyt-1 phenotype can be detected. However, since Lyt-2 staining of MLC lymphoblasts is very bright, it may be concluded, that MLC-induced blast cells differ in the fraction of Lyt-2$^+$ cells when different strains are tested. This finding accounts for the observed differences in efficiency of MLC-inhibition by αLyt-2. It should be noted, however, that of 16 strain combinations tested, none was blocked by αLyt-1 (data not shown); MLC blocking was mediated by αLyt-2 exclusively. Anti-Lyt-2 antibodies also blocked

TABLE XIII

Typing of Cells Proliferating in MLC of Different Strain Combinations

| Strain combination | Percent of T cells* | |
| --- | --- | --- |
| | Lyt-1 | Lyt-2 |
| C57BL/6J anti-BALB/c | 50 | 77 |
| C57BL/6J anti-AKR/J | 81 | 90 |
| BALB/c anti-C57BL/6J | 40 | 53 |
| BALB/c anti-AKR/J | 80 | 49 |
| BALB/c anti-CBA/J | 72 | 60 |
| DBA/2J anti-C57BL/6J | 50 | 23 |
| DBA/2J anti-AKR/J | 70 | 41 |
| DBA/2J anti-CBA/J | 70 | 41 |
| CBA/J anti-C57BL/6J | 54 | 62 |
| DBA/J anti-BALB/c | 75 | 45 |
| AKR/J anti-C57BL/6J | 84 | 31 |
| AKR/J anti-BALB/c | 75 | 54 |

* Cells were stained with 31-11 (αThy-1), αLyt-1, and αLyt-2. The fraction of Lyt-1- and Lyt-2-positive cells was calculated as percentage of the Thy-1-positive cells. Only large lymphoblasts were analyzed.

TABLE XIV

Inhibitory Effect of αLyt-2 Antibodies on MLC of Congenic Strains*

| Strain combination | Percent inhibition by αLyt-2 |
| --- | --- |
| BALB/c anti-BALB/B | 48 |
| BALB/c anti-BALB/K | 46 |
| BALB/B anti-BALB/c | 62 |
| BALB/K anti-BALB/c | 45 |

* Mice with BALB background were used. BALB/c :H-2$^d$, BALB/B: H-2$^b$, BALB/K: H-2$^k$.

MLC when congenic BALB combinations were studied (Table XIV), indicating that Lyt-2 antibodies are effective both in MLC induced by H-2 differences alone or by H-2 in combination with other possible allogeneic differences.

## Discussion

Previous attempts to block allogeneic killer cells by alloantibodies and xenoantibodies to various T cell surface antigens have generally failed (16, 17), although blocking of allogeneic cytotoxicity by Lyt-2 antibodies has been recently reported (18, 19). In the present study we succeeded in blocking effector function of killer cells by Lyt-2 antibodies in the absence of complement. Both short term killing of allogeneic cells and longer syngeneic cytotoxic responses against lymphoma or sarcoma target cells were inhibited. The blocking is at the level of the effector cells which bind the antibodies, rather than the target cells, which do not react with the antibodies. The discrepancy between the present observation and previous reports that Lyt-2 antibodies do not inhibit CML (16, 17) may be explained by differences in the antibodies. We used rat monoclonal antibodies, which recognize a constant region determinant of mouse Lyt molecule, whereas other investigators used conventional alloantisera, directed to the Ly alloantigens 1 and 2. Xenoantibodies against constant region

determinants may be more potent in inhibition of CML than the alloantibodies used in the past. Nevertheless, it has been recently reported that after screening of individual sera, few anti-Lyt-1 alloantisera were found to inhibit CML (18, 19). Our observations reinforce these findings and corroborative data (W. Seaman et al. unpublished observations.) demonstrating allogeneic cytotoxicity blockade on P815 cells with rat anti-Lyt-2 (53-6.72) and anti Lyt 3 (53-5.8), indicating the significance of CML inhibition by Lyt-2 antibodies.

The significance of Lyt-2 in certain T cell responses is further stressed by the blocking effect of Lyt-2 antibodies on MLC. The finding that Lyt-1 antibodies do not interfere with MLC is of particular interest. Lyt-1$^+$ cells are required as helper cells for the proliferation of Lyt-2$^+$ cells and for the generation of Lyt-2$^+$ killer cells. Thus, pretreatment of responder spleen lymphocytes with $\alpha$Lyt-1 and complement results in poor response to allogeneic stimuli. Whatever the helper mechanism of Lyt-1$^+$ cells, antibodies to Lyt-1 have no effect on the helper function. If cells of Lyt-1 phenotype proliferate in MLC, they do so only during early stages of the MLC. We demonstrated that although unstimulated spleen cells are strongly positive for both Lyt-1 and Lyt-2, after 4 d in culture the large cells are predominantly Lyt-2 positive, but negative or weakly positive for Lyt-1. Thus, in the allogeneic MLC which we studied there is selection for proliferation of Lyt-2$^+$ cells. This proliferation is inhibited by Lyt-2 antibodies. Whether these proliferating Lyt-2$^+$ cells are Lyt-1 negative or weakly positive, remains undetermined. It has been recently suggested that differences in expression of Lyt-1 antigen are quantitative rather than qualitative and therefore the designation high vs. low expression of Lyt-1 should be used instead of Lyt-1$^+$ vs. Lyt-1$^-$, even with regard to cytotoxic cells (19).

The observation of marked differences in proportions of Lyt-2$^+$ cells in different strains was surprising. However, recent studies with monoclonal anti-Lyt-2 antibodies (26), similarly demonstrated a high proportion of Lyt-2$^+$ cells in C57BL/6 as compared to BALB/c mice. This finding should call attention to studies on relative proportions and interactions of T cell subsets, when different mouse strains are studied.

The significance of Lyt-2 in certain T cell responses is not contradicted by the differences in Lyt-2$^+$ cell proportion manifested by different strains. Functions of Lyt-2$^+$ cells, such as cell-mediated cytotoxicity and cell proliferation are blocked by anti-Lyt-2 antibodies in all strains. The differences in Lyt-2$^+$ cell proportions is only quantitatively reflected in our experiments by the efficiency of blocking MLC with $\alpha$Lyt-2. Obviously, in cultures which contain 50% Lyt-2$^+$ cells, only 50% of the activity would be blocked by $\alpha$Lyt-2. It should be noted, however, that even in cultures with relatively low proportions of Lyt-2$^+$ cells, $\alpha$Lyt-1 antibodies had no inhibitory effect, further emphasizing the significance of Lyt-2 molecules in T cell functions.

Although the $\alpha$Thy-1 and $\alpha$Lyt-1 antibodies used in our studies were of the same rat $\gamma$2A immunoglobulin class as the Lyt-2 antibodies, had high titers, and their binding to T cells was as strong or stronger than that of Lyt-2 antibodies, they had little or no effect on CML or on MLC proliferation. The strong blocking activity of Lyt-2 antibodies therefore suggests a functional significance of Lyt-2 molecules.

The mechanism of inhibition of CML and MLC is yet unclear. The possibility that agglutination could prevent T cells from reacting with their stimulator or target cells seems unlikely, because other antibodies, such as $\alpha$Thy-1, showed comparable if not stronger binding and agglutinating capacity, but had no inhibitory effect. For the

same reason it is unlikely (although possible) that binding of antibodies alters cell surface in a way which prevents normal function. Such an explanation would imply a peculiar nature of Lyt-2 molecules, which following binding of antibodies cause membrane alteration not mediated by the reaction of antibodies with Lyt-1 or Thy-1 cell membrane components.

Another possibility is that Lyt-2 molecules, though distinct from the antigen-receptor, are directly involved in the killing of target cells, either by release of mediators or by local interaction with the target cells. This explanation may provide a correlation between the Lyt-2$^+$ phenotype and function of killer cells. However, it does not account for the observation that Lyt-2 antibodies inhibit cell proliferation in MLC.

Lyt-2 antibodies may interfere with T cell function at the level of T cell receptors. If Lyt-2 determinants are adjacent to T cell receptors, antibodies bound to Lyt molecules may block the receptors by steric hindrance. An alternative possibility is that Lyt-2 is an integral part of the antigen-receptors. In this case, Lyt-2 determinants would likely be components of a constant portion of the receptor. The finding that genes for K-light chain are closely linked to the Lyt-2 genes (27-29) may imply a model whereby the receptor of the killer T cell is composed of Lyt-2 determinants as a constant portion and $V_k$ determinants as a variable part. Since helper cells are Lyt-1$^+$2$^-$, and since a role of Ig heavy chain-linked gene products in the antigen-combining sites of these helper cells has been suggested (7-9), one would have to speculate that different subsets of T cells use different gene products for their receptors.

Con A, a polyclonal activator, stimulates T cells to undergo proliferation events similar to those induced by specific antigen. However, in contrast to inhibition of MLC by Lyt-2 antibodies, the same antibodies had no blocking effect on activation induced by Con A. This lack of inhibitory effect cannot be attributed to resistance of Lyt-2$^+$ cells to Con A, because it has been reported that Con A activates Lyt-1 and Lyt-2 subclasses to the same degree (30), and we have demonstrated that lymphoblasts activated by Con A express both Lyt-1 and Lyt-2 antigens. Con A stimulation is, however, blocked by Thy-1 antibodies, when high antibody concentrations are added. It may be that Thy-1, as a prominent T cell membrane component, is directly or indirectly associated with T cell mitogenesis; hence, when all Thy-1 determinants are coated by anti-Thy-1 , mitogen-induced proliferation is blocked. Elsewhere we have demonstrated that monoclonal anti-Thy-1 antibodies are extremely potent in the inhibition of proliferation of several neoplastic T cell lymphomas, a phenomenon we ascribe to inhibition of triggering of T-lymphoma mitogen receptors (21). It is possible that these T lymphomas are neoplastic counterparts of some Con-A responsive T cell subsets. Whatever the mechanism of Con A blocking by $\alpha$Thy-1 is, the fact that an antigen driven stimulation is sensitive to Lyt-2 antibodies, whereas nonspecific stimulation is resistant to the same antibodies, is in favor of the hypothesis that Lyt-2 antibodies interfere with T cell functions at the level of T cell antigen-receptor.

## Summary

Monoclonal anti-Lyt-2 antibodies blocked effector function of cytotoxic thymus-derived (T) cells in the absence of added complement. Cytolysis of both allogeneic cells and syngeneic lymphoma or sarcoma target cells was inhibited at the level of the effector lymphocytes. Anti-Lyt-1 and anti-Thy-1 antibodies did not block killer cells.

Proliferation of T cells in mixed lymphocyte culture was also inhibited by anti-Lyt-2, but not affected by anti-Lyt-1 or anti-Thy-1 antibodies. Although Lyt-1$^+$ lymphocytes were required in the mixed lymphocyte reaction as helper cells for proliferation of Lyt-2$^+$ lymphocytes, their helper function was not affected by the presence of Lyt-1 antibodies. Thus, although anti- Lyt-1, anti-Lyt-2 and anti-Thy-1 were of the same γ2A immunoglobulin class, had high titers, and interacted with T cells to the same extent, only anti-Lyt-2 blocked T cell functions. Polyclonal activation of T lymphocytes by concanavalin A, in contrast to activation by alloantigens, was not inhibited by Lyt-2 antibodies, suggesting that Lyt-2 antibodies interfere with T cell function at the level of the T cell antigen-receptor. The role which Lyt-2 molecules may play in T cell function is discussed.

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