Involvement of CD8$^+$RT1.B$^+$ and CD4$^+$RT1.B$^+$ Cells of Cervical Lymph Nodes in the Immune Response After Corneal Transplantation in the Rat

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Abstract: Graft rejection reactions have been observed with concomitant lymphocyte infiltrations after allogenic corneal transplantation, although the cornea is considered to be relatively protected from the systemic immune response. In order to characterize the lymphocytes that accumulate in cervical lymph nodes following transplantation, we used a model of orthotopic penetrating keratoplasty in inbred rats. After grafting, the time course of the pathological scoring was monitored, and subpopulations of CD4$^+$RT1.B$^+$ and CD8$^+$RT1.B$^+$ cells were analyzed in the cells harvested from the cervical lymph nodes. The number of CD8$^+$RT1.B$^+$ cells increased 1 week after grafting, reaching the maximum at 3 weeks; whereas CD4$^+$RT1.B$^+$ cells were induced 1 week after the grafting and remained constant during the next 3 weeks. There were four times as many CD4$^+$RT1.B$^+$ cells as CD8$^+$RT1.B$^+$ cells 1 week after grafting when there was no rejection. Therefore, it appears that CD8$^+$RT1.B$^+$ and CD4$^+$RT1.B$^+$ cells in the cervical lymph nodes do participate in ocular immunologic responses. **Jpn J Ophthalmol 41:209-216** © 1997 Japanese Ophthalmological Society

Key Words: CD4$^+$RT1.B$^+$ cell, CD8$^+$RT1.B$^+$ cell, cervical lymph nodes, penetrating keratoplasty, two-color-flow cytometry.

Introduction

Corneal transplantation is one of the most successful transplantation procedures in humans, but immunological graft rejection remains the leading cause of corneal graft failure. It is generally agreed that T cell-mediated immune reactions are primarily responsible for corneal allograft rejection.[1,2] A number of studies have supported the idea that CD4$^+$ T cells as well as CD8$^+$ T cells participate in the corneal allograft rejection reaction.[3] However, it is not clear which T cell population is playing the pivotal role.

Currently, inbred rats are widely used for immunological study of orthotopic corneal transplantation. In rejected corneas from rats,[4] class II antigen expression was found to increase markedly. This

change was accompanied by increases in both CD4$^+$ and CD8$^+$ T cell infiltration. However, the question of whether these infiltrating CD4$^+$ and CD8$^+$ T cells express class II antigens remains unanswered. The number of infiltrating cells in the operative cornea seems to be small and we hypothesized that cells in the ipsilateral cervic lymph nodes might provide meaningful information. We undertook the present study to determine if it is possible to analyze those cells by flow cytometry. We therefore analyzed cells from the cervical lymph nodes after corneal transplantation in the rat using a class II (RT1.B) antibody and flow cytometry.

Materials and Methods

Animals

Ninety-one male inbred DA (RT1^{avl}) rats purchased from Japan SLC, Inc., Hamamatsu, and 28 male inbred Fisher (F344, RT1^{lvl}) rats purchased

from Charles River Japan, Ltd., Yokohama, were used in this study. The rats were between 6 and 9 weeks of age and weighed about 200 g.

The DA rats were used as unoperated control animals, recipients for the syngeneic and allogeneic groups, and donors for the syngeneic group. The F344 rats were used as donors for the allogeneic group. All animals were treated in accordance with the National Institutes of Health guidelines on the use of laboratory animals.

Experimental Groups

The rats were divided into three groups: a syngeneic group, an allogeneic group, and an unoperated control group. Both syngeneic and allogeneic groups were composed of 28 animals, and the unoperated control group consisted of seven animals for a total of 63 animals. Seven animals each from the syngeneic and allogeneic groups were killed on days 7, 14, 21, and 28.

Corneal Grafting

A penetrating keratoplasty was performed according to the technique of Williams and Coster,[5] with several modifications. Preoperatively, one drop of 1% atropine sulfate and one drop of 0.5% tropicamide were instilled into the operative eye of each animal. The animal was then anesthetized with 40 mg/kg pentobarbital intraperitoneally. Only one cornea from each recipient rat was transplanted.

The donor animals were given an overdose of pentobarbital by intraperitoneal injection. A central corneal button was then prepared from the donor using an operating microscope. The central corneal button was cut from the donor eye using a trephine 3 mm in diameter, and the excision was completed with corneal scissors. The button was stored in an eye storage medium (EP-II®; Kaken Pharmaceutical Co., Ltd., Tokyo) until used for grafting.

The recipient cornea was prepared with a trephine measuring 2.5 mm in diameter, and the excision was completed with corneal scissors. One drop of sodium hyaluronate (Opegan®; Santen Pharmaceutical Co., Ltd., Osaka) was then instilled into the anterior surface of the lens. The donor corneal button was secured with eight interrupted 10-0 monofilament nylon sutures (Alcon Surgical, Inc., Fort Worth, TX, USA). The loose ends were cut as short as possible and the knots were left exposed. The cornea and lens were kept moist with a sterile balanced salt solution throughout the operation. At the end of the procedure, the anterior chamber was reformed by an air bubble.

One drop of 1% atropine sulfate and one drop of 0.3% gentamicin sulfate were applied to the grafted eye immediately after the operation. Animals were examined on the first postoperative day; those with surgical failures due to wound dehiscence, iris prolapse, and hyphema were excluded from the study and killed. Atropine sulfate 1% and 0.3% gentamicin sulfate were instilled into the same eye twice daily for the first postgraft week. The sutures were not removed postoperatively.

Pathological Evaluation

The corneal grafts were examined by slit-lamp microscopy every other day throughout the observation period, and compound pathological scores were computed. The pathological scores were defined as previously described.[6] This score was based on the sum grades 0–4 for opacity, 0–2 for edema, and 0–3 for vascularization. The scoring system for clarity was 0, transparent; 1, slight; 2, details of the iris obscured, but pupil margin still clearly visible; 3, pupil margin obscured; and 4, a whitish opaque cornea. The scoring for edema was 0, none; 1, slight; and 2, marked with an elevated margin of the graft. The scoring for vascularization was 0, none; 1, present but does not pass the suture; 2, passed the suture; and 3, vascularization of the entire graft. A graft having a total score of 4 or more was recorded as rejected on that day.

Two-Color Flow Cytometry

Flow cytometry was performed according to the technique of Harada et al,[7] with several modifications. Three ipsilateral cervical lymph nodes of the recipient rat were removed and dispersed in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 5% fetal calf serum (FCS) (Gibco), and single-cell suspensions were prepared. After being washed in RPMI 1640 medium + 5% FCS, the mononuclear cells were obtained by Ficoll-Paque® density centrifugation (Pharmacia LKB, Uppsala, Sweden) and washed twice in RPMI 1640 medium + 5% FCS. Contaminating red blood cells (RBCs) were lysed in a Tris RBC lysing buffer for 5 minutes. The cells were then washed twice, suspended in 1 mL of PBS + 0.1% BSA + 0.1% NaN$_3$. One mL of the cell suspension was divided into 0.1 mL and 0.9 mL quantities. The cells in 0.1 mL were stained with propidium iodine and counted.

The cells in 0.9 mL were exposed to fluorescein isothiocyanate (FITC)-labeled and phycoerythrin (PE)-labeled antibodies at 4°C for 30 minutes. Primary monoclonal antibodies (Table 1) were used.

Table 1. Monoclonal Antibodies

Monoclonal Antibody	Specificity
RT1.B (OX-6)	MHC class II
CD4 (W3/25)	T-helper/Inducer Lymphocytes
CD8 (OX-8)	T-suppressor/Cytotoxic Lymphocytes
CD45R (OX-33)	B Lymphocytes
CD11b/c (OX-42)	Macrophage/Monocytes

The combinations of reagents used were: (1) FITC anti-rat CD4 (W3/25) antibody (Cedarlane, Hotnby, Ontario, Canada) and PE anti-rat RT1.B (OX-6) antibody (Pharmingen, San Diego, CA, USA); (2) FITC anti-rat CD8 antibody (OX-8) (Cedarlane) and PE RT1.B antibody; (3) FITC anti-rat CD45R (OX-33) antibody (Cedarlane) and PE CD4 antibody (Serotec, Indianapolis, IN, USA); (4) FITC CD45R antibody and PE CD8 antibody (Serotec); and (5) FITC anti-rat CD11b/c (OX-42) antibody (Cedarlane) and PE anti-mouse IgG_1 (Becton-Dickinson, Mountain View, CA, USA). Immunofluorescence of the membrane was evaluated by flow cytometry (EPICS ELITE; Coulter Immunology, Hialeah, FL, USA). Forward scatter (FS) was set to exclude dead cells and debris. Electronic gating was performed on all cell samples. Gate A in Figures 1A, 1C, 2A, and 2C
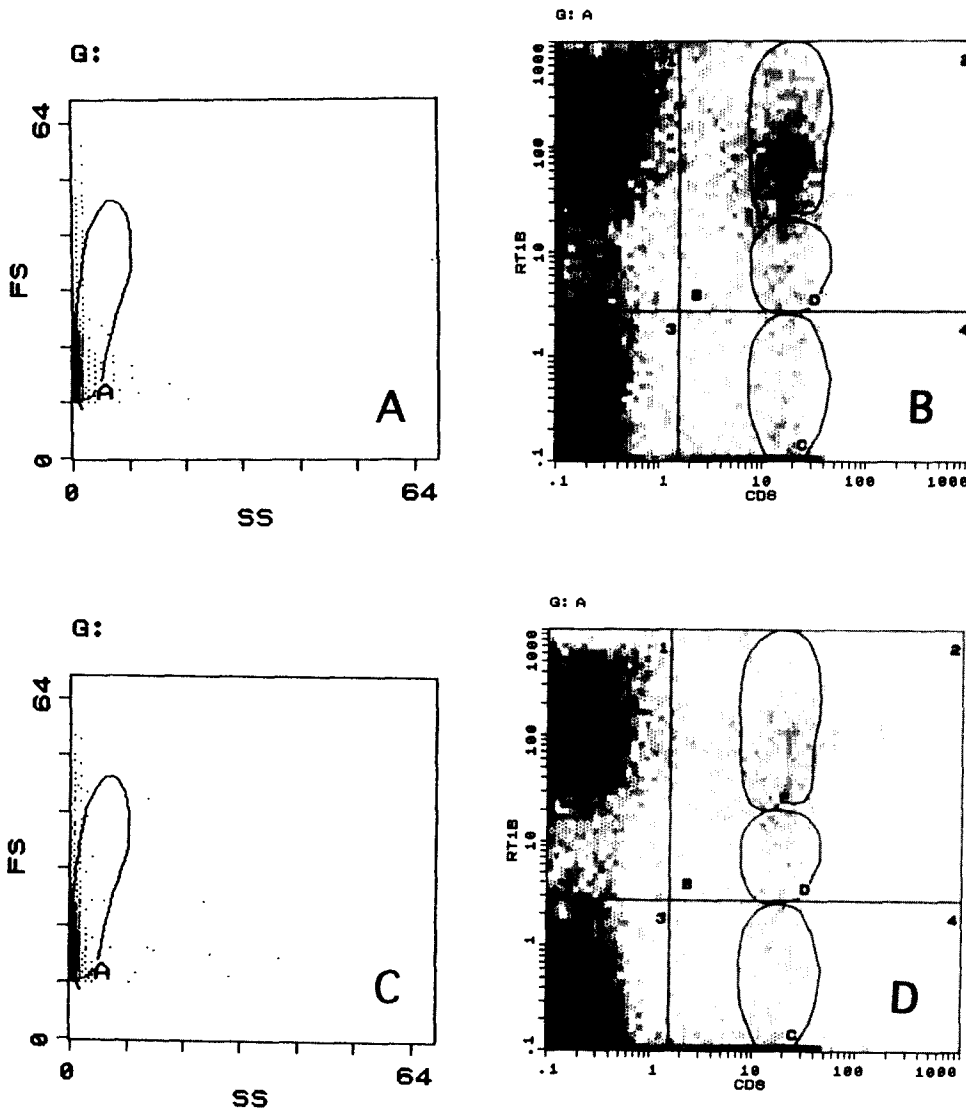
Figure 1. Two-color analysis of lymph node cells with FITC anti-CD8 antibody and PE anti-RT1.B antibody at week 3 after grafting. Analysis of the allogeneic group (**A, B**) and the syngeneic group (**C, D**). (**A**) and (**C**) depict gate A used to distinguish lymphocytes from the other cells. Gate E in (**B**) and (**D**) separated the strongly immunopositive CD8⁺ and RT1.B positive cells; gate D in (**B**) and (**D**) separated the weakly immunopositive CD8⁺ and RT1.B cells.

was used to distinguish lymphocytes from other cells. The proportion of lymphocytes was consistently more than 98% of all analyzed cells. Two-color flow cytograms with FITC staining (x-axis, log scale) and PE staining (y-axis, log scale) are presented in Figures 1B, 1D, 2B, and 2D). The percentage of immunopositive cells was calculated by flow cytometry. Immunopositive cells were calculated using the following formula:

number of positive cells = number of cells in 0.1 mL × 10 × % of positive cells × 1/100

Statistical Analysis

The data are reported as means ± standard deviation. Group differences in the number and percent of cells were compared using an unpaired Student's t-test. A P value < 0.05 was accepted as statistically significant.

Results

Pathological Evaluation

In the syngeneic grafts, vascularization of the peripheral donor graft was often seen around the su-
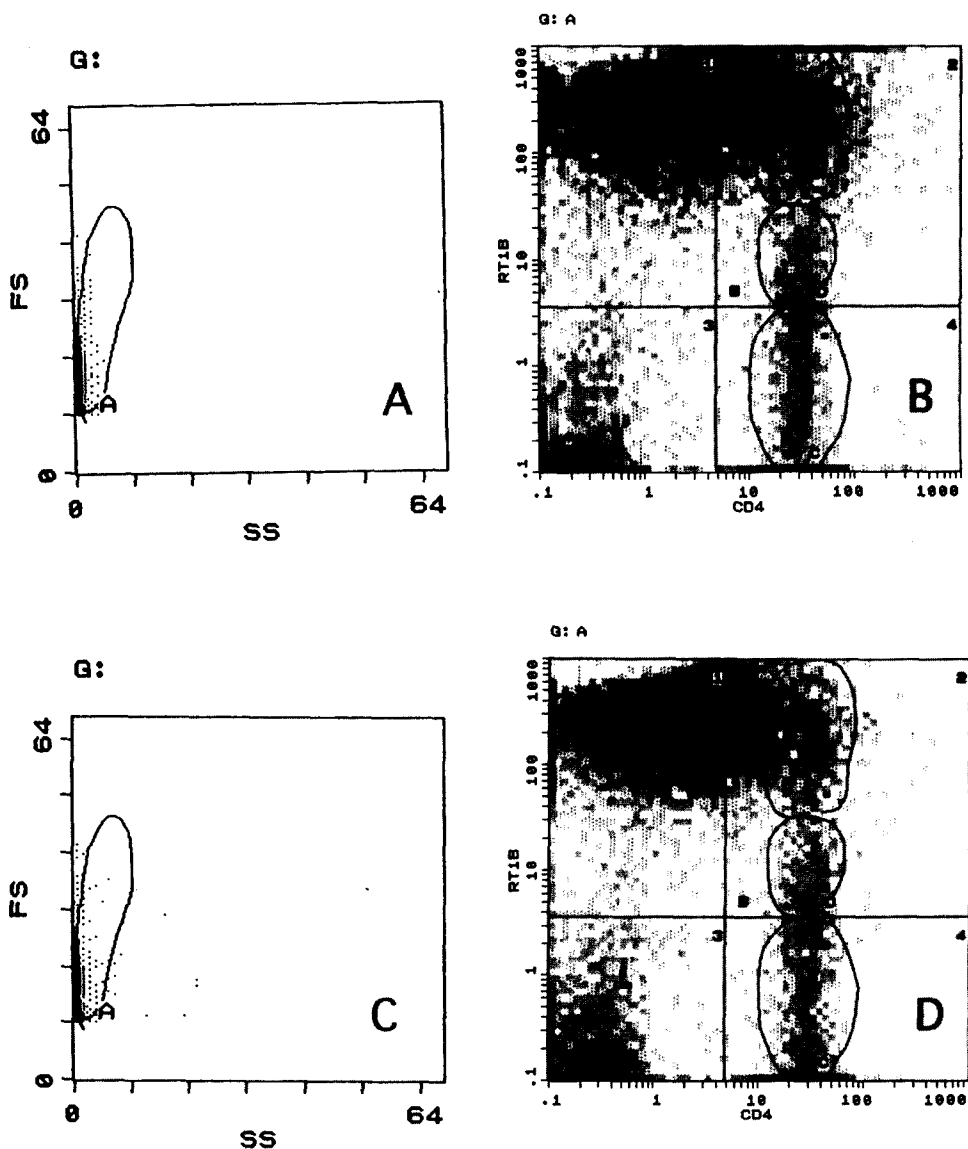
Figure 2. Two-color analysis of lymph node cells with FITC anti-CD4 antibody and PE anti-RT1.B antibody at week 3 after grafting. Analysis of the allogeneic group (**A, B**) and the syngeneic group (**C, D**). (**A**) and (**C**) depict gate A used to distinguish lymphocytes from the other cells. Gate E in (**B**) and (**D**) separated the strongly immunopositive CD4⁺ and RT1.B cells. Gate D in (**B**) and (**D**) separated the weakly immunopositive CD4⁺ and RT1.B cells.

tures. No rejection was observed in syngeneic grafts during the 4-week observation period. In the allogeneic group, rejection occurred 8–10 days after grafting (mean survival time: 8.8 days; median: 9 days). These results are summarized in Figure 3.
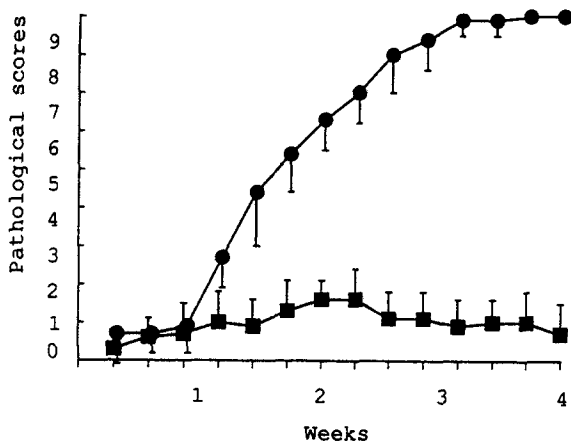
$CD8^+RT1.B^+$ Cells in Cervical Lymph Nodes

As shown in Figure 1B and 1D, a gradation of immunostaining for RT1.B was apparent. RT1.B immunopositive cells could be divided into two patterns (Figure 1B and 1D); strongly immunopositive cells (gate E) and weakly immunopositive cells (gate D), according to the primary conditions of the flow cytometry.
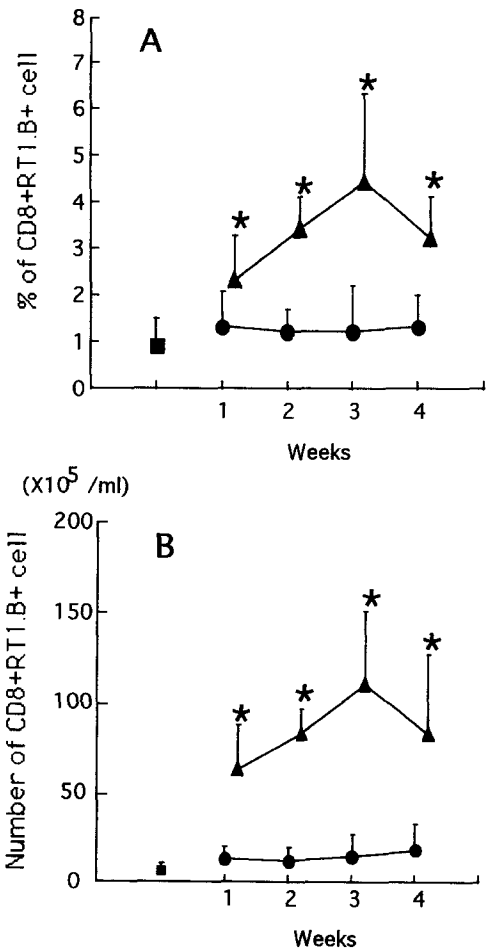
In the syngeneic group, the number and percentage of CD8⁺RT1.B⁺ cells remained constant, and there were no significant differences between the unoperated control group and syngeneic groups throughout the study. In the allogeneic group, the number and percentage of CD8⁺RT1.B⁺ cells peaked at 3 weeks postgraft and then dropped back to the 2-week level at 4 weeks postgraft. The increase in the number and proportion of CD8⁺RT1.B⁺ cells in the allogeneic group was statistically significant when compared to the syngeneic group. The percentages and the numbers of CD8⁺RT1.B⁺ cells in three groups are shown in Figure 4.

$CD4^+RT1.B^+$ Cells in Cervical Lymph Nodes

The immunostaining pattern of CD4⁺RT1.B⁺ cells was similar to the CD8⁺RT1.B⁺ cells. RT1.B

Figure 4. CD8⁺RT1.B⁺ cells in the cervical lymph nodes. **(A)** Percentage of CD8⁺RT1.B⁺ T cells. **(B)** Number of CD8⁺RT1.B⁺ T cells. Unoperated control (■), Syngeneic (●), Allogeneic (▲). Each point represents the mean ± SD (n = 7). In the allogeneic group, a significant increase (*P < 0.05) in the number and percentage of CD8⁺RT1.B⁺ T cells was observed compared with the syngeneic group during the 4-week observation period. There were no significant differences in the number or percentage of CD8⁺RT1.B⁺ T cells between the unoperated control group and syngeneic groups.

Figure 3. Pathological scores of the graft. Syngeneic (■), Allogeneic (●). The sum of the scores in the allogeneic group increased and reached a plateau at week 3. Scores of the syngeneic group did not exceed 4 during the 4-week observation period.
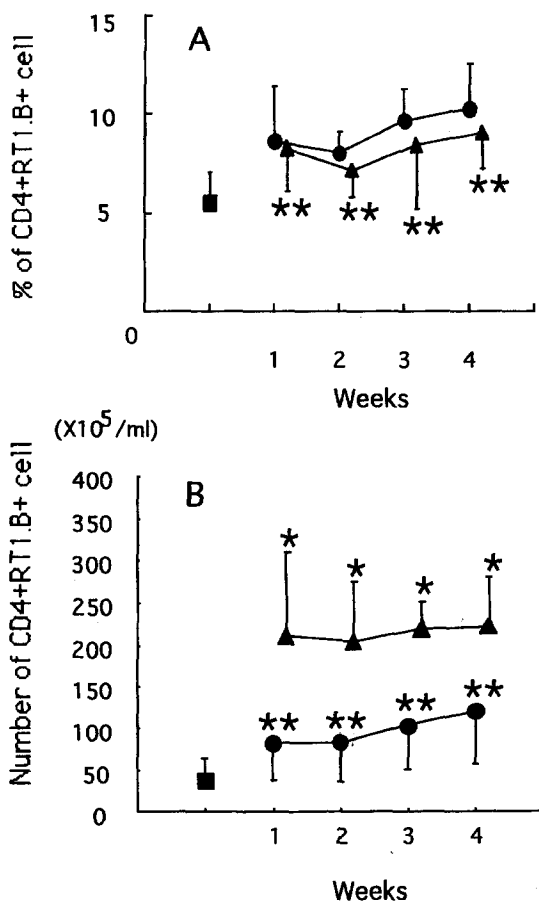
immunopositive cells could also be divided into two patterns (Figure 2B and 2D): strongly immunopositive cells (gate E) and weakly immunopositive cells (gate D), according to the primary conditions of the flow cytometry.

The percentage of CD4⁺RT1.B⁺ cells decreased slightly 2 weeks after the surgery and then increased slightly in both allogeneic and syngeneic groups. There were no significant differences between the allogeneic and syngeneic groups. Compared with the unoperated control group, there were significant increases in both groups each week. The percentages

of CD4$^+$RT1.B$^+$ cells in the syngeneic and allogeneic groups at various time points after the surgery are shown in Figure 5A.

In the syngeneic group, the number of CD4$^+$RT1.B$^+$ cells increased significantly after the corneal grafting compared with the unoperated control group. The number of immunopositive cells continued to increase during the observation period. In the allogeneic group, the number of CD4$^+$RT1.B$^+$ cells increased significantly to 2–3 times greater than the syngeneic group at each time point tested, and re-

mained elevated at 6–7 times the naive values after the corneal grafting. The numbers of CD4$^+$RT1.B$^+$ cells in the three groups are shown in Figure 5B.

CD45R$^+$CD4$^+$ Cells in Cervical Lymph Nodes

The percentages of CD45R$^+$CD4$^+$ cells in the allogeneic and syngeneic groups did not exceed 0.6%. There were no differences in either group compared to the unoperated control group.

CD45R$^+$CD8$^+$ Cells in Cervical Lymph Nodes

The percentages of CD45R$^+$CD8$^+$ cells in the allogeneic and syngeneic groups did not exceed 0.8%. There were no differences in either group compared to the unoperated control group.

CD11b/c$^+$ Cells in Regional Lymph Nodes

The percentages of CD11b/c$^+$ cells in the allogeneic and syngeneic groups did not exceed 2.8% during the 4 weeks. There were no differences in either group compared to the unoperated control group.

Discussion

In this report, the cells that were analyzed by two-color flow cytometry are considered lymphocytes because the proportion of the monocytes that were gated was more than 98%. Studies using monoclonal phenotypic markers for mononuclear cell subsets are complicated by overlaps in cell function that cannot be reflected by the cell marker. For example, markers for T-helper lymphocyte populations can cross-react with mononuclear phagocytes and dendritic cells[8]; however, in this study, the percentage of CD11b/c$^+$ cells was no greater than 2.8% in the three groups. It is, therefore, likely that the CD4$^+$ cells are T-helper lymphocytes. It is also well known that B cells express class II antigens; however, the percentage of CD45R$^+$CD4$^+$ and CD45R$^+$CD8$^+$ cells was no greater than 0.8% and 0.6%. Thus, the cell population of the gates set for analysis of CD4$^+$RT1.B$^+$ and CD8$^+$RT1.B$^+$ cells was likely to be T cells, not B cells. Specifically in the present study, we demonstrated that class II antigen was detected on T cells obtained from ipsilateral cervical lymph nodes after corneal transplantation using a two-color analysis method of flow cytometry.

It is well known that the rat model, which allows corneal transplantation with a constant histocompatibility barrier, can be useful in studies of rejection.[5,9] Young et al[10] suggested that class II antigens acti-

Figure 5. CD4$^+$RT1.B$^+$ T cells in the cervical lymph nodes. Unoperated control (■), Syngeneic (●), Allogeneic (▲). Each point represents the mean ± SD (n = 7). (A) Percentage of CD4$^+$RT1.B$^+$ T cells. There were no significant differences between the syngeneic and allogeneic groups. When compared to the unoperated control group, there was a significant increase (**P < 0.05) in both the syngeneic and allogeneic groups each week. (B) Number of CD4$^+$RT1.B$^+$ T cells. A significant increase (*P < 0.05) was seen in the allogeneic group compared to the syngeneic group during the 4-week observation period. There was a significant increase (**P < 0.05) in both the syngeneic and allogeneic groups as compared to the unoperated control group each week.

vated by cytokines play a critical role in rejection of allografted corneas. In addition, an immunohistopathologic study[11] showed that class II antigen expression was more extensive in the allogeneic grafts than in the syngeneic grafts. Another study[4] demonstrated that increased class II antigen expression correlated with a relative increase in CD8⁺ T cell and CD4⁺ T cell in grafted corneas. We speculate that the CD4⁺RT1.B⁺ and CD8⁺RT1.B⁺ cells in the cervical lymph nodes detected in our study participate in the rejection process of corneal grafts.

Early studies[12,13] demonstrated that rejection of grafts occurred in the absence of any detectable delayed-type hypersensitivity response, however, cytotoxic T lymphocyte (CTL) reactivity was elicited. More recently, treatment with monoclonal antibodies (mAb) has been done in experimental corneal transplantation as a consequence of the considerable progress of hybridoma technology. Conversely, some recent studies of treatment with anti-CD4 antibodies or anti-CD8 antibodies[14,15] suggested that CD8⁺ T cells were not required for rejection, whereas CD4⁺ T cells played a critical role. Pleyer et al[16] stressed the importance of CD4⁺ cells as potential targets for immunomodulation in corneal transplants. Roelen et al[17] reported that primed CTL was less inhibited by anti-CD8 mAb compared with naive CTL. More attention is being given to understanding the functions of both the CD4⁺ and CD8⁺ T cells. In this study, when rejection had not occurred at 1 week, the number of CD4⁺RT1.B⁺ cells reached a plateau, whereas the number of CD8⁺RT1.B⁺ cells was minimal. The ratio of CD4⁺RT1.B⁺ cells to CD8⁺RT1.B⁺ cells was about 4:1. Thus, CD4⁺RT1.B⁺ cells in cervical lymph nodes appear to play a more dominant role than CD8⁺RT1.B⁺ cells in cervical lymph nodes in the early phase of rejection.

After the rejection occurred, both the number and percentage of CD8⁺RT1.B⁺ cells increased considerably and peaked at 3 weeks. This change appears to be in accordance with the observations of our previous reports[6,18,19] in which we demonstrated that CTL activities in the host splenocytes were greatest at 3 weeks using the same combination of rats. Peeler et al[13] reported that rats experiencing rejection of allografts developed CTL activities that drained the lymph nodes and spleen, and the CTL responses from spleen or lymph node effector cells were similar in magnitude after rejection. Therefore we believe that CD8⁺RT1.B⁺ cells and CTL play a similar part after rejection. Holland et al[4] described a penetrating keratoplasty model in the rat in which CD8⁺ (T-suppressor/cytotoxic) cells became the ma-

jor infiltrating cell in allografts after rejection. Holland et al[4] also described how class II antigens were seen on inflammatory cells. It is, therefore, possible that CD8⁺RT1.B⁺ cells in cervical lymph nodes are active after corneal allograft rejection rather than before rejection.

References

1. Khodadoust AA, Silverstein AM. Induction of corneal graft rejection by passive cell transfer. Invest Ophthalmol 1976;15: 89–95.
2. Pepose JS, Nestor MS, Gardner KM, Foos RY, Pettit TH. Composition of cellular infiltrates in rejected human corneal allografts. Graefe's Arch Clin Exp Ophthalmol 1997;222:128–33.
3. Gebhardt BM. Cell-mediated immunity in the cornea. Transplantation 1988;46:273–80.
4. Holland EJ, Olsen TW, Chan CC, Bergstrom L, Palestine AG, Nussenblatt RB. Kinetics of corneal transplant rejection in the rat penetrating keratoplasty model. Cornea 1994;13: 317–23.
5. Williams KA, Coster DJ. Penetrating corneal transplantation in the inbred rat: A new model. Invest Ophthalmol Vis Sci 1985;26:23–30.
6. Minamoto A, Sakata H, Okada K, Fujihara M. Suppression of corneal graft rejection by subconjunctival injection of FK-506 in a rat model of penetrating keratoplasty. Jpn J Ophthalmol 1995;39:12–9.
7. Harada H, Kawano MM, Huang N, Harada Y, Iwato K, Tanabe O, et al. Phenotypic difference of normal plasma cells from mature myeloma cells. Blood 1993;81:2658–63.
8. Moscicki RA, Amento PE, Krane SM, Kurnick JT, Colvin RB. Modulation of surface antigens of a human monocyte cell line, U937, during incubation with lymphocyte-conditioned medium: Detection of T 4 antigen and its presence on normal blood monocytes. J Immunol 1983;131:743–8.
9. Treseler PA, Sanfilippo F. The expression of major compatibility complex and leukocyte antigens by cells in the rat cornea. Transplantation 1986;41:248–54.
10. Young E, McMillan BA. "Lymphocyte-defined" antigens on corneal fibroblasts. Curr Eye Res 1986;5:273–81.
11. Holland EJ, Chan CC, Wetzig RP, Palestine AG, Nussenblatt RB. Clinical and immunohistologic studies of corneal rejection in the rat penetrating keratoplasty model. Cornea 1991;10:374–80.
12. Callanan D, Peeler J, Niederkorn JY. Characteristics of rejection of orthotopic corneal allografts in the rat. Transplantation 1988;45:437–43.
13. Peeler J, Niederkorn J, Matoba A. Corneal allografts induce cytotoxic T cell but not delayed hypersensitivity responses in mice. Invest Ophthalmol Vis Sci 1985;26:1516–23.
14. Ayliffe W, Alam Y, Bell EB, Hutchinson IV. Prolongation of rat corneal graft survial by treatment with anti-CD4 monoclonal antibody. Br J Ophthalmol 1992;76:602–6.
15. He Y, Ross J, Niederkorn JY. Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. Invest Ophthalmol Vis Sci 1991;32:2723–8.
16. Pleyer U, Milani JK, Dukes A, Chou J, Lutz S, Ruckert D, Thiel HJ, Mondino BJ. Effect of topically applied anti-CD4 monoclonal antibodies on orthotopic corneal allografts in a rat model. Invest Ophthalmol Vis Sci 1995;36:52–61.

17. Roelen DL, Van Beelen E, Van Bree SPMJ, Van Rood JJ, Völker-Dieben H, Claas FHJ. The presence of activated donor HLA class I-reactive T lymphocytes is associated with rejection of corneal grafts. Transplantation 1995;59:1039–42.

18. Minamoto A, Sakata H, Tsumura K, Yamamoto M, Kusunoki K. Induction of cytotoxic T lymphocytes from splenocytes after orthotopic penetrating keratoplasty in the rat. Ophthalmologica 1994;208:105–9.

19. Okada K, Sakata H, Minamoto A, Fujihara M. Effect of FK 506 administered topically versus intramuscularly on suppression of the corneal immune reaction in rats. Ophthalmologica 1996;210:175–9.