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

Koji Okada,* Hiromu K. Mishima,* Michio M. Kawano,† Hideaki Mizote* and Atsushi Minamoto*

*Department of Ophthalmology, Hiroshima University School of Medicine; †Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan

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 cervical 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 (RT1av) rats purchased from Japan SLC, Inc., Hamamatsu, and 28 male inbred Fisher (F344, RT1b1) 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, 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. 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, 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% NaN₃. 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

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Specificity</th>
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<tbody>
<tr>
<td>RT1.B (OX-6)</td>
<td>MHC class II</td>
</tr>
<tr>
<td>CD4 (W3/25)</td>
<td>T-helper/Inducer Lymphocytes</td>
</tr>
<tr>
<td>CD8 (OX-8)</td>
<td>T-suppressor/Cytotoxic Lymphocytes</td>
</tr>
<tr>
<td>CD45R (OX-33)</td>
<td>B Lymphocytes</td>
</tr>
<tr>
<td>CD11b/c (OX-42)</td>
<td>Macrophage/Monocytes</td>
</tr>
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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 (Sero-tec, Indianapolis, IN, USA); (4) FITC CD45R antibody and PE CD8 antibody (Sero-tec); and (5) FITC anti-rat CD11b/c (OX-42) antibody (Cedarlane) and PE anti-mouse IgG1 (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:

\[
\text{number of positive cells} = \text{number of cells in } 0.1 \text{ mL} \times 10 \times \% \text{ of positive cells} \times \frac{1}{100}
\]

**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-

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**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.
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 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 the number or percentage of CD8^+RT1.B^+ T cells between the unoperated control group and syngeneic groups.

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.
of CD4+RT1.B+ cells in the syngeneic and alloge-
neic 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 in-
crease during the observation period. In the allogeneic group, the number of CD4+RT1.B+ cells in-
creased 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.

**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 signifi-
cant 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.**

**CD45R+CD4+ Cells in Cervical Lymph Nodes**

The percentages of CD45R+CD4+ cells in the al-
logeneic 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 al-
logeneic 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 al-
logeneic and syngeneic groups did not exceed 2.8% dur-
ing 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 be-
cause 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
er T-helper lymphocyte populations can cross-
react with mononuclear phagocytes and dendritic
cells; 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 de-
tected 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 histocompat-
ibility barrier, can be useful in studies of rejection.5,9
Young et al10 suggested that class II antigens acti-
vated by cytokines play a critical role in rejection of allografted corneas. In addition, an immunohisto-
pathologic study\textsuperscript{11} showed that class II antigen ex-
pression was more extensive in the allogeneic grafts than in the syngeneic grafts. Another study\textsuperscript{4} demonstrated that increased class II antigen expression correlated with a relative increase in CD8\textsuperscript{+} T cell and CD4\textsuperscript{+} T cell in grafted corneas. We speculate that the CD4\textsuperscript{+}RT1.B\textsuperscript{+} and CD8\textsuperscript{+}RT1.B\textsuperscript{+} cells in the cer-
vical lymph nodes detected in our study participate in the rejection process of corneal grafts.

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

After the rejection occurred, both the number and percentage of CD8\textsuperscript{+}RT1.B\textsuperscript{+} cells increased consid-
erably and peaked at 3 weeks. This change appears to be in accordance with the observations of our previous reports\textsuperscript{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\textsuperscript{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\textsuperscript{+}RT1.B\textsuperscript{+} cells and CTL play a similar part after rejection. Holland et al\textsuperscript{2} described a penetrating keratoplasty model in the rat in which CD8\textsuperscript{+} (T-suppressor/cytotoxic) cells became the ma-

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