T Cell Depletion Increases Susceptibility to Murine Cytomegalovirus Retinitis

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To study the effect of immunosuppression on the development of murine cytomegalovirus (MCMV) retinitis, BALB/c mice were immunosuppressed with methylprednisolone (a corticosteroid) and/or with antibodies against CD4+ and CD8+ T cells and inoculated with low-dose MCMV (5 × 102 plaque-forming units) by the supraciliary route. Nonimmunosuppressed mice inoculated with low-dose MCMV by the supraciliary route did not develop necrotizing retinitis. By contrast, 78-100% of immunosuppressed mice developed retinitis after inoculation of low-dose MCMV. To study the effect of depletion of individual T cell subsets, mice were depleted of either CD4+ or CD8+ T cells and inoculated with low-dose MCMV by the supraciliary route. The frequency of retinitis in CD8-depleted mice (30%) was not significantly different from that of nonimmunosuppressed control mice (0%). The frequency of retinitis in the CD8-depleted group (80%) was similar to that observed in mice immunosuppressed with corticosteroid alone (90.9%), with antibodies to both T cell subsets (100%), or with steroid and both T cell subset antibodies (100%). These results support the conclusion that the CD8+ T cell subset is responsible for control of ocular MCMV infection. Furthermore, these results suggest that the CD8+ T cell subset may be important in preventing ocular CMV infection in immunosuppressed patients. Invest Ophthalmol Vis Sci 33:3353-3360, 1992

Necrotizing retinitis caused by cytomegalovirus (CMV) infection is an increasingly important complication in patients with the acquired immune deficiency syndrome (AIDS).14 Although antiviral chemotherapy has been effective in halting the progression of the retinitis,2-7 very little is understood about the pathogenesis of the initiating infection or about the mechanism of tissue destruction. To study this, animal models for CMV retinitis might be useful in elucidating mechanisms of pathogenesis.

Recently, we described a mouse model of necrotizing retinitis caused by the Smith strain of murine CMV (MCMV).8 Supraciliary injection of immunocompetent BALB/c mice with high-dose MCMV (5 × 104 plaque-forming units [PFU]) results in necrotizing retinitis associated with cytomegalic cells in the retina and retinal pigment epithelium, transition zones between involved and uninvolved retina, and progression to full-thickness retinal necrosis in 60.3% of the injected eyes. In this model of primary ocular MCMV infection, the retinal pigment epithelium and the epithelium of the ciliary body appear to be the initial target tissues for the virus, followed by spread of the infection to retinal neurons. Intraocular replication of the virus correlates with the development of typical necrotizing retinitis.

However, in humans, CMV retinitis occurs only in immunodeficient hosts. Although patients who are immunosuppressed from chemotherapy, an underlying hematologic disorder, or after organ transplantation occasionally develop CMV retinitis, this disease most commonly occurs in patients with AIDS who have severely depressed T cell function.8-12 Therefore, we desired to investigate the role of the T cell immune response in modulating the frequency and pattern of necrotizing retinitis in our murine model. We hypothesized that, in immunocompetent mice, the presence of T cells contributes to their protection from necrotizing retinitis. To test this hypothesis, we performed experiments in which mice were immunosuppressed with methylprednisolone and/or cytotoxic antibodies specific for the CD4+ and CD8+ T cell subsets. In these experiments, injection of low-dose MCMV (5 × 102 PFU) by the supraciliary route failed to induce necrotizing retinitis in immunocompetent mice. By contrast, injection of low-dose MCMV into immunosuppressed mice by the supraciliary route resulted in a high frequency of necrotizing retinitis.
Furthermore, depletion of the CD8\(^+\) T cell subset alone was sufficient to permit development of retinitis after supraciliary inoculation of low-dose MCMV.

**Materials and Methods**

**Mice**

Eight- to 12-wk-old female MCMV-seronegative BALB/c mice were used for all experiments, except for propagation of viral stocks (described later). All animals were purchased from Taconic (Germantown, NY). The mice were given unrestricted access to food and water; the animals were maintained in a 12 hr light cycle alternating with a 12 hr dark cycle. All animal procedures were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

**Virus**

The Smith strain of MCMV was used in all experiments. Virus stocks were made by in vivo passage of virus in weanling BALB/c mice. The mice were injected intraperitoneally with \(1 \times 10^2\) PFU of MCMV; 2-3 wk later, all mice were killed, and their salivary glands were removed and homogenized. An aliquot of each virus stock was titered by plaque assay on duplicate cultures of mouse embryo fibroblast cells grown in Dulbecco's modified Eagle's medium (DMEM) tissue culture medium containing 10% fetal calf serum. All virus stocks were stored at -70°C. A fresh aliquot of stock virus (8 \(\times\) 10\(^6\) PFU/ml) was thawed, diluted to the appropriate concentration in serum-free DMEM, and used for a single experiment.

**Supraciliary Inoculation of MCMV**

The animals were anesthetized with pentobarbital (0.65 mg/10 g), and \(5 \times 10^2\) PFU of MCMV (low-dose) in a volume of 2 \(\mu\)l was injected via the supraciliary route as previously described.\(^8\)

**Monoclonal Antibodies**

Hybridomas producing monoclonal antibodies GK 1.5 and 2.43 were obtained from the American Type Culture Collection (Rockville, MD). GK 1.5 secretes a rat anti-mouse CD4-specific antibody of the immunoglobulin (Ig) G2b isotype,\(^1\) and 2.43 secretes a rat anti-mouse CD8-specific antibody also of the IgG2b isotype.\(^4\) Both hybridomas were grown in pristane-primed athymic mice; the ascites was harvested when tense abdominal swelling became apparent. For some experiments, the IgG fraction was partially purified by precipitation with saturated ammonium sulfate followed by dialysis against phosphate-buffered saline.\(^5\) Total protein in ascites and in partially purified IgG fractions was determined by the Bio-Rad protein assay (Richmond, CA). The amount of rat antibody in ascites or purified IgG fractions was determined by enzyme-linked immunosorbent assay as previously described.\(^16\)

**Quantitation of T Cell Depletion**

Several dilutions of the GK 1.5 and 2.43 antibodies were tested in vivo for their ability to deplete the appropriate T cell population. The extent of depletion at each dilution was assessed by flow cytometric analysis as described subsequently.\(^17\) The minimum dose of antibody that resulted in depletion of >98% of the appropriate T cell subset 24 hr later was used in subsequent immunodepletion experiments (1.0 mg of GK 1.5 or 2.43 per animal given on day -2 and day 0). On day 8 postinjection, the spleen was removed from each animal, and the number of splenic T cells was determined. A flow cytometric method was utilized to determine both the percentage and total number of residual T cells and subsets in each experimental animal. Whole spleens were removed and strained through a nylon mesh; the recovered splenocytes were resuspended in 10 ml of serum-free RPMI-1640. A quantitative aliquot from each sample was removed and diluted 1:10 (corticosteroid-treated groups) or 1:100 (controls or antibody-treated groups). The splenocytes were pelleted at 1500 \(\times\) g, and 40 \(\mu\)l of fluorescein isothiocyanate (FITC)-labeled Thy 1.2 (pan-T cell marker), FITC-labeled anti-CD4 (gift of Dr. Thomas Malek, University of Miami School of Medicine, Miami, FL), or FITC-labeled anti-CD8 (Becton Dickinson, San Jose, CA) was added to the pellet. After 30 min, the samples were washed once in RPMI and resuspended in RPMI containing 0.2% paraformaldehyde for 30 min. After one additional wash, the cells were resuspended in 1.0 ml of RPMI containing 1.0 mg/ml RNAse (Sigma, St. Louis, MO), 50 \(\mu\)g/ml of propidium iodide (Sigma), and 1% Tween 20 (Sigma). The total cell concentration within the sample and the percentage of FITC-positive cells were determined for each sample using a Becton Dickinson FACScan analyzer as previously described.\(^17\) The total number of T cells (Thy 1.2\(^+\) cells), CD4\(^+\) T cells, or CD8\(^+\) T cells was determined for each spleen by the formula:

\[
\text{Total no. of T cells in subset} = \text{no. of cells/ml} \times \% \text{FITC-positive for subset marker} \times \text{inverse of the original dilution.}
\]
For ease of interpretation, the results are presented as the percent depletion as calculated by the formula:

\[
\% \text{ depletion} = \frac{\text{Mean no. T-cell subset in experimental group}}{\text{Mean no. T-cell subset in control group}} - 1
\]

(2)

**Histopathologic Preparation and Evaluation**

Eyes for histopathologic examination were enucleated, fixed in buffered formaldehyde, and embedded in paraffin. Each eye was sectioned at six different levels to ensure that a focal area of retinal necrosis would not be overlooked. Each section was stained with hematoxylin and eosin. Microscopic examination of all specimens was performed in a masked fashion. Two types of scoring were used. First, the sections were scored for the presence or absence of necrotizing retinitis to determine the frequency of retinitis within a treatment group. Second, because our experimental protocol altered the severity and the frequency of retinitis, a semiquantitative scoring system was developed. The anterior segment changes in each section were scored according to the following criteria:

- **0** = normal.
- **1** = mild—inflammatory cells but minimal cytomegaly of the iris and ciliary body (CB).
- **2** = moderate—inflammation and cytomegaly of the iris and CB, but minimal necrosis.
- **3** = Severe—cytomegaly or significant necrosis of one side of the CB.
- **4** = Extreme—cytomegaly or significant necrosis of both sides of the CB.

The posterior segment changes in each section were also evaluated semiquantitatively according to the following criteria:

- **0** = normal or injection artifact.
- **1/2** = mild atypical retinopathy—absence of cytomegaly plus retinal folds or vascular cuffing involving less than three-quarters of the section.
- **1** = moderate atypical retinopathy—absence of cytomegaly plus mild changes plus photoreceptor atrophy or retinal infiltration by leukocytes involving more than one-quarter of the section.
- **2** = mild necrotizing retinitis—cytomegaly of retinal cells plus partial or full-thickness necrosis extending beyond a one-eighth section from the CB but less than a one-quarter section; or optic nerve inflammation with peripapillary retinal necrosis.
- **3** = moderate necrotizing retinitis—cytomegaly plus full-thickness retinal necrosis involving one-quarter to three-quarters of the section.
- **4** = severe necrotizing retinitis—cytomegaly with full-thickness necrosis involving the entire retina in that section.

Other unusual features, such as choroidal necrosis or atypical anterior segment involvement, were also noted. All six sections of each eye were scored for the extent of both the anterior segment and retinal involvement using the grading scale. The scores for all sections of each eye were summed (with a maximum score of 24 for the anterior or posterior segment). The median score and the percentage of the maximum score were calculated for each experimental group. The frequency of retinitis results were analyzed for significance by the chi-square test. Because the grading scores were ordinal rather than continuous measurements, the anterior and posterior segment results were analyzed for significance by the Mann-Whitney U test.

**Experimental Protocols**

**Experiment 1:** In the first experiment, four groups of animals (9–11 mice/group) were used. Mice in group 1 were immunosuppressed with methylprednisolone alone (2 mg intramuscularly on days -2, +2, and +6). Mice in group 2 were injected with monoclonal antibodies to both CD4 + and CD8 + T cells (1 mg of each antibody on days -2 and 0). Mice in group 3 were treated with both steroid and monoclonal antibodies to T cells (at the times and dosages described for groups 1 and 2), and mice in group 4 (untreated controls) were injected intraperitoneally with phosphate-buffered saline. An isotype-matched irrelevant antibody was not used because it has been demonstrated that isotype-matched irrelevant monoclonal antibodies do not deplete T cell subsets. All mice were injected with 5 × 10^2 PFU of MCMV via the supraciliary route on day 0. On day 8 postinjection, all mice were killed, and the injected eye of each animal was removed for histopathologic examination. The spleen also was removed, and the extent of residual T cell depletion was assessed by flow cytometry.

**Experiment 2:** Experiment 2 was performed to determine whether one or both T cell subsets was responsible for retinal protection after supraciliary inoculation of MCMV. Groups of ten BALB/c mice were depleted of CD4 + T cells (group 1), CD8 + T cells (group 2), or both CD4 + and CD8 + T cells (group 3). Mice in groups 1–3 were treated with antibodies on days -2 and 0 as described previously. Two groups of mice were repeated from experiment 1: group 4 received steroid and group 5 received steroid and both
antibodies. Mice in group 6 received only an intraperitoneal injection of phosphate-buffered saline. All mice were injected with $5 \times 10^2$ PFU of MCMV via the supraciliary route on day 0. On day 8 postinjection, the animals were killed, the injected eye of each mouse was enucleated for histopathologic study, and the spleen was removed for flow cytometry.

**Results**

**Supraciliary Inoculation of Low-Dose MCMV in Nonimmunosuppressed Mice**

We have demonstrated previously that inoculation of $5 \times 10^4$ PFU of MCMV (Smith strain) by the supraciliary space of euthymic BALB/c mice results in necrotizing retinitis in 60.3% of the animals. Injection of eyes of euthymic mice with lower dilutions of MCMV via the supraciliary route revealed that inoculation of $5 \times 10^2$ PFU (low-dose) produced only anterior segment inflammation without necrotizing retinitis (Table 1). Mice inoculated with this dose of virus had minimal posterior segment disease, characterized by nonspecific retinal folding without progression to retinal necrosis such as typically occurs after inoculation of $5 \times 10^4$ PFU of virus (Fig. 1).

**Supraciliary Inoculation of Low-Dose MCMV in Immunosuppressed Mice**

**Experiment 1:** To investigate the role of the immune system in protecting the eyes from necrotizing retinitis, mice were immunosuppressed before inoculation of MCMV via the supraciliary route. Mice were immunosuppressed using methylprednisolone (steroid), cytotoxic antibodies to CD4+ and CD8+ T cells, or methylprednisolone and antibodies to both T cell subsets. Flow cytometry revealed that residual T cell depletion at day 8 was similar among the three immunosuppressed groups, and the absolute number of T cells in the spleen remained severely diminished in all of the experimental groups (Table 1).

Microscopic evaluation of the retinas of the mice in the four experimental groups for the presence of necrotizing retinitis revealed that 78–90% of the immunosuppressed mice developed focal necrotizing retinitis characterized by cytomegalic cells in the retina and full-thickness retinal necrosis (Fig. 2). The frequency of focal necrotizing retinitis was not significantly different among the three immunosuppressed groups.

By contrast, none of the nonimmunosuppressed animals (group 4) had evidence of necrotizing retinitis, and the retinas of these animals appeared to be essentially normal (Fig. 1).

**Experiment 2:** Because depletion of both T cell subsets in the absence of supplemental steroid therapy resulted in necrotizing retinitis after injection of a low dose of the virus, the contribution of individual T cell subsets in protecting the retina from necrosis was ex-

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**Table 1. Immunosuppression results in retinitis following inoculation of $5 \times 10^2$ PFU of MCMV**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>T cell depletion (day 8 p.i.)</th>
<th>Necrotizing retinitis (retinitis/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Steroid*</td>
<td>67%</td>
<td>90% (9/10)†</td>
</tr>
<tr>
<td>2</td>
<td>t CD4 + t CD8†</td>
<td>75%</td>
<td>78% (7/9)‡</td>
</tr>
<tr>
<td>3</td>
<td>Steroid + t CD4</td>
<td>92%</td>
<td>82% (9/11)‡</td>
</tr>
<tr>
<td>4</td>
<td>PBS</td>
<td>0%</td>
<td>0% (0/10)</td>
</tr>
</tbody>
</table>

* Methylprednisolone, 2 mg, day -2, +2, and +6.
† 1 mg of each antibody, day -2 and day 0.
‡ Significantly different than PBS-control group, $P = 0.002.$
Table 2. CD8 \( ^+ \) T Cells protect against necrotizing retinitis

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Frequency of necrotizing retinitis (retinitis/total)</th>
<th>Median anterior segment score (range) % maximum</th>
<th>Median posterior segment score (range) % maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( aCD4^* )</td>
<td>30% (3/10)</td>
<td>10.5 (6-18)</td>
<td>4.5 (2-11) §</td>
</tr>
<tr>
<td>2</td>
<td>( aCD8^* )</td>
<td>80% (8/10) $\dagger$</td>
<td>43.8</td>
<td>18.8</td>
</tr>
<tr>
<td>3</td>
<td>( aCD4 + aCD8^* )</td>
<td>100% (10/10) $\ddagger$</td>
<td>15 (6-20) §</td>
<td>7.75 (1-15) §</td>
</tr>
<tr>
<td>4</td>
<td>Steroid $\dagger$</td>
<td>90.9% (9/10) $\ddagger$</td>
<td>62.5</td>
<td>7.75 (1-15) §</td>
</tr>
<tr>
<td>5</td>
<td>Steroid + ( aCD4 + aCD8 )</td>
<td>100% (10/10) $\ddagger$</td>
<td>15 (6-20) §</td>
<td>10.25 (6.5-20) §</td>
</tr>
<tr>
<td>6</td>
<td>PBS</td>
<td>0% (0/10)</td>
<td>3 (0.5-4.5) &amp;</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* 1.0 mg of \( aCD4 \) and/or \( aCD8 \), day -2 and day 0.
† Methylprednisolone, 2 mg, day -2, +2, +4.
‡ Significantly different than PBS-control group, \( P \leq 0.001 \) (chi-square).

In the posterior segment, the severity and extent of the retinal changes correlated with the type of immunosuppression. The median posterior segment score in the control mice was relatively low (12.5% of the maximum), which correlated with the absence of necrosis and minimal-to-mild nonspecific inflammatory and degenerative changes observed in this group. Extensive retinal changes were noted in the eyes of mice that received combined steroid and antibody immunosuppression (group 5). The eyes from this group exhibited widespread retinal necrosis, which was accompanied frequently by choroidal necrosis (Fig. 3); the median severity in this group was 42.7%. Similar histopathologic findings, including cytomegaly of retinal cells and focal retinal necrosis, were observed in both the CD8-depleted group (Fig. 4) and in the group

Fig. 3. Photomicrograph of the retina 8 days after supraciliary inoculation of \( 5 \times 10^2 \) PFU of MCMV into a BALB/c mouse treated both with cytotoxic antibodies to CD4\(^+\) and CD8\(^+\) T cells and with steroid. The entire retina and some of the underlying choroid are necrotic. Numerous, cytomegalic cells are seen within what remains of the substance of the retina (hematoxylin and cosin, original magnification \( \times 108 \)).
depleted of both CD4+ and CD8+ T cells (Fig. 2). Mice depleted of only CD4+ T cells developed one of two patterns of retinitis. Seventy percent of the eyes of CD4-depleted mice developed a pattern similar to that seen in the controls, and 30% developed a very mild retinitis accompanied by focal retinal destruction (Fig. 5). The median severity score in this group was 18.8% of the maximum.

Discussion

The experiments presented herein present evidence that: (1) immunosuppression increases susceptibility to necrotizing retinitis after supraciliary inoculation of MCMV and (2) inoculation of low-dose MCMV, which does not induce disease in nonimmunosuppressed mice, produces fulminant retinal disease in immunosuppressed mice. In addition, our results indicate that T cells are critically important for protecting the BALB/c mice injected with low-dose MCMV by the supraciliary route from necrotizing retinitis. Interestingly, there was no significant difference in the frequency of retinitis between groups depleted of CD8+ T cells and mice depleted of both CD4+ and CD8+ T cells. By contrast, only a small percentage of the CD4-depleted mice developed retinitis, and within this group, eyes with retinitis had a very mild form of necrosis that was both qualitatively and quantitatively different than that observed in mice depleted of CD8+ T cells alone or both CD4+ and CD8+ T cells.

In interpreting these results, we cannot rule out the possibility that incomplete depletion of the CD4 population might have permitted the persistence of some antiviral CD4 effector or helper T cells, thereby exaggerating the effectiveness of CD8+ T cells. Although our depletion protocol resulted in nearly 100% depletion of the appropriate T cell subset in the spleen within 24 hr, by day 8 postinjection, 25% of the CD4+ population had been reconstituted. Because complete depletion did not last for the duration of the experiment, low levels of antiviral effector cells may have been present at day 8 postinjection. Nevertheless, using anti-Thy 1.2 antibody to monitor changes in the total T cell population and enumerating the total subset population ensured that the majority of the CD4+ population had actually been eliminated and not merely underestimated because of downregulation of the CD4 surface molecule itself.

The association between CD8 depletion and development of necrotizing retinitis suggests that the CD8+ T cell is important in preventing replication or spread of MCMV in the eyes of nonimmunosuppressed BALB/c mice injected with MCMV by the supraciliary route. The CD8+ T cell subset has been shown to be responsible for anti-MCMV effector activity, presumably by preventing virus-mediated histopathologic changes,19 and other studies have demonstrated that CD4+ T cells are not required for generation of anti-MCMV CD8-mediated effector function.20 In addition, specific antiviral antibody is not required for termination of MCMV infection in most tissues.21 Therefore, it is unlikely that an inability to produce anti-MCMV antibody in T cell-depleted mice would affect the outcome of low-dose MCMV infection. In this ocular model, CD4+ T cells did not appear to be responsible for protecting the retina. Other investigators have demonstrated that CD4+ T cells are important in protecting and limiting virus replication in MCMV-infected mice. In adoptive transfer experiments of T cell subsets into athymic (nu/nu) mice,
Shanley demonstrated that MCMV-specific L3T4 (CD4) T cells alone limited virus replication in the adrenal glands after inoculation of virus by the intranasal route. By contrast, transfer of MCMV-specific Lyt-2 (CD8) T cells had no effect on the titer of virus in the adrenal glands. Differences in the MCMV-protective ability of individual T cell subsets between the current studies and those of Shanley may be the result of the route of inoculation (supraciliary versus intranasal), differences in the overall experimental approach (depletion of individual T cell subsets in nonimmune euthymic mice versus adoptive transfer of single populations of MCMV-immune T cells to athymic mice), or possibly, incomplete depletion of the CD4+ T cell subset.

Although in these experiments the frequency of necrotizing retinitis was similar among all immunosuppressed groups except the CD4-depleted group, important qualitative differences were noted. The extent of retinal involvement, including the choroid, was more severe in the groups of mice treated with steroid, and frequently, the eyes of steroid-treated mice had marked choroidal thickening and choroidal necrosis. In addition, many different leukocytic subpopulations were probably influenced by the steroid therapy. The spleens of steroid-treated mice were extremely atrophic, and preliminary data suggest that, in addition to T cell depletion, the number of B-cells and monocytes was also diminished in steroid-treated mice. Although we did not specifically assess the number of neutrophils and other leukocytic subpopulations, it is also possible that depletion of these cells may have also affected nonspecific antiviral immune capabilities, resulting in more severe disease in steroid-treated groups.

The results of these studies suggest that MCMV retinitis in the immunodepleted mouse might serve as a model of primary ocular CMV infection in the immunosuppressed patient. Many questions regarding the pathogenesis of this model remain to be answered. Although the site of virus infection can be inferred from the location of cytomegalic cells (presumptive evidence of MCMV infection) and our preliminary results indicate that virus replication in the eyes of CD8-depleted mice was higher than in nondepleted mice (Atherton SS, Newell CK, Kanter MY, and Cousins SW, unpublished observations, 1991), further studies are required to define the relationship between viral infection of the retina and the pathogenesis of MCMV retinal necrosis, especially in the setting of CD8 depletion. In nonocular tissue in immunodepleted mice, MCMV-induced tissue damage appears to be mediated mainly by viral replication and associated viral cytopathic effects; there is little cellular infiltrate. Unlike herpes simplex virus type 1 infections of the mouse cornea and retina where an antiviral T cell immune response contributes to viral pathogenesis, MCMV infection in immunocompetent mice is controlled by CD8+ T cells, and CD8+ lymphocytes prevent virus-induced tissue destruction by a mechanism which does not require CD4+ T cells. The finding that CD8+ T cells are important in the control of ocular MCMV infection may have implications for ocular CMV infection in immunosuppressed patients. The results from these studies suggest that CD8+ T cells might also be a controlling factor in patients with AIDS and CMV retinitis and that, perhaps, the absolute number of CD8+ T cells or of a specific subset of CD8+ cells should be determined in patients who are at risk for CMV retinitis.

Key words: retinitis, murine cytomegalovirus, BALB/c mouse, immunosuppression, T cell subsets

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References


