

# Experimental Autoimmune Keratitis Induced in Rats by Anti-Cornea T-cell Lines

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**PURPOSE.** Idiopathic inflammation of the cornea, keratitis, has been proposed to result from an autoimmune process, but thus far no convenient animal model of keratitis exists. An attempt was made to establish an animal model for keratitis, to investigate possible autoimmune mechanisms.

**METHODS.** T-cell lines were established from lymph node cells removed from rats immunized with bovine corneal epithelium (BCE) extract. After restimulation in vitro with BCE or a specific corneal antigen, the cells were transferred by intraperitoneal injection into naive rats, rats subjected to total body irradiation, or rats in which only one eye was irradiated.

**RESULTS.** Neither direct immunization with corneal antigens nor transfer of activated anti-corneal T-cells into naive rats gave any signs of keratitis. Irradiation alone did not induce corneal inflammation. Transfer of corneal-specific activated T cells into irradiated rats produced keratitis starting around day 4 and culminating around day 8. The disease was self-limiting and the severity dependent on the dose and site of radiation. Keratitis was characterized by corneal haze, conjunctival and episcleral hyperemia, episcleral hemorrhages, chemosis, corneal infiltrates, and vascularization. Immunohistochemistry showed T-cell and macrophage infiltration of epithelium and stroma in the affected corneas.

**CONCLUSIONS.** Thus, keratitis may be produced by T cells reactive to corneal antigens, provided that the target tissue has been made susceptible by irradiation. The effectiveness of T-cell vaccination in preventing adoptive keratitis suggests that systemic as well as local tissue factors may regulate the disease process. (*Invest Ophthalmol Vis Sci.* 1999;40:2191-2198)

Idiopathic inflammation and ulceration of the cornea characterizes a group of disorders that usually become chronic and may result in visual impairment or even blindness. These types of keratitis occur either as separate disease entities, in association with episcleritis or other ocular disorders, or as a manifestation of a systemic connective tissue disease such as rheumatoid arthritis, Wegener's granulomatosis, or relapsing polychondritis. The absence of a causative microorganism, the bilateral nature of the disease, and the efficacy, although limited, of immunosuppressive medication has led to the hypothesis that these disorders may result from an autoimmune process.<sup>1,2</sup> Antibodies to corneal and conjunctival tissues, circulating immune complexes, T-cell proliferative responses to corneal extracts, and immunoglobulin depositions

and plasma cell infiltrates in the lesions have been described in patients.<sup>3-5</sup> However, none of these findings proves an autoimmune pathogenesis. Indeed, several findings seem to argue against a role for circulating autoantibodies in the immunopathology of idiopathic keratitis. First, the presence of corneal autoantibodies is not restricted to patients with idiopathic keratitis; cornea-specific autoantibodies can be found in some types of uveitis, in connective tissue disease without keratitis, in Beçhet's disease, and in some healthy individuals.<sup>6-9</sup> Second, although the cornea contains several putative autoantigens,<sup>10-15</sup> it has not been possible to induce autoimmune keratitis in experimental animals. Immunization of rats to corneal extracts or purified antigens such as class 3 aldehyde dehydrogenase (3-ALDH) does not result in keratitis despite the induction of cornea-specific autoantibodies.<sup>16</sup>

In this article, we report the mediation of keratitis by cornea-specific T-cell lines.

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Supported by funds of the Netherlands Organization for Scientific Research (CV) and the Haagsch Oogheekundig Fonds (CV) and by a grant from the Minerva Foundation. Irun R. Cohen is the incumbent of the Mauerberger Chair in Immunology and the Director of the Robert Koch-Minerva Center for Research in Autoimmune Diseases.

Submitted for publication July 20, 1998; revised February 5, 1999; accepted March 3, 1999.

Proprietary interest category: N.

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## MATERIALS AND METHODS

### Animals

Inbred female Lewis rats were purchased from Harlan Olac (Bicester, UK) and maintained at the Weizmann Institute of Science animal care facilities or from HSD/CBP (Harlan Sprague-Dawley, Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands) and housed at The Netherlands Ophthalmic Research Institute under standard conditions. Rats were maintained on laboratory chow and water ad libitum and were used at the age of 8 to 12 weeks for active

immunization or as recipients for adoptive transfer. Experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Antigens

Corneal antigens were prepared from bovine eyes, obtained from a local slaughterhouse, enucleated immediately after exsanguination, and kept on ice. The eyes were rinsed in cold water to remove blood, and the corneal epithelium was scraped off and placed for processing in ice-cold 0.01 M Tris/HCl, pH 8.0, containing 0.5 M NaCl, 20 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The corneal tissue was extracted overnight at 4°C by head-over-head rotation, and the insoluble material was removed by centrifugation (30 minutes, 14,000g). The soluble BCE extract was dialyzed against phosphate-buffered saline (PBS) for 48 hours at 4°C and stored at -20°C (8 mg/ml, determined using Bradford's reagent<sup>17</sup>). 3-ALDH, identical with bovine corneal protein 54 kDa (BCP54),<sup>18</sup> was prepared from bovine corneal epithelium by gel filtration followed by anion-exchange chromatography as described previously.<sup>10</sup> The purity of the 3-ALDH was ascertained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using "PhastGel" minigels (10% to 15% gradient gel; Pharmacia LKB Biotechnology, Uppsala, Sweden), followed by silver staining. Concanavalin A was purchased from Miles Yeda (Rehovot, Israel), and guinea pig myelin basic protein (MBP) was prepared as described previously without anion-exchange chromatography.<sup>19</sup>

### T-Cell Lines

T-cell lines specific for BCE or 3-ALDH were established as described previously.<sup>20</sup> Briefly, 11 days after immunization, cell suspensions were prepared from popliteal lymph nodes. Cells ( $5 \times 10^6$ /ml) were cultured for 3 days with BCE or 3-ALDH (10  $\mu$ g/ml) in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml), 2-ME ( $5 \times 10^{-5}$  M), 1% (vol/vol) MEM-Eagle nonessential amino acids (GIBCO, Gaithersburg, MD), and 1% fresh autologous serum. T-cell blasts were isolated by density centrifugation (Ficoll-Paque; Pharmacia), washed once in PBS, and propagated in DMEM containing 10% (vol/vol) fetal calf serum (GIBCO) and 10% (vol/vol) supernatant of spleen cells stimulated with concanavalin A (1.25  $\mu$ g/ml), used as a source of T-cell growth factors.<sup>20</sup> After 5 days, T cells ( $4 \times 10^5$ ) were restimulated in the presence of antigen and irradiated syngeneic thymocytes ( $10^7$ /ml) and propagated as described above. The cycles of restimulation and rest were repeated at least 5 to 7 times. A control anti-MBP T-cell line was prepared as described previously.<sup>20</sup>

### Keratitis Induction

BCE or 3-ALDH was diluted in PBS to 0.5 mg/ml and emulsified in an equal volume of complete Freund's adjuvant (CFA). CFA was prepared by adding 4 mg/ml of heat-killed *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI) to incomplete Freund's adjuvant (Difco).

Lewis rats were injected in both hind foot pads with 50  $\mu$ l of emulsion. Immunized rats were observed daily for development of keratitis or were used on day 11 after immunization as donors of lymph node cells to raise T-cell lines.

Adoptive transfer was performed by intraperitoneal injection of  $10^7$  activated T cells reactive to BCE or 3-ALDH, with or without prior irradiation of the rats (2.0–7.5 Gy). T cells were activated 3 days before injection by incubation with antigen and irradiated thymocyte antigen presenting cell (APC) as described below. For irradiation, rats were anesthetized systemically with 75  $\mu$ l Hypnorm (fluanisone, 10 mg/ml; and fentanyl citrate, 0.315 mg/ml; Janssen Pharmaceutica, Gooise, The Netherlands) and 50  $\mu$ l diazepam (5 mg/ml). Anesthetized rats were placed on a backscatter block and irradiated with a Siemens Stabilipan x-ray generator, operated at 250 kV and 14 mA, and a 0.5-mm copper filter was used. The dose rate at 52-cm focus skin distance was 94.9 cGy/min for total body irradiation (TBI; aperture  $17 \times 17$  cm) and 88.7 cGy/min for irradiation of the left eye only ( $6 \times 5$  cm aperture). TBI or TBI-excluding the head by a 2-mm shielding device was done with 50% of the dose applied dorsally and 50% ventrally. In some rats, only the left eye was irradiated, using a 2-mm lead shielding device over head and body, with a 9-mm diameter hole located above the eye. In an attempt to circumvent irradiation, splenectomy was performed under Hypnorm anesthesia, 7 days before adoptive transfer of BCE-specific T cells. Control rats underwent sham splenectomy. Some rats were treated with cyclophosphamide (60 mg/kg IP) 48 hours before transfer of T cells. To induce systemic resistance to T-cell-mediated keratitis, naive rats were first injected intraperitoneally with  $10^7$  activated T cells from an anti-BCE T-cell line (BC-1), 8 days later the rats were irradiated (7.5 Gy) and inoculated intraperitoneally with a second dose of  $10^7$  activated BC-1 cells.

Intracorneal injections were performed as described previously.<sup>21</sup> Briefly, rats were anesthetized systemically by 50  $\mu$ l Hypnorm and locally by one drop oxybuprocaine (0.2%). The eyes were immobilized with a forceps, and 5  $\mu$ l activated T cells ( $10^7$ /ml) in PBS, either BCE-specific or MBP-specific, was injected into the corneal stroma (in the center of the cornea) using a 30-gauge needle attached to a Hamilton gas-tight 50- $\mu$ l syringe.

The inoculated rats were examined daily by slit-lamp for signs of keratitis. Inflammation of the cornea and adjacent conjunctiva was scored as follows: hyperemia (0–2), conjunctival/episcleral hemorrhages (0–2), corneal haze (0–2), corneal vascularization (0–2), chemosis (0–1), and (sub-)epithelial corneal infiltrates (0–1); 0 = absent, 1 = mild, and 2 = intense. The clinical score per rat was the sum of the six parameters for both eyes. The maximal possible score per eye was 10, per rat 20. Results are expressed as the arithmetic mean  $\pm$  SEM.

### Proliferation Assay

T cells ( $5 \times 10^5$ /ml) and irradiated thymocytes (APCs,  $5 \times 10^6$ /ml) were cultured in stimulation medium containing BCE, 3-ALDH, or MBP (10  $\mu$ g/ml) in 96-well round-bottomed microplates (Greiner, Nürtingen, Germany). Assays were performed in triplicate, using stimulation medium without added antigen as a negative control. Plates were incubated at 37°C in a humidified incubator containing 7% CO<sub>2</sub> for 72 hours. Sixteen hours before termination, each well was pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (sp. act. 41,000  $\mu$ Ci/mmol; Nuclear Research Centre, Negev, Israel). Cultures were harvested on glass microfiber filters, and [<sup>3</sup>H]thymidine incorporation was measured as counts per minute (cpm). Results are expressed as the arithmetic mean cpm of triplicate samples  $\pm$  SD.

**TABLE 1.** Monoclonal Antibodies Used in this Study

Clone	Source	CD/Specificity
OX6	Sera-Lab MAS46g	Rat RT1 <sup>b</sup> (MHC class II)
W3/13	Sera-Lab MAS010c	CD43: polymorphonuclear cells/plasma cells/T lymphocytes
OX19	Sera-Lab MAS099b	CD5: surface marker of T lymphocytes
OX8	Sera-Lab MAS041c	CD8 $\alpha$ : T cytotoxic/suppressor cells/ subset of natural killer cells
ED1	Serotec MCA341	Macrophages, monocytes, and dendritic cells (cytoplasmatic antigen)
ED2	Serotec MCA342	Resident macrophages (cell surface marker)
ICAM-1	Serotec MCA1333	CD54: ligand for LFA-1(CD11a) and MAC-1(CD11b)

CD, cluster differential; LFA, lymphocyte function-associated antigen; MAC, macrophage integrin also complement receptor (CR3).

### Immunohistochemistry

Animals were killed for immunohistologic evaluation 0, 3, 7, or 12 days after disease induction. Eyes were removed and embedded in gelatin capsules containing Tissue-Tek (OCT Compound 4583; Sakura Fine Tek Europe BV, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Routine histopathology (hematoxylin and eosin [H&E] staining) and immunoperoxidase staining was carried out as described by Verhagen et al.<sup>21</sup> on serial cryostat sections, 8- $\mu$ m-thick, cut across the center of the cornea along the optical axis. Mouse anti-rat antibodies were used as primary antibodies (Table 1). Peroxidase-labeled goat anti-mouse IgG antibody (Dakopatts, Glostrup, Denmark) was used as the second antibody and visualized (brown staining) using diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. Antibodies were diluted in PBS containing 1% normal rat serum. Immunopositive cells infiltrating the tissues were quantified as follows: - (absent),  $\pm$  (very few positive cells), + (some positive cells), ++ (many positive cells), and +++ (very many positive cells). Positively stained cells were scored in three different areas: limbus, peripheral cornea, and central cornea.

## RESULTS

### Proliferative Responses and Characterization of T-Cell Lines to BCE and 3-ALDH

To obtain cornea-specific T-cell lines, Lewis rats were injected with BCE or 3-ALDH emulsified in CFA. BC-1 cells, a cell line obtained from BCE/CFA-immunized rats, showed proliferative responses to BCE and 3-ALDH but not to MBP (Table 2). A T-cell line obtained from rats immunized to 3-ALDH/CFA (AL-1) manifested similar proliferative responses (data not shown). Both BC-1 and AL-1 were found to express CD4 and the  $\alpha\beta$  T-cell receptor.

### Adoptive Transfer of Experimental Autoimmune Keratitis

Keratitis was not inducible in naive rats by active immunization with BCE or 3-ALDH (Table 3, groups 2 and 3). In naive rats,

BC-1 sporadically produced mild signs of keratitis on the fourth day after adoptive transfer lasting 1 day only (Table 3, group 4). Pretreatment of recipient rats by either cyclophosphamide or splenectomy did not render them susceptible to T-cell line-induced keratitis (Table 3, groups 7 and 8). However, activated T-cell lines, BC-1 or AL-1, could adoptively transfer keratitis in irradiated recipient rats (Table 3, groups 9 and 10). An anti-MBP-specific T-cell line could not induce experimental autoimmune keratitis (EAK) in either naive or irradiated rats (Table 3, groups 6 and 11).

To learn whether recipient rats might acquire resistance to keratitis, we undertook T-cell vaccination. Naive rats were inoculated with activated BC-1 cells, and 8 days later irradiated and inoculated with a second dose of activated BC-1 T cells. Slit-lamp analysis of these rats revealed no signs of EAK (Table 3, group 12). Thus, prior exposure of rats to BC-1 T cells without irradiation induced resistance to later attempts to induce EAK by irradiation and adoptive T cell transfer.

To investigate whether irradiation of the recipient was required systemically or could be directed only at the target organ, we experimented with selective shielding of the rats. No keratitis developed in recipient rats when only the body was irradiated, and not the head including the eyes (Table 4). It appeared that irradiation of the eye itself was sufficient. In rats treated with TBI, keratitis was induced in both eyes, whereas in rats in which the whole body was shielded with lead, with the exception of the left eye, keratitis developed only in the left eye after adoptive transfer of BC-1 T cells (Table 4).

### Dose of Irradiation and Timing of Cell Transfer in EAK

EAK varied with the dose of irradiation and the time of T cell transfer. To examine the effect of the irradiation dose, groups of rats were irradiated with 2.0, 4.0, 6.0, or 7.5 Gy and injected on the same day with 10<sup>7</sup> activated BC-1 T cells derived from the same culture batch. Slit-lamp biomicroscopy showed a progressive increase in the signs and the duration of EAK with increasing irradiation (Table 5). Rats receiving 2.0 Gy displayed a delayed onset of EAK with only mild signs of hyperemia and corneal haze. One rat remained without signs of EAK.

The effect of irradiation was further analyzed by varying the time interval between the irradiation and the adoptive transfer of activated BC-1 T cells. There was a considerable difference in the severity and kinetics of the disease in the different treatment groups (Fig. 1). When the irradiation (7.5 Gy) and T cells were given on the same day, the EAK started on day 3 and peaked at day 6. When the rats were irradiated 3 days

**TABLE 2.** Proliferative Responses of the BC-1 T-Cell Line

Antigen	[ <sup>3</sup> H]Thymidine Uptake, cpm $\times 10^{-3} \pm$ SD
None	0.6 $\pm$ 0.4
BCE	58.5 $\pm$ 13.0
3-ALDH	30.8 $\pm$ 9.1
MBP	0.2 $\pm$ 0.1
Con A	100.6 $\pm$ 2.6

Proliferation assays with antigen and [<sup>3</sup>H]thymidine uptake were performed after 5 to 7 cycles of restimulation and rest.

**TABLE 3.** Incidence of EAK after Active Immunization or Adoptive Transfer of Activated T Cells into Lewis Rats

Group	Rats	Inoculum	Incidence*	Onset, d	Duration, d
1	Naive	PBS/CFA†	0/4	—	—
2	Naive	BCE/CFA†	0/8	—	—
3	Naive	3-ALDH/CFA†	0/4	—	—
4	Naive	BC-1‡	2/8	4	1
5	Naive	AL-1‡	0/4	—	—
6	Naive	MBP line	0/10	—	—
7	Cy treated§	BC-1	0/4	—	—
8	Splenectomized	BC-1	0/5	—	—
9	Naive, irradiated¶	BC-1	9/10	3-5	2-10
10	Naive, irradiated	AL-1	6/8	4-5	2-5
11	Naive, irradiated	MBP line	0/5	—	—
12	Vaccinated, irradiated#	BC-1	0/4	—	—

\* Number of rats with EAK in both eyes/number of rats inoculated. The experiments in groups 2, 4, 6, 9, and 10 were done both in Rehovot and Amsterdam with identical results.

† PBS, BCE, or 3-ALDH mixed with CFA, 50  $\mu$ l injected into each hind foot pad.

‡  $10^7$  T cells from cell lines activated in vitro 3 days prior to transfer, injected intraperitoneally.

§ Cyclophosphamide (60 mg/kg IP) given 48 hours before T cell transfer.

|| Splenectomy was performed 1 week before to T cell transfer.

¶ 7.5 Gy TBI to naive Lewis rats on the day of activated T cell transfer.

#  $10^7$  activated BC-1 intraperitoneally 8 days before TBI (7.5 Gy) and second injection of  $10^7$  activated BC-1.

prior to T cell transfer, EAK also started on day 3 but peaked at day 4 and was less severe compared with the EAK developing in rats that had been irradiated and inoculated with T cells on the same day. When the irradiation was given 6 days or more before adoptive T cell transfer, EAK did not develop. Thus, the susceptibility to EAK induced by irradiation is transient and effective only for about 3 days.

### Clinical Evaluation of EAK

The onset of keratitis varied between 3 and 5 days after local or total body irradiation and adoptive transfer of activated T cells. The first signs of disease consisted of episcleral and conjunctival hyperemia and peripheral corneal haze, followed by corneal edema, chemosis, and episcleral hemorrhages (Fig. 2A). Vascularization of the cornea started on day 3 to day 4 after onset, and the number of episcleral hemorrhages increased during the period of disease. Spontaneous recovery was associated with superficial infiltrates scattered over the entire corneal surface and fading of the corneal blood vessels. The eye regained its normal appearance within 11 days after the onset of the disease. Rats irradiated (7.5 Gy) without transfer of T

cells had some transient corneal edema lasting less than 1 day but no signs of corneal inflammation.

When anti-BCE-specific T-cell lines or control anti-MBP T-cell lines were injected directly into the corneal stroma of either naive or irradiated rats (7.5 Gy TBI), no signs of keratitis developed. Local injection caused a bleb that disappeared after a few hours. The site of injection remained somewhat opaque for at least 10 days. The opacity was caused by the injected cells, and no difference was observed between anti-BCE T-cell lines and control anti-MBP T-cell lines. Corneas injected with sterile PBS regained total transparency within a few hours.

### Histopathology of EAK

Histopathologic and immunohistochemical analyses of diseased corneas were performed on days 0, 3, 7, and 12 after irradiation and intraperitoneal injection of activated BC-1 T cells. H&E-stained sections showed inflammation of the cornea, mainly in the anterior stroma. Inflammation was maximal on day 7 (Fig. 2B), mild on day 3, and had greatly subsided by day 12.

**TABLE 4.** EAK after Adoptive Transfer of Activated T Cells\* into Rats with Irradiation of Different Parts of the Body

Rat No.	TBI (7.5 Gy)		TBI with Shielded Head (7.5 Gy)		Irradiation of Left Eye Only (7.5 Gy)	
	OS	OD	OS	OD	OS	OD
1	14	19	0	0	17	0
2	18	22	0	0	19	0
3	3	7	0	0.5	9	0.5
4	15	19	0	0	17	1
5	16	19	0	0	23	0
Mean $\pm$ SD	13.2 $\pm$ 5.9	17.2 $\pm$ 5.8	0	0.1 $\pm$ 0.2	17 $\pm$ 5.1	0.3 $\pm$ 0.4

T cells ( $10^7$ ) from BC-1 cell line in vitro stimulated with BCE three days prior to transfer. Cumulative keratitis scores per eye. OS, left eye; OD, right eye. Rats were examined from day 4 to 10.

**TABLE 5.** Effect of Dose of Irradiation on the Clinical Signs of EAK Induced by Adoptive Transfer of BCE-Activated T Cells in Lewis Rats

Dose, Gy	Incidence*	Onset, d	Duration, d	Maximum Score,† Mean ± SEM
0	1/4	4	1	2 (0.5 ± 0.5)
2.0	3/4	5-6	2-3	3 (2.1 ± 0.4)
4.0	4/4	4	6-7	8 (6.4 ± 0.9)
6.0	4/4	4	8	8.5 (5.7 ± 1.5)
7.5	4/4	4	8-9	13.5 (11.4 ± 1.0)

All the T cells were derived from the same *in vitro* stimulation.

\* Number of rats with EAK in both eyes/number of rats injected with T cells.

† Combined score for left and right eyes.

In naive control rat eyes, no T cells were seen, but some OX6 and ED1, and many ED2-positive cells were observed in the limbal area (data not shown). Similar findings were observed on day 0, the day of irradiation and T cell transfer (Table 6). An influx of T cells and macrophages was visible by day 3. The infiltrate greatly increased by day 7, and decreased again by day 12 (Table 6 and Figs. 2C through 2K). At the same time points, we studied ocular sections from rats with irradiation of only but without injection of T cells. These eyes showed mild transient edema (<1 day) but no subsequent signs of inflammation or corneal defects on slit-lamp examination. Some pyknotic cells were observed in the corneal epithelium and around the pupillary area. Autopsy of rats that had received TBI, and activated BC-1 T cells showed atrophy of the spleen and thymus due to the irradiation, but no additional pathology.

## DISCUSSION

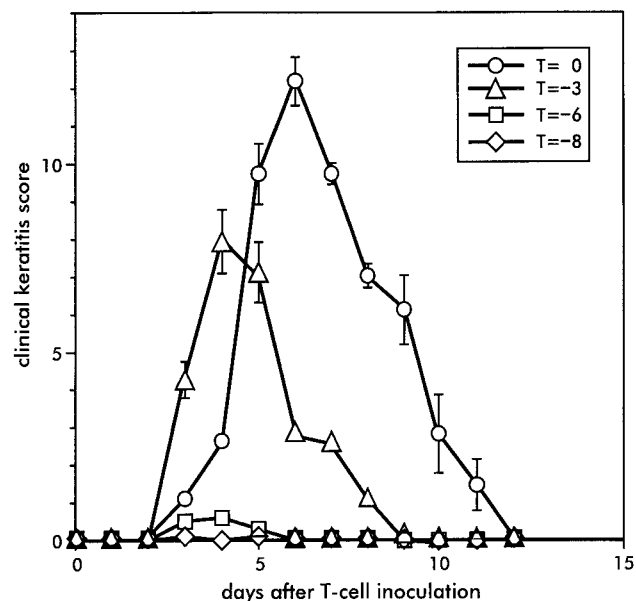
This study provides direct evidence that keratitis can be produced in rats by T cells reactive to corneal antigens, crude BCE, or purified 3-ALDH. The disease required that recipient rats be irradiated in the eye. T cells raised against crude BCE appeared to induce disease of longer duration and in a higher percentage of animals than did T cells raised to purified 3-ALDH. This suggests that more than one antigen may be involved in causing disease. Be that as it may, 3-ALDH, identical to BCP54,<sup>18,22</sup> is now identified as a purified corneal antigen that can trigger keratitis mediated by T cells.

Patient and animal studies have hinted at 3-ALDH as a possible corneal auto-antigen.<sup>10,23,24</sup> 3-ALDH constitutes approximately 30% of the total amount of soluble proteins of the corneal epithelium. It is also found in the corneal stroma and endothelium, in the lens epithelium, and in the conjunctiva.<sup>14</sup> Functional 3-ALDH is a dimer composed of identical subunits of 51 to 54 kDa.<sup>25</sup> The enzyme is constitutively expressed and localized in the cytosol. Corneal 3-ALDH is similar, if not identical to, the major ALDH isozymes expressed in the stomach,<sup>26</sup> in the urinary bladder, and in hepatocellular carcinomas.<sup>27</sup> However, we do not know why the T-cell lines produced detectable lesions only in the cornea and not in these other organs. Type I diabetes mellitus is another example of an organ-specific autoimmune disease that seems to be associated with target antigens that are not restricted to the target organ.<sup>28</sup>

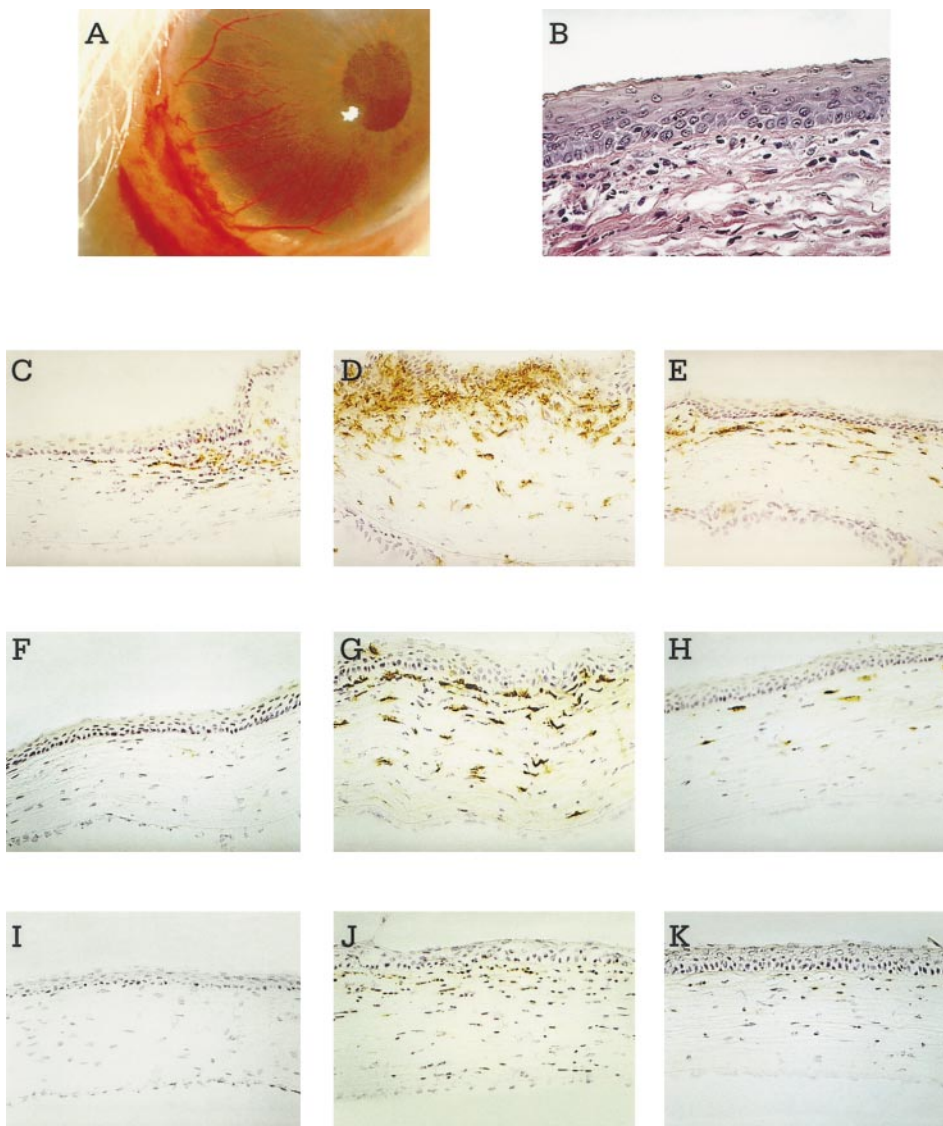
Use of functional T-cell lines to study cellular mechanisms involved in EAK is advantageous; these lines are homogeneous cell populations that lend themselves to cloning, characteriza-

tion, and manipulation. T-cell lines capable of adoptively transferring disease were first developed for experimental autoimmune encephalomyelitis<sup>29</sup> and extended to experimental autoimmune thyroiditis,<sup>30</sup> adjuvant arthritis,<sup>31</sup> and type I diabetes.<sup>32</sup> The technology has been adopted for induction of other experimental autoimmune diseases of the eye such as experimental autoimmune uveitis using different retina-specific antigens.<sup>33,34</sup>

In these various experimental autoimmune diseases, pathogenic T cells reach their target organ through the blood stream. Indeed, it appears that the T cells may be required to traverse blood vessels to acquire virulence. EAE can be observed clinically after injection of pathogenic T cells specific for central myelin antigens by intravenous, intraperitoneal, intramuscular, or subcutaneous routes. The only site of injection that does not always lead to disease appears to be the central nervous system itself; Wekerle and associates reported



**FIGURE 1.** Timing of irradiation and adoptive T cell transfer. The effect of the interval between the irradiation (7.5 Gy) and the intraperitoneal injection of activated BCE-specific T cells on the clinical severity of EAK. Rats ( $n = 3/\text{group}$ ) were irradiated on days 0, -3, -6, and -8, and given  $10^7$  T-cells intraperitoneally on day 0. The clinical keratitis score is the sum of scores for the left and right eyes per rat (maximal score per eye is 10, per rat 20).



**FIGURE 2.** (A) Rat eye with EAK. Eye of a Lewis rat 7 days after TBI (7.5 Gy) and intraperitoneal injection of  $10^7$  activated BCE-specific T cells. Note the hemorrhages in the conjunctiva and the neovascularization and haziness of the cornea. (B) Histopathology of eye with EAK. H&E-stained section of a cornea from a Lewis rat 7 days after TBI (7.5 Gy) and intraperitoneal injection of  $10^7$  activated BCE-specific T cells. Note influx of lymphocytes: few in the corneal epithelium and many in the anterior stroma (original magnification,  $\times 200$ ). (C through K) Immunohistochemistry of eyes with EAK. Immunohistochemistry of corneas with EAK: 3 (C, F, I), 7 (D, E, J), and 12 (E, H, K) days after disease induction. The positive cells stain brown with diaminobenzidine. (C, D, E) ED1<sup>+</sup> cells in the peripheral cornea; (F, G, H) ED2<sup>+</sup> cells in the peripheral cornea; and (I, J, K) W3/13<sup>+</sup> cells in the central cornea (original magnification,  $\times 128$ ).

this observation after intrathecal inoculation,<sup>35</sup> and we have noted that clinical disease also fails to develop after inoculation into the white matter of the brain itself (unpublished observations). For experimental autoimmune uveitis both local and systemic injections of activated antigen-specific T cells induced uveitis.<sup>36</sup> The finding that keratitis did not develop after direct injection into the cornea suggests that traversing the vascular compartment may be required for potential virulent T cells to acquire their full effector potential. It is possible that integrins and other receptor molecules activated in T-cell migration<sup>37</sup> may influence the state of the T cell. If that is the case, then blood-tissue “barriers” are not merely physical boundaries but “avenues”<sup>35</sup> of T-cell differentiation.

How circulating autoimmune lymphocytes are able to recognize their target tissue in naive hosts is still unknown. The need for activation of T cells before transfer suggests that T-cell migration may depend on inducible molecules. Indeed, it has been shown that activated T cells of any antigen-specificity can penetrate the central nervous system but that only T cells specific for antigens present in the tissue remain there.<sup>35</sup> For experimental autoimmune uveitis Prendergast et al.<sup>38</sup> have shown that distribution is independent of T-cell specificity

within the first 24 hours; however, a second peak of T-cell influx is observed 96 to 120 hours after inoculation only when activated experimental autoimmune uveitis-inducing T cells were injected. Furthermore, Zhang<sup>39</sup> has shown that after the encounter *in vivo*, transferred antigen-specific T cells expand 10- to 15-fold followed by a decline in number due to activation-induced apoptosis. Zhang also showed that the remaining transferred cells were fully unresponsive *in vitro* when cultured with their antigen, even in the presence of added IL-2 and IL-4.<sup>39</sup> Whether these observations on T-cell traffic apply to the cornea remains to be investigated, because the cornea lacks blood vessels and MHC class II expression.<sup>40</sup> It is therefore not clear how cornea-specific T-cell lines might enter the cornea and mediate keratitis. Irradiation of the eye may therefore be needed to cause damage that “opens” the barrier between the cornea and circulating immune cells. Liu<sup>23</sup> found that keratitis developed in rabbits immunized to corneal antigens only if accompanied by uveitis produced by injection of cytokines into the eye. Induction of uveitis contributes to the breakdown of blood-ocular barriers and abrogation of ocular immune privilege. In uveitis, reduced levels of TGF- $\beta$ 2 are found in ocular fluids,<sup>41</sup> and TGF- $\beta$ 2 is a central factor for the

TABLE 6. Immunohistological Analysis of Inflammatory Cells in Experimental Autoimmune Keratitis

	MAB		Day 0	Day 3	Day 7	Day 12
MHC class II	OX6	Limbus	+*	±/++	++	++
		Peripheral cornea	±*	±/-	+++	++
		Central cornea	-	-†	+‡	++
PMC, plasma cells, T cells	W3/13	Limbus	-	++/-	+	-
		Peripheral cornea	-	-	+++‡	+
		Central cornea	-	-	-	±
T cells	OX19	Limbus	-	++/-	+	±
		Peripheral cornea	-	-	+++‡	+
		Central cornea	-	-	-	±
CD8 <sup>+</sup> T cells, NK cells	OX8	Limbus	-	-	+	±
		Peripheral cornea	-	-	+	±‡
		Central cornea	-	-	±	-
Macrophages, monocytes, and dendritic cells	ED1	Limbus	±*	±	++	+
		Peripheral cornea	-	-	++	++
		Central cornea	-	-	±	+
Resident macrophages	ED2	Limbus	+++*	++	+++	++
		Peripheral cornea	-	-	++	+
		Central cornea	-	-	-	-
Adhesion molecules	ICAM1	Limbus	-	+§	+++§	+§
		Peripheral cornea	-	-	+	+
		Central cornea	-	-	-	±

Three rats per time point.

\* This pattern is also observed in naive rats. The scoring system is listed in the Materials and Methods section.

† Slight coloring of corneal epithelium.

‡ Positive cells are seen only in the anterior third of the corneal stroma.

§ Corneal epithelium and limbal vessels are positive.

induction of anterior chamber-associated immune deviation.<sup>42</sup> Likewise, local irradiation of the eye, associated with development of transient edema, may break down ocular barriers and increase the permeability of corneal stroma, allowing the influx of macromolecules and possibly cells. Induction of cytokine production by corneal cells could also be implicated in the immunopathogenesis. It has been reported that 5 hours after sublethal irradiation (7.0 Gy) in mice, upregulation of gene expression for IL-1 $\beta$ , IL-3, IL-6, and G-CSF could be detected mouse spleens.<sup>43</sup> Note that the ability of T-cell lines or clones to cause arthritis in rats also required irradiation of the recipients.<sup>44</sup> In the arthritis studies, however, it was not feasible to discriminate between the effects of local and systemic irradiation.

Although the intact cornea may be impenetrable by T cells, this tissue does seem to be accessible to antibodies. In rats, corneal 3-ALDH (BCP54) has been shown to be a target for antibodies in vivo; circulating anti-corneal antibodies could reach the corneal stroma and sclera but not the corneal epithelium.<sup>45</sup> However, for antibodies present in the tear film, an intact corneal epithelium seems to present an impenetrable barrier.<sup>45</sup>

In addition to local resistance to EAK, which is radiosensitive, the existence of systemic immune regulation of T cells mediating keratitis is suggested by the finding that BCE-specific T cells could vaccinate against the subsequent adoptive transfer of disease. This induced resistance was not abolished by TBI. The mechanism of this resistance remains to be elucidated. Other experimental models of autoimmune diseases have also been susceptible to prevention or therapy using T-cell vaccination.<sup>29-32,46</sup> Thus, immune modulation of the autoimmune process is feasible.<sup>47,48</sup> The fact that only an anti-cornea-specific T-cell line can cause inflammation of the

cornea in rats suggests that autoimmune T cells might be pathogenic in human keratitis too. If confirmed, this could provide a new insight into the pathogenesis and treatment of this group of destructive conditions, which are difficult to treat and often lead to permanent visual impairment.

### Acknowledgments

The authors thank Ad C. Breebaart, Department of Ophthalmology, University of Amsterdam, for continuous support and valuable suggestions and discussion, Socha H. W. van der Kracht and Hans M. Rodermond for their help with the irradiation of the rats, and Ton Put for his help with the color photography.

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