Cathepsin G, Acid Phosphatase, and α1-Proteinase Inhibitor Messenger RNA Levels in Keratoconus Corneas

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Purpose. Keratoconus is characterized by thinning and scarring of the central region of the cornea. The authors have shown, in corneas obtained from patients with keratoconus, that lysosomal enzyme activities are elevated, whereas levels of protease inhibitors such as α1-proteinase inhibitor (α1-PI) are reduced. This study was undertaken to examine further the gene expression of cathepsin G, acid phosphatase, and α1-PI in keratoconus corneas.

Methods. Corneal buttons were collected from patients with keratoconus, normal subjects, and patients with other corneal diseases. In situ hybridization was performed on paraffin sections using a tritium-labeled probe for cathepsin G or α1-PI. Competitive polymerase chain reaction (PCR) was used to determine the messenger RNA (mRNA) levels for lysosomal acid phosphatase and α1-PI in epithelial and stromal cells of keratoconus corneas.

Results. Silver grains, indicative of positive in situ hybridization products, were observed in all three cell types of normal corneas for both DNA probes. Compared with normal and other diseased controls, the labeling was enhanced for cathepsin G but was diminished for α1-PI in the epithelium of keratoconus corneas. Competitive PCR showed that the mRNA level for acid phosphatase was higher and that the mRNA level for α1-PI was lower in keratoconus corneas.

Conclusions. These results indicate that the mRNA level for degradative enzymes is increased and that for α1-PI it is reduced in keratoconus corneas. This study provides the first evidence that the altered expression of multiple enzymes and inhibitors in keratoconus occurs at the gene level. Furthermore, it implicates a possible role of coordinated transcriptional regulation of gene expressions in keratoconus.

Keratoconus is a progressive ocular disease characterized by thinning and scarring of the central portion of the cornea, resulting in eventual visual impairment. The exact cause is unclear, although evidence suggests that a genetic component probably is involved. Certain behaviors, such as excessive eye rubbing and contact lens wear, may be associated with the disease.

Previous protein studies, both in corneal specimens from affected persons and in cultured cells, indicated that there is a reduction in the overall amount of protein in keratoconus corneas compared with normal controls, even though protein synthesis proceeds normally in some cases. This led to the formulation of the hypothesis that the abnormality in keratoconus may lie in the degradative pathway of macromolecules. Recent data supported the degradation hypothesis, demonstrating increased levels of degradative enzymes and decreased amounts of protease inhibitors in keratoconus corneal specimens compared with normal and other corneal disease controls. Because interacting systems of enzymes and their inhibitors are normally under tight regulatory controls, any alteration in the enzyme-inhibitor balance may have a profound impact on the integrity of the cornea.

The degradative enzymes that have been found enhanced in keratoconus corneas include acid esterase, acid phosphatase, acid lipase, and cathepsin G. The inhibitors that are reduced include α1-proteinase inhibitor (α1-PI) and α2-macroglobulin. The total amount of matrix metalloproteinases and their physical forms detected by zymography are unaltered, whereas the net activities of gelatinases are found to be higher, possibly because of the decreased levels of tissue inhibitor of metalloproteinase and α2-macroglobulin in corneas with keratoconus.

Because an alteration pattern in enzymes and inhibitors is established in keratoconus at the protein level, questions arise whether changes in mRNAs also exist, whether alterations at the protein level parallel those in mRNAs, and what may be the causes of the observed alterations. To address the issues related to mRNAs, we performed in situ hybridization in this study to detect and specific mRNA species and to localize their expression in tissue sections.

In addition, a polymerase chain reaction (PCR)-based quantitative approach (competitive PCR) was chosen to measure more precisely the quantities of mRNAs in various corneal tissues. This approach entails the use of an exogenous template as an internal standard. The target sequence and the standard sequence compete for the same primers. The PCR amplification, therefore, takes place in a competitive fashion. In our experiments, a dilution series was made of the standard sequence, and a constant amount of...
our sample was added to each of the reaction tubes. Quantitation was performed after competitive amplification of the entire series of reactions and was achieved by distinguishing the two PCR products from each tube by differences in sizes. Our results indicated that in keratoconus corneas, the mRNA levels were altered. The mRNA results corroborated the previous protein data and suggested that the changes at the protein level may be related to alterations in mRNA levels.

MATERIALS AND METHODS. Twenty-one normal human corneas were obtained from the Illinois Eye Bank (Chicago). Donors did not have any known ocular diseases, and all the corneas excised were clear. Fourteen of these corneas were used for in situ hybridization. The median (± SD) donor age of the corneas was 33 ± 20 years (range, 2 to 68 years), and the median (± SD) postmortem time was 34 ± 23 hours (range, 6 to 82 hours). Seven other normal corneas, with a median (± SD) donor age of 48 ± 20 years (range, 17 to 73 years) and a median (± SD) postmortem time of 24 ± 13 hours (range, 3 to 48 hours), were used for competitive PCR experiments.

Half-corneal buttons from 27 patients with clinical features typical of keratoconus were collected at the time of transplantation. Nineteen corneal buttons (patient age, 37 ± 13 years; range, 14 to 57 years; duration of disease, 15 ± 10 years; range, 1 to 33 years) were used for in situ hybridization. The other eight corneal buttons (patient age, 38 ± 15 years; range, 19 to 73 years; duration of disease, 14 ± 10 years; range, 2 to 30 years) were used for competitive PCR studies.

Half-corneal buttons from 16 patients with other corneal diseases were obtained to serve as another set of controls. Six patients (63, 66, 70, 74, 77, and 87 years of age) had pseudophakic bullous keratopathy, three (46, 70, and 77 years of age) had aphanic bullous keratopathy, three (66, 75, and 77 years of age) had Fuchs’ corneal dystrophy, two (6 and 65 years of age) had corneal scars, one (34 years of age) had granular corneal dystrophy, and one (71 years of age) had lattice corneal dystrophy.

In Situ Hybridization. Corneal specimens were fixed in 4% formaldehyde for 24 hours, processed, and embedded in paraffin. For in situ hybridization experiments, 5-μm-thick sections were baked at 65°C overnight, deparaffinized, and rehydrated. The sections were digested with proteinase K (10 μg/ml; Promega, Madison, WI) for 15 minutes at 37°C and were postfixed with 4% paraformaldehyde for 10 minutes. The slides were treated with 0.25% acetic anhydride in 0.1 M triethanolamine–HCl buffer, pH 8. After washing in 2× sodium chloride–sodium citrate buffer, the slides were air dried.

Genomic DNA clone pUC-cathepsin G (3.0 kb) and cDNA clone phAT85 (1.4 kb) specific for α1-PI were obtained from American Type Culture Collection (Rockville, MD). The tritium-labeled probes were made using [3H]-dATP and a random-primed labeling kit, both from Amersham (Arlington Heights, IL). The tissue sections were prehybridized. Hybridization was carried out with a probe activity of 2 × 10⁶ cpm/ml (0.1 μg/ml) overnight at 45°C in a moist chamber. For negative controls, unlabeled cDNA (10 μg/ml) was added to the hybridization mixture. In addition, some sections were treated with 40 μg/ml ribonuclease A at 37°C for 60 minutes before prehybridization.

After hybridization, the slides were washed as previously described and were treated with SI nuclease (960 U/ml; Sigma, St. Louis, MO) for 30 minutes at room temperature. The sections were dehydrated, coated with 50% Kodak NT-2 nuclear emulsion (Eastman Kodak, Rochester, NY), and exposed in the dark at 4°C for 4 to 6 weeks. Coated slides were developed in Kodak D-19 developer, counterstained in Meyer’s hematoxylin (Sigma), and mounted. Slides then were examined and photographed under dark-field microscopy (Zeiss, Thornwood, NY).

Competitive Polymerase Chain Reaction. The central region of normal human corneas was obtained using a 7.5-mm trephine and was cut in half. These tissues and the half-corneal buttons collected from patients were dissected into epithelial, stromal, and endothelial layers. The epithelial and endothelial tissues were placed directly in TriReagent (Molecular Research Center, Cincinnati, OH) and hand-homogenized using a shaped pellet pestle to grind up the soft tissues. The stroma was quick-frozen in liquid nitrogen and pulverized mechanically before its addition to TriReagent. Total RNA was isolated through a modified guanidinium thiocyanate–phenol procedure and was stored at −70°C. Approximately 10 μg of total RNA, as determined by absorbance at 260 nm, was obtained from the epithelial and the stromal layers. The RNA isolated from the endothelium was at a much lower level (approximately 2 μg), and, hence, competitive PCR was not performed.

The primer sequences for lysosomal acid phosphatase, α1-PI, and β2-microglobulin, designed through the use of the program Oligo version 4.1 (National Bionics, Plymouth, MN) were, respectively:

1. upstream (US): CACGGCTTCTCAAAACACCTC,
downstream (DS): TGTCTCCGGGCTCTCGTCT;
2. US: GCAATGGCCTGTTCCTCA,
DS: CTTCTCCTCCTCGTCT;
3. US: CTCGGGCTACTCTCTTTCTGG,
DS: GCCCTCCACGTCTGACCCCTTTAA.

The expected size of each PCR product was 255, 275, and 335 base pairs (bp), respectively. Experiments for β2-microglobulin, a housekeeping gene,
were performed to normalize the mRNA quantifications across different specimens.

A MIMIC Construction Kit (Clontech, Palo Alto, CA) was used to develop internal PCR standards for each mRNA species. All were designed based on the neutral DNA fragment (633 bp, BamH I/EcoR I fragment of the v-erbB gene), and the validated priming site information was contained in the kit. Chimeric primer sets, consisting of a fusion of our target-specific primer sequences and selected primer sequences on the neutral DNA fragment, were postulated for each target sequence and evaluated by Oligo version 4.1. These chimeric primers were used under moderately stringent PCR conditions for 20 cycles (93°C, 45 seconds; 60°C, 45 seconds; 72°C, 90 seconds) to amplify the neutral DNA, producing a DNA sequence of known size whose ends were bounded by our target-specific priming sites. A subsequent round of PCR with target primers allowed the generation of large amounts of the desired construct. The size of the MIMIC standard for acid phosphatase, α1-PI, and β2-microglobulin were, respectively, 320, 156, and 322 bp.

All MIMIC standards were constructed and experimentally examined to ensure that they amplify with the same conditions, primer set, and kinetics as the target sequence; amplify with high fidelity through multiple PCR rounds, both alone and when in competition with target sequence; and have sufficient size differences in their PCR products from the target ones to permit resolution through gel electrophoresis. The β2-microglobulin MIMIC and target product required the use of MetaPhor agarose (FMC, Rockland, ME) to resolve the small size differential.

Total RNA from each corneal tissue specimen was reverse transcribed into a cDNA population using the RNA PCR Core Kit (Perkin-Elmer, Foster City, CA) and oligo-dT primers. Competitive PCR was carried out in a series of PCR reactions with serial tenfold dilutions (three to five tubes) of MIMIC standard and a constant amount of cDNA obtained by reverse transcription from each corneal specimen. The products were electrophoresed on 1.4% agarose gels, stained with either ethidium bromide (Sigma) or Sybr Green I (Molecular Bioprobes, Eugene, OR), photographed using Polaroid 667 film (Polaroid, Cambridge, MA), and evaluated using an Intelligent Quantifier densitometry system from Biolmage (Ann Arbor, MI). The tenfold dilution results were followed by a more precise twofold dilution series of competitive PCR for each primer pair. The range of the twofold dilution series was determined by analysis of band intensity ratios of target—MIMIC from the preceding tenfold series experiments using the same MIMIC standard. All twofold dilution series were replicated at least three times to ensure reproducible results. All PCR reactions were carried out on a Perkin-Elmer model 480 thermocycler using AmpliTaq polymerase and thin-walled PCR tubes (Mid-West Scientific, St. Louis, MO). The basic cycling profile (93°C, 45 seconds; 65°C, 45 seconds; 72°C, 80 seconds) was tailored to the optimal annealing temperature and cycle number for each primer pair.

RESULTS. The diagnosis of keratoconus was confirmed by pathologic examination. Typical features of keratoconus, such as breaks in Bowman’s membrane, thinning in the central cornea, positive iron staining for Fleischer’s ring, and fibrous scar, were seen.

In situ hybridization for cathepsin G mRNA was performed using a tritium-labeled probe on 10 keratoconus, 9 normal, and 10 other diseased corneal specimens. Silver grains, indicative of positive hybridization products, were observed in the epithelial, stromal (Fig. 1), and endothelial layers of normal corneas. The postmortem time did not seem to affect the in situ hybridization results. In addition, all specimens used for negative controls uniformly showed only background hybridizations. In keratoconus corneas (Fig. 1A), the labeling was enhanced visibly in the epithelium compared with the counterparts from normal controls (Fig. 1B) and from patients with other corneal diseases. No alteration could be discerned in the stromal and endothelial layers of keratoconus corneas. The enhancement in the epithelial layer was observed, to varying degrees, in 9 of the 10 keratoconus specimens examined. The degree of alterations did not correlate with clinical features, including the duration of the disease and the degree of scarring.

For α1-PI, in situ hybridization experiments were performed on 14 keratoconus, 12 normal, and 11 other diseased corneas. Labeling in the epithelium of keratoconus corneas (Fig. 1D) was diminished compared with normal human (Fig. 1E) and other diseased controls in 12 of the 14 specimens examined.

The mRNA levels for acid phosphatase and α1-PI in 19 samples (eight keratoconus, eight normal, one pseudophakic bullous keratopathy, one Fuchs' corneal dystrophy, and one granular corneal dystrophy) were examined by the competitive PCR technique. These experiments, in conjunction with in situ hybridization, confirmed our previous finding that mRNA for α1-PI is present in the cornea. In addition, they demonstrated the presence of mRNAs for cathepsin G and acid phosphatase.

Figure 2 shows a typical gel electrophoretogram after PCR for acid phosphatase. The gel displayed two PCR product bands, the 320-bp band for the expected MIMIC product and the 255-bp band as the expected product for acid phosphatase. With a decreasing amount of MIMIC added to the reaction tubes, the intensity of the former band decreased and that of the latter band increased concurrently.

Photographs of agarose gels, after electrophoresis, were subjected to densitometry. An intensity value for each PCR product was obtained, and the relative intensities of the target and the MIMIC product in each sample were determined. A graph was constructed that plotted the ratio of target—MIMIC intensities as a function of the introduced concentration of MIMIC standard. The initial
FIGURE 1. In situ hybridization of corneas for cathepsin G (A to C) and α1-proteinase inhibitor (C to E) mRNAs. For cathepsin G experiments, corneal sections were from a 20-year-old patient with keratoconus (A) and a 17-year-old normal human donor (B, C). A [3H]-labeled genomic DNA probe specific for cathepsin G was used. C shows a serial section of B treated with ribonuclease A before hybridization as a negative control. Note that the number of grains in the epithelial layer of keratoconus corneas (A) was increased visibly compared to B. For α1-proteinase inhibitor experiments, corneas were from a 41-year-old patient with keratoconus (D, F) and a 41-year-old normal human donor (E). F shows a serial section of D hybridized in the presence of an unlabeled DNA probe as a negative control. Compared with controls (E), the number of grains, indicative of the mRNA level, was decreased in keratoconus corneas (D). Dark field; original magnification, ×40.

amount of the target equaled that of the MIMIC standard added when the molar ratio of final target–MIMIC amplicon was 1. This maneuver was carried out for acid phosphatase, α1-PI, and β2-microglobulin for each sample. β2-microglobulin is a housekeeping gene constitutively expressed in the cornea. Concentrations of β2-microglobulin can thus serve as an indicator of cDNA concentrations and a reflection of the initial total RNA concentrations undergoing reverse transcription.

The competitive PCR data, expressed as the mRNA level for acid phosphatase or α1-PI relative to that of β2-microglobulin, are summarized in Table 1. The mRNA level for acid phosphatase in the epithelium and stroma of keratoconus corneas was approximately 1.4- to twofold higher, whereas the mRNA level for α1-PI was approximately one third to one fifth that found in the normal control sample and in other diseased control samples.

DISCUSSION. Using in situ hybridization, we examined the gene expression of cathepsin G and α1-PI in keratoconus corneas. Cathepsin G is a neutral serine protease. It is known to degrade proteoglycans and collagens of articular cartilage and to enhance elastase activity in vitro. Immunostaining experiments in our laboratory have found an abnormally high level of cathepsin G in keratoconus corneas. The level of α1-PI, one of the major protease inhibitors in the plasma, was reduced markedly, especially in the epithelial layer of keratoconus corneas. Our current in situ hybridization results paralleled those of previous protein studies. The epithelium of keratoconus corneas appeared to contain abnormal levels of mRNAs for the enzyme cathepsin G and the inhibitor α1-PI.

Competitive PCR was performed to provide quantifiable information on the expression patterns of mRNAs.

FIGURE 2. A representative gel electrophoretogram of a competitive polymerase chain reaction (PCR) for acid phosphatase. Each tube contained the primer set for acid phosphatase, cDNAs from a tissue specimen, and a known amount of MIMIC internal standard. After PCR, the acid phosphatase products (255 bp, arrowhead) were separated from the MIMIC products (320 bp, arrow) on 1.4% agarose gels. The cDNAs were from the corneal stroma (lanes 1 to 3) and epithelium (lanes 4 to 6) of a 62-year-old normal subject and from the stroma (lanes 7 to 9) and epithelium (lanes 10 to 12) of a 17-year-old normal subject. The amount of MIMIC standard added, in attomoles, is 200 (lanes 1, 4, 7, 10), 20 (lanes 2, 5, 8, 11), and 2 (lanes 3, 6, 9, 12). Lane 13 shows the φX174 Hae III-digested DNA markers. As the concentration of MIMIC standard is reduced, its PCR amplicon band intensity decreases whereas that of acid phosphatase increases.
TABLE 1. Messenger RNA Levels for Acid Phosphatase and α1-Proteinase Inhibitor Measured by Competitive Polymerase Chain Reaction Assays

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Acid Phosphatase</th>
<th>α1-Proteinase Inhibitor</th>
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<tbody>
<tr>
<td></td>
<td>Epithelium</td>
<td>Stroma</td>
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<tr>
<td>Keratoconus</td>
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<tr>
<td>1.41</td>
<td>2.42</td>
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<tr>
<td>2.50</td>
<td>3.09</td>
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<td>3.23</td>
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<td>3.50</td>
<td>3.90</td>
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<tr>
<td>2.33</td>
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<tr>
<td>3.90</td>
<td>2.72</td>
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<td>2.26</td>
<td>2.02</td>
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<tr>
<td>3.26</td>
<td></td>
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<tr>
<td>Keratoconus as a group</td>
<td>2.80 ± 0.29*</td>
<td>3.28 ± 0.44†</td>
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<td>(n = 8)</td>
<td>(n = 7)</td>
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<tr>
<td>Control</td>
<td>2.00 ± 0.30</td>
<td>1.66 ± 0.23</td>
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<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
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Data (mean ± SEM) are expressed as mRNA levels for either acid phosphatase or α1-proteinase inhibitor relative to that for β2-microglobulin. The control group included specimens from seven normal individuals, one patient with PBK, one with Fuchs' corneal dystrophy, and one with granular corneal dystrophy. The significance of the data was evaluated by nonparametric Mann-Whitney tests.

* P = 0.0564 (marginally significant) versus controls.
† P < 0.0123 versus controls.
‡ Not significantly different from controls.

This technique attempts to circumvent many of the factors that interfere with PCR quantification through the introduction of an internal MIMIC standard at a known amount that coamplifies with the target sequence. The MIMIC standard is added in known decreasing concentrations to a series of otherwise identical PCR reactions. At the point when the MIMIC standard coamplifies equally with the target sequence, the unknown starting concentration of target, in theory, is equal to that of the introduced known concentration of the internal standard.

Our initial attempts to construct a MIMIC standard for cathepsin G failed for unknown reasons, although it may most likely be related to difficulties inherent in the method chosen for MIMIC construction. Acid phosphatase was therefore selected as an alternate lysosomal enzyme candidate and the MIMIC standard was successfully constructed. This enzyme, like cathepsin G, has been shown to be elevated in keratoconus as well.

Competitive PCR, though elegant in theory, presents a number of difficulties when put into practice. It is extremely labor intensive and costly, especially when the number of samples increases, because replications are performed for each sample to determine data reproducibility. Furthermore, despite attempts to obviate the variables that hinder quantification by PCR, problems still exist in competitive PCR. The ideal result, a decreasing MIMIC product that smoothly coincides with an increasing target product over the MIMIC standard dilution range, is often difficult to achieve. The results also tend to vary slightly each time PCR is performed, necessitating multiple iterations to ensure reproducibility. In addition, measurements through densitometry can be reliable only when the band intensities of the PCR products are in a linear (detectable, but not saturated) range. The limitation imposed by the densitometric range did prevent us from obtaining results from some of the keratoconus samples.

The heterogeneity in keratoconus demonstrated previously underscores the importance of examining each keratoconus specimen individually. In competitive PCR studies, we found that the mRNA levels, although altered from controls, varied considerably among the keratoconus samples studied. Heterogeneity in keratoconus could account for some of the observed variability. Moreover, corneal buttons were received when transplantation was judged medically necessary and when the inherent variability in disease progression might have contributed to the variations.

Despite the drawbacks, the competitive PCR data agree and better quantify the results of in situ hybridization. In keratoconus corneas, the acid phosphatase mRNA levels are significantly higher, and those of α1-PI are lower, than in normal and other diseased controls. This is entirely consistent with the previous protein findings. In α1-PI, the magnitude of variation at the RNA (one third to one fifth the control value) level even agrees well with that of the protein (one fourth to one sixth) alteration in keratoconus versus controls.

This study provides the first evidence that gene control may be involved in keratoconus. Altered expression of multiple genes, including α1-PI, acid phosphatase and cathepsin G, whose products are involved in corneal macromolecular degradation, is demonstrated. The alteration...
of these genes as a group points to the possibility that corneal cells, particularly those in the epithelial layer, may exert some form of coordinated transcriptional control to produce the characteristic disease phenotype. Gene regulation studies of keratoconus is a logical extension of these results. Future lines of pursuit may include examination of regulatory elements such as transcription factors, which are capable of coordinating the expression of a variety of genes in response to external factors or conditions. Additionally, a systematic evaluation of external agents, including cytokines, is warranted for their expression in keratoconus corneas, their regulatory effects on levels of enzymes and inhibitors in the cornea, and their receptors.

Key Words
degradative enzymes, keratoconus, in situ hybridization, protease inhibitor, quantitative polymerase chain reaction

References


The Antigen-Bearing Eye and the Spleen Are Indispensable in Maintaining Anterior Chamber-Associated Immune Deviation

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Purpose. To investigate the role of the eye and the spleen in maintaining suppression of delayed-type hypersensitivity (DTH) after anterior chamber (AC) inoculation of allogeneic splenocytes.

Methods. Suppression of DTH response was tested in BALB/c mice after AC inoculation of allogeneic B10.D2 splenocytes. Seven days after AC injection, the antigen-inoculated eyes were enucleated or the spleens were removed. After enucleation or splenectomy at different time intervals, DTH responses in groups of the BALB/c mice were examined. Splenec components obtained from BALB/c mice that had been primed by B10.D2 splenocytes in the AC 7 days earlier were transferred intravenously to groups of naive syngeneic acceptors. At various intervals after adoptive transfer, variations of DTH responses were tested.

Results. Inoculation of B10.D2 splenocytes to the AC of BALB/c mice induced antigen-specific suppression of DTH. Either enucleation of the antigen-inoculated eyes or splenectomy weakened the DTH-suppressive effect within 5 weeks and abolished it within 9 weeks, whereas the mice retaining both antigen-inoculated eyes and spleens maintained longstanding DTH suppression. Adoptive transfer of spleen components to syngeneic acceptors demonstrated DTH suppression for only 3 weeks.