

Brief Report

**THERAPY WITH A PURIFIED
PLASMINOGEN CONCENTRATE
IN AN INFANT WITH LIGNEOUS
CONJUNCTIVITIS AND HOMOZYGOUS
PLASMINOGEN DEFICIENCY**

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LIGNEOUS conjunctivitis is a rare disease characterized by acute or chronic recurrent conjunctivitis in which the conjunctival membranes acquire a wood-like consistency, due primarily to deposits of fibrin.^{1,2} Corneal involvement and chronic obstruction of the eye may lead to blindness. The disease is frequently associated with nasopharyngitis, tracheobronchial obstruction, otitis media, vulvovaginitis, and defective wound healing.²⁻⁹ Pseudomembranous conjunctivitis was first described in 1847 by Bouisson,¹⁰ and the term "conjunctivitis lignosa" was introduced by Borel in 1933.¹¹ More than 100 cases have been reported in the literature, but no satisfactory treatment has yet been found. The results of therapy with hyaluronidase eye drops, corticosteroids, cyclosporine, and antiviral agents are generally disappointing.^{6,7,12-14} Surgical treatment often causes accelerated recurrence of pseudomembranes.^{2,4-6,14}

Family studies have suggested that the disorder is caused by a genetic defect with an autosomal recessive pattern of inheritance.³ A case of ligenous conjunc-

tivitis with gingival and peritoneal lesions was reported after treatment with the antifibrinolytic agent tranexamic acid,¹⁵ suggesting a relation between ligenous conjunctivitis and impaired fibrinolysis. Although the syndrome may have more than one cause, it has recently been linked to severe plasminogen deficiency.¹⁶⁻¹⁸ Plasminogen-deficient mice generated by targeted gene disruption show signs of ligenous conjunctivitis, defective wound healing, and internal hydrocephalus.^{19,20}

In this report, we describe a patient with homozygous plasminogen deficiency in whom ligenous conjunctivitis developed soon after birth. Other symptoms included hyperviscosity of tracheobronchial and nasopharyngeal secretions, impaired wound healing, and internal hydrocephalus. Replacement therapy with lys-plasminogen (lysine-conjugated plasminogen) led to rapid regression of the pseudomembranes and normalization of respiratory tract secretions and wound healing.

CASE REPORT

The child was the second son of parents of Turkish origin, who were first cousins. Progressive internal hydrocephalus was diagnosed by prenatal ultrasound examination, and the child was delivered by elective cesarean section at a gestational age of 35 weeks. The findings on physical examination were normal except for a bulging fontanelle and macrocephalus (head circumference, 36.5 cm, above the 97th percentile). Bilateral inflammation of the palpebral portion of the conjunctiva, with hypersecretion and formation of pseudomembranes, was noticed three days after birth. Within two weeks, a thick, yellowish-white, fibrous, woody pseudomembranous layer of conjunctival proliferation had developed, spreading from the inner side of the upper and lower eyelids and completely closing both eyes (Fig. 1). The pseudomembranes were removed surgically several times but regrew rapidly. Local treatment with antibiotics, corticosteroids, and cyclosporine did not result in improvement, and there was a risk of complete loss of vision.

Computed tomography and magnetic resonance tomography of the head and ultrasound examination showed an extended, symmetric internal hydrocephalus with a Dandy-Walker malformation, hypoplasia of the cerebellum, and a hypoplastic corpus callosum. The hydrocephalus was drained by ventriculoperitoneal shunting three weeks after birth. Wound-healing complications subsequently developed, with wound dehiscence and markedly retarded regeneration of the skin, which was generally dry and eczematous. Furthermore, recurrent airway infections developed, with hyperviscosity of the nasopharyngeal and tracheobronchial secretions. Visual evoked potentials on an electroencephalogram were normal; the results of hearing tests, including auditory evoked potentials, were abnormal, indicating a hearing deficiency at the middle-ear level.

Family studies showed that the patient's parents and older brother had decreased levels of plasminogen antigen and activity, suggesting heterozygous plasminogen deficiency. None of the family members had signs of venous or arterial disease, ligenous conjunctivitis, or other symptoms. In the patient, the level of functional plasminogen was below the limit of detection (<1 percent on chromogenic assay), as was that of plasminogen antigen (<1 percent on laser nephelometry, and <0.5 percent on immunoblotting). A diagnosis of homozygous plasminogen deficiency was therefore proposed. After written informed consent from the parents and approval from the local ethics committee of Mannheim University Hospital were obtained, replacement therapy was started (the patient was six months old at the time), with continuous intravenous

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Figure 1. Initial Appearance of a Child with Homozygous Plasminogen Deficiency, Showing the Typical Features of Ligneous Conjunctivitis.

infusion of 1000 caseinolytic units (CU) of lys-plasminogen (corresponding to 180 CU per kilogram of body weight) per 24 hours. One CU is defined as the activity of enzyme that liberates 450 μ g of trichloroacetic acid-soluble tyrosine from casein in one hour under defined conditions.²¹ After two weeks of continuous infusion, lys-plasminogen was given as bolus injections of 2000 CU (corresponding to approximately 350 CU per kilogram) every 48 hours for two weeks. Maintenance therapy with daily bolus injections of 1000 CU (corresponding to 180 CU per kilogram at first, decreasing to 125 CU per kilogram as the child's weight increased) was then initiated and has been continued up to the present.

METHODS

Histologic Findings

Specimens of conjunctival pseudomembranes were snap-frozen in liquid nitrogen, and 4-to-5- μ m-thick sections were cut and processed for staining with hematoxylin and eosin and for immunohistologic analysis. Immunofluorescence staining for plasmin and plasminogen deposits in the granulation tissue was performed with use of previously described murine antihuman antibodies to plasmin and plasminogen²² and fluorescein isothiocyanate-labeled antimouse IgG antibodies.²³

Analysis of Mutations

Genomic DNA was prepared from blood samples drawn from the patient and from healthy family members (his parents and elder brother). The DNA samples were amplified by the polymerase chain reaction (PCR) with use of a set of primer pairs flanking all 19 exons that include intron boundaries of the human plasminogen gene,²⁴ with primer sequences and PCR conditions as previously described.²⁵ To screen for mutations in the plasminogen gene, we used single-strand conformation polymorphism analysis.²⁶ Amplified and subsequently denatured PCR segments underwent electrophoresis for seven hours at 10 W with a nondenaturing 5 percent polyacrylamide gel (Roth, Karlsruhe, Germany) containing 5 percent glycerol at room temperature. The gel was dried and exposed to Kodak XAR-S films (Eastman Kodak, Rochester, N.Y.). Variant bands of single-stranded DNA fragments that differed in their migration patterns were excised from dried gels, dissolved in buffer, purified with MicroSpin columns (Pharmacia, Freiburg, Germany), and reamplified. These PCR products were again purified with MicroSpin columns and were directly cycle-sequenced by the dideoxy chain-termination method with use of [³⁵S]deoxyadenosine triphosphate and the Exo(-)Pfu Cyclist DNA Sequencing Kit (Stratagene, Heidelberg, Germany). Samples were electrophoresed on 6 percent polyacrylamide-8.3 M urea sequencing gels (Roth), dried, and exposed to Kodak XAR-S films.

Direct Detection of Mutations

To detect the Glu460Stop mutation directly, once it had been identified in the patient, amplified PCR products of exon 11 of the plasminogen gene (263-bp fragments) were digested with *Mbo*I (New England Biolabs, Beverly, Mass.) according to the manufacturer's recommendations and electrophoresed on a 2 percent agarose gel for analysis of the restriction-fragment-length pattern.

Laboratory Analyses

The functional activity of plasminogen was measured by chromogenic assay (Boehringer Mannheim, Mannheim, Germany). Plasminogen antigen was measured by laser nephelometry with use of polyclonal rabbit antiserum and reagents (Behring Diagnostics, Marburg, Germany). Plasminogen antigen was also detected by immunoblotting after sodium dodecyl sulfate-polyacrylamide-gel electrophoresis of whole plasma with use of polyclonal rabbit antiserum against human plasminogen (Behring) and an alkaline phosphatase-labeled monoclonal antibody against rabbit IgG (Sigma, Deisenhofen, Germany). A solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate substrate was used for visualization of enzyme-labeled antibodies.

Thrombin-antithrombin complex was measured by enzyme immunoassay (Enzygnost TAT, Behring). Tests for fibrin monomers (Enzymun-Test FM), D-dimer antigen (TINAquant), and fibrinogen were performed with use of reagents from Boehringer Mannheim. The functional activity of antithrombin was measured by chromogenic assay (Boehringer Mannheim).

Lys-Plasminogen

A sterile, freeze-dried concentrate of lys-plasminogen purified from human plasma was obtained from Immuno (Vienna, Austria). The product was supplied in vials containing 1000 CU for reconstitution with 10 ml of sterile water. Per milliliter, the reconstituted solution contains 100 ± 20 CU of lys-plasminogen, 4 to 6 mg of total protein, 7.2 to 10.8 mg of sodium chloride, 0.8 to 1.2 mg of trisodium citrate, 0.14 to 0.26 mg of lysine, and 0.8 mg of dibasic sodium citrate. This concentrate has been used in other clinical studies²⁷ and is not licensed in Germany. The safety of the vapor heating process used for inactivation of virus was demonstrated in an international study in which no cases of product-related transmission of hepatitis viruses or human immunodeficiency virus occurred.²⁸

RESULTS

Histologic Evaluation

The hematoxylin–eosin staining of parts of the conjunctival pseudomembranes revealed fibrin-rich granulation tissue with a mixed neutrophil-rich perivascular infiltrate. Immunophenotyping confirmed the mixed character of the infiltrate, with predominantly neutrophilic granulocytes (which are elastase-positive), monocytes and macrophages (CD68-positive), and a few scattered T cells (CD3-positive). Staining with an antibody specific for fibrin and fibrinogen revealed diffuse fibrin deposits; no staining was evident when antibodies against plasmin and plasminogen were used.

Molecular Genetic Findings

In single-strand conformation polymorphism analysis, a sequence difference of only a single base (point mutation) alters the secondary structure of DNA and thus its mobility in electrophoresis. Altered single-strand band patterns were found in the patient's genomic DNA only when primers specific for exon 11 of the plasminogen gene were used (Fig. 2, lane 2).

Direct sequencing of fragments of exon 11 from the patient (Fig. 3, right) demonstrated a homozygous point mutation at position 1511 (G→T), leading to a stop codon (TAA) at position 460 (Glu460Stop). This mutation abolishes the catalytic domain of plasmin. The healthy brother (Fig. 2, lane 5) and the healthy parents (Fig. 2, lanes 3 and 4) were shown to be heterozygous for this mutation, whereas it was not present in a healthy control (Fig. 2, lane 1). The

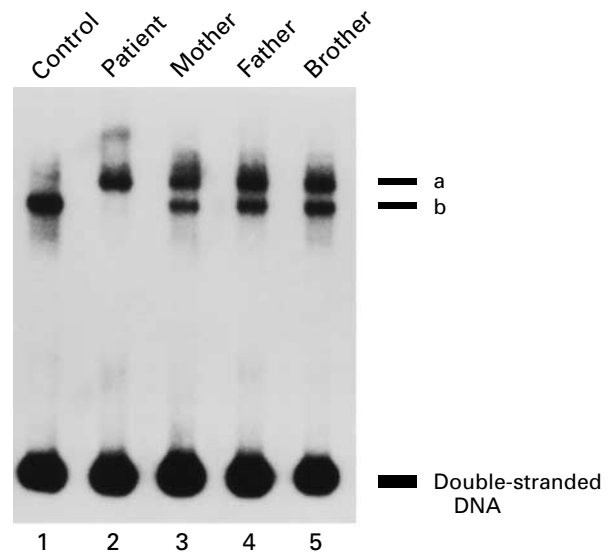


Figure 2. Results of Single-Strand Conformation Polymorphism Analysis of Exon 11 of the Plasminogen Gene in the Patient, His Parents, and His Brother.

Radiolabeled single-stranded PCR products of exon 11 of the plasminogen gene were electrophoresed on a nondenaturing 5 percent polyacrylamide gel as described in the Methods section. Lane 1 shows amplified DNA from a healthy control subject; lane 2, DNA from the patient with ligneous conjunctivitis; lane 3, DNA from the healthy mother; lane 4, DNA from the healthy father; and lane 5, DNA from the healthy brother; a denotes the mutant single strand of exon 11, and b the normal single strand.

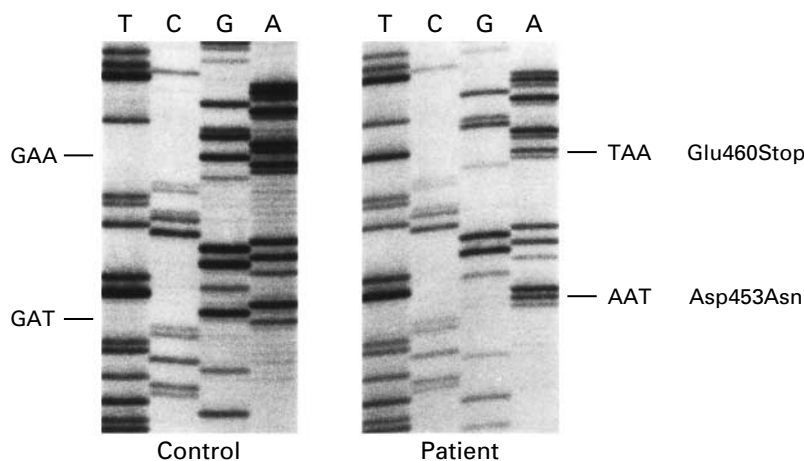


Figure 3. Sequence Analysis of Exon 11 of the Plasminogen Gene in the Patient and a Healthy Control Subject.

The coding strand is shown in Figure 2. Single-stranded DNA fragments with a variant migration pattern in single-strand conformation polymorphism analysis were excised from polyacrylamide gel and sequenced as described in the Methods section. The numbering of the nucleotides was carried out according to the method of Forsgren et al.,²⁹ and that of amino acids according to the method of Petersen et al.²⁴

healthy control had a homozygous single-base-pair substitution (Fig. 3, left) at position 1490 (A→G), leading to an amino acid substitution at position 453 (Asn453Asp), which probably represents only a polymorphism in the normal population.²⁴ We found the Asn453Asp substitution in four additional healthy subjects, all of whom had normal plasminogen antigen concentrations and functional plasminogen activity (data not shown).

Restriction-Fragment–Length Analysis

The Glu460Stop mutation disrupts a restriction site for the restriction enzyme *Mbo*II (GAAGA) in exon 11 of the plasminogen gene (Fig. 4A). Therefore, subjects who are homozygous or heterozygous for the Glu460Stop mutation can be identified with use of agarose-gel electrophoresis to detect differences in the restriction-fragment–length pattern of their *Mbo*II-digested PCR fragments (Fig. 4B). The patient had only one band of 263 bp, a finding that was compat-

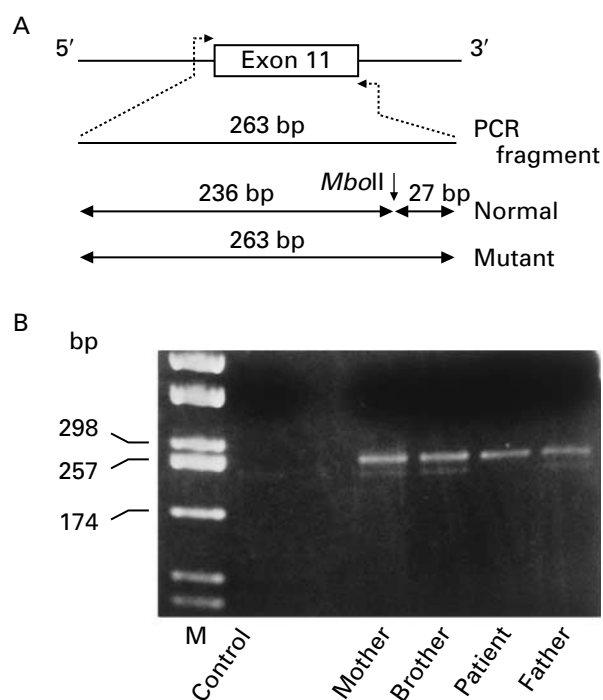


Figure 4. Detection of the Glu460Stop Mutation at Exon 11 of the Plasminogen Gene by Digestion with the Restriction Enzyme *Mbo*II.

Panel A shows the amplified portion of exon 11 (263-bp DNA fragments). The position of the *Mbo*II site abolished by the Glu460Stop mutation is indicated. In Panel B, electrophoresis of *Mbo*II-digested PCR fragments of exon 11 on a 2 percent agarose gel shows the undigested 263-bp mutant allele in the sample from the homozygous patient, both the normal 236-bp allele and the mutant 263-bp allele in the samples from three heterozygous relatives, and normal 236-bp alleles in a sample from a healthy control. The molecular-size marker (M) was pUC181HaeIII.

ible with both alleles' containing the Glu460Stop mutation and that indicated homozygosity. The mother, the elder brother, and the father each had both the mutant allele (263 bp) and the shorter normal allele (236 bp), indicating heterozygosity. The healthy control, as well as 50 other unrelated healthy subjects (data not shown), had only the normal allele. These data indicate that the Glu460Stop mutation does not represent a polymorphism.

Recovery and Half-Life of Lys-Plasminogen

The patient's plasminogen level began to increase within 3 hours after the start of the initial continuous intravenous infusion of lys-plasminogen (1000 CU per 24 hours); the level approached the normal value within 24 hours, fluctuated, and then leveled off at between 40 percent and 50 percent of normal after one week (Fig. 5).

For the evaluation of the in vivo half-life of lys-plasminogen, serial blood samples were obtained from the patient before and 30 minutes and 1, 3, 6, and 24 hours after bolus injection of lys-plasminogen on three separate occasions (a sample was also obtained after 48 hours on the first occasion). Early during the course of therapy, the administration of 2000 CU of lys-plasminogen increased the plasminogen level from 0 to 150 percent of normal within 30 minutes, with 7 percent residual activity after 24 hours and 1 percent after 48 hours (data not shown). After approximately five months of replacement therapy, the administration of 1000 CU of lys-plasminogen increased the plasminogen level on one occasion to 74 percent of normal after 30 minutes, with 4 percent residual activity after 24 hours, and on another occasion to 80 percent of normal, with 6 percent residual activity after 24 hours. The half-life of lys-plasminogen was approximately five hours.

Effect of Lys-Plasminogen on Measures of Fibrinolysis and Coagulation

Whereas D-dimer antigen was absent in pretreatment blood samples, indicating the absence of endogenous plasmin-dependent fibrinolysis, the levels rose sharply after the start of treatment, peaking at six hours and then decreasing gradually (Fig. 5). This pattern showed that fibrinolytic activity had resulted in the release of fibrin split products.

The level of thrombin–antithrombin complex increased three hours after the start of the continuous infusion of lys-plasminogen and reached maximal levels after six hours (Fig. 5); this pattern indicated that thrombin, which is inhibited by covalent binding to antithrombin, had been formed or released. Thrombin–antithrombin complex levels returned to normal within 24 hours. Fibrin monomers (measured with the Enzymun Test FM) were absent in pretreatment samples, indicating the absence of an activation state of coagulation; their level paralleled the rise in

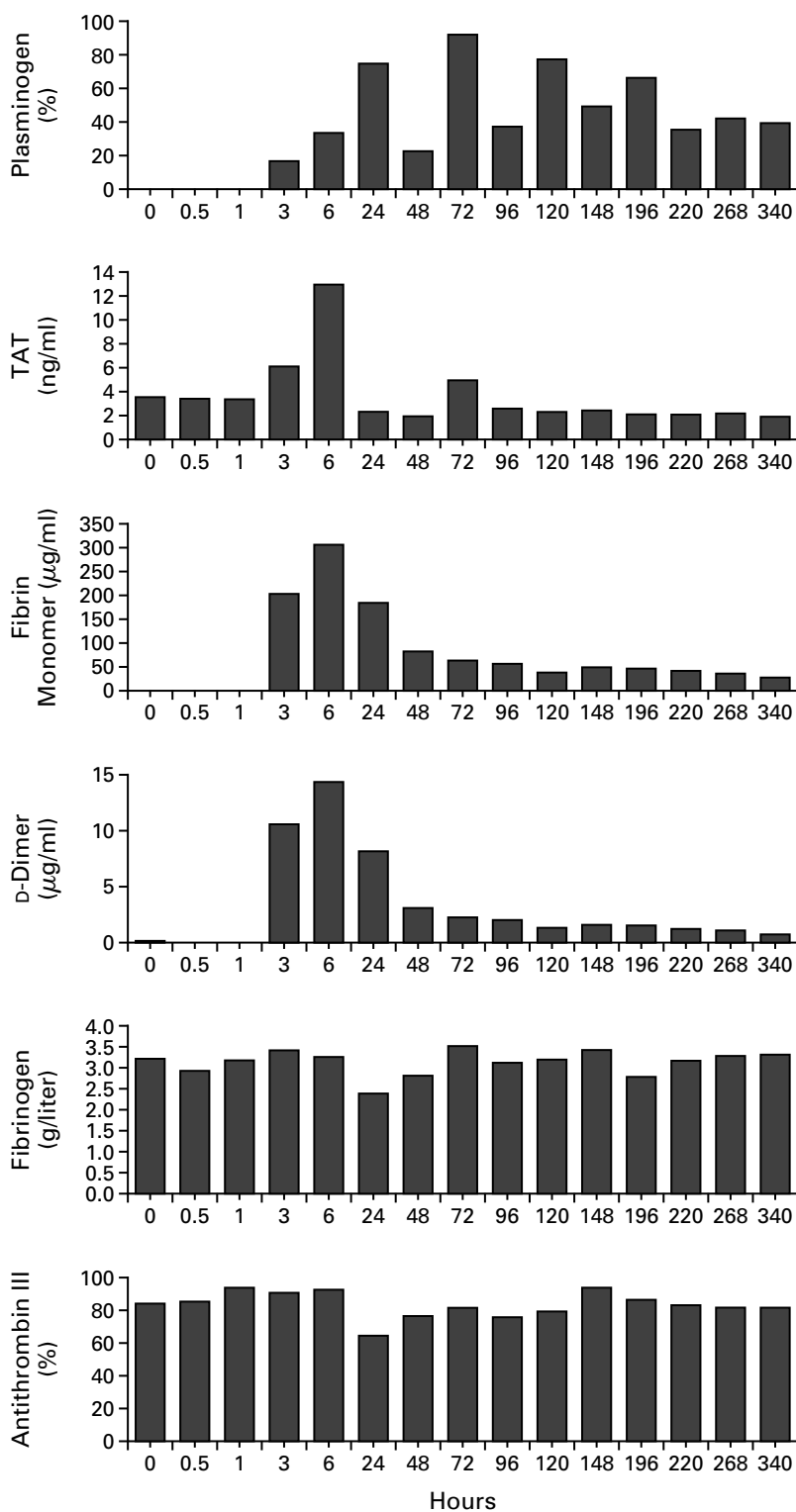


Figure 5. Levels of Plasminogen, Thrombin–Antithrombin Complex (TAT), Fibrin Monomer, D-Dimer, Fibrinogen, and Antithrombin III during the Initial 340 Hours of Continuous Intravenous Lys-Plasminogen–Replacement Therapy in a Patient with Homozygous Plasminogen Deficiency.

Hours are shown on a nonlinear scale indicating when measurements were made. Plasminogen and antithrombin III levels are expressed as percentages of normal values.

thrombin–antithrombin complex, but decreased only slowly over the following 10 to 12 days (Fig. 5). Fibrinogen and antithrombin activity remained virtually within the normal range throughout treatment, indicating the absence of consumption coagulopathy (Fig. 5). Thus, coagulation appeared to be somewhat activated as a result of fibrinolysis, without the consumption of fibrinogen or measurable antithrombin activity.

Similarly, in the pharmacokinetic study with the administration of a bolus of 2000 CU of lys-plasminogen during the initial phase of therapy, D-dimer and thrombin–antithrombin complex levels showed slight increases, suggesting fibrinolytic activation and the release of small amounts of thrombin. However, no effects were seen after the administration of 1000 CU of lys-plasminogen later in the course of therapy.

Clinical Course

Both the viscosity of the nasopharyngeal and tracheobronchial secretions and wound healing improved markedly within three days after the start of replacement therapy and subsequently became normal. After approximately two weeks, the conjunctival pseudomembranes softened and could easily be drawn off, although slight bleeding was observed on two occasions after removal. After four weeks of treatment, both eyes were largely free of membranes except for a small remnant, and vision was restored. Symptoms and fibrin deposits on the cornea reappeared when therapy was interrupted for more than 48 hours and disappeared again when plasminogen treatment was reinstated. After seven months of treatment (through a central venous catheter), only minor residues of liginous conjunctivitis remained (Fig. 6).

DISCUSSION

The fibrinolytic activity of all body fluids clears fibrin deposits. In the tear fluid of the eye, plasminogen activators released by the cornea³⁰ convert the zymogen plasminogen into the fibrinolytic enzyme plasmin, which rapidly clears the cornea of fibrin deposits. The absence of plasmin activity results in the formation of the fibrin-rich viscous or membranous material typically seen in patients with liginous conjunctivitis and in mice with targeted disruption of the plasminogen gene.^{19,20,31} An inflammatory reaction combined with activation of inflammatory cells and fibroblasts, with a drying out of the fibrin, results in the wood-like appearance of the conjunctival lesions. In a double-gene knockout-mouse model, the combination of plasminogen deficiency and fibrinogen deficiency prevents corneal fibrin deposits and associated inflammatory reactions and restores normal wound healing.^{19,20,32} Tracheobronchial fibrin deposition has been observed in our patient, in others with liginous conjunctivitis,^{2,5,13,33} and in plasminogen-deficient mice.³⁴ The fibrin deposits impair the



Figure 6. Patient with Homozygous Plasminogen Deficiency and Liginous Conjunctivitis after Seven Months of Replacement Therapy with Lys-Plasminogen.

ciliary system of the tracheobronchial tree and support bacterial growth, predisposing the patient to multiple sinobronchial infections. The ear involvement in our patient and others⁸ is attributable to fibrin deposition in the middle ear. The internal hydrocephalus reported in patients with liginous conjunctivitis^{4,12,13,16} and in plasminogen-deficient mice¹⁹ appears to be related to plasminogen deficiency. The pathophysiologic mechanism may be fibrin deposition in the cerebral ventricular system, causing impaired circulation of the fluid in the aqueduct region.

It is striking that no intravascular thromboembolic episodes occur in liginous conjunctivitis,¹⁷ despite a severe deficiency of the key zymogen of the fibrinolytic system. Heterozygous plasminogen deficiency also appears not to be a risk factor for thrombosis,^{35,36} despite several reports to the contrary.³⁷⁻⁴⁰ It remains a matter of speculation which mechanisms are involved in keeping the vasculature free of thrombosis even with

virtually complete plasminogen deficiency. Experiments with plasminogen-deficient mice have shown a 10 percent rate of spontaneous clot lysis after eight hours,⁴¹ suggesting the action of fibrinolytic proteases other than plasmin. Electrophoretic analysis of plasma obtained from our patient before treatment with lys-plasminogen concentrate showed the presence of small amounts of fibrin derivatives that did not react with antibodies against epitopes exposed by plasmin cleavage of fibrin.⁴² This suggests the presence of an alternative system for the dissolution of intravascular fibrin, which apparently is not active in the extravascular compartments.

Our patient had a homozygous inactivating mutation of the plasminogen gene that abolished the catalytic domain of plasmin and caused severe type I plasminogen deficiency. Because of this mutation, messenger RNA transcription, the half-life of messenger RNA of the mutant plasminogen allele, or both may be markedly decreased. As a result, the liver may synthesize a truncated plasminogen molecule that is rapidly cleared from the circulation, or secretion of the mutant plasminogen molecules may be impaired. In a recent study using genetic engineering techniques, the latter mechanism has been demonstrated in vitro for type I plasminogen deficiency caused by a Ser572Pro mutation.⁴³ In addition to the Glu460Stop plasminogen mutation, two other homozygous mutations (Arg216→His and Trp597Stop) have recently been identified in two unrelated Turkish girls with ligneous conjunctivitis and severe type I plasminogen deficiency.¹⁷ These molecular genetic findings confirm assumptions by others^{4,23,29} that ligneous conjunctivitis is inherited, probably in an autosomal recessive manner.

Mingers et al.¹⁸ were the first to try low-dose lys-plasminogen concentrate for the short-term treatment of patients with homozygous plasminogen deficiency, but they did not observe any definite effect on clinical symptoms. In our patient, long-term plasminogen-replacement therapy at a dose 2 to 10 times as high as that used by Mingers et al. led to complete regression of the ligneous conjunctivitis and normalized both the hyperviscous secretions in the respiratory tract and wound healing. The regression of symptoms with replacement therapy and their reappearance when therapy was interrupted demonstrate the pivotal role of plasminogen in extravascular fibrinolysis and confirm that severe plasminogen deficiency causes ligneous conjunctivitis.

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