myeloma proteins have antibody activity for specific antigens (*27*). These proteins are the secreted immunoglobulin products of malignant plasma cells, a mature cell in the B-cell lineage. Antibody activity of immunoglobulin from B-cell CLL or B-cell tumors, which are immature relative to the plasma cell, do not secrete immunoglobulins, and a candidate antigen that might be responsible for the initial or chronic stimulation is not readily apparent. Application of hybridoma technology allowed us to produce the immunoglobulin from the B-cell CLL, and HTLV-I was the obvious candidate antigen.

The results of our studies lead us to hypothesize a role through an indirect mechanism for HTLV-I in B-cell leukemogenesis in these cases (patients I.C. and L.L.), in contrast to the postulated direct leukemogenic role of HTLV-I in ATL (*1, 2*). The reactivity of the tumor-associated immunoglobulin to HTLV-I–specific proteins, p24 in the first case, and large envelope in the second case, suggests specific antigen commitment of the leukemic B cells to retroviral antigens. These data are highly indicative that B-cell CLL may, in some cases, be a tumor of an antigen-committed B-cell clone.

In addition, we speculate that infection of the T cells (*10*), in these cases, may play a contributory role in the leukemogenic process by altering the normal immunoregulatory milieu. T cells and B cells infected with HTLV-I have been shown to produce a variety of lymphokines, including B-cell growth factor (*28, 29*). These lymphokines may act in concert with antigen stimulation to effect expansion of an antigen responsive B-cell clone which undergoes malignant transformation. Alteration in normal T-cell–B-cell interaction and regulatory processes may also be affected when T cells are infected with HTLV-I. In this regard, we have directly demonstrated that HTLV-I infection of antigen-specific T-cell clones alters their function and recognition patterns (*30*). Alteration of T-cell function by HTLV-I infection may lead to immunosuppression. Immunosuppressed individuals (transplant recipients, patients with AIDS, the acquired immune deficiency syndrome) are known to have a higher incidence of B-cell lymphomas than that expected in the general population. Thus, HTLV-I infection in patients I.C. and L.L. may have provided the antigenic stimulus and altered T-cell regulatory process that results in B-cell malignancy. This indirect role of a retrovirus in leukemogenesis has substantial implication for the search for other retroviruses that may be linked to some human lymphomas where a virus cannot be directly demonstrated by probing the tumor.

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Human Amnion Membrane Serves as a Substratum for Growing Axons in Vitro and in Vivo

GEORGE E. DAVIS,* SCOTT N. BLAKER, EVA ENGVALL, SILVIO VARON, MARSTON MANTHORPE,† FRED H. GAGE

The epithelial cell layer of human amnion membrane can be removed while the basement membrane and stromal surfaces remain morphologically intact. Such a preparation has been used as a substratum for the in vitro culture of dissociated neurons. Embryonic motor neurons from chick ciliary ganglion attached to both surfaces but grew extensive neurites only on the basement membrane. On cross sections of rolled amnion membranes, regenerating axons of cultured neurons were guided along pathways of basement membrane that were immunoreactive with an antibody to laminin. In addition, when rolled amnion membranes were implanted into a lesion cavity between the rat septum and hippocampus, cholinergic neurons extended axons through the longitudinally oriented implant into the hippocampus. Thus, this amnion preparation can serve as a bridge to promote axonal regeneration in vivo in damaged adult brain.

WHEN PROVIDED WITH AN APpropriate growth environment in vivo, central nervous system neurons will extend axons. Such an environment normally exists for developing neurons and can be experimentally provided to damaged adult neurons by grafts of fetal brain (*1*) or peripheral nerve (*2*). However, most of these grafts are neural tissues, and their supply is often limited and sometimes con-

G. E. Davis, S. Varon, M. Manthorpe, Department of Biology (M-001), University of California, San Diego, La Jolla, CA 92093.
S. N. Blaker and F. H. Gage, Department of Neurosciences (M-024), University of California, San Diego, La Jolla, CA 92093.
E. Engvall, Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, CA 92037.

*Present address: Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
†To whom correspondence should be addressed.

troversial, particularly for use in humans. Several purified proteins, including collagen, fibronectin, and laminin, promote the in vitro adhesion of neural cells and stimulate axonal elongation from both peripheral and central nervous system neurons (*3*). Collagen, fibronectin, and laminin are components of the extracellular matrix, and laminin, the most potent in stimulating axonal growth in vitro (*4*), is concentrated in all basement membranes (*5*). The success of peripheral nerve grafts in promoting axonal regeneration in vivo may be due, in part, to their longitudinally oriented basement membranes (*6*).

To test directly whether basement membranes promote neuritic regeneration, we have utilized acellular preparations of human amnion membrane matrix that have a morphologically intact basement membrane on one side and a stromal surface on the other (*7*). The basement membrane contains laminin, collagen type IV, and heparan sulfate proteoglycans as major constituents, while the stromal surface contains interstitial collagens and proteoglycans. We show in vitro that the basement membrane surface stimulates and guides growing neurites from cholinergic chick ciliary ganglion motor neurons. We also report that, when implanted in vivo into a cavity produced by a lesion that disconnects the adult rat septum from the hippocampus, the amnion membrane will serve as a "bridge" for cholinergic axons regenerating to the hippocampus.

Human fetal membranes were obtained from full-term placentas within 24 hours of normal delivery. The amnion membrane was separated from the chorion (*7*); rinsed in phosphate-buffered saline containing penicillin, streptomycin, and Fungizone; and incubated in 0.1% ammonium hydroxide for 10 to 15 minutes. The epithelial cells were removed by gentle brushing and repeated rinsing of the membranes. To study the influence of the amnion membrane on in vitro neuritic growth responses by ciliary ganglion neurons, pieces of membrane were bound to nitrocellulose paper with the basement membrane (side originally facing the epithelium) or the stromal surfaces facing up, and the neurons were cultured on either surface. Alternatively, the membrane was carefully rolled up, frozen, and cross-sectioned, and the sections were adsorbed to nitrocellulose to yield immobilized, patterned arrays of basement membrane and stroma on which the neurons were then cultured. A diagram of the three preparations and a representative photograph of neurons cultured on them are shown in Fig. 1. Chick ciliary ganglion neurons, visualized by immunoperoxidase staining with a monoclonal antibody to neurofilaments (*8*),

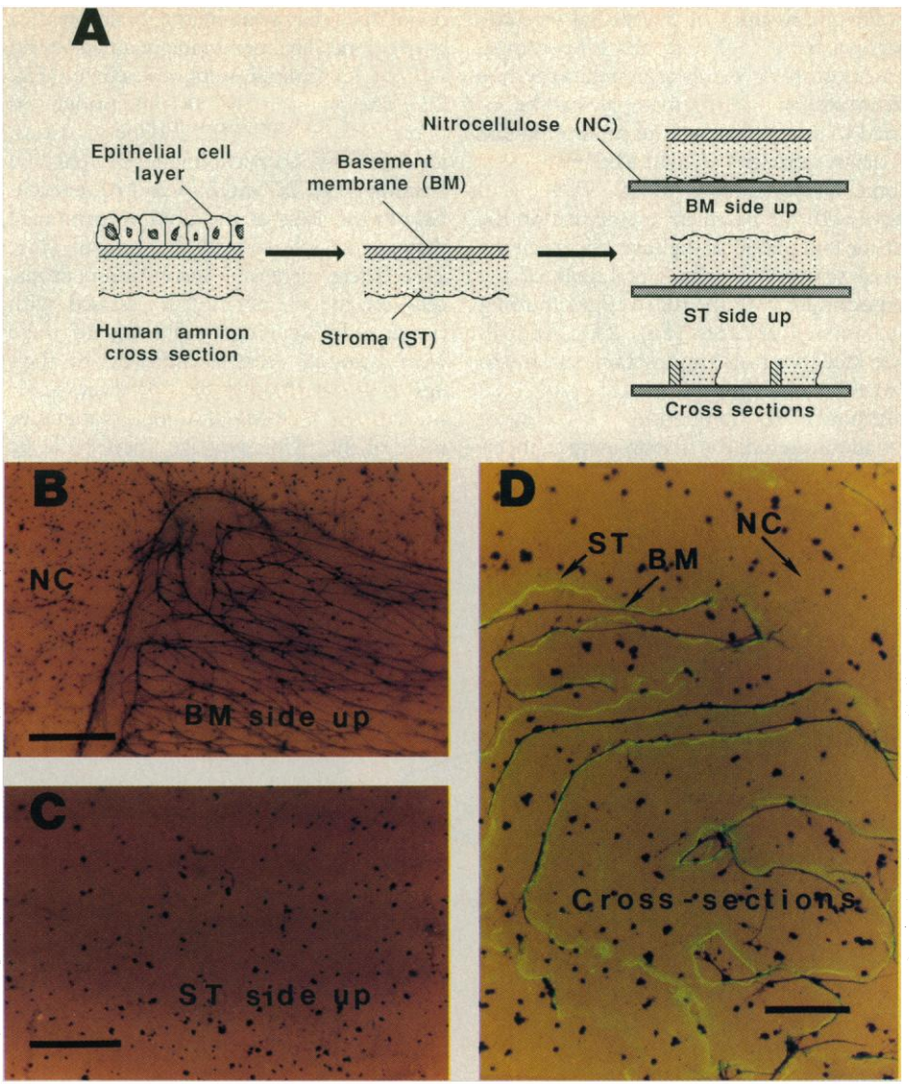

Fig. 1. The human placental amnion membrane extracellular matrix as a substratum for the regeneration of cultured neurons. (**A**) Pieces of acellular human amnion membrane were anchored to nitrocellulose paper (NC) with the basement membrane (BM) or stromal (ST) sides up. Alternatively, the membrane was coiled, frozen, and cross-sectioned, and the frozen sections were anchored to nitrocelluose. Purified embryonic (8-day) motor neurons from chick ciliary ganglia were prepared and cultured in serum-free medium (*11*) in 16-mm wells containing one of the anchored amnion membrane preparations. After 24 hours, the cultures were fixed and neuronal somata and axons stained black with peroxidase with the RT97 antibody to neurofilaments (*9*). Cross sections were further stained with antiserum to human laminin (*9*). An extensive neurite outgrowth occurred from neurons cultured on the BM surface (**B**) but not on the underlying nitrocellulose paper. Also, neurites did not grow on the ST surface (**C**). On cross-sectioned amnion membrane (**D**), only those neurons originally attaching to the areas staining for laminin (yellow) have extended neurites (black), which appear to have been guided along the laminin-containing BM pathways. (B and C) Bar, 300 μm; (D) bar, 500 μm.

attached to both the basement membrane (Fig. 1B) and stromal (Fig. 1C) surfaces of the amnion membrane but grew extensive neurites only on the basement membrane side. The neurons that contacted the basement membrane portion of the cross-sectioned membranes, identified by immunostaining for laminin (*9*), extended neurites that appeared to have followed the contours of this narrow pathway (Fig. 1D). Little neurite growth was observed on the juxtaposed cross-sectioned stroma or on the nitrocellulose paper.

The neurite-promoting and guiding influence of the amnion membrane for neurons in vitro was next applied to adult central nervous system neurons in vivo. We chose as a model the septo-hippocampal system of the adult rat because septal innervation of the hippocampus can be experimentally interrupted, and the axotomized septal neurons exhibit essentially no spontaneous regeneration back into the denervated hippocampus except in certain transplantation procedures (*1, 10*). When the axons in the fornix-fimbria pathway projecting from the

cholinergic neurons of the medial septum–diagonal band region to the hippocampal formation are surgically aspirated, a cavity is left in which bridging materials can be inserted to assess their neurite promoting and guidance capacities (*1, 10*). The human amnionic membrane was rolled as described above with the basement membrane on the outside and placed in the cavity between the dorsal septum and the septal pole of the hippocampus with the rolled edges abutting the lesioned surfaces (Fig. 2A). Animals were killed for histological examination 2 to 8 weeks after surgery.

Although no immunosuppressive agent was administered, the human amnion membrane persisted in the rat brain with no greater immunological reaction in the host brain than that seen in the brains with a cavity alone. The membrane retained strong staining for laminin immunoreactivity (Fig. 2B). The amnion membrane coil bridge was in contact with numerous cholinergic axons, identified by acetylcholinesterase (AChE) staining (Figs. 2C and 3, A and B), particularly in the areas of the amnion membrane that are heavily stained for laminin (Fig. 2D). There were also many non-neuronal cells on the membrane, as assessed with cresyl violet staining, and some of these were astroglial cells, as indicated by their immunoreactivity for glial fibrillary acidic protein. In the denervated hippocampus, some of the AChE-positive fibers could be seen to abandon the amnion membrane (Fig. 3, C and D) and to enter the hippocampus and distribute in a laminated pattern in the inner molecular layer of the dentate gyrus (Fig. 3, E through H). Some fibers could be traced from the lesioned septum through the amnionic membrane into the hippocampus. It remains possible that other sources of cholinergic fibers could contribute to the observed AChE-positive growth on the amnion membrane.

Thus, human amnion membrane can stimulate neurite elongation in vitro and in vivo. Large quantities of this membrane can be obtained sterilely from human placentas normally discarded in hospitals; it does not contain living cells, has a consistency that allows convenient cutting, folding, coiling, stretching, and suturing, and can promote neurite extension, an activity that is stable in

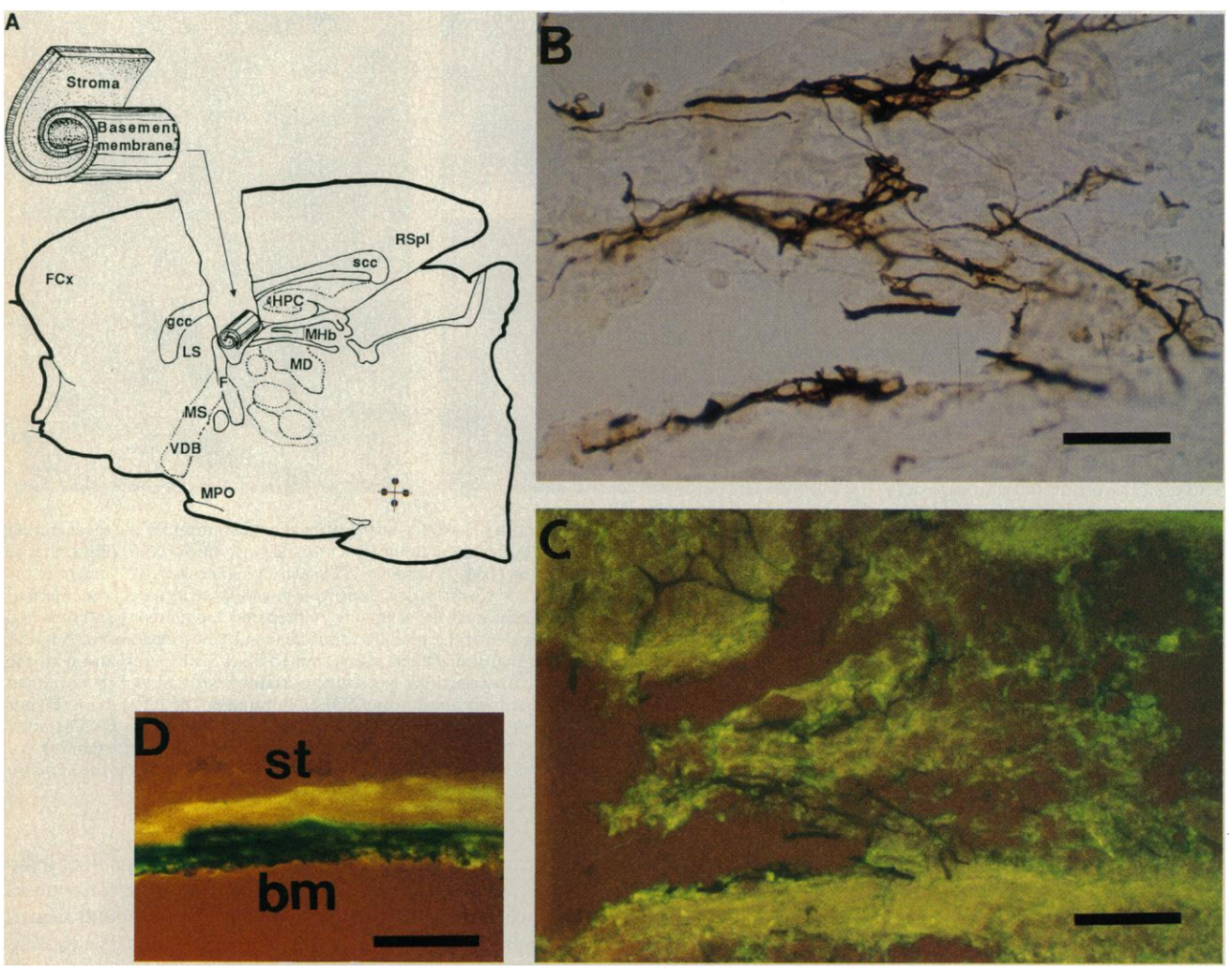

Fig. 2. Human placental amnion membrane extracellular matrix as a bridge for the regeneration of transected adult rat brain septal cholinergic axons. (**A**) Schematic drawing of a sagittal section through the rat brain. The cavity is depicted, as well as the approximate orientation of the membrane implanted in the cavity. After implantation (for 2 to 8 weeks), the brains were stained for AChE and laminin immunoreactivity in the same section (*12*), and with cresyl violet for Nissl substance, and, in independent, adjacent sections, with antiserum to glial fibrillary acidic protein for astroglial cells. (**B**) Photomicrograph with transmitted light source of AChE-positive fibers oriented horizontally between the septum and the hippocampus in the amnionic membrane bridge. (**C**) Photomicrograph with an epi-illumination fluorescent light source of immunoreactivity to laminin in the amnionic membrane of the same section as (B). (**D**) Photomicrograph of combined AChE and laminin staining within the same section. FCx, frontal cortex; RSpl, retrosplenial cortex; gcc, genu of corpus callosum; HPC, hippocampus; MHb, medial habenula; MD, medial dorsal thalamus; F, fornix; MS, medial septum; VDB, vertical limb of the diagonal band of Broca; MPO, medial preoptic area; LS, lateral septum; scc, splenium of the corpus callosum; bm, basement membrane side of human amnion membrane; st, stroma side of amnion membrane. (C) Bar, 100 μm; (B and D) bar, 50 μm.

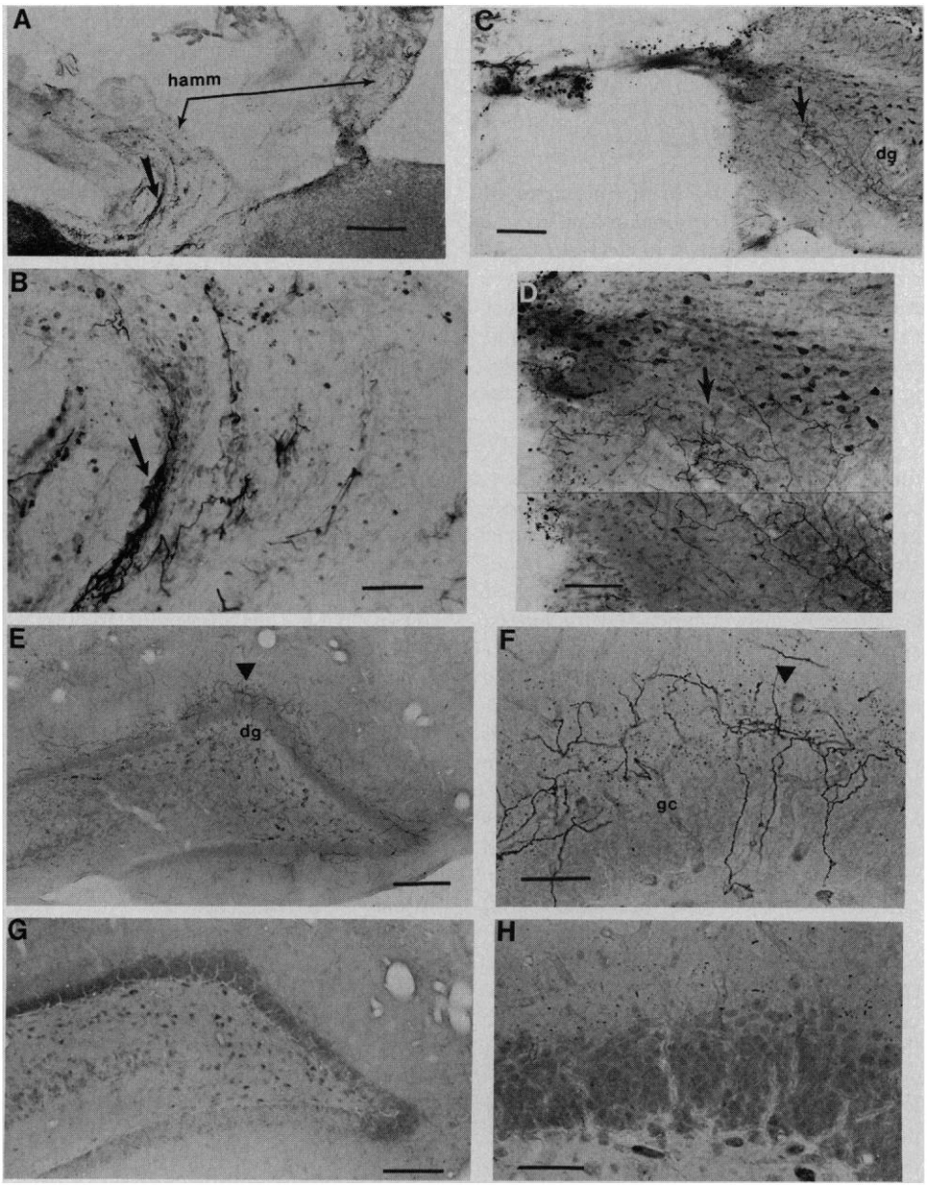

Fig. 3. Growth of acetylcholinesterase (AChE)-positive fibers extending on the human amnion membrane matrix (hamm). (**A** and **B**) Photomicrographs of AChE-positive fibers extending from the septum onto the coiled hamm. Thick arrows indicate AChE fibers and thin arrows indicate amnion membrane; (A) bar, 300 μm; (B) bar, 120 μm. (**C** and **D**) AChE-positive fibers extending from the hamm into the previously denervated dentate gyrus of the hippocampal formation. Arrows indicate AChE fibers; dg, dentate gyrus; (C) bar, 300 μm; (D) bar, 120 μm. (**E** and **F**) AChE-positive fibers within the previously denervated dentate gyrus 2 weeks after implantation of hamm into the fornix-fimbria cavity. Arrowheads indicate AChE fibers; gc, granule cells; (E) bar, 300 μm; (F) bar, 50 μm. (**G** and **H**) AChE-stained dentate gyrus 2 weeks after fornix-fimbria transection without hamm implantation; (G) bar, 300 μm; (H) bar, 50 μm. Of ten animals with hamm implants examined in our study, all had at least partially intact hamm implants and AChE-positive fibers on the hamm, and eight of the ten animals had fiber ingrowth into the hippocampus. However, the number of AChE-positive fibers on the hamm and the degree of ingrowth was variable between animals. Four animals were used as lesion controls with no implants; none of these animals had any AChE-positive growth into the dentate gyrus of the denervated hippocampus. Also, correlations were not apparent either between the amount of fiber growth on the hamm and the ingrowth into the hippocampus or between the number of weeks examined following hamm implantation and the degree of ingrowth.

vitro for more than 6 months at 4°C. In in vivo experiments, the membrane does not cause inflammation, even when grafted into an adult rat brain. Thus, this acellular amnion membrane preparation offers advantages over the use of living tissues as a prosthetic bridge in experimental regeneration studies of the peripheral or central nervous system and as a tool to help elucidate the mechanisms by which an extracellular matrix stimulates axonal growth and cell migration from neural tissue.

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