HTLV-I–Associated B-Cell CLL: Indirect Role for Retrovirus in Leukemogenesis

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Serum containing antibodies to the human T-lymphotropic virus type I (HTLV-I) has been observed at a higher than expected frequency in patients with B-cell chronic lymphocytic leukemia (CLL) in an area endemic for HTLV-I. An attempt was made to determine whether the cells from patients with this leukemia were HTLV-I antigen–committed B cells that had undergone malignant transformation. Cells from two HTLV-I seropositive Jamaican patients with CLL were fused with a human B-lymphoblastoid cell line. The hybridoma cells that resulted from the fusion of CLL cells from patient I.C. produced an immunoglobulin (IgM) that reacted with the p24 *gag* protein from HTLV-I, HTLV-II, and HTLV-III (now referred to as HIV), but showed preferential reactivity with HTLV-I. The specific immunoglobulin gene rearrangement (IgM, κ) in the CLL cell was demonstrated in the hybridoma cell line, indicating that the captured immunoglobulin was from the CLL cells. The IgM secreted by the fusion of CLL cells from patient L.L. reacted only with HTLV-I–infected cells and with the HTLV-I large envelope protein (gp61) on Western blots. The CLL cells from these patients appear to be a malignant transformation of an antigen-committed B cell responding to HTLV-I infection, suggesting an indirect role for this retrovirus in leukemogenesis.

AN AGGRESSIVE FORM OF ADULT T-cell leukemia (ATL) is associated with infection by the human T-lymphotropic virus type I (HTLV-I) [for reviews, see (*1, 2*)]. In patients with ATL, HTLV-I sequences are integrated in the tumor cell DNA, and antibodies to viral proteins are found in these patients' serum (*1, 2*). Antibodies to HTLV-I are also found in the serum of patients with other lymphoid malignancies (*3–5*). However, HTLV-I sequences have not been found in non-ATL lymphomas from these patients (*6*). Thus far, there has been no report of direct, integrated HTLV-I infection in tumor cells of lymphoid or hematopoietic origin except in T lymphocytes, such as in ATL.

Epidemiological studies in the West Indies, an area endemic for HTLV-I, have documented the frequent occurrence of HTLV-I seropositivity in patients with ATL (*3, 7*) as well as the presence of monoclonally integrated HTLV-I sequences in tumor cells from these patients (*6, 8*). These studies have also shown that the frequency of HTLV-I seropositivity is higher in patients with chronic lymphocytic leukemia (CLL) than in the general West Indian population (*3*). These data suggest that HTLV-I may, by some indirect mechanism, effect the pathogenesis of CLL.

A number of models have been suggested whereby retroviruses may indirectly effect leukemogenesis. These include: binding of virus to receptors which in turn stimulate and expand a cell population that undergoes malignant transformation (*9*); malignant transformation of regulatory cells in a cell population responding to viral infection (*10*); and a "hit and run" mechanism wherein retroviruses rearrange and activate cellular proto-oncogenes (*11*). Another hypothesis, which does not involve retroviruses specifically, is that an antigen–committed B-cell clone expands and undergoes malignant transformation (*12*). The epidemiologic data prompted us to explore the possibility that one or more of these models might be relevant to the B-cell CLL observed in patients with HTLV-I infection.

Table 1. Binding of ^{125}I-labeled p24 protein from HTLV-I to CLL cells from six patients. CLL cells from these patients were cryopreserved as described. The cells were thawed and placed in culture for 48 hours in RPMI 1640 containing 10% heat-inactivated fetal bovine serum. Labeled p24 (10,000 counts per minute) was added to 10^5 cells in a total volume of 100 μl of the above medium. Quadruplicate samples from each individual were incubated for 2 hours at 37°C and washed three times with 50 volumes of medium. The cells were centrifuged and washed, and the radioactivity was determined by means of a gamma counter. Unlabeled p24 was added at protein concentrations of 1, 5, and 10 μg together with the ^{125}I-labeled p24 and the test was carried out as described above. Only the results of the incubation with 10 μg of p24 are shown. Values are in counts per minute (± SEM). ND, not determined.

CLL cell source	Direct binding of ^{125}I-labeled p24 to CLL cells	Effect of addition of 10 μg of unlabeled p24
I.C.	1258 ± 204	312 ± 114
H.P.	102 ± 86	116 ± 80
L.L.	123 ± 44	102 ± 54
F4706	118 ± 72	ND
F4528	92 ± 34	ND
F0859	106 ± 60	92 ± 36

In previous experiments, we were able to cause secretion, in culture, of cell surface immunoglobulin of CLL cells by fusing these cells with a human B-lymphoblastoid line (*13*). The clonality of this product was documented by demonstrating a specific idiotypic marker shared by the cell surface and "captured" immunoglobulin. We have applied the same methodology in the present study, using CLL cells from two patients with serum antibodies to HTLV-I proteins, to determine whether the immunoglobulins from the CLL reacted with the virus.

The two Jamaican patients (I.C. and L.L.) with CLL that were studied had high levels of serum antibody to HTLV-I proteins. One patient (H.P.) with CLL from the United States was seronegative (*3, 14*). Peripheral blood lymphoctyes (PBL) were obtained from these patients by venipuncture, isolated by the standard Ficoll-Hypaque gradient technique, and cryopreserved. Greater than 97% of the cells from all three patients appeared morphologically as CLL, and had cell surface markers as well as immunoglobulins characteristic of B-cell CLL (*14, 15*). In our previous analysis, we documented that the CLL cells from the antibody-positive patient I.C. lacked integrated virus in the B cells, whereas a T-cell line established from this patient demonstrated a pattern of polyclonal virus integration (*15*). The CLL cells from the serum antibody positive patient L.L. also lacked detectable HTLV-I (*6*).

Cryopreserved PBL to be used for fusion were thawed and placed in culture for 24 hours in RPMI 1640 medium containing 10% fetal bovine serum, 25 m*M* Hepes, 2 n*M* L-glutamine, and 100 m*M* hypoxanthine. These cells were fused with the hypoxanthine, quinine phosphoribosyltransferase negative B-lymphoblastoid cell line WI-L2-729-HF2 (Techniclone International, California) at a 5:1 ratio with the use of 50% polyethylene glycol. The fused cells were distributed over 264 wells in microculture

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plates and grown in 200 μl of RPMI 1640 medium supplemented as described above with 1 μg/ml of azaserine substituted for hypoxanthine to establish the growth conditions needed to select hybridoma cells (*16*). The culture supernatants were tested for immunoglobulin production (IgM, IgD, IgA, IgG) by using a double-sandwich enzyme-linked immunoassay (ELISA) comparing culture fluids to known concentrations of the immunoglobulins with the different isotypes (*17*). Immunoglobulin M was the only isotype found to be produced by the hybridomas from the fusion of the CLL cells from the three patients. The individual cultures producing IgM were expanded and tested for the presence of HTLV-specific antibodies by flow cytometry with intact cells infected with HTLV-I, HTLV-II, and HTLV-III (now referred to as HIV), or by ELISA with concentrated HTLV-I, -II, and -III virus particles (*18*).

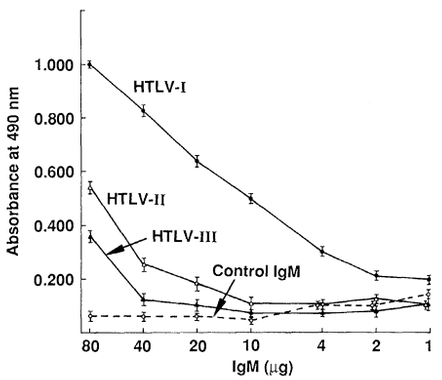

Fig. 1. Virus particles from cell lines C10-MJ infected with HTLV-I, C3-44 infected with HTLV-II, and H9 infected with HTLV-III were concentrated from culture supernatants. Virus particles were partially disrupted by multiple freeze-thaw cycles and diluted in 0.06*M* carbonate-bicarbonate buffer, *p*H 9.6, to a final protein concentration of 4 μg/ml. The viral components were attached to polyvinyl chloride assay plates (Costar) by addition of 100 ng of protein per well and incubation at room temperature for 12 hours. The plates were washed and 95% methanol added for 30 minutes. After further washing with RPMI 1640 containing 10% fetal bovine serum, dilutions of the culture supernatants from the I.C. and H.P. hybrids were added to the virus-coated plates. The avidin-biotin horseradish peroxidase system (ABC Vectastain Kit, Vector Laboratories) was used as described by the manufacturer to develop the reactions. Human antisera known to react with each virus were used as controls as was a rabbit serum that detects a common antigen on p24 from HTLV-I, -II, and -III (*21*). The enzyme reaction was developed by the addition of 100 μl of the substrate solution [50 m*M* citrate buffer, *p*H 4.0, containing 1 mg/ml of orthophenylendiamine (Sigma) and 5 μl/ml of 30% H_2O_2] to each of the wells. After incubation at room temperature for 60 minutes the reaction was stopped by the addition of 50 μl of 2.0*M* H_2SO_4 per well. The tests were measured with an automatic ELISA reader (Dynatech Laboratories) and the absorbance at 490 nm was recorded. The IgM used for control was produced by fusing CLL cells from the HTLV-I seronegative patient H.P. Data points represent the mean ± SEM of quadruplicate tests.

Significant binding to HTLV-I was detected when we used as little as 4 μg/ml of the IgM (Fig. 1). Binding of this immunoglobulin to HTLV-II particles was also observed, but higher concentrations of IgM (20 μg/ml) were required. Immunoglobulin binding to HTLV-III required IgM concentrations of 80 μg/ml or more. The control IgM used in these and other studies was produced by the fusion product of CLL cells from the HTLV-I seronegative patient (H.P.).

The reactivity pattern of this antibody to the three different HTLV types demonstrated the existence of a shared epitope. Because the greatest degree of genomic homology among the three types of viruses is in the *gag* region (*19*), we used purified p19 and p24 *gag* proteins from each virus to test for reactivity with the immunoglobulin.

The *gag* proteins were purified from concentrated virus preparations from the cell lines C10-MJ (HTLV-I), C3-44 (HTLV-II), and H9 (HTLV-III). The virus was solubilized with 0.5 m*M* phenylmethylsulfonylfluoride and 50 m*M* tris-HCl (*p*H 7.9). Nucleic acids were removed by batch absorption with DEAE cellulose, and the p19 and p24 proteins were isolated by elution from a phosphocellulose column that gave a single band on gel electrophoresis (*20*).

The IgM reacted against p24 proteins of all three virus strains but bound preferentially to the p24 of HTLV-I (Fig. 2). No binding was observed to the p19 proteins. The antibody binding patterns with the p24 from HTLV-II and -III were as would be predicted from the binding patterns observed when whole virus particles are used; HTLV-II p24 reacted to a higher degree than the HTLV-III p24, but neither were recognized as well as the HTLV-I p24 protein. These reaction patterns were similar to those previously observed with a polyclonal serum (*21, 22*). Thus, the immunoglobulin produced by the CLL–B-lymphoblastoid hybridoma derived from patient I.C. appears to have antibody activity to an epitope that is shared by HTLV-I, -II, and -III on a protein product of the region of these viruses that, in the proviral DNA, has the highest degree of homology.

To ensure that the IgM with antibody activity to the p24 *gag* proteins was a product of immunoglobulin genes of the CLL cell and not of a non-CLL peripheral blood B cell, we determined specific immunoglobulin gene rearrangement in the CLL cell, the B-lymphoblastoid cell line (WI-L2-729-HF2), and the fusion product of these two cells. High molecular weight DNA was prepared from the three cell sources, digested with restriction endonucleases, and, after electrophoresis, was blotted to a nylon membrane and hybridized to light and heavy chain J region probes. The results for patient I.C. are shown in Fig. 3, A and B. With restriction enzymes Hind III, Bam HI, and Sac I, the specific VDJ rearrangement observed in the CLL cells was found in the hybridoma cell line. These results establish the clonality of the CLL cells and demonstrate the presence of the rearranged genes in the hybridoma cell line that produced the immunoglobulin that reacted with the p24 protein.

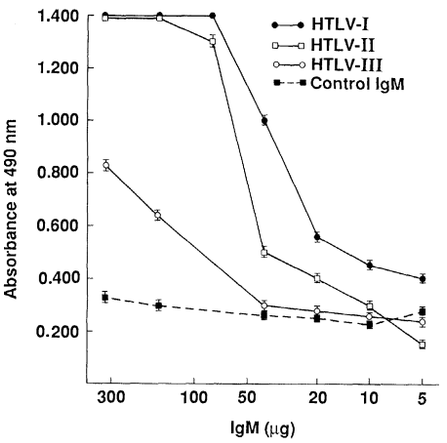

Fig. 2. The p24 *gag* proteins were purified from HTLV-I, -II, and -III as described in the text. The purified p24 was attached to polyvinyl chloride assay plates at a concentration of 100 ng of protein per well and used for ELISA as described in Fig. 1. Data points represent the mean ± SEM of quadruplicate tests.

We therefore conclude that the product of the CLL cells was an immunoglobulin with specific antibody activity to the p24 protein. To confirm this conclusion, we conducted studies to determine if the HTLV-I p24 protein bound specifically to the CLL cells from patient I.C. HTLV-I p24 was radiolabeled with ^{125}I as described (*20*), and incubated with CLL cells from six patients, two from an HTLV-I endemic area (I.C. and L.L.) and four from the United States (H.P., F4706, F4528, and F0859). As shown in Table 1, the CLL cells from I.C. bound significantly more ^{125}I-labeled p24 than did the CLL cells from other patients. The addition of 10 μg of unlabeled p24 inhibited 75% of the binding of the labeled p24 and had no effect on the low levels of binding of the labeled p24 to other CLL cells.

The IgM captured by fusion of CLL cells from patient L.L. had a pattern of reactivity different from that seen with the I.C. fusion. Low levels of reactivity in the ELISA assay were observed only with the isolated HTLV-I. No reactivity was detected with HTLV-II or HTLV-III or the p19 and p24

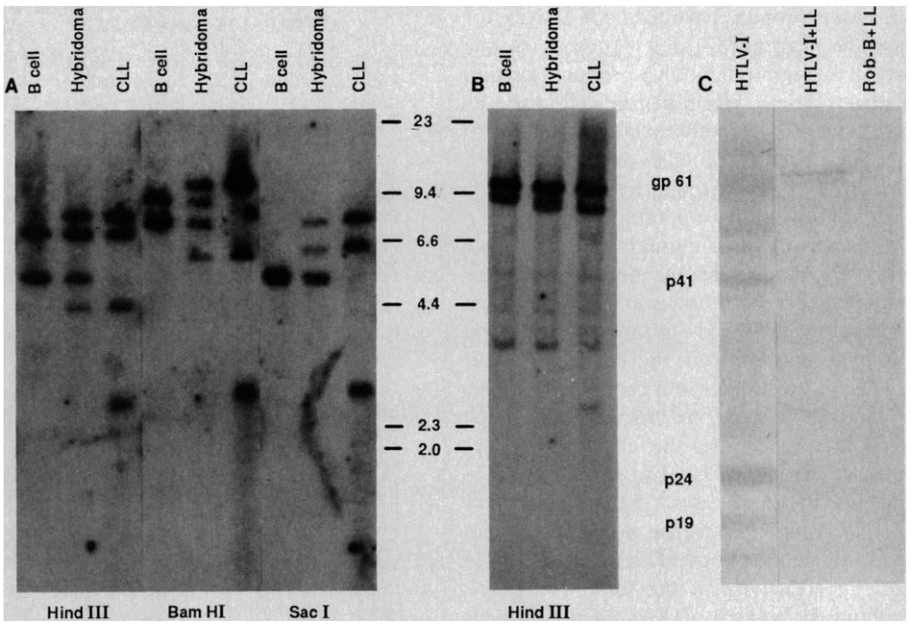

Fig. 3. (A and B) Autoradiograms of Southern blots of endonuclease-digested genomic DNAs from the B-lymphoblastoid line (WI-L2-729-HF2), the I.C.-derived hybridoma, and the CLL cells from patient I.C. after hybridization to molecular probes for the germline human immunoglobulin light and heavy chain J regions. Genomic DNAs (10 μg) from the indicated cells were digested with Hind III, Bam HI, or Sac I, subjected to electrophoresis on a 0.7% agarose gel, and transferred to a nylon membrane (GeneScreen, New England Nuclear, NEN). The resulting blots were hybridized (as described by NEN) with ^{32}P-labeled nick-translated DNA probes (*23*): (**A**) κ light chain J region probe, a 1.8-kb Sac I fragment cloned into pBR322 containing sequences encompassing the five κ light chain J regions (*32*) and (**B**) heavy chain J region probe, a 6.0-kb Bam HI–Hind III fragment cloned into pBR322 containing the six J regions of the immunoglobulin heavy chain (*33*). The hybridized blots were washed twice with 2× SSC at room temperature for 5 minutes, twice with 2× SSC containing 1% SDS at 65°C for 30 minutes, and twice with 0.1× SSC at room temperature for 30 minutes, and then exposed for 22 hours at −70°C with an intensifying screen. Fragment sizes of coelectrophoresed radiolabeled Hind III–digested DNA are indicated in kilobases. The 2.7-kb band in the DNA digest from CLL cell is interpreted to be artifactual, because hybridization occurred when different enzymes were used with both the κ light and heavy chain J region probes and was seen only when DNA was prepared from cryopreserved cells. (**C**) SDS–polyacrylamide gel electrophoresis, Western transfer, and immunodetection of a viral protein with immunoglobulin produced by the L.L.-derived CLL hybrid [for methods, see (*24, 34*)]. The lane labeled HTLV-I is the electrophoretic separation of 25 μg of protein from viral particles concentrated from culture supernatants from the MJ-T cell line. The HTLV-I–specific proteins are designated by molecular weight. The lane labeled Rob-B is the electrophoretically separated protein (25 μg) from the culture supernatants that banded at the same density as did the virus. Addition of the immunoglobulin from the L.L.-derived hybrid to transferred proteins and development with antibody to human IgM demonstrates reaction with the 61-kD protein from HTLV-I and no reactions with proteins from Rob-B.

gag proteins from these viruses at IgM concentrations of 200 μg/ml. The supernatants from L.L.-derived IgM-producing hybrids were tested for reactivity on intact, viable, HTLV-I–, -II–, and -III–infected cells by flow cytometry, with the use of a fluorescence-activated cell sorter (FACS-II, Becton-Dickinson). We used the same lines as those described above, as well as C2-39 cord blood lymphocytes from a single individual infected with both HTLV-I (C239TK) and HTLV-II (C2-39Mo). HTLV-I–infected B-cell lines CF and HS, as well as noninfected B-cell lines and HTLV-I–infected T-cell lines established from patients with ATL, were also tested. Samples of 10^6 cells were exposed to 100 μl of tissue culture supernatants (100 μg of IgM per milliliter), washed, and centrifuged, and the pellet was exposed to 10 μl of fluorescein-conjugated rabbit antibody to human IgM. As shown in Table 2, the supernatant fluid from the L.L.-derived hybrid reacted only with those cells infected with HTLV-I. The specificity of the reaction of the immunoglobulin produced by the L.L.-derived hybrid was tested on Western blots after SDS–polyacrylamide gel electrophoresis of viral particles concentrated from the supernatants from the HTLV-I producing cell line MJ-T, and of proteins from supernatant from the HTLV-I negative cell line Rob-B that banded at the same density as the virus on sucrose gradients (*23, 24*). The immunoglobulin produced by the L.L.-derived hybrid reacted with a 61-kD protein from the HTLV-I preparation and showed no reaction with the Rob-B proteins (Fig. 3C). The flow cytometry results together with the results of the Western blots are consistent with the interpretation that this immunoglobulin reacts with the HTLV-I large envelope protein.

Table 2. Binding of immunoglobulin produced by the hybrid cell lines resulting from the fusion of CLL cells from the HTLV-I seropositive patient L.L. and the seronegative patient H.P. to HTLV-I–, -II–, and -III–infected T cells and Epstein-Barr virus (EBV)–infected B cells. HUT-102b, Rob-B, MJ-T, and MJ-B are, respectively, T- and B-cell lines derived from patients Rob and M.J. All cell lines with a "C" prefix were derived from umbilical cord blood lymphocytes. Cell lines CF and HS are HTLV-I– and EBV-infected B-cell lines that have been described (*31*).

Cell designation	Virus infection	Number reacting (%)	
		L.L.-derived hybrid	H.P.-derived hybrid
HUT-102b (T cell)	HTLV-I	28.0	0.6
Rob-B (B cell)	EBV	0.3	0.0
MJ-T (T cell)	HTLV-I	84.0	0.3
MJ-B (B cell)	EBV	0.1	0.2
C10-MJ (T cell)	HTLV-I	58.0	0.1
C33-Mo (T cell)	HTLV-II	0.2	0.0
H9 HTLV-III (T cell)	HTLV-III	0.3	0.2
C2-39 (T cell)	None	0.1	0.3
C239TK (T cell)	HTLV-I	57.0	0.4
C2-39Mo (T cell)	HTLV-II	0.5	0.2
CF (B cell)	HTLV-I, EBV	48.0	0.2
HS (B cell)	HTLV-I, EBV	53.0	0.6

These results fit, in part, with several of the models cited above. The B-cell CLL from patient I.C. bound the p24 protein, thus the cell surface immunoglobulin with antibody activity is the receptor for this viral component. B cells, the progenitor of the CLL, can be considered immune regulatory cells that respond to viral infection. The concept that best fits our results was proposed by Salmon and Seligmann (*12*) and was based on the finding of rheumatoid-like antibody activity on the surface of CLL cells during a period of blast crises (*25, 26*). In addition, there has been evidence that some

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

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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-

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