Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 646–650, January 1999
Immunology

cDNA cloning of FRIL, a lectin from *Dolichos lablab*, that preserves hematopoietic progenitors in suspension culture

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Contributed by Maarten J. Chrispeels, October 29, 1998

ABSTRACT ***Ex vivo* culture of hematopoietic stem cells is limited by the inability of cytokines to maintain primitive cells without inducing proliferation, differentiation, and subsequent loss of repopulating capacity. We identified recently in extracts of kidney bean and hyacinth bean a mannose-binding lectin, called FRIL, and provide here evidence that this protein appears to satisfy properties of a stem cell preservation factor. FRIL was first identified based on its ability to stimulate NIH 3T3 cells transfected with Flt3, a tyrosine kinase receptor central to regulation of stem cells. Molecular characterization from polypeptide sequencing and identification of the cDNA of hyacinth bean FRIL shows 78% amino acid identity with a mannose-binding lectin of hyacinth beans. Treatment of primitive hematopoietic progenitors in suspension culture with purified hyacinth FRIL alone is able to preserve cells for 1 month without medium changes. *In vitro* progenitor assays for human hematopoietic cells cultured 3 weeks in FRIL displayed small blast-like colonies that were capable of serial replating and persisted even in the presence of cytokines known to induce differentiation. These results suggest that FRIL is capable of preserving primitive progenitors in suspension culture for prolonged periods. FRIL's clinical utility involving procedures for stem cell transplantation, tumor cell purging before autologous transplantation, and *ex vivo* cultures used for expansion and stem cell gene therapy currently are being explored.**

The process of producing a balanced supply of blood cells starts with a rare and mostly quiescent population of stem cells in the adult bone marrow. Signals from cell–cell contact and soluble regulators activate stem cells to generate either identical daughter cells (self-renewal) or to begin an irreversible process that ends with the production of functional blood cells. Differentiation is coupled to proliferation as stem cells give rise to progenitors that progressively become committed to producing only one blood cell type. The enormous expansion of progenitors needed to meet the body's daily requirement for hundreds of billions of new mature cells is directed by potent soluble regulators [colony-stimulating factors (CSFs) and cytokines].

Stem cells and primitive progenitors do not readily respond to strong inducers of proliferation and differentiation acting alone. *In vitro* studies have demonstrated that single early-acting cytokines do not promote proliferation; instead, a combination of several cytokines is needed to activate primitive cells. Central to stem cell regulation are two ligands of the two related tyrosine kinase receptors Kit and Flt3 (1). These ligands recruit quiescent stem cells into cycle and enhance their proliferative capacity when combined with early-acting synergistic regulators [interleukin 1 (IL-1), IL-4, IL-6, IL-11, IL-12, IL-12, leukemia inhibitory factor] or CSFs [IL-3, granulocyte–macrophage (GM)-CSF, G-CSF, M-CSF, Tpo]. The Flt3 ligand (FL), in particular, prolongs maintenance of highly primitive human bone marrow cells in long-term stromal cocultivation cultures (2) and of primitive human cord blood cells in stromal-free suspension cultures (3, 4). Using engraftment in nonobese diabetic/severe combined immunodeficient (SCID) mice to assess the *in vivo* repopulating capacity of highly enriched cord blood cells, FL-containing cultures maintain SCID-repopulating cells up to 8 days in suspension cultures (4, 5). However, these culture conditions induce extensive proliferation and differentiation, resulting in the loss of repopulating capacity of primitive cells.

Better control of stem cells *ex vivo* is needed to facilitate clinical applications to expand the number and quality of stem cells and progenitors for transplantation, to purge tumor cells for autologous transplantation, and to improve efficiency of transferring corrective genes into stem cells. *Ex vivo* preservation of stem cells allowing slow turnover without differentiation would (*i*) facilitate the timing and extent of *ex vivo* expansion of primitive cells, (*ii*) keep stem cells quiescent while exposing tumor cells to higher levels of chemotherapy, and (*iii*) synchronize the cell cycle of stem cells to improve efficiency of gene transfer.

We recently identified a new plant lectin in red kidney bean (*Phaseolus vulgaris*) extracts that appears to satisfy properties of a stem cell preservation factor (6). The protein was identified in a biological screening assay based on its ability to stimulate the proliferation of NIH 3T3 cells transfected with Flt3 but not cells transfected with the related Fms receptor or untransfected cells. The new lectin was named Flt3 receptor-interacting lectin (FRIL) based on its functional properties. During purification, FRIL-containing fractions were tested for activity on cord blood cells enriched for primitive cells based on CD34 selection. FRIL fractions maintained a small population of viable cells that uniformly expressed CD34 after 2 weeks in suspension culture containing IL-3 without medium changes. Neither FL, other early-acting cytokines, nor other plant lectins have been reported to maintain $CD34^+$ cells in culture. This observation led to the hypothesis that FRIL acts in a dominant manner to preserve progenitors by preventing their proliferation and differentiation even in the presence of a cytokine known for its strong induction of proliferation and differentiation.

FRIL obtained from red kidney beans is composed of two different polypeptides with molecular masses of 18 kDa and 15 kDa. Amino acid sequencing of these two proteins indicated

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Abbreviations: CB mnc, cord blood mononuclear cells; DLL, *Dolichos lablab* lectin; FRIL, Flt3 receptor-interacting lectin; FL, Flt3 ligand; PHA, phytohemagglutinin; CSF, colony-stimulating factor; IL, interleukin; SCID, severe combined immunodeficient; RACE, 3′ rapid amplification of cDNA ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. frlgb AF067417).

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significant amino acid identity with an already reported mannose-binding lectin isolated from hyacinth bean (*Dolichos lablab*) (7). To further characterize this hyacinth bean homolog, we purified and characterized its constituent polypeptides, examined its biological properties, and isolated the cDNA. FRIL, isolated from hyacinth beans, maintains hematopoietic progenitors in suspension culture up to 4 weeks. In contrast to early-acting cytokines, FRIL functionally selects cells (most cells die) that presumably express Flt3 and maintains them in a quiescent state. Consequently, the medium did not require replenishment during the month of culture. Cells cultured for 3 and 4 weeks in methylcellulose colony assays generated colonies consisting of undifferentiated cells. Cells harvested from these colonies and replated in colony assays generated small colonies consisting of undifferentiated cells. These results support the conclusion that FRIL preserves primitive hematopoietic cells in suspension culture for prolonged periods.

MATERIALS AND METHODS

Plant Material and FRIL Purification. Seeds of hyacinth bean (*Dolichos lablab*) were obtained from Stokes Seeds, (Buffalo, NY) and grown in a greenhouse. Dry seeds were ground in a coffee mill and the powder was extracted with 5 volumes of 50 mM Tris·HCl containing 1 mM $MgCl_2$ and $CaCl_2$ each, for 4 h at 4°C. After centrifugation at 20,000 × *g* for 20 min, the supernatant was acidified to pH 4.0 with acetic acid, centrifuged again to clarify, and readjusted to pH 8.0. This crude extract was stored frozen. Single-step purification of FRIL was achieved by binding to a mannose-Sepharose matrix (Sigma) and elution with 200 mM α-methyl α-D-mannoside. The gel was tumbled with the crude extract (see above) for 4–12 h at 4°C, carefully washed several times with 50 mM Tris·HCl containing 1 mM $MgCl_2$ and $CaCl_2$, and then eluted. FRIL purified in this manner was used for all subsequent experiments.

RNA Isolation and cDNA Synthesis. Total RNA was prepared from midmaturation *Dolichos lablab* seeds stored at −70°C following the procedure reported by Pawloski *et al.* (8). Poly(A)$^+$ RNA was obtained from this total RNA by using the PolyATract mRNA Isolation System (Promega) according to the manufacturer's instructions. Avian myeloblastosis virus reverse transcriptase (Promega) was used to generate cDNA from 0.5 or 3.0 μg of poly(A)$^+$ or total RNA, respectively, using 1 mg of oligo(dT) in the standard reaction conditions (9). In the cDNA synthesis for the 3′ rapid amplification of cDNA ends (RACE), the oligo(dT) anchor primer was used instead of the oligo(dT), at the concentration of 32.5 μM, in the standard conditions.

PCR and cDNA Cloning. Based on the amino acid sequence published by Gowda *et al.* (7), two degenerate oligonucleotide primers were designed by using the *Phaseolus vulgaris* codon usage. A 500-bp product was amplified by 30 cycles of PCR, each cycle comprising 40 s at 94°C, 40 s at 50°C, 60 s at 72°C, and an extension step at 72°C for 10 min. Reactions were performed in 50 μl containing 30 pmol of each primer, 0.2 mM deoxyribonucleotides, and 0.5 unit of Ampli-*Taq* polymerase (Perkin–Elmer) in the corresponding buffer.

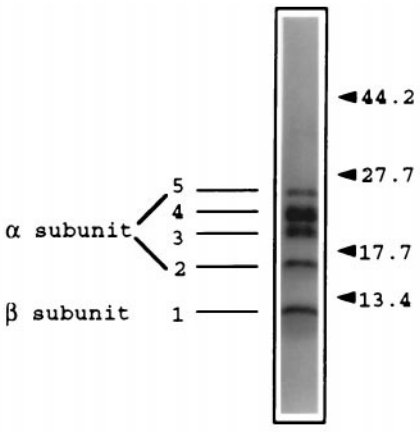
The 500-bp product obtained by PCR was cloned in the T/A plasmid, pCR2.1 (Invitrogen), and sequenced by sequenase dideoxy chain termination (United States Biochemical). Based on the sequence of the amplified product, a specific primer was designed and used in combination with a degenerate primer corresponding to the first 5 aa of the published sequence. A 480-bp product was amplified by 30 PCR cycles using the same conditions reported above. The amplified fragment was cloned in the pCR2.1 vector and sequenced. The 3′ end of the clone was obtained through RACE by PCR (RACE-PCR) by using the 5′/3′ Racekit (Boehringer Mannheim) used according to the manufacturer's instructions. Nested PCR amplifications were performed by using the PCR-Anchor primer with the specific primer. The 900-bp product obtained was subcloned in pCR2.1 and sequenced. To obtain the full-length cDNA clone, a specific primer corresponding to the first 5 aa at the 5′ terminus was used in combination with the anchor primer.

Cord Blood Assay. Umbilical cord blood from healthy donors was collected in 100 units/ml of heparin under general consent from the Department of Obstetrics and Gynecology, St. Vincent's Hospital, New York. Cord blood mononuclear cells (CB mnc) were isolated within 4 h of collection by Ficoll/Paque (Pharmacia), following the manufacturer's directions, and washed in X-VIVO 10 (BioWhittaker). CB mnc were cultured in six-well plates (Corning) at a concentration of 200,000 cells/ml in a volume of 4 ml X-VIVO 10. FRIL and recombinant *Escherichia coli* Flt3-L (recFL; BioSource International, Camarillo, CA) were added at a concentration of 40 ng/ml at the outset. Cultures were incubated in humidified chambers without medium changes for up to 29 days.

The progenitor capacity of cultured CB mnc was assayed by washing harvested cells in X-VIVO 10, determining viable cell number by Trypan blue exclusion (Sigma), and plating cells in fetal bovine serum-free methylcellulose colony assay medium containing recombinant cytokines (StemCell Technologies, Vancouver).

RESULTS

Protein Purification and Amino Acid Sequences of the Polypeptides of FRIL. FRIL was purified from a crude extract of hyacinth beans using a mannose affinity column that was eluted with α-methyl mannoside. When the eluted protein was fractionated by SDS/PAGE, we obtained 5 polypeptide bands in the 10- to 20-kDa size range (Fig. 1). Amino-terminal sequencing of the polypeptides shows that the smallest polypeptide starts with the sequence AQSLS and corresponds to the β-subunit of the hyacinth bean lectin of Gowda *et al.* (7). The three largest polypeptides start with DSSTS and contain the sequence TT-TKA. This is also the start of band 2. These results indicate that the 4 larger bands are variants of the same polypeptide. Such variation in size can be caused by different numbers of *N*-glycans,

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Band 5   DSSTS EXQTT TKAAS SNVVA
Band 4   DSSTS EXQTT TKA
Band 3   DSSTS EXQTT TKAAS SNVVA
Band 2            TT TKAAS SNVVA VEFKT YLN

Band 1   AQSLSF FSFTK FDPNQ EDLIF QHATS TNNV
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FIG. 1. Fractionation of purified hyacinth bean FRIL by SDS/PAGE and amino-terminal amino acid sequences of the constituent polypeptides.

differential processing of *N*-glycans, or C-terminal amino acid processing. The presence of the sequence SNVV in three of these polypeptides indicates that they represent the α-subunit of the hyacinth bean lectin. Since the DSSTS sequence does not occur in the amino acid sequence of the hyacinth bean lectin sequence obtained by Gowda *et al.* (7), we decided to isolate the cDNA to try to understand the relationship between FRIL and the lectin obtained by Gowda *et al.* (7).

Isolation of a cDNA Clone of FRIL. To obtain a cDNA clone of FRIL we carried out PCR and PCR-RACE as described in *Materials and Methods*. This resulted in the isolation of a nearly complete coding sequence of FRIL. The nucleotide sequence has been submitted to GenBank (accession no. frlgb AF067417), and the derived amino acid sequence without the partial putative signal peptide is shown in Fig. 2 together with the determined amino acid sequence obtained by Gowda *et al.* (7) for the *Dolichos lablab* lectin (DLL) protein and two of the peptide sequences of purified FRIL shown in Fig. 1. The sequence AQSLS of the β-subunit (Fig. 1) was found between amino acids 21 and 26 of the derived sequence. Since all seed lectins of legumes have signal sequences we assumed that the amino acids that are upstream of AQSLS constitute the signal sequence. However, as the derived amino acid sequence does not start with a methionine residue, we assumed that the RACE-PCR did not result in a clone that included the entire signal peptide. Repeated attempts to obtain a clone with the entire signal peptide were not successful.

A comparison of the derived amino acid and the sequence obtained by Gowda *et al.* (7) via protein sequencing shows that three additional peptide domains are present in the derived sequence. These include 5 aa at the C terminus, a linker peptide of 12 aa that joins the α- and β-subunits, and a peptide of 8 aa near the N terminus (amino acids 27–34 of the mature protein). The loss of amino acids at the carboxyl terminus is a common occurrence in lectins and other vacuolar proteins (10). The loss of a linker peptide between subunits also occurs frequently in vacuolar seed proteins that are proteolytically processed (11). Processing into subunits takes place through the action of an endopeptidase that cleaves specifically on the carboxyl side of Asn (12, 13). Such cleavage may be followed by the action of carboxypeptidase C, a vacuolar protein. The presence of an 8-aa peptide near the amino terminus of FRIL, seen by amino-terminal sequencing of the native protein and derived from the predicted amino acid sequence of the cDNA, appears to represent an in-frame insertion. This difference in FRIL and the DLL may be the result of using different cultivars.

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        1                                                   50
FRIL    AQSLSFSFTK FDPNQEDLIF QGHATSTNNV LQVTKLDSAGN PVSSSAGRV
DLL     AQSLSFSFTK FDPNQEDLIF QGTATS... .... KLDSAGN PVSSSAGRV
β-Pep   AQSLSFSFTK FDPNQEDLIF QGHATSSTNN V

        51                                                  100
FRIL    LYSAPLRLWE DSAVLTSFDT IINFEISTPY TSRIADGLAF FIAPPDSVIS
DLL     LYSAPLRLWE DSAVLTSFDP TIY..IFTNY TSRIADGLA. FIAPPDSVIS

        101                                                 150
FRIL    YHGGFLGLFP NANTLNNSST SENQTTTKAA SSNVVAVEFD T.YLNPDYGD
DLL     YHGGFLGLFP NA..... .....AESGIA ESNVVAVEFD TDYLNPDYGD
α2-Pep  .......... ......DSST SEXQTTTKAA SSNVV
α1-Pep  .......... ......... .....TTTKAA SSNVVAVEFK T YLN

        151                                                 200
FRIL    PNYIHIGIDV NSIRSKVTAK WDWQNGKIAT AHISYNSVSK RLSVTSYYAG
DLL     PNYIHIGIDV NSIRSKVTAS WDWQNGKIAT AHISYNSVSK RLSVTTYYPG

        201                                                 250
FRIL    SKPATLSYDI ELHTVLPEWV RVGLSASTGQ DKERNTVHSW SFTSSLWTNV
DLL     RGKPATSYDI ELHTVLPEWV RVGLSASTGQ NIERNTVHSW SFTSSLWTNV

        251          265
FRIL    AKKENENKYI TRGVL
DLL     AKVGVASISG .....
```

FIG. 2. Amino acid sequence alignments of the determined amino acid sequence of the hyacinth bean lectin [see Gowda *et al.* (7)], the derived amino acid sequence obtained from the FRIL cDNA, and the sequences of two peptides determined with the purified protein obtained from the same beans. The underlined tripeptides represent potential N-linked glycosylation sites.

Three potential glycosylation sites are present in the linker peptide of FRIL that connects the two subunits: Asn-Asn-Ser, Asn-Ser-Ser, and Asn-Gln-Thr. One of these appears to be used at least some of the time because sequencing of the α2 peptide (Fig. 1) showed that there was no signal (marked by X) in the seventh position. This position corresponds to Asn in the derived sequence. The glycoprotein nature of the protein was confirmed by using a polyclonal serum that reacts specifically with the complex Asn-linked glycans found on plant glycoproteins (data not shown). Antibodies in this serum react primarily with the β,1–2 xylose epitope of plant complex glycans (14).

FRIL Preserves Progenitors for 4 Weeks in Culture. In earlier work we demonstrated that FRIL isolated from red kidney bean has the unusual property of maintaining a small population of cord blood cells expressing the $CD34^+$ phenotype for 2 weeks in suspension cultures containing the cytokine IL-3 (15). FRIL-containing cultures did not show the effects of IL-3's strong induction of proliferation and differentiation (high cell number and exhausted medium); instead, low cell number at the end of the culture period resulted in persistence of cells in the context of cell death. The ability of FRIL fractions to act in a dominant manner over IL-3 led to the hypothesis that FRIL preserves $CD34^+Flt3^+$ progenitors and prevents their proliferation and differentiation.

Since proteins obtained from kidney bean might be contaminated by traces of the potent mitogenic lectin PHA-L, known to be abundantly present in kidney beans, and to avoid any possible contamination, we tested our hypothesis about FRIL's hematopoietic activity using hyacinth bean as the source of lectin. CB mnc were cultured in serum-defined medium containing either FRIL, recombinant *E. coli* FL (recFL), FRIL and recFL, or medium only as a control, for up to 29 days. No medium changes were made during culture. Harvested cells were washed free of FRIL or recFL and assessed for their progenitor capacity in a methylcellulose colony assay containing serum-defined medium and IL-3, granulocyte–macrophage CSF, and kit ligand (Table 1). Colonies derived from mature myeloid and erythroid progenitors formed from cells cultured for 15 days in either FRIL or FRIL + recFL; 25-fold fewer mature colonies formed from cells cultured in recFL alone; and no colonies appeared if neither was present. After 21 days of culture, myeloid and erythroid colonies formed only from cells exposed to FRIL. The frequency of myeloid colonies in FRIL-alone cultures (based on the initial number of CB mnc) decreased by 2.7-fold from 1 in 774 after 2 weeks in culture to 1 in 2,067 after 3 weeks; erythroid colonies decreased in frequency by 9.6-fold from 1 in 11,940 to 1 in 114,286 (Table 1 and Fig. 4). Further studies

Table 1. FRIL but not recFL preserves progenitors in suspension culture

Day	Medium	Myeloid	Erythroid	Mix	Blast
15	FRIL	1,033 ± 12	67 ± 12	7 ± 12	0
	recFL	40 ± 69	0	0	0
	FRIL + recFL	933 ± 250	167 ± 95	0	0
	No addition	0	0	0	0
21	FRIL	387 ± 83	7 ± 12	0	167 ± 64
	recFL	0	0	0	0
	FRIL + recFL	473 ± 133	53 ± 42	0	300 ± 34
	No addition	0	0	0	0
29	FRIL	0	0	0	80 ± 72
	recFL	0	0	0	0
	FRIL + recFL	0	0	0	40 ± 20
	No addition	0	0	0	0

Cord blood mononuclear cells (800,000) were cultured in X-VIVO 10 containing native FRIL (40 ng/ml) or recFL. No medium changes were made during culture. Cells harvested from liquid culture were plated in triplicate. Data are reported as ±SD of three values. The reported experiment is representative of four experiments.

are needed to determine whether the decline in cell numbers is a result of suboptimal culture conditions or FRIL's limited ability to maintain mature progenitors in suspension culture. In addition to myeloid and erythroid colonies, day 21 cultures contained small colonies consisting of undifferentiated cells (Fig. 3*A*). Only blast-like colonies were observed when cells were cultured in FRIL for 29 days (Table 1 and Fig. 3*B*). The frequency of blast-like colonies cultured in FRIL alone decreased by 2.1-fold from day 21 to 29, from 1 in 4,790 to 1 in 10,000, and 7.5-fold in FRIL + recFL cultures from 1 in 2,667 to 1 in 20,000 of the initial CB mnc cells cultured (Fig. 4). Again, further experiments are needed to determine the decrease in frequency of progenitors from week 3 to 4.

The progenitor capacity of the blast-like colonies was examined further for cells initially cultured for 3 weeks in either FRIL or FRIL + recFL and then without these regulators for an additional 6 weeks in a methylcellulose colony assay. Serial replating experiments were not possible for cells initially cultured in either recFL or the medium control for 21 days since no colonies were detected. Viable cells harvested from cells initially cultured in FRIL were replated in a colony assay for an additional 4 weeks. A schematic diagram of the experiment is shown in Fig. 4. Small, diffuse, blast-like colonies appeared at a frequency of 1 in 67 (900 colonies/60,000 mnc) exclusively in dishes of cells initially cultured in FRIL alone, and at a frequency of 1 in 132 (990 colonies/131,000 mnc) for cells cultured in FRIL + recFL. An example is shown in Fig. 3*C*.

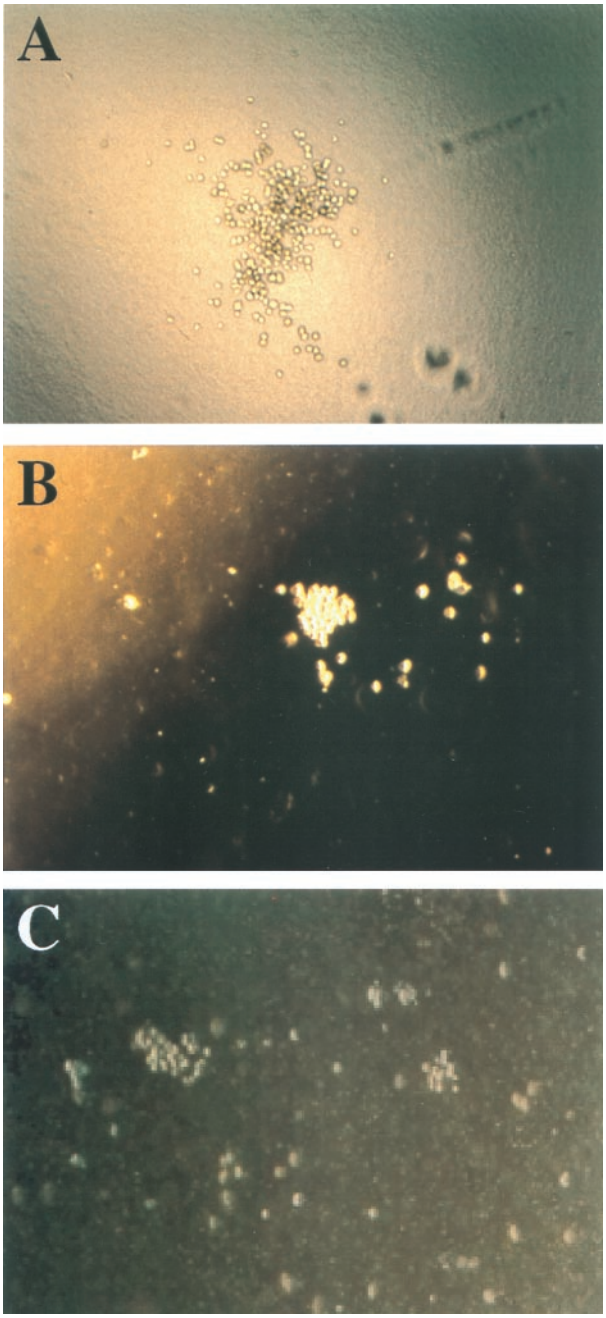

FIG. 3. Colonies derived from cord blood cells cultured in FRIL. Blast-like colonies formed in methylcellulose colony assays after exposure of cord blood mononuclear cells to FRIL for 3 weeks (*A*) or 4 weeks (*B*). Colonies that formed after 3 weeks of FRIL generated yet more blast-like colonies (*C*).

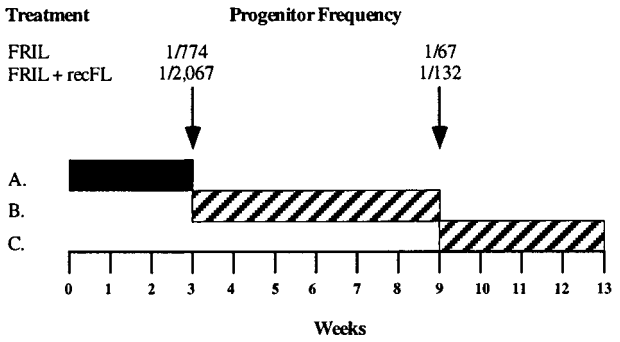

FIG. 4. Serial replating of progenitors cultured in FRIL. (*A*) CB mnc were cultured for 3 weeks in suspension culture in FRIL (solid box). (*B*) Harvested cells were assessed for progenitor capacity in methylcellulose colony assay (striped box) for 6 weeks. (*C*) Cells harvested from the colony assay were replated in a second colony assay for an additional 4 weeks. Progenitor frequencies were determined both for cells after 3 weeks of suspension culture and for cells harvested after an additional 6 weeks of methylcellulose culture (indicated by arrows). ND, not detected.

There are two interpretations for the observation of the persistence of progenitors that still give rise only to small colonies consisting of undifferentiated cells (Fig. 3*C*) after exposure for 6 weeks to the strong inducers of proliferation and differentiation in a methylcellulose colony assay. Either FRIL's progenitor-preserving properties can persist for prolonged periods after removal of the lectin from culture in a dominant manner over cytokines, or the progenitors that FRIL preserves do not respond to cytokines, or both. Further studies are needed to characterize the phenotypic and functional properties of these FRIL-preserved progenitors.

DISCUSSION

In this report we show that the mannose-binding lectin FRIL obtained from the seeds of the hyacinth bean preserves human cord blood progenitors for up to a month in suspension culture without medium changes. Cells cultured for a month in FRIL generate small blast-like colonies in the context of strong inducers of proliferation. Further, the primitive nature of these colonies not only persists in replating experiments but the progenitor frequency of replated cells is enriched 71-fold over that in suspension cultures (Fig. 4). The unusual biological property of this plant lectin confirms our previous preliminary work (15) with a homologous mannose-binding lectin from a different legume species (red kidney or common bean).

Lectins, proteins that bind sugar moieties of glycolipids and glycoproteins, occur in all organisms and are especially abundant in the leaves, bark, and seeds of many plants. Plant lectins probably have multiple roles related to their ability to act as recognition molecules (16). Their specificity appears directed primarily to carbohydrate moieties outside the plant cell. A role in host defense (17)—protection of the plant against predators—is inferred from toxicity of these proteins toward

insects (18), growth inhibitory effects on fungi, and interference with signaling pathways in mammalian cells (19). Phytohemagglutinin (PHA), a red kidney bean lectin, recognizes epithelial cells in the luminal surface of the gastrointestinal tract of mammals and activates hyperplasia of the small intestine (19). PHA also activates human T cells to proliferate and secrete high levels and a broad range of cytokines. Conditioned medium collected from these cells, commonly called PHA leukocyte-conditioned medium (PHA-LCM), has been used in hematopoiesis for more than two decades as a positive control in colony assays.

FRIL was first identified while searching for Flt3 3T3 stimulatory activities in PHA-leukocyte conditioned medium (6). Amino acid sequencing of the two constituent polypeptides of the active protein showed the sequences to be lectin-related and to share considerable identity with the amino termini of the two polypeptides of the mannose-binding lectin DLL of the hyacinth bean. The results suggest that kidney bean seeds contain at least three different lectins: PHA-E, PHA-L, and FRIL. To further characterize the hyacinth bean protein, we purified it, confirmed and extended the amino-terminal sequences, and isolated the cDNA. Comparing the amino acid sequence derived from the cDNA with the determined amino acid sequence of DLL reported by Gowda *et al.* (7), we found 78% identity. Some of this difference is caused by posttranslational processing at the C terminus and internally near the cleavage site of the two subunits. Amino acids that are lost during processing are present in the derived sequence, but absent from the determined sequence. The presence of an 8-aa in-frame insertion near the amino terminus of FRIL represents a distinct difference. The same insert also was observed in FRIL isolated from red kidney beans (data not shown). Studies are underway to determine whether the insert relates to FRIL's function. Preliminary crystallographic data on FRIL indicate that the insert is exposed at the surface of the protein (T. Hamelryck, unpublished observation).

Does the carbohydrate-binding domain of FRIL interact with a carbohydrate moiety on the Flt3 receptor? Preliminary studies show that the addition of either excess mannose or α-methyl α-D-mannoside prevents binding of ^{125}I-labeled FRIL to Flt3 3T3 cells (data not shown). Studies to characterize the carbohydrate-binding properties of FRIL have revealed that the lectin most effectively accommodates a nonreducing terminal α-D-mannosyl unit (20). FRIL's recognition of the trimannosyl core of N-linked glycosylation in mammals is consistent with the notion that the lectin interacts with primitive cells. Further studies are needed to confirm the nature and specificity of FRIL's binding to Flt3. Using site-directed mutagenesis to inactivate the carbohydrate-binding activity of FRIL will help clarify whether the lectin exerts its biological activity via carbohydrate binding. Previously we have shown that mutant PHA-L without carbohydrate-binding activity also does not have mitogenic activity, indicating that for PHA, lectin activity is required for biological activity (21). Similar studies, combined with classic binding studies, should confirm FRIL's interaction with Flt3.

Although FRIL and FL activate proliferation of Flt3 3T3 cells, the two proteins act differently on hematopoietic progenitors. FL, as other early-acting cytokines, does not sustain stem cells or primitive progenitors *in vitro* by itself. Instead, a combination of early-acting cytokines is required. Although FL-containing cytokine cocktails maintain primitive hematopoietic cells *in vitro*, this is achieved in the context of proliferation and differentiation. FRIL, in contrast, preserves primitive progenitors without support of exogenous cytokines. Studies are underway to characterize the mechanism by which FRIL mediates it unique functions.

Ex vivo manipulation of stem cells for clinical applications currently is limited by the inadequacy of cytokines to effectively control these cells. FRIL's ability to functionally select and preserve primitive hematopoietic cells may significantly extend the range and time for manipulating these cells. Preliminary studies indicate that cells preserved by FRIL may include stem cells. Using the nonobese diabetic/SCID murine assay to assess *in vivo* properties of human repopulating cells, defined as SCID-repopulating cells, Kollet and colleagues found human cord blood cells cultured in FRIL for 2 weeks engrafted in nonobese diabetic/SCID mice (O. Kollet, J.G.M., R. Aviram, H. Ben-Hur, A. Ben-Arie, A. Nagler, L. Schultz, M.F., and T. Lapidot, unpublished results).

Experiments are underway to explore whether FRIL can improve applications in stem cell transplantation, treatment of leukemia, and gene therapy. Since FRIL acts without exogenous cytokines to preserve a small population of cells, the lectin provides an effective method to functionally select primitive cells. If alloreactive cells are greatly reduced or eliminated during culture, the resultant cells may have a higher likelihood of engrafting and generating a chimeric immune system and avoid a serious graft-versus-host reaction. FRIL's ability to preserve primitive cells (*in vivo* or *ex vivo*) may also facilitate using a higher dose of chemotherapy for shorter periods. The functional selection and cell-preservation properties of FRIL may help synchronize cell populations for more efficient gene transfer and subsequent manipulations of stem cells for gene therapy.

This work was supported by a grant from the U.S. Department of Energy to M.J.C. We thank Mick Bhatia from the Robarts Research Institute in London, Ontario, Canada, for his careful reading and critical comments on this manuscript.

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