synthesis as noted from the results of protein assays.

Flunarizine is a lipophilic, weakly basic diphenylalkylamine $Ca^{2+}$ channel antagonist that has been used to treat vertigo, epilepsy, migraine headaches, and peripheral vascular insufficiency (*11*). Experimentally, flunarizine has a cerebral protective effect in an ischemic-hypoxic model in rats (*12*), possibly because of its ability to block intracellular entry of $Ca^{2+}$ (*13, 14*). The inhibitory effect of flunarizine on contractile smooth muscle protein has an intracellular site of action (*15*). Flunarizine also inhibits the important intracellular regulatory protein calmodulin at concentrations greater than 10 μM (*11*). Secondary inhibition by flunarizine of calmodulin-phosphodiesterase may alter levels of adenylate cyclase with potential beneficial effects. Our data indicate that neuronal death after neurotrophic deprivation can be prevented by flunarizine in vivo and in vitro. In cell culture, the dose required for protection (20 to 40 μM) was much higher than required for block of the voltage-dependent $Ca^{2+}$ channels (3 to 10 μM) (*11*). Studies in sympathetic neurons in culture with various $Ca^{2+}$ entry-blocking agents at concentrations capable of blocking the voltage-dependent channels offered no protection from neuronal death (*16*). Similar high concentrations of nimodipine in DRG cell culture failed to protect neurons. On the basis of the concentration of flunarizine required for protection and the lack of protection afforded by the $Ca^{2+}$ channel blocker nimodipine, we believe an alternative intracellular mechanism of action may be responsible. The protective ability of flunarizine in the in vivo and in vitro models provides opportunities to study the mechanisms involved in neuronal death after injury and trophic factor deprivation. Such pharmacological agents have promise in future clinical approaches.

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# Inhibition of Factor VIIa–Tissue Factor Coagulation Activity by a Hybrid Protein


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**Lipoprotein-associated coagulation inhibitor (LACI) appears to inhibit tissue factor (TF)–induced blood coagulation by forming a quaternary inhibitory complex containing factor Xa, LACI, factor VIIa, and TF. A genetically engineered hybrid protein consisting of the light chain of factor Xa and the first Kunitz-type inhibitor domain of LACI is shown to directly inhibit the activity of the factor VIIa–TF catalytic complex. Unlike inhibition of factor VIIa–TF activity by native LACI, inhibition by the hybrid protein is not dependent on factor Xa. In an assay of TF-induced coagulation, 50% TF inhibition occurs with hybrid protein at 35 nanograms per milliliter, whereas LACI at 2.5 micrograms per milliliter is required for an equivalent effect. γ-Carboxylation of glutamic acid residues in the factor Xa light chain portion of the hybrid protein is required for inhibitory activity, indicating that the first Kunitz-type domain of LACI alone is not sufficient for inhibition of factor VIIa–TF.**


BLOOD COAGULATION CAN BE INITIated when factor VII or VIIa, a plasma protease, binds to its cell membrane–associated cofactor, tissue factor (TF), and proteolytically activates its substrates, factors IX and X (*1*), triggering a cascade of events that leads to the formation of a fibrin clot. TF-initiated coagulation has been implicated in the pathogenesis of thrombohemorrhagic disorders associated with tissue damage, sepsis, malignancy, and obstetrical complications (*2*). Thus, an agent that inhibits factor VIIa–TF activity may be useful as a specific antithrombotic agent.

Plasma contains a lipoprotein-associated coagulation inhibitor (LACI) that inhibits activated factor X (Xa) directly and, in a factor Xa–dependent fashion, inhibits factor VIIa–TF activity, presumably by forming a quaternary inhibitory complex consisting of factor Xa, LACI, factor VIIa, and TF (*3, 4*). LACI contains three Kunitz-type serine protease inhibitory domains (Kunitz domains) (*5, 6*), of which both the first and second are necessary for inhibition of the factor VIIa–TF complex. We proposed that in the putative quaternary inhibitory complex, the first Kunitz domain of LACI is bound to the active site of the factor VIIa, whereas the second Kunitz domain is bound to the active site of factor Xa (*7*). Coagulation factor X consists of two peptide chains covalently linked by a disulfide bridge. Proteolytic release of a peptide from the heavy chain of factor X by the factor VIIa–TF catalytic complex produces the active enzyme factor Xa. Factor X can also be activated by factor IXa with its cofactor VIIIa. The heavy chain of factor Xa contains the catalytic site. The light chain of factor Xa (and X) contains the γ-carboxyglutamic acid (gla) domain, which is responsible for $Ca^{2+}$ binding, followed by two growth factor–like domains, which may in part mediate the interaction with specific coagulation cofactors (Fig. 1A). Chymotrypsin treatment cleaves the gla domain from the $NH_2$-terminus of factor Xa. LACI binds to and inhibits factor Xa without the gla domain, but, in the presence of factor Xa without the gla domain, LACI does not inhibit factor VIIa–TF activity (*3*).

Since neither factor Xa nor LACI alone significantly inhibit factor VIIa–TF activity, the binding of factor Xa to the second Kunitz domain in LACI may serve to juxtapose the gla domain of the factor Xa light


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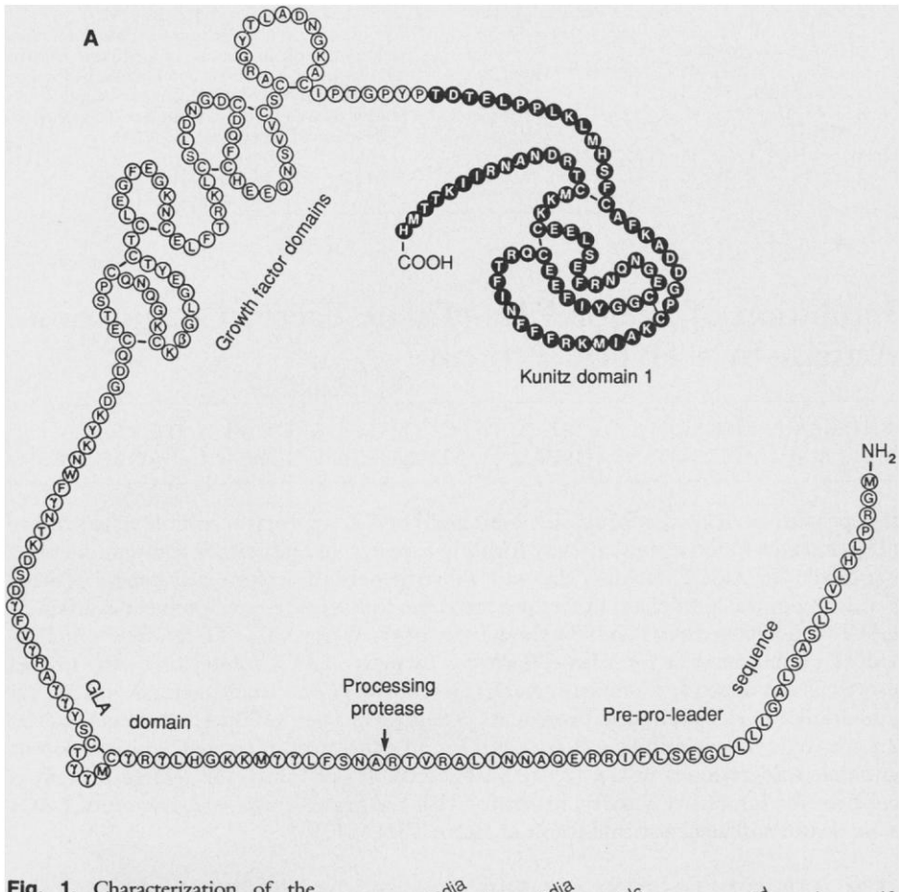


**Fig. 1.** Characterization of the $X_{LC}LACI_{K1}$ hybrid protein. (**A**) Schematic diagram of $X_{LC}LACI_{K1}$. Figure of the factor X light chain is from Leytus *et al.* (*12*). The LACI-derived portion of the molecule is shaded. The arrow indicates the probable cleavage site used to generate the mature protein. (**B**) Protein immunoblots showing the expression of the recombinant hybrid protein. Samples were five-fold-concentrated serum-free conditioned medium (25 μl) from the cloned cell lines C10.1, 40 ng of purified LACI, and 60 ng of factor X. After SDS-PAGE and transfer to nitrocellulose, the filter was probed with a rabbit polyclonal antibody to a synthetic peptide of the $NH_2$-terminus of LACI (anti-LACI), or with a mouse monoclonal antibody to the factor Xa light chain (anti-X). The filter was then probed with the appropriate second antibody conjugated to alkaline phosphatase and developed colorimetrically (*6*). (**C**) SDS-PAGE and silver staining of 250 ng of purified $X_{LC}LACI_{K1}$ (*13*). The reduced sample contained 1.25% 2-mercaptoethanol. Relative molecular size (×1000) of protein standards are shown.

chain and the first Kunitz domain of LACI. A hybrid gene was designed that encodes the factor X pre-pro-leader sequence (which directs the γ-carboxylation of glutamic acids) and light chain fused to a sequence encoding the first Kunitz domain of LACI (*8*) (Fig. 1A). This gene was inserted into a bovine papilloma virus expression vector and transfected into mouse C127 fibroblasts (*9*). Secretion of the hybrid protein, designated $X_{LC}LACI_{K1}$, into the media by a transfected cell line, C10.1, was detected by protein immunoblot analysis. Both a monoclonal antibody to the factor Xa light chain and a polyclonal antibody to the $NH_2$-terminal region of LACI bound proteins migrating at 30 kD and 28 kD (Fig. 1B). A diffuse protein band of 52 kD was also observed but its identity is unclear.

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining showed that the purified hybrid protein consists predominantly of a 30-kD band with minor bands of 31 kD and 28 kD. This staining pattern was also observed under reducing conditions, indicating the multiple bands are not simply due to alternative disulfide bonding (Fig. 1C).

The expressed $X_{LC}LACI_{K1}$ was tested for the ability to inhibit factor VIIa–TF–mediated release of the activation peptide from factor IX (*6*). After SDS-PAGE of conditioned media from C10.1 cells, gel slices were extracted and assayed, showing that functional inhibitory activity comigrates with the protein of an apparent size of 30 kD. No inhibitory activity was observed comigrating with the 28-kD or 52-kD proteins (*10*). As previously observed (*3*), the inhibition of factor VIIa–TF activity by LACI is dependent on the presence of factor Xa (Fig. 2A). In contrast, inhibition of factor VIIa–TF activity by the $X_{LC}LACI_{K1}$ hybrid does not depend on factor Xa (Fig. 2B).

$X_{LC}LACI_{K1}$ was compared to LACI for the ability to inhibit TF-induced coagulation of normal plasma as measured in a modified prothrombin time coagulation assay (*11*). A 50% reduction in TF activity

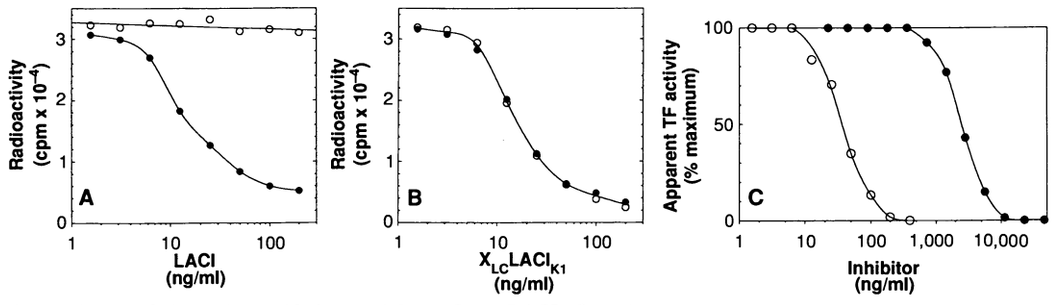


**Fig. 2.** (**A**) Inhibition of factor VIIa–TF activity by purified LACI (*14*) in the presence (●) or absence (○) of factor X (0.1 μg/ml) (which is converted to factor Xa during the assay) (*15*). (**B**) Inhibition of factor VIIa–TF activity by $X_{LC}LACI_{K1}$ eluted from barium sulfate in the presence (●) or absence (○) of factor X at 0.1 μg/ml (*15*). In (A) and (B), radioactivity represents [$^3$H]activation peptide released. (**C**) Inhibition of TF-induced coagulation of plasma by purified $X_{LC}LACI_{K1}$ (○) or LACI (●) (*11*).

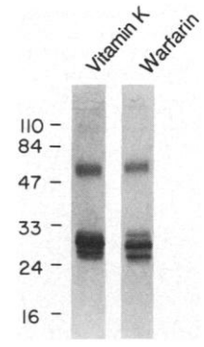


**Fig. 3.** Protein immunoblot showing the presence of $X_{LC}LACI_{K1}$ in the medium of C10.1 cells cultured with vitamin K or warfarin. Twenty microliters of each concentrated medium was electrophoretically fractionated by SDS-PAGE, transferred to nitrocellulose, probed with anti-X followed by the appropriate second antibody conjugated to alkaline phosphatase, and developed colorimetrically (*6*).

**Table 1.** Effects of vitamin K or warfarin on inhibitory activity expressed by C10.1 cells and the ability of barium sulfate to adsorb this activity (*16*).

| Sample | Expressed inhibitory activity (LACI equivalents, ng/ml) |
|---|---|
| Vitamin K | |
| Medium | 590 |
| $BaSO_4$ nonadsorbed | 3 |
| $BaSO_4$ adsorbed | 420 |
| Warfarin | |
| Medium | 7 |
| $BaSO_4$ nonabsorbed | 1 |
| $BaSO_4$ adsorbed | 0 |

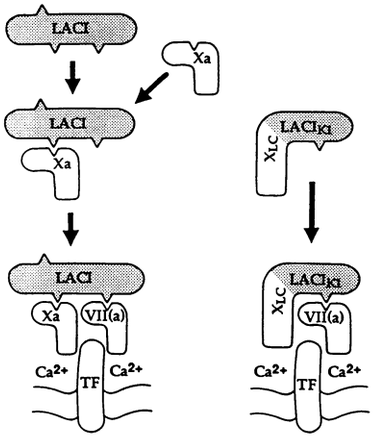


**Fig. 4.** A schematic diagram illustrating the formation of the putative quaternary inhibitory complex (factor Xa-LACI–factor VIIa–TF) and the hybrid protein inhibitory complex ($X_{LC}LACI_{K1}$–factor VIIa–TF). Indentations represent the active sites for factor VIIa and factor Xa; the protrusions represent the three Kunitz domains of LACI. In the normal quaternary inhibitory complex, the active site of factor Xa is bound to the second Kunitz domain of LACI, and the active site of factor VIIa is abound to the first Kunitz domain of LACI. The gla domain of factor Xa interacts in a $Ca^{2+}$-dependent fashion with an unidentified site of the factor VIIa–TF complex. The $X_{LC}LACI_{K1}$ hybrid protein most likely substitutes for the factor Xa–LACI complex.

occurs with $X_{LC}LACI_{K1}$ at 35 ng/ml, whereas LACI at 2.5 μg/ml is required for an equivalent effect (Fig. 2C). The greater inhibitory activity in this system of $X_{LC}LACI_{K1}$ probably reflects its ability to directly inhibit factor VIIa–TF, in contrast to LACI, which requires the generation of factor Xa before its inhibition of factor VIIa–TF becomes manifest. Further, the inhibitory effect of LACI in the TF-induced coagulation assay is at least partly due to its direct inhibition of factor Xa, because at the same concentrations LACI, but not $X_{LC}LACI_{K1}$, also prolongs surface contact–activated coagulation of normal plasma, as measured in an activated partial thromboplastin time (aPTT) coagulation assay (*10*).

The γ-carboxylation of glutamic acid residues on the factor X light chain requires a vitamin K–dependent process, which is inhibited by warfarin. Replacement of vitamin K with warfarin in the C10.1 culture medium results in a reduction in inhibitory activity to <2% of that with vitamin K (Table 1), while reducing the quantity of $X_{LC}LACI_{K1}$ in the medium by about 50% (Fig. 3). Barium sulfate, which selectively adsorbs gla-containing molecules, binds the inhibitory activity expressed by C10.1 cells grown in the presence of vitamin K, indicating that the functional $X_{LC}LACI_{K1}$ is γ-carboxylated (Table 1). Thus, the γ-carboxylation of $X_{LC}LACI_{K1}$ appears essential for its ability to inhibit factor VIIa–TF activity. In addition, neither factor Xa nor inactivated factor Xa (treated with dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone) inhibit factor VIIa–TF activity (*10*), although each contains the factor Xa light chain present in the $X_{LC}LACI_{K1}$ hybrid. These results indicate that both the factor Xa light chain and the LACI Kunitz domain of the hybrid protein are necessary for inhibition of factor VIIa–TF activity.

We propose that $X_{LC}LACI_{K1}$ mimics the factor Xa–LACI complex in its ability to bind to and inhibit the factor VIIa–TF catalytic complex (Fig. 4). Inhibition of factor VIIa–TF activity by LACI occurs through a novel feedback inhibition mechanism, which requires the generation of factor Xa, a product of factor VIIa–TF enzymatic activity (*3*). In contrast, the $X_{LC}LACI_{K1}$ hybrid protein is a direct inhibitor of factor VIIa–TF. This property suggests that $X_{LC}LACI_{K1}$ may be useful as an antithrombotic agent in clinical conditions where TF-initiated coagulation plays a pathological role.

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8. A plasmid, pGEM (Promega, Madison, WI), containing a cDNA insert encoding human factor X in which the naturally occurring Eco RI site was eliminated by a modification that did not alter the encoded amino acid sequence, was used to derive $pGEMX_{LC}$. $pGEMX_{LC}$ contains a portion of cDNA encoding the light chain of factor X linked through an Apa I site to complementary synthetic oligomers. The synthetic oligomers encode additional factor X light chain sequence, followed by LACI $NH_2$-terminal sequence, which precedes the first Kunitz domain and includes an Nsi I site, then two stop codons and convenient restriction site palindromes. A modified LACI cDNA insert, previously used for expression (*6*), was transferred into M13 and site-directed mutagenesis was used to create an Nsi I site between the first and second Kunitz domain coding sequences. A naturally occurring Nsi I site exists upstream from the first Kunitz domain coding sequence. After Nsi I digestion, the region encoding the LACI first Kunitz domain was isolated and ligated into the Nsi I site of $pGEMX_{LC}$ to create $pGEMX_{LC}LACI_{K1}$.
9. The insert from $pGEMX_{LC}LACI_{K1}$ was excised with Bam HI and ligated into the bovine papilloma virus expression vector pMON1123. The resulting plasmid, $pMONX_{LC}LACI_{K1}$, was used along with pSV2neo to cotransfect C127 mouse fibroblasts (*6*).
10. T. J. Girard *et al.*, unpublished observation.
11. TF-induced coagulation was measured by a modified prothrombin time assay in a fibrometer (BBL, Cockeysville, MD). Rabbit brain cephalin (60 μl) was incubated with 60 μl of crude EDTA-washed TF [G. J. Broze, Jr., and P. W. Majerus, *J. Biol. Chem.* **255**, 1242 (1980)], 10 μl of sample, and 60 μl of citrated normal human plasma at 37°C. After 30 s, 60 μl of 25 mM $CaCl_2$ was added, and the time required to clot was determined. When there were no inhibitors, the clotting time was 29 s. A standard curve of TF concentration as a function of clotting time (log-log plot) was used to determine apparent TF activity at various concentrations of inhibitor. Data points are the mean of duplicate measurements, which did not deviate more than 5%.
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13. $X_{LC}LACI_{K1}$ was purified from serum-free conditioned medium from C10.1 cells by barium sulfate adsorption and elution with 0.2 M sodium citrate [W. Kisiel and E. W. Davie, *Biochemistry* **14**, 4928 (1975)] followed by anhydrotrypsin affigel affinity chromatography [S. Ishii, H. Yokosawa, T. Kumakazi, I. Nakamura, *Methods Enzymol.* **91**, 378 (1983)] and mono Q anion-exchange chromatography.
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15. $X_{LC}LACI_{K1}$ was partially purified from 200 ml of serum-free conditioned medium from C10.1 cells by barium sulfate adsorption and elution (*13*), concentrated, and dialyzed into TS buffer (100 mM NaCl and 50 mM tris-HCl, pH 7.4) to a final volume of 1 ml. We assayed the inhibition of factor VIIa–TF activity by observing the release of the activation peptide from $^3H$-labeled factor IX as described (*6*) using factor VIIa rather than factor VII. Heparin (2 units/ml) was also present in the assay system. In this assay the activation of factor IX by factor VIIa–TF is measured by the release of the trichloroacetic acid (TCA)–soluble, tritiated activation peptide from radiolabeled factor IX. Inhibition of factor VIIa–TF activity results in decreased TCA-soluble radioactivity (counts per minute). LACI equivalents (nanograms) were determined from a standard LACI concentration curve. Data points are the mean of duplicate measurements, which deviated less than 10%.

16. At confluence, C10.1 cells expressing $X_{LC}LACI_{K1}$ were washed and then fed serum-free medium containing vitamin K (1 μg/ml) or warfarin (20 μg/ml). The culture medium was replaced on days 0, 1, and 2 and the conditioned medium was harvested on day 5. A sample (10 ml) of each medium was adsorbed and eluted as described (*13*). The barium sulfate–adsorbed and eluted materials, barium sulfate–nonadsorbed materials, and 10 ml of additional conditioned medium were each dialyzed into TS buffer and concentrated to a final volume of 1 ml. Inhibitor activity and amounts of LACI equivalents (nanograms) were determined as described (*15*).

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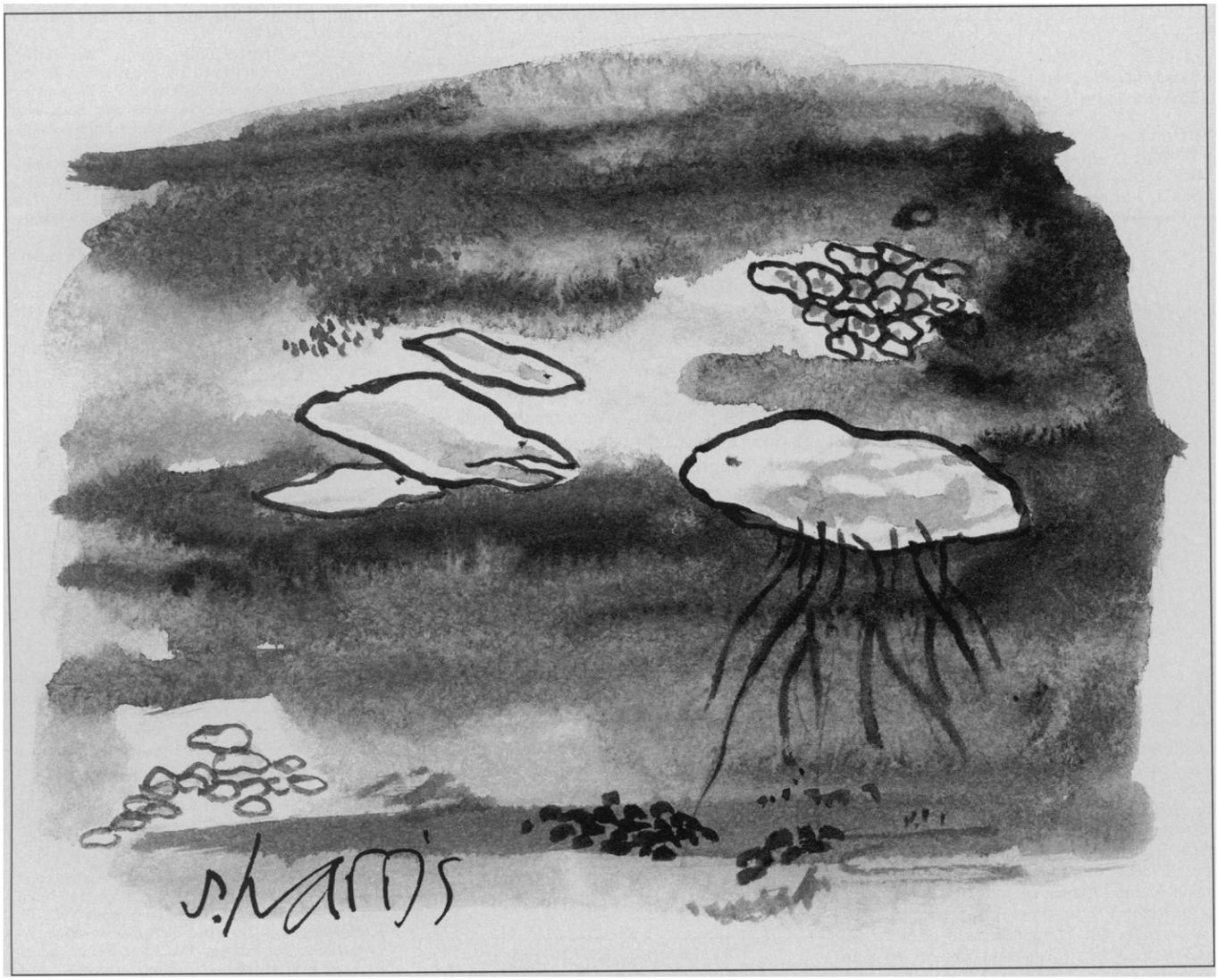

***" You may have good intentions, but you just* can't *start an epidemic by yourself."***