37°C for 4 to 12 hours. RNAs (sense and antisense) were synthesized in vitro in the presence of [^{3}H]uridine triphosphate on a full-length angiotensinogen cDNA template (*2*), to a specific activity of 1.3×10^8 dpm/pmol. After limited base hydrolysis (to a length of 50 to 100 nucleotides), RNA probes were added directly to the prehybridization solution at 10 to 15 p*M*; the sense RNA generates a background control. Alternatively, a 42-residue oligonucleotide complementary to the region of rat angiotensinogen mRNA encoding the amino-terminal tetradecapeptide Ang I-Leu-Tyr-Tyr-Ser (DRVY-IHPFHLLYYS; abbreviations are D, Asp; F, Phe; H, His; I, Ile; L, Leu; P, Pro; R, Arg; S, Ser; V, Val; and Y, Tyr) was end-labeled with [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate to a specific activity of 5×10^6 dpm/pmol and added directly to the prehybridization solution at 1.0 to 1.5 n*M*; a 20- to 50-fold molar excess of unlabeled oligonucleotide was added to some sections as a background control (*3*). After addition of the probe, the sections were returned to 37°C for 18 to 56 hours. Sections were then rinsed through decreasing concentrations of salt solutions (*3*), with a final rinse at 45°C for 20 min. At this point, tissues hybridized with tritiated ribroprobe were incubated with ribonuclease A (20 μg/ml) for 45 min at 37°C, followed by a 45 min incubation at 37°C with the ribonuclease buffer alone. Then sections were postfixed in 4% paraformaldehyde for 30 min and rinsed before following standard immunocytochemistry procedures. Nonspecific antibody binding was blocked with a 1-hour incubation in 5% bovine serum albumin in 50 m*M* tris-saline (*p*H 7.4). Sections were rinsed and incubated overnight at 4°C in primary antibody [1:2000 dilution of a mouse monoclonal antibody to MAP-2 (*9*); or a 1:500 dilution of a mouse monoclonal antibody to GFAP (Boehringer Mannheim). The anti-GFAP is a commercially available antibody first developed and tested as the clone G-A-5 (*10*), which selectively stains astrocytes in rat brain sections]. Sections were rinsed and incubated in rabbit antibody to goat immunoglobulin G (1:100). Sections were then incubated in goat peroxidase-antiperoxidase (PAP) (1:200), rinsed, and reacted with 0.04% diaminobenzidine in 50 m*M* tris buffer and 0.0075% hydrogen peroxide and 10 m*M* imidazole. After further rinsing, sections were mounted onto gelatin-coated slides, dehydrated through a graded alcohol series, and dipped in Kodak NTB2 emulsion. Exposure time was 4 to 8 weeks.

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Metabolic Correction of Defects in the Lipid Anchoring of Thy-1 in Lymphoma Mutants

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Many plasma membrane proteins, including Thy-1, are anchored by a carboxyl terminal glycophospholipid. This unit is absent from the Thy-1 of several lymphoma mutants that synthesize the Thy-1 polypeptide but fail to express it at the cell surface. Recessive mutants of complementation groups A to C, E, and F contain Thy-1 mRNA of normal size, which suggests that their Thy-1 polypeptide is normal. To identify possible metabolic lesions, each mutant was grown with various supplements. The class F and B mutants exhibited a reversible induction of surface lipid anchored Thy-1 when grown with the aminoglycoside G418. Other aminoglycosides, sugars, and ethanolamine were inactive. These unexpected observations are discussed in the context of lipid anchor biosynthesis.

MEMBRANE PROTEINS SUCH AS Thy-1 are anchored to the plasma membrane by a complex moiety composed of ethanolamine phosphate, mannose, glucosamine, *N*-acetylgalactosamine, and phosphatidylinositol (PI) (*1*) that is sensitive to PI-specific phospholipase C (*2*). This unit is added to the COOH-terminus of Thy-1 after the completion of polypeptide synthesis and excision of the most COOH-terminal 31 amino acids, which includes a putative membrane-spanning sequence (*3*). A preassembled unit including most or all of the "anchor components" may be involved (*4*), and dolichol-phosphorylmannose appears to be a precursor of some of the mannose residues (*5*); however, none of the metabolic paths and enzymes involved in anchor assembly or addition have been definitively identified.

There is a family of Thy-1–negative murine T lymphoma mutants (*6*) that synthesizes immunoprecipitable Thy-1 of slightly abnormal gel mobility and expresses essentially none of the antigen on the plasma membrane. These mutants do, however, express normal amounts of transmembrane proteins. Cell fusion studies have assigned the recessive mutants that synthesize Thy-1 to six complementation groups.

Each of the recessive mutants does not add the lipid anchor to Thy-1, and two of them (classes B and E) secrete a hydrophilic form of Thy-1 (*5, 7*). Thus, these cells may have (i) metabolic defects that interrupt anchor biosynthesis or cause anchor hydrolysis, or (ii) mutations that interrupt the normal splicing and processing of the Thy-1 pre-mRNA, thereby producing a variant form of Thy-1 that cannot accept an anchor.

We extracted RNA from wild-type and mutant cell lines to determine the size of each Thy-1 mRNA species (Fig. 1). Each cell line produced an mRNA of 1.8 kb, the same size as in selected wild-type lymphoid and nonlymphoid cells (*8*). Thus, as previously suggested (*6*), the cell-associated Thy-1 of each of these cells may have the same primary structure. The amount of Thy-1 mRNA is, however, variable among the cells examined.

Since several mammalian cell mutations that affect post-translational glycosylation

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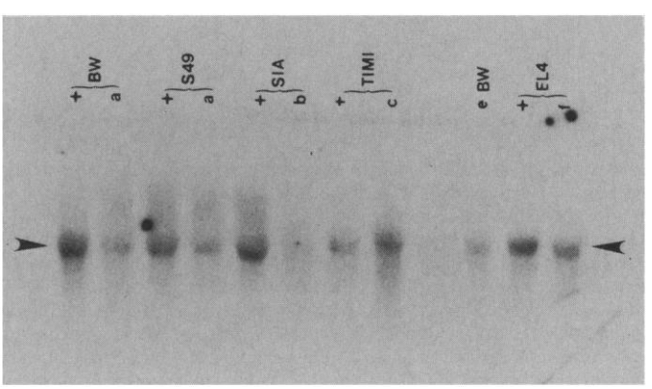

Fig. 1. Analysis of Thy-1 mRNA. RNA was extracted with guanidine isothiocyanate and purified by sedimentation through CsCl from wild-type (+) Thy-1 expressing cells (S49, BW5147, SIA, T1M1, and EL4) and mutant (−) cells (S49-a, BW5147-a, SIA-b, T1M1-c, BW5147-e, and EL4-f). Samples (20 μg) were fractionated on a 1.5% agarose formaldehyde gel, blotted to GeneScreen, and probed with a single-stranded ^{32}P-labeled DNA transcript of a Sac I fragment of the Thy-1 gene (*27*) that had been cloned into M13. The probe was purified and eluted from a low temperature–melting agarose gel before use.

are corrected by inclusion of amino sugars in their medium (*9*), and some cultured cells require ethanolamine for growth (*10*), we monitored surface Thy-1 expression on mutant cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with sugars and ethanolamine. Each of the mutants (A to C, E, and F) (*11*) was grown for 2 days (three doubling times) in culture medium supplemented with either ethanolamine, glucosamine, galactosamine, *N*-acetylglucosamine, or *N*-acetylgalactosamine. In no case was an increase in surface Thy-1 detected (*12*).

We also added more complex molecules that include cyclitols, the aminoglycosides (*13*). When the aminoglycoside antibiotic geneticin sulfate (G418) was added, cell surface expression of Thy-1 increased for the majority of class F EL4 mutant cells, as detected by flow cytometry (Fig. 2A), microscopic examination, or by panning procedures (*14*). As with wild-type cells (*2*), most of the surface fluorescent signal could be removed by treatment with PI-specific phospholipase C. The induction of Thy-1 expression was a time- and concentration-dependent response (Fig. 2B). Cell survival, judged by exclusion of Trypan blue, was ~80%. For most further investigations, a 2- to 3-day period of incubation with G418 (0.75 mg/ml) was therefore selected.

The mean fluorescent cell surface signal for Thy-1 of class F cells cultured in G418 was consistently 5 to 10% of that observed for wild-type EL4 cells. Higher levels of surface Thy-1 were not observed after incubation with G418 (0.3 mg/ml) for 1 to 2 weeks. However, as expected from the previous demonstration that Thy-1 turnover is an order of magnitude faster in the mutants than in wild-type cells (*6, 7*), G418 treatment for 4 to 10 days led to a substantial increase in the total amount of cell-associated Thy-1 (*15*).

The induction of surface Thy-1 expression on the class F mutant was reversible (Fig. 2C). After 2 days exposure to G418, cells that were washed and returned to culture in G418-free medium continued to increase their surface Thy-1 expression for 1 day, but then progressively lost surface Thy-1.

Class F mutant cells were the most responsive to G418, although class B mutants (and, to a slight extent, class A mutants) also exhibited induction after 2 to 3 days. Class C mutants were killed by the drug over this period of time.

Although we saw no morphologic differences between wild-type EL4 and class F mutant cells that were examined by transmission electron microscopy after 2 days of incubation with G418 (*16*), incorporation of [^{3}H]leucine into protein by both EL4 wild-type and class F mutant cells was inhibited by this dose of G418 (*17*). Inhibition of [^{3}H]leucine incorporation was also observed with gentamycin and neomycin (0.75 mg/ml); however, neither of these agents nor low doses of cycloheximide, which allow cell survival for 2 days but reduce [^{3}H]leucine incorporation, resulted in cell surface Thy-1 expression by class F (*17*).

Thy-1 expression on the class F mutant was not induced after 3 days of culture with the aminoglycosides (0.75 mg/ml) amikacin, gentamycin, hygromycin, kanamycin, neomycin, streptomycin, and tobramycin (*18*). Acid hydrolysis of G418 (6 hours in 6*N* HCl at 100°C) eliminated its activity.

The responsive class F mutant was obtained by nitrosoguanidine mutagenesis and negative immunoselection of an EL4 lymphoma (*6*). Thy-1 synthesized by this mutant is sufficiently hydrophobic to bind Triton X-114 but lacks glycophospholipid anchor components (*7*). Since the Thy-1 mRNA of this mutant has a normal size, it is likely that the protease that normally cleaves Thy-1 between residues 112 and 113 does not act in these cells, possibly because a preassembled anchor unit (*4*) is not synthesized. The aminoglycoside G418 (Fig. 3) is often used in conjunction with eukaryotic expression vectors encoding genes whose products inactivate G418 (*19*); however, the mechanism by which G418 exerts its slow toxicity is not known. What is striking is that (i) a part of the structure of G418 (*20*) resembles that of glycophospholipid anchors (Fig. 3), and (ii) a limited structural change of G418 converts the drug into gentamycin C (*21*), which lacks Thy-1 inducing activity. Neomycin, and possibly other aminoglycosides, binds polyphosphoinositides (*22, 23*) and inhibits PI-specific phosphodiesterase (*23, 24*). Because of their positive charge, aminoglycosides surely bind many negatively charged molecules.

There are several ways to rationalize the effect of G418: (i) G418 binds a PI-containing anchor precursor, thereby making it an acceptable substrate for a mutated enzyme whose normal counterpart is responsible for the step in anchor biosynthesis that is blocked in the mutant; (ii) G418 inhibits a phosphodiesterase that is abnormally active in the mutant and destroys an anchor precursor or hydrolyzes the anchor as soon as it has been added to Thy-1; (iii) a G418 metabolite serves as an anchor precursor; and (iv) G418 causes sufficient misreading of the genetic code (*13*) to restore activity to a limited number of copies of the translation product of the mutant gene.

Evaluation of these several explanations must wait for elucidation of the normal path

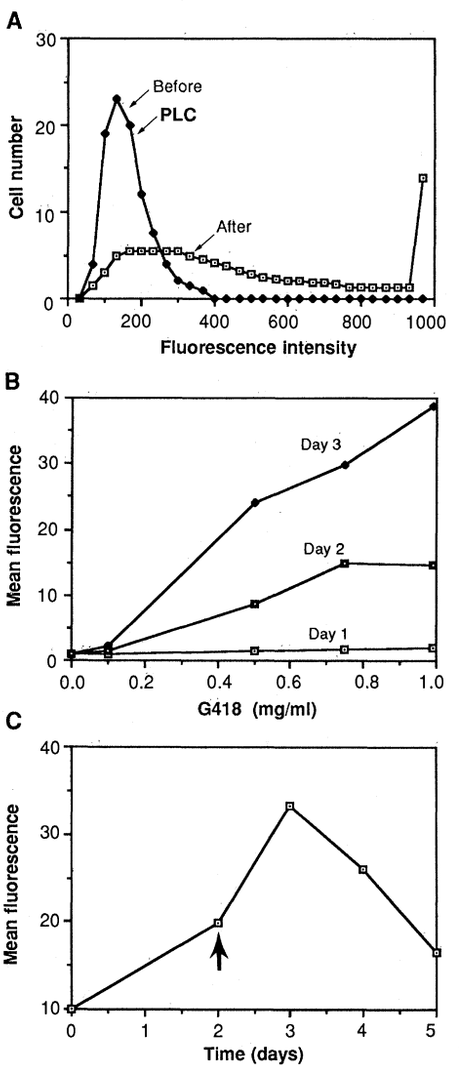

Fig. 2. Quantitative evaluation of Thy-1 expression on class F mutant cells in a typical experiment. A subclone of the mutant was grown for 1 to 3 days in the absence or presence of G418 in DMEM supplemented with 10% horse serum and penicillin-streptomycin. The cell concentration was readjusted to 2.5×10^5 cells per milliliter each day. Cells were stained with a rat monoclonal antibody (MAb) to Thy-1 (M5/49) (*28*), and then with the fluoresceinated goat antibody to rat Ig (Fisher Biotech), and examined by flow cytometry (Becton-Dickinson Cytofluorograph 2S). (**A**) Flow cytometry of mutant cells before (◆) and after (⊡) 2 days of culture in G418 (0.75 mg/ml). The signal from cells cultured with G418, and then treated with PI-PLC (PLC) was identical to (◆). (**B**) Flow cytometry of cultures stained after 0 to 3 days in G418 (0.1 to 1.0 mg/ml). (**C**) Flow cytometry of cultures equivalent to (B) (0.75 mg/ml, 2 days) that were washed (arrow) and recultured for 1 to 3 days before staining. Induction of surface Thy-1 expression was also detected with the use of the Thy-1.2–specific MAb 30-H-12 (*29*). The response of the uncloned class F mutant was indistinguishable from that illustrated. In (A) the spike at the extreme right of the horizontal axis corresponds to the summation of all fluorescent events more intense than 999 linear units. The PI-PLC used for panel A was from *Bacillus thuringiensis*.

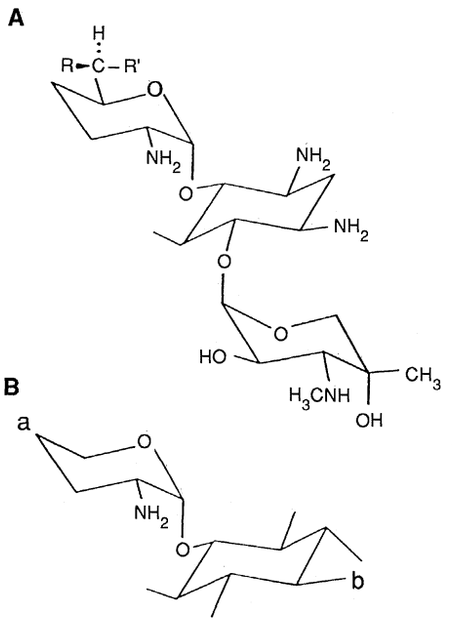

Fig. 3. Comparison of the structure of G418 and gentamycin (**A**) to part of the anchor unit of rat brain Thy-1 (**B**). In (A) R = −OH for G418, −H for gentamycin C_{1a}, and $-CH_3$ for gentamycins C_2 and C_1; R′ = $-CH_3$ for G418, $-NH_2$ for gentamycins C_{1a} and C_2, and $-NHCH_3$ for gentamycin C_1. In (B) carbon a is in glycosidic linkage to mannose and carbon b is in phosphodiester linkage to glycerol (*1*).

of anchor biosynthesis. Considering the large number of membrane proteins that bear lipid anchors (*25*), the fact that lesions in the biosynthetic pathway of these anchors appear to underlie the human disease paroxysmal nocturnal hemoglobinuria (*26*), and because of the widespread use of G418 and other aminoglycosides, the present observations may be of both therapeutic and practical importance.

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11. Lymphomas obtained from R. Hyman of the Salk Institute were as follows: Wild-type lines BW5147.G.1.4.OUA^R.1, S49.1TB.2.3, T1M1.4G.1.3, and EL4.G.1.4. Mutant cell lines: group A (S49.1TB.2/θ5x.3.2OUA^R.1, AKR1/Thy 1.1 2x.1, and BW5147.G1/θ3x.6), group B (SIA.TB.4.8.2/θ10x.3), group C (T1M1.7BU.2), group E (BW5147.3/Thy 1.1-10G.OUA^R.1), and group F (EL4.G.1N/Thy 1.2-2x.1.OUA^R.1). The wild-type lymphoma SIA.TB.4.8.2 was obtained from American Type Culture Collection.
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16. For electron microscopic examination, cells were fixed in 1% glutaraldehyde, 0.1*M* cacodylate-HCl, *p*H 7.3, followed by OsO_4, and embedding in Spurr resin. Examination of thin sections did not reveal changes due to G418 (Gupta, Tartakoff, Tisdale, unpublished observation).
17. Wild-type EL4 or class F mutant cells were labeled 30 to 120 min with 10 μCi/ml [^{3}H]leucine (Du Pont, Biotechnology Systems), washed, and samples precipitated on Whatman cellulose filter disks with 10% trichloroacetic acid, washed with 95% ethanol and ether, and counted. Cells were labeled (i) in the absence of G418, (ii) in G418 (0.75 mg/ml), or (iii) in G418 after 2 days of culture in G418. The groups were normalized to (i), and incorporation of [^{3}H]leucine into the cells of groups (ii) and (iii) was inhibited 37 and 44%, respectively, for wild type and 60 and 95%, respectively, for the mutant. Comparable inhibition of [^{3}H]leucine incorporation was observed in experiments with neomycin or gentamycin in place of G418. Since parallel experiments with wild-type cells showed that G418 inhibited [^{3}H]ethanolamine and [^{3}H]palmitate incorporation into both lipids and proteins, it was not meaningful to study [^{3}H]ethanolamine labeling of the mutant.
18. The composition and source of the aminoglycosides were as follows: amikacin and gentamycin (30% C1, 30% C1a, and 40% C2), kanamycin (5% A and 95% B), neomycin (90 to 95% B, and 5 to 10% C); streptomycin and tobramycin were from Sigma, G418 was from Gibco, and hygromycin B was from Calbiochem.
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