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14. The methylation was carried out as follows. A residue obtained on evaporation of the solvent from the methanolic effluent was dissolved in 0.1*N* HCl. The free sulfate ester was immediately extracted with ethyl acetate after the saturation of the acidic solution with NaCl. Then an ethereal solution of diazomethane was added to the organic phase.
15. The sulfate was synthesized by reaction of 7-HMBA (5.0 mmole) with freshly redistilled chlorosulfonic acid (5.5 mmole) in anhydrous pyridine (20 ml) at room temperature. After 3 hours the mixture was titrated with an aqueous NaOH (11.5 mmole) solution. The neutralized mixture was diluted with ether (ten volumes) in three portions. The white precipitate that resulted was collected by filtration, washed thoroughly with ether, and suspended in ethanol (10 ml). The ethanol-insoluble inorganic salts were removed by filtration, and the filtrate was concentrated to dryness in vacuo at 20°C. The residue was redissolved in ethanol (5 ml), and the solution filtered through. The sodium salt of the sulfate ester was precipitated from the filtrate by the addition of ether (ten volumes), collected by filtration, washed with ether, and dried in vacuo. Overall yield of the sulfate was 70 percent. Spectroscopic results for 7-HMBA sulfate (Na) are: IR ν_{max}^{KBr} cm^{-1} = 3040, 2949, 2860, 1638, 1495, 1269, 1230, and 1066; UV λ_{max}^{EtOH} nm (ϵ) = 212 (24,503), 264 (18,197), 272 (22,909), 282 (34,674), 293 (34,674), 340 (7,692), 356 (10,440), and 374 (8,791); NMR δ_{ppm} in CD_3OD = 3.16 (methyl H, *s*), 5.43 (methylene H, *d*, *J* = 7,7), and 7.20 to 8.45 (aromatic H, *m*).
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20. The sulfates were synthesized from the corresponding arylmethanols by the method described for 7-HMBA sulfate (*14*). Benzyl sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3050, 3012, 2875, 1497, 1465, 1250, 1201, and 1080; UV λ_{max}^{EtOH} nm (ϵ) = 212 (2686) and 257 (124); NMR δ_{ppm} in CD_3OD = 5.06 (methylene H, *s*) and 7.43 (aromatic H, *s*). 1-Hydroxymethylnaphthalene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3000, 2850, 1592, 1506, 1251, and 1210; UV λ_{max}^{EtOH} nm (ϵ) = 224 (73666), 270 (4613), 280 (5390), and 290 (4194); NMR δ_{ppm} in CD_3OD = 5.53 (methylene H, *d*, *J* = 7.4) and 7.53 to 8.43 (aromatic H, *m*). 2-Hydroxymethylnaphthalene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3050, 2850, 1595, 1500, 1245, and 1213; UV λ_{max}^{EtOH} nm (ϵ) = 230 (73714), 275 (4629), 282 (5411), and 298 (4208); NMR δ_{ppm} in CD_3OD = 5.55 (methylene H, *d*, *J* = 7.6) and 7.60 to 8.54 (aromatic H, *m*). 1-Hydroxymethylpyrene sulfate (Na): IR ν_{max}^{KBr} cm^{-1} = 3020, 2900, 1606, 1584, 1450, 1265, 1235, and 1070; UV λ_{max}^{EtOH} nm (ϵ) = 233 (8901), 241 (14256), 264 (5271), 274 (9541), 311 (2503), 326 (6120), and 342 (9179); NMR δ_{ppm} in CD_3OD = 5.40 (methylene H, *d*, *J* = 7.3) and 7.65 to 8.35 (aromatic, H, *m*).

9 June 1981; revised 12 August 1981

Expression of Bovine Leukemia Virus Genome Is Blocked by a Nonimmunoglobulin Protein in Plasma from Infected Cattle

Abstract. *Plasma of cattle infected with bovine leukemia virus contains a soluble factor that blocks the expression of the viral genome in cultured lymphocytes. The blocking factor is not present in plasma of bovine leukemia virus–free cattle or of cattle infected with common bovine viruses. Blocking of bovine leukemia virus expression by the plasma factor is reversible, and seems to be mediated by a nonimmunoglobulin protein molecule.*

Bovine leukemia virus (BLV) is regarded as the causative agent of the adult or enzootic form of bovine leukemia, which is the most common neoplasia of cattle (*1*). In naturally infected cattle, BLV has been detected only in the lymphocytes (*2*). However, regardless of whether the BLV-infected lymphocytes are neoplastic or not, they produce virus particles and express the major internal (p25) and glycoprotein (gp51) virion antigens only after cultivation in vitro for a few hours (*2–4*). Molecular hybridization studies in which a BLV complementary DNA probe was used failed to detect BLV-specific RNA in infected lymphocytes before cultivation in vitro. In contrast, significant amounts of viral RNA were detected in the same cells after cultivation (*2*).

These results suggest that, in vivo, the BLV genome is repressed at the transcriptional level. BLV is the only known virus responsible for spontaneous leukemia, which is present in the infected cells in a repressed state. It is likely that virus-neutralizing antibodies, which are present in virtually all BLV-infected cattle (*5*, *6*), interfere with virus production in the infected lymphocytes. However, it is less clear how viral antibodies block the expression of the BLV genome. The present study indicates that expression of the BLV genome is blocked by a protein factor present in the plasma of BLV-infected cattle.

The amount of p25 produced by BLV-infected lymphocytes during short-term cultivation in vitro is ten times higher than that of other virion proteins (*7*). Therefore, in this study the synthesis of p25 was used as the indicator of BLV genome expression.

Lymphocytes were obtained without hypotonic shock from the buffy coat of the blood of BLV-infected cattle, cultured for 24 hours under a variety of conditions, and tested for the presence of BLV p25 by competitive radioimmunoassay (*8*). As shown in Table 1, no BLV p25 was detected in infected lymphocytes cultured with 100 percent autologous plasma. In contrast, p25 was readily detected when these cells were cultured in 100 percent autologous serum or in 100 percent plasma or serum from a BLV-free cow. Recovery of viable cells was greater than 70 percent in all cultures. The absence of a detectable blocking effect in the serum of BLV-infected cattle indicates that the blocking factor is not an antibody. Further evidence supporting this conclusion was obtained in an experiment in which BLV-infected lymphocytes were cultured in the presence of BLV-negative bovine plasma supplemented with serum gamma globulin (30 mg/ml) from a BLV-infected animal. Even though this concentration of gamma globulin is equivalent to that normally present in cattle serum, no blocking of p25 expression was observed (Table 1).

The blocking factor was found in the plasma of nine of ten BLV-infected cattle. Expression was blocked by either autologous or homologous plasma from BLV-infected cattle. The blocking effect was also observed when the infected lymphocytes were cultured for 48 hours rather than 24 hours. No blocking was

Table 1. Expression of BLV p25 antigen in cultured lymphocytes from a BLV-infected cow. Lymphocytes were isolated (without hypotonic shock) from the buffy coat of heparinized peripheral blood and cultured at 37°C for 24 hours at a density of 3×10^6 cells per milliliter in 20 ml of 100 percent plasma or serum. Following incubation, the cells were harvested, washed twice, disrupted, and tested by competitive radioimmunoassay for BLV p25 (*8*).

Additions to BLV-infected lymphocytes in culture	BLV p25 in lymphocytes (nanograms per milliliter of cell extract)
100 percent autologous plasma	< 0.3
100 percent autologous serum	7
100 percent BLV-negative plasma	6
100 percent BLV-negative serum	7
100 percent BLV-negative plasma mixed with gamma globulin* from BLV-infected cow	6

*The titer of the gamma globulin at a concentration of 1 mg/ml was 1:1000 in the radioimmunoassay.

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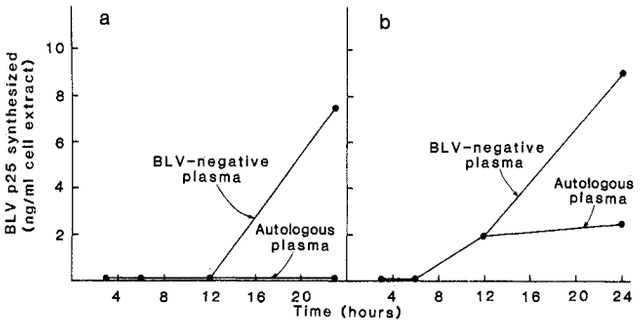

Fig. 1. Expression of BLV p25 antigen in BLV-infected lymphocytes cultured in (a) 100 percent autologous plasma or (b) 100 percent plasma from a BLV-free cow. At 3, 6, and 12 hours after incubation, cells were harvested and tested by competitive radioimmunoassay. (*8*). Cells were also harvested 12 hours after incubation from parallel cultures of (a) and (b) and each was divided into two parts. One part of the harvested cells was cultured in 100 percent autologous plasma and the other part was cultured in 100 percent plasma from a BLV-free cow. Following incubation for an additional 14 hours, the cells were harvested, washed, and tested by competitive radioimmunoassay (*8*).

detected in the plasma of any of the ten BLV-free animals examined or in the plasma of four BLV-free cattle that were infected with the foamy-like bovine syncytia virus.

To determine whether blocking of BLV expression by the plasma factor is reversible, lymphocytes from a BLV-infected cow were cultured in vitro for 12 hours in the presence of 100 percent autologous plasma, washed two times, and incubated for an additional 14 hours in 100 percent plasma from a BLV-free cow or in 100 percent autologous plasma. As shown in Fig. 1a, no BLV p25 antigen was detected during the first 12 hours of culture. Expression of BLV antigen readily occurred when the lymphocytes were transferred to plasma from a BLV-free cow, but continued to be blocked when the cells were maintained in autologous plasma.

We also investigated whether the plasma factor can block the synthesis of the BLV p25 antigen in lymphocytes that are already expressing the antigen. Figure 1b shows that BLV p25 was detected in the infected lymphocytes after 12 hours of culture in plasma from a BLV-free cow. Further synthesis of BLV p25 was blocked when the lymphocytes were washed and cultured in plasma from a BLV-infected cow, but not in plasma from a BLV-free cow.

As shown in Table 2, the blocking factor is sensitive to protease, but not to deoxyribonuclease, ribonuclease, lipase, or amylase. The factor was readily inactivated by heating at 56°C for 10 minutes, but was not affected by freezing and thawing. Since the factor is present in the plasma in a relatively small amount (titer 1:4), conventional biochemical and biophysical techniques, such as gel filtration chromatography and rate zonal centrifugation, could not be applied to determine its molecular weight.

Thus, the data indicate that the plasma of BLV-infected cattle contains a nonimmunoglobulin protein that blocks the expression of BLV genome in bovine lymphocytes and is somehow removed or inactivated during the clotting process. Preliminary studies indicate that the plasma factor blocks virus expression not only in BLV-infected lymphocytes, but also in BLV-infected monolayer cultures. However, the plasma factor does not block virus expression in monolayer cultures infected with Rauscher murine leukemia virus. Leong *et al.* (*9*) described a nonimmunoglobulin serum factor that neutralizes xenotropic murine leukemia virus. However, this serum factor clearly is different from the plasma factor described herein because it is insensitive to protease.

The mechanism by which the plasma factor blocks the expression of the BLV genome is not known. It is conceivable that this factor, like epidermal growth factor (*10*), reacts with specific receptors on the cell surface. Upon internalization, the receptor-factor complex would dissociate and the released factor would control expression by interacting with the cellular genome. Alternatively—as in the case of insulin (*11*) and luteinizing hormone (*12*)—the blocking factor, on interaction with cell surface receptors, could generate secondary messenger RNA that could control expression. It is important to point out that the plasma factor does not block BLV expression if the infected lymphocytes are subjected to hypotonic shock before cultivation in vitro. It is conceivable that, during hypotonic treatment, lymphocytes lose specific cell surface receptors for the blocking factor.

The finding that, in vivo, the BLV-infected lymphocytes usually harbor BLV genome in a covert, nonproductive state may account for the fact that BLV infection in cattle is persistent despite the continuous presence of antibodies to virus (*6, 13*). On the other hand, the presence of antibodies to virus in the infected animals is evidence that BLV antigens are expressed in vivo at least occasionally. This could occur as a result of cyclic fluctuations in the level of blocking factor in plasma. The reversibility of the blocking effect supports this idea. Our findings raise the possibility that the expression of cryptic leukemia viruses in other species are controlled by a similar inhibitor.

Table 2. Properties of blocking factor in the plasma of BLV-infected cattle. BLV-infected lymphocytes were cultured as described in the legend to Table 1. Following incubation the cells were harvested, washed, and tested for the presence of BLV p25 by competitive radioimmunoassay (*8*). Values are nanograms of p25 per milliliter of cell extract.

Treatment of plasma before cultivation	BLV p25 in lymphocytes after cultivation with treated	
	BLV-positive plasma	BLV-negative plasma
None	< 0.3	7
Enzymatic digestion*		
Protease-Sepharose	6	7
Deoxyribonuclease	< 0.3	6
Ribonuclease	< 0.3	6
Lipase	< 0.3	6
Amylase-polyacrylamide	< 0.3	6
Heating for 10 minutes at 56°C	7	7
Freezing and thawing three times	< 0.3	6

*Plasma from a BLV-infected or BLV-free cow was incubated for 2 hours at 37°C with the indicated enzymes at a concentration of 1 U/ml. The plasma treated with protease-Sepharose (Sigma) or amylase-polyacrylamide (Sigma) was centrifuged at 1000 rev/min for 5 minutes and then used for cell culture. Plasma treated with deoxyribonuclease (Worthington), pancreatic ribonuclease (Worthington), or lipase (Sigma) was used for cell culture without further manipulation.

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An Inhibitor of the Binding of Thyroid Hormones to Serum Proteins is Present in Extrathyroidal Tissues

Abstract. *Extrathyroidal tissues of man and the rat contain a potent inhibitor of the binding of thyroid hormones to serum proteins and to an anion-exchange resin. The inhibitor is heat-labile and nondialyzable. It acts by reducing the binding affinity of thyroid hormones to serum proteins, not by reducing the number of binding sites. The tissue inhibitor is similar in several characteristics to an inhibitor described previously in the serum of some critically ill patients, suggesting that the tissue inhibitor may leak into the circulation in severe illnesses.*

Recent studies (*1, 2*) suggest that a substance that inhibits binding of thyroxine (T_4) and triiodothyronine (T_3) to serum proteins is present in the circulation of some critically ill patients with nonthyroid illnesses (NTI). The presence of the inhibitor is often associated with a reduction in serum concentration of T_4 (and T_3) in patients with NTI. This is so marked in some patients that a battery of carefully monitored biochemical tests is needed to avoid erroneously diagnosing hypothyroidism (*1, 2*). The low T_4 in patients with NTI carries an ominous prognostic significance (*3*), but no data are available to suggest that the prognosis improves by administering thyroid hormones as it does in hypothyroidism.

The dialyzable (free) fraction of serum T_4 is usually normal or high in patients with NTI. This increase is often far beyond what would be expected from the moderate reduction in the serum concentration of T_4-binding globulin that is common in NTI (*1*). Curiously, however, the resin uptake of T_3, a simple test used routinely in clinical laboratories for assessing thyroid binding proteins, frequently fails to demonstrate as severe a reduction in serum binding of thyroid hormones as is shown by equilibrium dialysis (*1*). The basis for the difference is not fully understood. It has been suggested that the difference may lie in the ability of the thyroid hormone binding inhibitor to inhibit the binding of T_3 not just to serum proteins but also to resin, charcoal, isolated rat hepatocytes (*2*), and possibly other solid matrices.

There is no clear information on the nature of thyroid hormone binding inhibitor or the mechanism by which it appears in the circulation of seriously ill patients. In this study we have tested the hypothesis that the inhibitor is a normal component of extrathyroidal tissues and that it leaks into the circulation when the integrity of tissues is compromised in severe illnesses. In this report we describe the characteristics of a thyroid hormone binding inhibitor in tissues of the normal rat and of humans who died from NTI.

The presence of the inhibitory activity in tissues was tested by measuring the dialyzable fraction of serum T_4 in samples of pooled normal human serum in the presence or absence of a homogenate of the tissues. The method of determination of dialyzable fraction of T_4 has been described in detail elsewhere (*1, 4*). In brief, a 0.1-ml sample of pooled normal human serum, diluted to 5 ml with 0.15*M* phosphate buffer (*p*H 7.4), was labeled with dialyzed ^{125}I-labeled T_4 (specific activity, ~ 100 mCi/mg) and placed on one (A) side of an equilibrium dialysis cell (Technilab Inc.); 5 ml of unmodified buffer was placed on the other (B) side of the cell. The cell was incubated at 37°C for 20 hours, at which time the buffer on B side of the cell was removed and carrier T_4 was added; a magnesium chloride solution (*p*H 9.3) was then added to precipitate radioactive and nonradioactive T_4. The precipitate was washed several times with a magnesium chloride wash solution (*p*H 8.6) and the radioactivity in the precipitate was determined with a precision of ± 2 percent by means of a Tracor-analytic gamma scintillation counter. The dialyzable fraction of T_4 in the serum of 25 normal subjects tested by this method averaged (mean ± standard error) 0.033 ± 0.01 percent. The dialyzable fraction of T_3, as determined by using radioactive T_3 instead of radioactive T_4 in the method described above, averaged 0.34 ± 0.16 percent (N = 16). The coefficient of variation of measurements in eight replicates of a serum in an assay was 13 percent for dialyzable T_4 and 9 percent for dialyzable T_3.

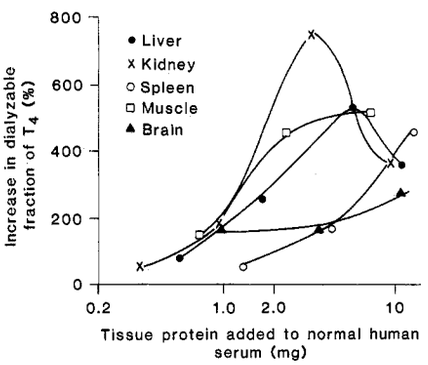

Fig. 1. Increases in the dialyzable fraction of T_4 caused by the addition of rat tissue homogenates to 0.1 ml of a pooled sample of normal human serum labeled with [^{125}I]T_4.

Human tissues obtained at autopsy or fresh tissues obtained from male Sprague-Dawley rats were homogenized in 3 volumes of 0.15*M* phosphate buffer, *p*H 7.4, with a Polytron (Brinkmann Instruments). Homogenates were filtered through four layers of gauze. The protein concentration of the homogenates was measured by the method of Bradford (*5*) with kits obtained from Bio-Rad Laboratories.

Addition of 0.2 to 1.7 mg of protein from a homogenate of human liver or kidney to normal human serum increased the dialyzable fraction of T_4 by 20 to 480 percent in a dose-dependent manner; addition of higher amounts of tissue protein was associated with less than maximum increases in dialyzable T_4, apparently because of an increasing effect of T_4-binding proteins in the homogenates on the assay for dialyzable T_4. Since there was a possibility of an interference resulting from postmortem autolysis of tissues or by the various drugs administered to ill patients prior to death, we examined the effect of fresh homogenates of various rat tissues on the dialyzable fraction of serum T_4. Even small amounts of tissue proteins substantially reduced the serum binding of T_4, as shown by the marked increases in dialyzable fraction of T_4 in normal human

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