ture endothelial cells isolated and grown in this manner, the establishment of strains is not difficult and has been accomplished. Once established, the strains can be preserved in liquid nitrogen and thus provide a reliable source of the same cells for investigational purposes. Each split can be made at a 6 : 1 ratio with a doubling time of approximately 40 hours. Although the primary cultures may contain a few cells which display a fibroblastic morphology, these are soon overgrown by the great predominance of endothelial cells which are present. In fact, by the third serial subculture the cultures appear to be composed of pure endothelial cells, and when compared to fibroblasts, the cultured endothelial cells are morphologically different.

Although replication is indicated by our ability to subculture these cells, it was also shown that cells cultured in this manner when treated with [^{3}H]-thymidine labeled 80 percent of the nuclei, providing further evidence that the cells were actively dividing.

When tested by the indirect fluorescent-antibody technique, these cultures of endothelial cells stained brilliantly with antibody against human thrombosthenin. Cultures of human foreskin fibroblasts did not. These results suggest that the endothelial cells produce a substance which reacts like thrombosthenin, but might not be monospecific. In addition, these endothelial cells were found to possess ABO blood group antigens, as demonstrated by rosette formation and absorption techniques. If the cultured endothelial cells from the umbilical vein of a type A infant were treated with anti-A serum, washed thoroughly, and type A erythrocytes were added, rosettes formed. No such rosettes were found when type B erythrocytes were added to type A endothelial cells after incubation of the type A endothelial cells with anti-B serum. Similarly, fibroblasts did not react with absorption of anti-A or anti-B antibodies. It had been observed earlier that human endothelial cells had ABO antigens and immunologic evidence of thrombosthenin (*3, 4*).

Electron microscopy of the cultured cells demonstrated tight intercellular junctions and typical morphologic features of endothelial cells, including Weibel-Palade bodies (*5*) (Figs. 1 and 2). Further evidence that the cells obtained after serial subculture were endothelial cells was provided by freeze-etch preparations which demonstrated caveolae in the plasma membrane (Fig. 3). These features are characteristics of endothelial cells prepared from kidney, pituitary, and lung tissues (*6*).

The results of the present study indicate that human endothelial cells can grow and replicate in vitro. This growth occurs at a rate which facilitates the harvest of adequate numbers of cells for investigative studies which, heretofore, had been considered improbable, if not impossible.

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27 April 1973

Feline Leukemia and RD-114 Virus Group-Specific Proteins: Comparison of Amino Terminal Sequence

Abstract. *The major internal virion polypeptide from feline and RD-114 type C viruses has been subjected to amino terminal sequence analyses with the Beckman automated sequencer. These proteins, as well as their homologs in rat and mouse viruses, begin with the sequence prolylleucylarginyl (Pro-Leu-Arg). Virus RD-114 differs from conventional feline type C viruses that show about 80 percent relatedness based on calculation of the minimum number of base changes to give equivalent coding for the protein segments analyzed. In addition, insertion of a gap in the RD-114 sequence is necessary to maintain positional homology. The difference between RD-114 and feline leukemia virus appears as great as the difference between mouse type C viruses and either of these two viruses. Thus, even though current evidence suggests that RD-114 is of feline origin, the sequence differences between RD-114 and conventional feline virus group-specific proteins is well beyond that based on one or a few point mutations.*

The type C viruses of mice, cats, rats, and hamsters are related both morphologically and immunochemically. The major internal polypeptide (gs protein) of viruses from these species (molecular weight about 30,000) contains a cross-reactive antigenic determinant (*1, 2*), originally designed group-specific protein-3 (gs-3) (*3*), in addition to species-specific antigenic determinants (*1*), originally designated gs-1 (*4*). Current evidence (*2, 5*) indicates that these designations will require modification to include results both from more detailed analyses of subprimate gs proteins and from studies of gs proteins of two primate isolates, woolly monkey and gibbon ape.

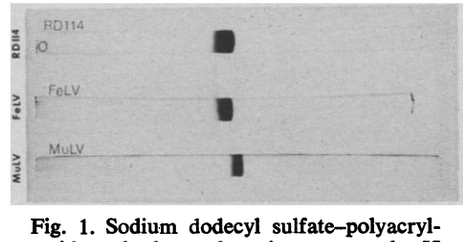

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis at neutral *p*H (*17*) of gs proteins purified by isoelectric focusing as described earlier for mouse (*18*), cat (*19*), and RD-114 (*8*) viruses. In the final electrofocusing, the gradients were *p*H 5 to *p*H 8 for mouse, and *p*H 7 to *p*H 10 for cat and RD-114 virus. Ampholine was removed by gel filtration through Bio-Gel P-10 or Bio-Gel P-100 (Bio-Rad), equilibrated with 0.33*M* ammonium acetate, and proteins were recovered by lyophilization; *O* indicates origin.

In order to more specifically define relationships among the various viruses, we have initiated NH_2-terminal sequence analysis of the gs protein from several viruses. In the course of this work a new virus, designated RD-114, was recovered from human rhabdomyosarcoma cells passaged through a fetal cat (*6, 7*). This virus was distinguished from known conventional feline type C viruses (FeLV) on the basis of the absence of feline gs-1 (*7, 8*) and the

Table 1. Amino terminal sequences of gs proteins. The stepwise degradation of proteins (*20*) in an automated Beckman protein sequencer (model 890) and the identification of individual residues by gas chromatography, amino acid analysis, and special color reactions were carried out (*13*). For thin-layer chromatography of the phenylthiohydantoin derivatives of amino acids, a recently developed micro method (*21*) was used. The mouse sequence has been reported previously and confirmed by independent analysis of a 0.12-μmole protein sample. The sequence analyses of cat and RD-114 gs proteins were performed on 0.13 and 0.11 μmole of material, respectively.

Virus	Position														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Mouse	*Pro*	*Leu*	*Arg*	Leu	*Gly*	Gly	*Asn*	Gly	Gln	Leu	*Gln*	*Tyr*	*Trp*	*Pro*	*Phe*
Cat	*Pro*	*Leu*	*Arg*	Glu	*Gly*	Pro	*Asn*	Asn	Arg	Pro	*Gln*	*Tyr*	*Trp*	*Pro*	*Phe*
RD-114	*Pro*	*Leu*	*Arg*	Thr	*	Val	*Asn*	Arg	Thr	Val	*Gln*	*Tyr*	*Trp*	*Pro*	*Phe*

* Indicates a gap.

presence of a unique determinant on its gs protein (*8*) and on the lack of feline-specific antigenic determinants associated with the virion reverse transcriptase (*9*). Furthermore, it does not share envelope determinants (*7*) with any of the three described feline viral envelope antigen subgroups (*10*), and showed minimum (<5 percent) cross-hybridization with feline type C viruses with the DNA product of reverse transcription and viral RNA (*11*). Nevertheless, recent evidence, based on nucleic acid hybridization and viral activation, has suggested that this virus represents a second family of feline type C viruses not normally expressed (*11, 12*).

Sequence analysis of the RD-114 gs protein was considered important in showing the extent of the difference of these proteins, since, to this point, all type C isolates within a single species share a common gs protein on the basis of both antigenic and biophysical properties. We present the results of our studies extending to the amino terminal 15 residues of FeLV and RD-114, determined by an automated sequencer (Beckman).

We reported previously (*13*) the amino terminal 15 residues of the gs protein from one strain (Rauscher) of murine type C virus. The same sequence has been found for the gs protein from a second strain (AKR) of murine type C virus. Tripeptide sequences from feline and RD-114 type C viruses, as obtained by manual sequence methods, were also reported. We have now subjected FeLV (Thielen strain) and RD-114 gs proteins to the automated sequencer, using described methods (*13*). The purity of these preparations and the NH_2-terminal amino acid sequences are shown in Fig. 1 and Table 1, respectively.

The significant results are as follows. (i) Each protein begins with the sequence Pro-Leu-Arg (*14*); in addition, initial analyses of the gs protein from a rat type C virus revealed the same tripeptide sequence. (ii) At positions 11 to 15 for murine leukemia virus (MuLV) and FeLV, identical residues appear, whereas in RD-114, the identical sequence of residues begins at position 10. Therefore, in order to match RD-114 with the other proteins, the insertion of a gap is required at some point between residues 3 and 10. On the basis of the Asn residue at position 7 in MuLV and FeLV, and position 6 in RD-114, the gap should most likely be placed between residues 4 and 6. We tentatively place the gap at position 5 since Gly is found at this position in each protein, including that of the rat (*15*), with the exception of RD-114. (iii) Residues 4 to 10 show considerable variability from protein to protein, with some of the differences necessitating two base changes (Table 2). (iv) While we originally reported Leu at position 3 of RD-114, Arg was unequivocally identified by automated sequencer analyses. The initial designation was evidently based on carry-over by the manual method, and thus residue 3 was not detected. Nevertheless, the RD-114 protein shows considerable difference from that of a conventional feline type C virus in that two Thr and two Val residues are found among the first ten positions of RD-114 and neither of these amino acid residues occurs in the 15 residues of the NH_2-terminal of feline virus.

Table 2. Mutational distances among amino terminal 15 residues of mouse, cat, and RD114-gs proteins. The minimum number of nucleotide changes to convert one gene segment to code for a heterologous protein was calculated from table 1 of Fitch and Margoliash (*22*). The gap in RD-114 is placed at position 5 and is excluded from this calculation.

Comparison	Dissimilar residues (No.)	Nucleotide changes (No.)
Mouse-cat	5	8
Mouse–RD-114	5	7
Cat–RD-114	5	9

The various type C virus gs proteins thus show clear evidence of sequence homology through the first 15 residues at the NH_2-terminal. This provides evidence for a common ancestral gene coding for this set of proteins. The RD-114 protein differed from the others in necessitating the insertion of a gap to maintain positional homology. The data also show that, even if RD-114 represents a second family of feline type C viruses, its relation to the commonly studied feline viruses is quite distinct. For example, excluding the gap, 8 to 10 base changes—depending on the location of the gap (out of 45)—are required to achieve similar coding for both proteins. In contrast to this difference between conventional FeLV and RD-114, gs proteins from several type C virus isolates from mice are very similar on the basis of immunological assays, and the two strains tested have identical NH_2-terminal sequences. Thus, the distinct serological specificity of the two proteins (*8*) is not explicable by a single point mutation at some key position in the molecule. The degree of relationship on the basis of the minimum number of nucleotide differences between FeLV and RD-114 gs proteins estimated from this short sequence (about 80 percent) is consistent with overall amino acid compositional data (*16*), and thus could be a reflection of the relationship over the total molecule.

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7 March 1973

6-Hydroxydopamine: Evidence for Superoxide Radical as an Oxidative Intermediate

Abstract. *Superoxide dismutase inhibited the autoxidation of 6-hydroxydopamine as measured by the rate of formation of a quinone and the rate of oxygen consumption. These observations demonstrate the formation of the superoxide radical during the autoxidation process. This finding may be relevant to the mechanism of adrenergic nerve terminal degeneration caused by 6-hydroxydopamine.*

Superoxide dismutase (*1*) is the enzyme that catalyzes the decay of the superoxide anion ($O_2^{\cdot-}$, a free radical) according to the reaction

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

In a recent review, Fridovich (*2*) discussed how the presence of $O_2^{\cdot-}$ in biological systems could be ascertained through the use of superoxide dismutase. It has been shown, for example, that $O_2^{\cdot-}$ was involved in oxygen-dependent hydroxylations (*3*), the mechanism of xanthine oxidase (*4*), the mechanism of tryptophan pyrrolase (*5*), hemolysis induced by dialuric acid (*6*), and the autoxidation of epinephrine at elevated *p*H (*7*).

6-Hydroxydopamine (6-OHDA) accumulates within catecholamine nerve terminals after injection into experimental animals and subsequently causes degeneration of these nerve terminals (*8*). It spontaneously reacts with oxygen to form a quinone (*9*) and H_2O_2 (*10*). We used superoxide dismutase to look for $O_2^{\cdot-}$ during the autoxidation of 6-OHDA. We found that $O_2^{\cdot-}$ is formed and that it catalyzes the autoxidation of 6-OHDA.

The rate of reaction between 6-OHDA and molecular oxygen was studied in two ways. First, the rate of formation of the quinone of 6-OHDA [or other oxidation products (*11*)] was measured by the increase in absorbancy at 490 nm on a Gilford model 300 spectrophotometer. This was done at 37°C with constant agitation in 0.05*M* potassium phosphate buffer at *p*H 7.4. Second, a Clark oxygen electrode (Yellow Springs Instruments) connected to a Honeywell Electronik 19 recorder was used to monitor O_2 consumption by 6-OHDA. The reaction was carried out in a closed chamber with continuous stirring at 37°C in 1 ml of the potassium phosphate buffer. Superoxide dismutase (100 μg/ml, 300 units per milliliter, Truett Laboratories) was added in some experiments. In both systems, the initial rates were calculated and the data analyzed statistically by using Student's *t*-test.

Table 1. Effect of superoxide dismutase on the initial rate of O_2 consumption by 6-OHDA ($10^{-4}M$). The experimental details are the same as for Fig. 1B. The data are given as mean ± S.D., with the number of experiments in parentheses. The buffers used were: *p*H 7.4 and 8.0, 0.05*M* potassium phosphate; *p*H 8.5, 0.05*M* tris(hydroxymethyl)aminomethane hydrochloride; and *p*H 9.0, 0.05*M* sodium carbonate.

*p*H	O_2 consumed (nmole/min)		Inhibition (%)
	Without enzyme	With enzyme	
7.4	31 ± 11 (6)	8 ± 4 (6)	74
8.0	45 ± 11 (7)	8 ± 5 (6)	82
8.5	173 ± 15 (4)	45 ± 6 (4)	74
9.0	203 ± 29 (4)	42 ± 5 (4)	79

The rate of formation of the quinone from $2.5 \times 10^{-4}M$ 6-OHDA was inhibited by superoxide dismutase (Fig. 1A). The initial rate in the absence of enzyme was 0.415 ± 0.148 absorbancy unit per minute [mean ± standard deviation (S.D.); $N = 5$], while in the presence of superoxide dismutase the initial rate was 0.027 ± 0.010 unit per minute ($N = 5$). Superoxide dismutase inhibited the rate of formation of the quinone by 93 percent ($P < .001$).

6-Hydroxydopamine spontaneously reacted with O_2 at a rapid rate (Fig. 1B). We showed previously with the use of the enzyme catalase (*12*) that O_2 consumption could be equated with H_2O_2 formation according to the reaction scheme

$$\text{6-OHDA} + O_2 \rightarrow Q + H_2O_2 \qquad (2)$$

where Q is the quinone of 6-OHDA. The initial rate of O_2 consumption by $2.5 \times 10^{-4}M$ 6-OHDA was 75 ± 11 nmole of O_2 per minute (mean ± S.D.; $N = 9$) in the absence of superoxide dismutase and 20 ± 1 ($N = 4$) in the presence of superoxide dismutase. Superoxide dismutase inhibited the rate by 73 percent ($P < .001$).

We also studied the reaction between 6-OHDA ($10^{-4}M$) and O_2 over the range of *p*H 7.4 to 9.0 (Table 1). The rate of O_2 consumption increased with increasing *p*H, but the effect of superoxide dismutase was similar over the *p*H range.

The data showed that $O_2^{\cdot-}$ was formed during the autoxidation of 6-OHDA. They also indicated that $O_2^{\cdot-}$ formed from 6-OHDA catalyzed the oxidation of 6-OHDA. For a decrease in the rate of reaction to have occurred after the addition of superoxide dismutase (Fig. 1, A and B), $O_2^{\cdot-}$ must have played a catalytic role in the overall reaction. This was the means by which Misra and Fridovich (*7*) showed that $O_2^{\cdot-}$ was an intermediate in the autoxidation of epinephrine at elevated *p*H and catalyzed the reaction. However, in the epinephrine system $O_2^{\cdot-}$ was detected only in a reaction catalyzed by ferrous ion at *p*H 9.0 or higher, or in a reaction catalyzed by base at *p*H 10.2. In contrast, the generation of $O_2^{\cdot-}$ at physiologic *p*H was