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Malignant Transformation of Erythroid Cells in Vivo by Introduction of a Nonreplicating Retrovirus Vector

Abstract. *DNA from a replication-defective spleen focus-forming virus (SFFV) was reconstructed and transfected into ψ-2 cells containing a packaging-defective mutant of Moloney murine leukemia virus. Replication-incompetent retrovirus particles (helper virus–free) containing genomes that express the transforming envelope gene of SFFV (gp52) transformed bone marrow cells in vitro and, after direct intravenous introduction of the vector, induced malignant erythroid disease in vivo. Disease induction was dependent on prior treatment of mice with phenylhydrazine, which probably increased the availability of erythroid target cells. Since there was no evidence of virus particle expression in mice with malignant disease, this study demonstrates the acute oncogenic potential of a limited number of erythroid cells expressing SFFV gp52. Direct inoculation of animals with nonreplicating retroviral vectors containing transforming genes may be useful in studying the oncogenic effects of such genes.*

Retroviruses can transfer their own genes into mammalian cells and can also be used to transfer genes that have been artificially inserted into them by genetic engineering. The successful use of retroviruses as vectors for the introduction of genes into hematopoietic cells has been demonstrated (*1–3*). With the development of helper cell lines that provide viral particles containing only the minimum genetic information required for integration and expression, genes can be directly introduced into recipient cells without the presence of replication-competent helper virus (*4, 5*). We used a helper cell line to produce viral particle preparations containing only the genome of the spleen focus-forming virus (SFFV), a replication-defective retrovirus, which—in the presence of replication-competent virus—causes an acute erythroleukemia (*6*). Our results show that helper cell lines can facilitate studies of transforming genes, as in the case of SFFV, by delivering them directly to cells in vitro or in vivo in the absence of a spreading infection.

The SFFV expresses a pathogenic 52-kilodalton (kD) glycoprotein encoded by a retroviral envelope gene (*env*) that is highly altered from its putative parent gene in Friend murine leukemia virus (F-MuLV) as a result of deletion, insertion, and recombination (*7*). This gene is essential for the acute erythroleukemia induced by this virus (*6, 8*), but it is unknown whether the replicating helper virus used to rescue the defective SFFV from cells plays a role in the disease process—either directly through expression of its own genes or indirectly through its ability to cause a systemic infection and, therefore, continual infection of new erythroid progenitor cells. To answer this question, we reconstructed SFFV DNA so that it could be efficiently expressed in helper cell lines and be incorporated into particles. Preparations derived this way were tested for their ability to induce transformation of bone marrow cells in vitro and to cause an erythroleukemia by direct inoculation in vivo.

DNA fragments derived from permuted clones of SFFV were used to prepare structures analogous to integrated proviruses that have a long terminal repeat (LTR) at each end (see Fig. 1). We prepared two constructs ($SFFV_A$-L and $SFFV_{AP}$-L) that would have different phenotypic characteristics in vitro—one that would induce abnormal erythroid proliferation and differentiation in the absence of exogenous erythropoietin (Epo) (analogous to the $SFFV_P$ strain), and one that would require Epo (analogous to the $SFFV_A$ strain) (*6*). The $SFFV_A$-L construct is entirely composed of DNA fragments derived from $SFFV_A$ (*9*). The 5′ end consists of a Cla I–Eco RI fragment from the full-size genomic clone (Cla I–Cla I), which has two internal LTR's (Fig. 1A); the 3′ end of the envelope gene and the 3′ LTR consist of Eco RI–Kpn I [1200 base pairs (bp)] and Kpn I–Bam HI (200 bp) fragments derived from a subclone of the same virus. The extra 5′ LTR was not removed during construction since it should be eliminated on formation of viral RNA; the message presumably initiates at the second 5′ LTR and terminates at a polyadenylation site in the 3′ LTR. The recombinant $SFFV_{AP}$-L is identical except that the Eco RI–Kpn I fragment is derived from $SFFV_P$. This Eco RI–Kpn I fragment was chosen for the recombinant virus because it has been suggested that sequences in the 3′ end of the envelope gene may be responsible for the phenotypic differences between $SFFV_P$ and $SFFV_A$ (*10*). When the structures were checked by restriction analysis and Southern blotting (see Fig. 1B), both were found to produce three similar LTR-hybridizing, Kpn I fragments, confirming the presence of three LTR's. The derivation of the Eco RI–Kpn I fragments in each of the constructions could be shown by the presence or absence of a unique Cla I site in the 3′ region of the virus; while the $SFFV_A$-L DNA was seen as a single band at 8.7 kilobases (kb) after Cla I digestion, the $SFFV_{AP}$-L remained supercoiled (Fig. 1B). Further double digestions and hybridization with a 5′ envelope probe (Fig. 1B) confirmed the structures depicted in Fig. 1A.

The phenotypic characterization in vitro and in vivo of helper virus–containing stocks of the constructed viruses has been carried out and will be the subject of another report. Briefly, $SFFV_A$-L (with F-MuLV) behaves in vitro like the original $SFFV_A$ (with F-MuLV) (*8*), inducing transformation of erythroid cells in the presence, but not in the absence, of Epo-containing preparations. The

Table 1. Induction of erythroid bursts by helper-free virus. Bone marrow cells from phenylhydrazine-treated mice were incubated for 2 hours at 4°C with the test preparation and then plated in methylcellulose by previously described procedures (*15*). Hemoglobinized (benzidine-positive) erythroid bursts were counted on day 5. Media from fibroblast cultures were tested undiluted.

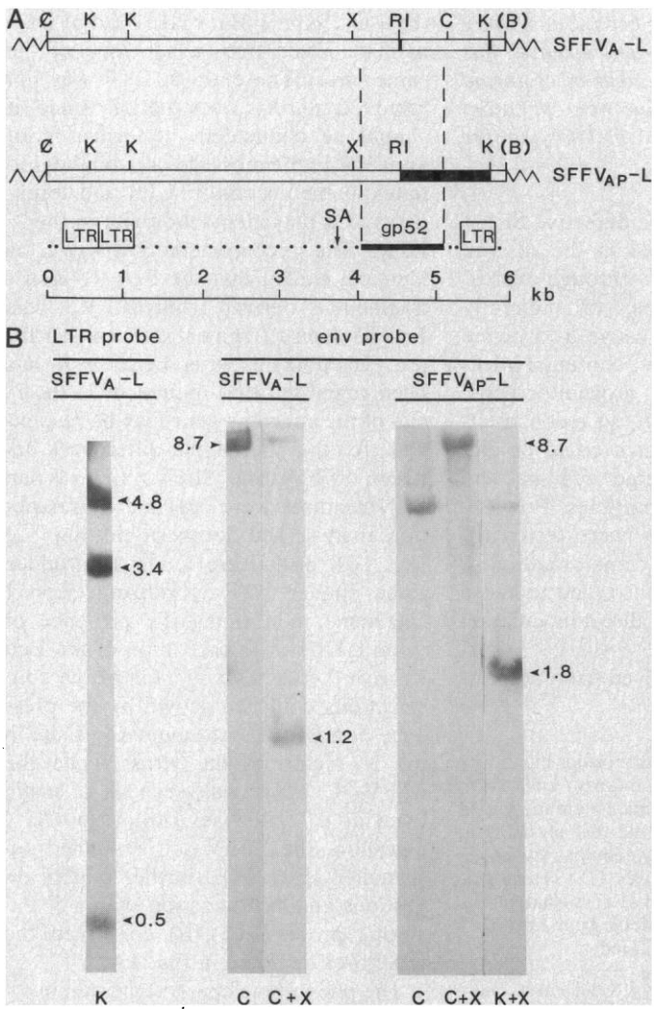
Test virus	Erythroid bursts per 10^6 cells	
	No Epo added	Epo added (0.2 U/ml)
None	0	0
F-MuLV/$SFFV_P$*	89	177
$SFFV_{AP}$-L (ψ-2)	23	55
$SFFV_A$-L (ψ-2)	0	>200
ψ-2	0	0

*Positive control virus produced by NIH 3T3 cells.

recombinant $SFFV_{AP}$-L (with F-MuLV), like the original $SFFV_p$ (with F-MuLV), transforms erythroid cells whether or not Epo is added to the cultures. In vivo, both virus complexes induce an acute erythroblastosis associated with a large increase in erythroid colony-forming cells (CFU-E), but only the CFU-E from $SFFV_{AP}$-L–infected mice can differentiate in vitro in the absence of exogenous Epo. As observed earlier with $SFFV_p$ (*11*), the peripheral blood of animals infected with $SFFV_{AP}$-L contains abnormally high levels of reticulocytes.

Helper virus–free stocks of $SFFV_A$-L and $SFFV_{AP}$-L were prepared by cotransfecting the DNA's with pSV2neo (*12*) into ψ-2 helper cells containing integrated packaging-defective mutants (pMOV-ψ$^-$) of Moloney murine leukemia virus (M-MuLV) (*4*, *13*). Individual clones resistant to the neomycin analog G418 were propagated and screened for expression of the SFFV *env* protein. As shown in Fig. 2A, two representative clones, 3B2 (containing integrated $SFFV_{AP}$-L) (lane 1) and 4C4 (containing $SFFV_A$-L) (lane 3), express both SFFV gp52 and the M-MuLV envelope precursor protein ($gPr85^{env}$). The virus released from these clones, however, contains only the SFFV genome, since NIH 3T3 cells infected with culture fluids expressed SFFV, but not MuLV, envelope proteins (Fig. 2B) and did not release reverse transcriptase (*14*). These results indicate that we prepared viruses capable of transferring the SFFV genome to new cells in the absence of replicating helper virus.

The biological activity of the helper-free SFFV's on hematopoietic cells was tested either by introducing the virus in vitro into bone marrow cells, which were then observed for erythroid transformation, or directly injecting the virus into mice. Although undiluted culture fluid containing the $SFFV_A$-L(ψ-2) virus was capable of transforming erythroid precursor cells in vitro, it was unable to induce in mice an acute erythroleukemia under the conditions used. The transfected $SFFV_A$-L DNA was not defective, however, since mice receiving this virus in combination with subsequent inoculations of helper virus did develop erythroleukemia. A preliminary study has also shown that concentrated culture fluid containing $SFFV_A$-L(ψ-2) can induce erythroleukemia. Culture fluid contain-



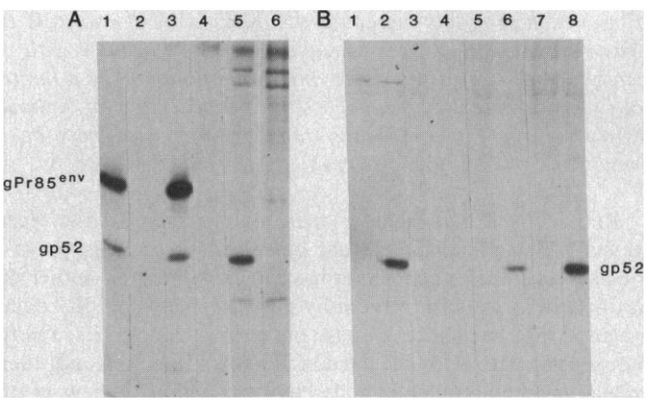


Fig. 1 (left). (A) Restriction endonuclease maps of constructed DNA's. SFFV DNA fragments were ligated and cloned into pUC9 at the Acc I and Bam HI sites. Locations of the gp52 coding region, splice acceptor (SA) and long terminal repeats (LTR's) are shown. Open bars represent $SFFV_A$ DNA from either clone 1310 or 6011/3 (*9*). The solid bar in $SFFV_{AP}$-L is DNA derived from $SFFV_p$ (Lilly-Steeves) clone 52-36 (*20*). B, Bam HI; C, Cla I; K, Kpn I; RI, Eco RI; X, Xma III. The Cla I restriction site, lost after ligation into the Acc I site of pUC9, is indicated by a C with a slash through it. Because there is more than one Bam HI site in each viral construct, the one shown is in parentheses. DNA fragments used for ligation were prepared from cesium chloride gradient–purified plasmid DNA by restriction endonuclease digestion and separation on agarose gels. (B) Confirmation of constructions by restriction endonuclease analysis and Southern blotting. DNA's were digested with the enzymes indicated at the bottom of each lane. A 550-bp Cla I–Kpn I LTR probe was derived from $SFFV_A$ clone 6011/3 (*9*). The *env* probe, a 600-bp Bam HI–Eco RI fragment subcloned from the original $SFFV_p$ genomic clone (*21*), corresponds to the 5′ end of the $SFFV_A$ *env* up to the RI site shown in this figure. Lighter unmarked hybridizing bands in this figure are incompletely digested DNA's that were not visible in the original ethidium bromide–stained agarose gel. Electrophoresis of DNA's, blotting, and hybridization with ^{32}P nick-translated probes were performed by standard techniques (*22*). Fig. 2 (right). Envelope protein expression in transfected ψ-2 cells. Cells were labeled with ^{35}S-methionine for 45 minutes. Labeling, immune precipitation of extracts, and electrophoresis on 7 percent sodium dodecyl sulfate–polyacrylamide gels were performed as described (*23*). Labeled extracts of ψ-2 cells transfected with either $SFFV_{AP}$-L (lanes 1 and 2) or $SFFV_A$-L (lanes 3 and 4) and control NIH 3T3 cells containing integrated $SFFV_A$-L (lanes 5 and 6) were precipitated with goat antiserum to Rauscher leukemia virus gp69/71 (lanes 1, 3, and 5) or with normal goat serum (lanes 2, 4, and 6). Cells used in labeling were prepared by cotransfecting viral DNA (3 μg) and pSV2neo DNA (0.3 μg) by the calcium phosphate technique (*24*). G418 resistance clones were screened for their ability to express gp52. (B) Expression of gp52 in NIH 3T3 cells incubated with culture fluids from transfected ψ-2 cells. Virus containing culture fluid was incubated with NIH 3T3 cells in the presence of polybrene (4 μg/ml) for 16 hours. After 5 days the cells were labeled with [^{35}S]methionine, and the extracts were immune precipitated. Extracts from control NIH 3T3 cells, containing integrated $SFFV_A$-L, (lanes 1 and 2) and cells incubated with culture fluids from either ψ-2 cells (lanes 3 and 4), $SFFV_{AP}$-L–transfected ψ-2 cells (lanes 5 and 6), or $SFFV_A$-L–transfected ψ-2 cells (lanes 7 and 8) were precipitated with normal goat serum (lanes 1, 3, 5, and 7) or with antiserum to RLV gp69/71 (lanes 2, 4, 6, and 8).

ing $SFFV_{AP}$-L(ψ-2) virus in contrast to those containing $SFFV_A$-L(ψ-2), produced striking effects both in vivo and in vitro as shown below.

Transforming effects of the helper virus–free SFFV's in vitro are summarized in Table 1. When bone marrow cells were incubated with culture fluid from $SFFV_{AP}$-L(ψ-2)–transfected cells and observed for colony formation in semisolid medium, bursts of erythroid cells could be detected after 5 days in culture with or without exogenous erythropoietin. This result is consistent with results obtained when helper-containing stocks of $SFFV_P$ were used (*15*). The activity in this culture fluid was reduced to zero after heat inactivation at 56°C for 60 minutes or clarification at 100,000*g*, procedures known to eliminate retroviral activity. Culture fluids from $SFFV_A$-L–transfected ψ-2 cells were also capable of inducing erythroid bursts; however, hemoglobinized bursts formed only in the presence of exogenously added Epo, a result typical for $SFFV_A$ complexes. In the assays presented here, no erythroid bursts were observed on day 5 after incubation of bone marrow cells with culture fluids from untransfected ψ-2 cells.

To assess the in vivo oncogenic potential of the recombinant $SFFV_{AP}$-L(ψ-2) in the absence of helper virus, NIH Swiss mice were injected intravenously with culture fluids from ψ-2–transfected cells. The results are presented in Table 2. More than 50 percent of adult NIH Swiss mice treated with phenylhydrazine and then given the $SFFV_{AP}$-L(ψ-2) virus developed grossly enlarged spleens (weighing as much as 1.94 g) 12 to 27 days after inoculation (Fig. 3A). Stained cytospin preparations of spleen cells from diseased mice showed an overproduction of erythroblasts that had basophilic cytoplasms and condensed nuclei (Fig. 3B), typical of spleen cells from mice infected with standard helper virus–containing $SFFV_p$ stocks (*11*). When examined for expression of SFFV proteins, the diseased spleens expressed high levels of gp52 (Fig. 3C).

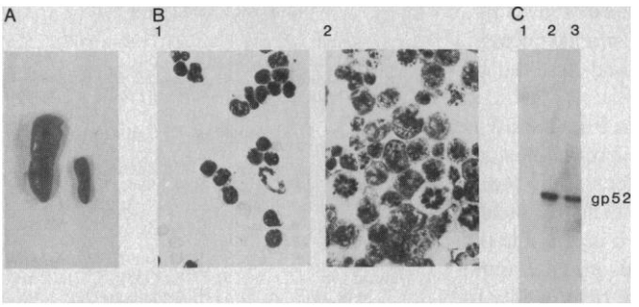


Fig. 3. (A) Enlarged spleen from a 7-week-old mouse that received an inoculation of $SFFV_{AP}$(ψ-2) at age 5 weeks compared with an age-matched normal mouse spleen. (B) Erythroblasts in a diseased spleen. Cytospin preparations from the spleen of a phenylhydrazine-treated control NIH Swiss mouse (B1) or a mouse receiving phenylhydrazine and $SFFV_{AP}$-L(ψ-2) (B2) were stained with a Wright-Giemsa stain (magnification, ×860). (C) Expression of gp52 in diseased spleens of animals receiving $SFFV_{AP}$-L(ψ-2). Spleen cells taken from a mouse with a 1.83-g spleen (20 days after inoculation) were labeled with [^{35}S]methionine and precipitated with normal goat serum (lane 1), antiserum to RLV gp69/71 (lane 2), or antiserum to gp69/71 made specific for gp52 or MCF envelope proteins by adsorption (*23*) (lane 3).

Mice that had not been treated with phenylhydrazine before introduction of the helper virus–free SFFV did not develop erythroid disease (Table 2). Previous studies have shown that phenylhydrazine treatment can lead to an increase in the number of SFFV target cells, proposed to be a late BFU-E (burst-forming unit, erythroid) (*16*). The particular regimen that the mice in this study were given has been shown to result in maximum levels of such cells in the peripheral blood at the time that we intravenously injected the virus (*17*). Thus, the induction of erythroleukemia by helper virus–free SFFV may depend on the presence of a high concentration of target cells in the circulation at the time of injection.

$SFFV_{AP}$-L(ψ-2) virus produced discrete foci on the spleens of infected mice 9 days after injection and, from the number of these foci, the titer of SFFV in the culture fluids could be estimated to be less than 10^2 focus-forming units per milliliter. This is considerably lower than that observed with helper virus–rescued stocks of $SFFV_{AP}$-L, which show a titer greater than 10^5 focus-forming units per milliliter in phenylhydrazine–treated mice. However, it may not be accurate to compare SFFV concentrations in helper-containing and helper-free stocks since the possible role of the helper virus in increasing the number of foci as a result of replication and spread is not clear. If $SFFV_{AP}$-L(ψ-2) virus is titrated in adult mice (not previously treated with phenylhydrazine) in the presence of helper virus, the titer is 10^3 to 10^4 foci

Table 2. Effects of nonreplicating $SFFV_{AP}$-L(ψ-2) in vivo. Undiluted culture fluid (0.5 ml) from $SFFV_{AP}$-L transfected or untransfected ψ-2 cells was tested in vivo by tail vein inoculation of 5-week-old female NIH Swiss mice. Some mice were treated with phenylhydrazine (60 mg/kg, intraperitoneally) 1 and 2 days before virus inoculation. Foci were counted after spleens were fixed in Bouin's solution (*19*). Animals were considered diseased if they had discrete splenic foci on day 12 or spleens weighing more than 0.4 g. Data representing the incidence of disease are given as the number of animals with SFFV disease per the number of animals tested. Abbreviations: P.I., postinoculation of virus; N.D., not determined; T.N.T.C., too numerous to count.

Virus	Day P.I.	Mice tested (No.)	Spleen weight (g)		Foci per spleen	SFFV disease
			Mean	Range		
			Phenylhydrazine-treated			
None	12	5	0.14	0.13–0.16	All negative	0/8
	20	3	0.12	0.11–0.13	N.D.	
ψ-2	12	5	0.18	0.13–0.35	All negative	0/8
	20	3	0.12	0.10–0.14	N.D.	
$SFFV_{AP}$-L (ψ-2)	12	3	0.35	0.15–0.60*	16, T.N.T.C., T.N.T.C.	
	20	5	1.12	0.14–1.83*	0, 0, T.N.T.C., T.N.T.C., T.N.T.C.	7/11
	27	3	0.73	0.10–1.94*	0, 0, T.N.T.C.	
			No pretreatment			
ψ-2	12	3	0.12	0.10–0.14	All negative	0/3
$SFFV_{AP}$-L (ψ-2)	12	5	0.10	0.09–0.10	All negative	0/13
	20	8	0.11	0.06–0.16	All negative	

*Individual weights in grams on day 12: 0.15, 0.31, 0.60; on day 20: 0.14, 0.19, 1.62†, 1.82†, 1.83; and on day 27: 0.10, 0.10, 0.16, 1.94. Animals found dead are designated by daggers next to weights.

per milliliter, only a factor of 10 less than the titer of $SFFV_{AP}$-L stocks prepared by helper-virus rescue and assayed under similar conditions.

Although the results in Fig. 2 show no evidence that recombination has occurred between the defective M-MuLV and SFFV in the ψ-2 cells to yield a replicating helper virus, it is possible that a low titer of such a virus may be amplified in the mouse and be responsible for the results obtained. This, however, does not appear to be the case since grossly diseased spleens from mice infected with $SFFV_{AP}$-L(ψ-2) showed no evidence of helper virus–encoded gene products (for example, see Fig. 3C) and gave negative results for reverse transcriptase. Furthermore, cell-free homogenates from these spleens were unable to transfer replicating virus to NIH 3T3 cells or to induce disease when injected into other mice.

These studies show that the envelope gene of SFFV is capable of producing a malignant transformation by conferring a strong proliferative potential on a relatively few progenitor cells. Affected cell populations containing transforming DNA expanded out of control and caused a fulminating disease. Previous work in vitro with helper-deficient virus preparations suggested that SFFV alone can transform hematopoietic cells (*18*), an observation that is extended by our experiments in vivo. It is now clear that the oncogenic potential of SFFV can be realized without the spread of virus and recruitment of cells.

The present studies demonstrate that under conditions in which sufficient target cells are accessible in vivo, a nonreplicating retrovirus vector containing a transforming gene can be introduced into animals by direct inoculation. Previously reported experiments have shown that nontransforming genes, such as the hypoxanthine phosphoribosyltransferase (HPRT) gene and a drug resistance gene (Neo^R), can be efficiently introduced into hematopoietic cells of mice by delivery of the genes first to bone marrow cells in vitro and then engrafting them in vivo (*2, 3*). Williams *et al.* (*3*) have been successful in applying this technique in combination with helper-free retroviral vectors. Their methods of introducing genes into mice, as well as those described in this report, may have application to studies of various types of transforming genes.

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Acid Rain: Statistical Analysis of Ionic Correlations Questioned

In analyzing data on the chemical composition of rainwater, Gorham *et al.* (*1*) claim that hydrogen ions are correlated "much more closely with sulfate than with nitrate" and also that the H^+ correlation with the sum of SO_4^{2-} and NO_3^- is slightly less than that between H^+ and SO_4^{2-} alone. We wish to point out several shortcomings in this analysis and thereby to suggest that less sweeping conclusions are appropriate.

First, for acid-base systems that are weakly buffered, such as rainwater, the relation between (H^+) and (anion) (*2*) is inherently nonlinear unless $(H^+) >>$ (other cations) (*3*). Rainwater often fails to satisfy this inequality, so that linear regression is inappropriate. In dilute systems (SO_4^{2-}) and (NO_3^-) increase in proportion to the amount of each acid added to a fixed volume of water; however, (H^+) may or may not increase in proportion depending on the presence of base (for example, NH_3) with which it can react. Indeed, this nonlinear functional relation of (H^+) and (anion) is well known as the classical titration curve of a weak base titrated by a strong acid. No data are presented in the report by Gorham *et al.* (*1*) which can be tested against this fundamental chemical principle.

Second, it is a fact that both HNO_3 and H_2SO_4, when added to rainwater, cause the *p*H to drop and that a given concentration (in equivalents per liter) of either acid will cause an identical drop in *p*H. The lower correlation of (H^+) with (NO_3^-) and other statistical calculations seem to be used by Gorham *et al.* to suggest that SO_2, as a precursor of H_2SO_4 in the atmosphere, is more likely to produce rainwater acidity than NO_x, the precursor of atmospheric HNO_3. Gorham *et al.* further claim that their "analysis also supports the focus of emission control upon SO_2 . . . ." We suggest that it is prudent to consider both H_2SO_4 and HNO_3 as important contributors of acidity to rainwater. The possibility exists that NO_x sources may be located close to sources of basic materials that might neutralize some of the HNO_3 that is formed. However, if NO_x were not emitted, these basic materials would be available to react with the H_2SO_4 formed from SO_2. Control of sources of HNO_3 would appear to be as important to rainwater *p*H as control of sources of H_2SO_4 on an equivalent-for-equivalent basis.

Third, the value of a correlation coefficient is limited by the individual variance of the variable as well as by the number of observations used. This is especially true when two or more correlations are compared. It is customary to report confidence limits about correlation coefficients so that their reliability is apparent. From the work of Gorham *et al.*, it is impossible to tell how confident one can be in calling $r^2 = 0.85$ statistically significant, let alone in concluding that the difference between an r^2 of 0.85 and 0.83 has any meaning whatever. In other words, if the linear correlations were indeed meaningful, the difference between the H^+/SO_4^{2-} $r^2 = 0.85$ and the H^+/NO_3^- $r^2 = 0.63$ really may not be significant and the difference between the H^+/SO_4^{2-} relation and the $H^+/(SO_4^{2-} + NO_3^-)$ may not even pass a hypothesis test at the 50 percent level. What might