in brain tissue. These results confirmed the presence of viral DNA in affected organs.

These observations clearly demonstrate the importance of this region of the viral genome for the oncogenicity of the virus. The deletion mutants described here have allowed us to identify a region of the *H. saimiri* genome required for expression of the lymphoma-inducing capacity of the virus. This region of the genome is apparently not necessary for replication of the virus; the deletion mutants grow as well as parental virus in cultured OMK and Vero cells. Also, the deletion mutant strains are able to infect New World primates, since virus can be recovered repeatedly after experimental inoculation. However, it is not known at what stage these deletion mutants are defective for oncogenic transformation. Studies of the ability of these *H. saimiri* strains and cloned DNA fragments to function in a variety of transformation systems in vitro may help us to understand the mechanisms underlying the oncogenicity of this virus. Recent results have shown that in permissively infected OMK cells, one minor and two major polyadenylated RNA's are affected by the S4 and 11att deletions (*9*). However, these polyadenylated RNA's were not detected in tumor cell lines or tumor biopsy samples. The only RNA detected in tumor cells from the leftmost 7.4 kbp of L-DNA was a 100-base-pair RNA; this 100-base-pair RNA was not detected in permissively infected cells. The role of these gene products in oncogenic transformation by *H. saimiri* remains to be investigated.

Note added in proof: We have recently developed a highly reproducible assay for in vitro immortalization of New World primate T-lymphocytes with *H. saimiri* strain 11. The S4 and 11att deletion mutants do not immortalize in this assay system.

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11. Supported by PHS grants 31363 (R.C.D.) and 26225 (L.A.F.) awarded by the National Cancer Institute, by a special fellowship from the Leukemia Society of America (R.C.D.) and by grant RR00168 to the New England Regional Primate Research Center from the Division of Research Resources, National Institutes of Health. We thank R. Byington for laboratory assistance.
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1 October 1984; accepted 3 December 1984

Hypomethylation of DNA from Benign and Malignant Human Colon Neoplasms

Abstract. *The methylation state of DNA from human colon tissue displaying neoplastic growth was determined by means of restriction endonuclease analysis. When compared to DNA from adjacent normal tissue, DNA from both benign colon polyps and malignant carcinomas was substantially hypomethylated. With the use of probes for growth hormone, γ-globin, α-chorionic gonadotropin, and γ-crystallin, methylation changes were detected in all 23 neoplastic growths examined. Benign polyps were hypomethylated to a degree similar to that in malignant tissue. These results indicate that hypomethylation is a consistent biochemical characteristic of human colonic tumors and is an alteration in the DNA that precedes malignancy.*

Changes in the genome have long been proposed to play a role in abnormal cell growth (*1*). Genomic abnormalities including rearrangements (*2*), mutations (*3*), amplifications (*4*), deletions (*5*), and alterations in methylation (*6*) have all been reported in neoplastic cells. Some or all of these aberrations may result in abnormal gene expression. Two crucial questions from both a mechanistic and a clinical standpoint are when, during the process of neoplasia, can a particular change in DNA structure first be detected? and how often does this change occur in tumors of a given type? In this report, these questions were addressed with regard to alterations in DNA methylation by comparing DNA from a large number of benign and malignant human colon neoplasms with DNA from adjacent, normal colonic tissue obtained from the same patients.

DNA methylation is a covalent modification of the genome that occurs in many

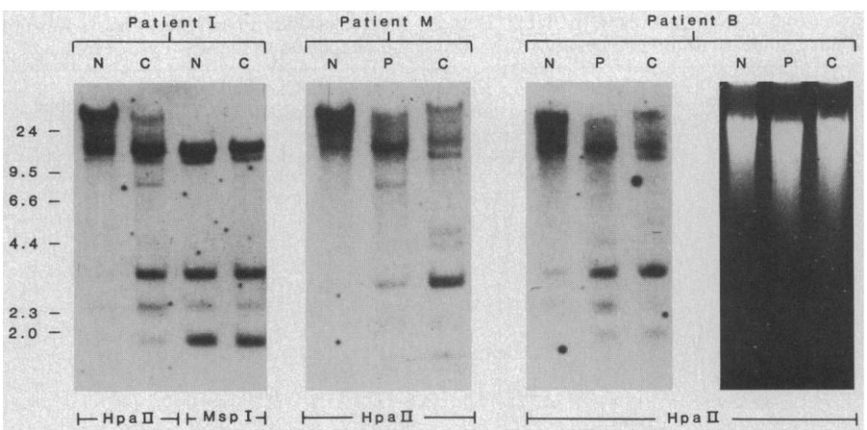

Fig. 1. Analysis of the methylation state of the crystallin gene in normal and in neoplastic colonic tissue. DNA was prepared (*23*) and each sample (5 μg) was digested with Hpa II, Msp I, or Hha I (each at 5 U/μg) at 37°C for 12 to 16 hours. The samples were fractionated by electrophoresis on 1.0 percent agarose gels, stained with ethidium bromide (*24*), and photographed. The gels were then washed (*23*) and the DNA transferred to nitrocellulose essentially as described (*25*). Prehybridizations and hybridizations (12 to 20 hours) were performed (*24*), and the filters were washed as described (*26*) except that the first three washes were for 30 minutes and the fourth and fifth washes were in 0.3× standard saline citrate and 3 percent sodium dodecyl sulfate (for 45 minutes each). Autoradiography was for 16 hours with intensifying screens. Since the Msp I patterns were identical for all 23 neoplasms studied, the Msp I pattern from only one patient is displayed. Molecular weight markers were bacteriophage λ-DNA digested with Hind III. N, normal; P, benign polyp; C, malignant cancer.

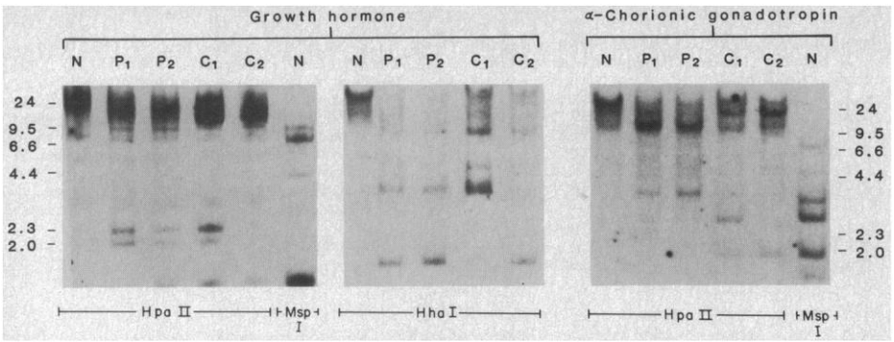

Fig. 2. Analysis of methylation patterns of the growth hormone and α-chorionic gonadotropin genes in four neoplastic growths from patient C. Samples for hybridization with growth hormone and with α-chorionic gonadotropin were separated on 0.9 and 0.8 percent gels, respectively. The marker was λ-DNA cut with Hinf I. N, normal; P, benign polyp; C, malignant cancer.

prokaryotes and eukaryotes. In mammals it occurs almost exclusively at the dinucleotide CG (cytosine, guanine) with about 70 percent of the cytosines being methylated in normal, differentiated cells (*7*). Although the role of DNA methylation in gene regulation is not fully understood, many studies have shown that a decrease in methylation is associated with gene activation (*7*). Because methylation changes occur in the normal course of differentiation, and because malignant cells appear, in some ways, to have an altered developmental program, methylation patterns in neoplastic cells are of considerable interest. Changes in DNA methylation occur in a variety of transformed cells and neoplastic tissues (*6, 8*), including primary human tumors (*9*), but it is not clear whether this phenomenon is an early or a late event in the neoplastic process or whether hypomethylation is an invariable property of tumors of a given type.

To investigate these questions we examined the methylation state of DNA from human neoplastic colonic tissue. Colon was chosen for a number of reasons. First, it is one of the most common forms of human cancer; there were 130,000 new cases of colon cancer in the United States in 1984 (*10*), with a 50 percent mortality (*11*). Second, an excellent control tissue is afforded by the adjacent normal colonic mucosa, the tissue from which the tumors arise. The cell-cycle kinetics of the normal mucosa are similar to those of the tumors (*12*). Third, benign adenomatous colonic polyps represent a premalignant stage of neoplastic growth; not all polyps become malignant but nearly all malignant carcinomas are thought to originate from these polyps (*11*). Finally, the pathology of colonic neoplasia is well established, and normal mucosa, adenomatous polyps, and malignant carcinomas can readily be distinguished.

Two methylcytosine-sensitive restriction endonucleases were used to study DNA methylation. Hpa II and Hha I cleave DNA at their recognition sites (CCGG and GCGC, respectively) only if these sites are not methylated (*13*). However, Hpa II will not cleave DNA that is methylated at the internal C and Hha I will not cleave DNA that is methylated at either C residue within its recognition sequence. With each enzyme, approximately 6 percent of the possible methylation sites (CG dinucleotides) can be examined. The enzyme Msp I provides a control for Hpa II since it cleaves CCGG regardless of the methylation state of the internal cytosine (*14*). No such methylation-insensitive isoschizomer of Hha I has been found.

In a typical experiment, DNA from normal or neoplastic colonic tissue from a given patient was digested with one of the restriction endonucleases described above, separated on agarose gels, transferred to nitrocellulose, and probed with a particular gene. The probes selected for use detected genes that should not be expressed in normal colonic epithelium and thus were likely to be highly methylated in the normal tissue (*7*). Ten genes, located on at least six different chromosomes, were each examined in five randomly selected tumors. The results obtained with these ten probes (*15*) indicated that DNA from neoplastic tissue was hypomethylated and that this hypomethylation was not random. Of the ten genes initially screened, eight showed at least some decrease in methylation in neoplastic tissue: four were usually hypomethylated to a significant degree (γ-crystallin, growth hormone, α-chorionic gonadotropin, and γ-globin), four were occasionally hypomethylated and to a lesser degree (insulin, pro-opiomelanocortin, β-chorionic gonadatropin, and platelet-derived growth factor), and in two no change was seen (α-fetoprotein and parathyroid hor-

Table 1. Quantitation of the extent of hypomethylation displayed by 23 colon neoplasms. The restriction pattern of DNA from neoplasms was compared with DNA from normal tissue from the same patient. The restriction patterns were quantitated as follows: (−), slight or no change between DNA from normal and neoplastic tissue; (+), clear qualitative decrease in the density of the major high molecular weight bands with a concomitant increase in the density of the lower molecular weight bands; (++), most of the DNA in the large bands in the normal tissue was cleaved to smaller fragments. For examples of this scoring compare the autoradiographs of Figs. 1 to 3 with the scores in the table. Data for the γ crystallin probe/Hha I digests was not included since no difference between the normal and neoplastic tissue was ever seen.

Patient	Tissue	γ-Crystallin	Growth hormone		α-Chorionic gonadatropin		γ-Globin	
		Hpa II	Hpa II	Hha I	Hpa II	Hha I	Hpa II	Hha I
A	Polyp	+	+	++	++	−	−	−
	Cancer	++	++	+	++	−	+	+
B	Polyp	++	++	++	++	++	++	−
	Cancer	++	++	++	++	+	−	−
C	Polyp 1	++	+	++	++	+	+	++
	Polyp 2	++	+	++	++	+	+	++
	Cancer 1	+	+	++	++	−	+	+
	Cancer 2	++	+	++	++	+	++	++
D	Cancer	+	+	+	++	+	+	+
E	Cancer	+	+	+	+	+	++	+
F	Polyp	++	+	+	+	+	+	+
G	Polyp 1	++	++	++	++	−	++	++
	Polyp 2	++	++	++	++	−	++	++
	Polyp 3	++	++	++	++	−	++	++
H	Cancer	++	+	+	++	+	++	−
I	Cancer	++	++	++	++	++	++	++
J	Polyp	+	++	++	+	−	+	+
	Cancer	++	+	++	++	−	++	++
K	Cancer	++	++	++	++	++	+	++
L	Cancer	+	++	++	++	−	++	+
M	Polyp	++	+	++	+	−	+	+
	Cancer	++	+	+	++	−	++	+
N	Cancer	++	++	+	++	++	++	+

mone). The four genes frequently hypomethylated in the initial screening were chosen for a more detailed analysis with all 23 neoplasms available.

When the methylation state of DNA from normal tissue, polyps, and cancers was examined with the ^{32}P-labeled γ-crystallin probe, the resulting autoradiographs showed that DNA from both the benign polyps and the malignant tumors was substantially hypomethylated when compared to DNA from normal tissue from the same patients (Fig. 1). The crystallin genes appeared to be highly methylated in normal colon tissue. However, in the DNA from the various neoplasms, the high molecular weight fragments in the normal tissues were in large part replaced by several fragments of lower molecular weight. In fact, the DNA from some of the neoplastic samples was almost completely unmethylated at all Hpa II sites [that is, showed a pattern similar to that seen with Msp I (Fig. 1, patient I)]. Changes similar to those noted in Fig. 1 were seen in every neoplasm studied, although the degree of hypomethylation varied from tumor to tumor. As shown in Table 1, DNA from benign polyps was approximately as hypomethylated as DNA from the malignant tissues. Moreover, neither the extent nor the pattern of methylation seemed to be dependent on the histologic grade or on the stage of invasion of the neoplasms. Thus, it appears that alterations in the methylation state of colonic tumor DNA are not a result of malignancy, but, in contrast, precede it.

Measures of total genomic methylation may not accurately reflect alterations in methylation affecting protein coding genes. In patient B there were marked changes in the crystallin, growth hormone, and α-chorionic gonadotropin genes (Fig. 1 and Table 1) whereas the ethidium bromide–stained Hpa II digest of DNA (a measure of DNA methylation at all CCGG sites) from this patient showed a definite but much less marked decrease in methylation (Fig. 1). By means of ethidium bromide staining, such decreases in total methylation could be detected in 22 of the 23 neoplasms examined, although the differences were often slight (for example, see Fig. 1).

An explanation for the discrepancy between the small methylation changes seen when total DNA was examined and the sometimes striking differences seen when individual genes were examined lies in the observation that the hypomethylation was selective. As already noted, four out of the ten genes initially examined were usually hypomethylated while the other six genes were affected either occasionally or not at all. Moreover, when repetitive sequences were examined with a probe to satellite DNA, the methylation patterns of DNA from neoplastic and normal tissue were not significantly different (*16*). Since repetitive sequences account for a disproportionately high amount of the methylated C residues in mammalian DNA (*7*), an assay for changes in specific genes (for example, Southern blots) may be a more revealing method for determining methylation changes in tumor tissue than an assay of total methylcytosine levels.

Despite the fact that all 23 neoplasms showed significant degrees of hypomethylation, there was substantial heterogeneity among the restriction patterns obtained, even in different tumors from the same patient. Figure 2 shows the methylation patterns of two genes in DNA from patient C from whom two polyps and two malignancies were obtained. The DNA was cleaved with Hpa II, Msp I, or Hha I and was hybridized with the probe for growth hormone or for α-chorionic gonadotropin. In all four neoplasms from this patient, both genes appeared to be hypomethylated when compared to the normal tissue. However, the changes in the four neoplasms were far from identical. For example, in the region of the growth hormone gene, the DNA from polyp 1 had a slightly different pattern from that of polyp 2 when digested with Hpa II, but the same pattern as polyp 2 when digested with Hha I. The pattern for cancer 1 differed from cancer 2 in both the Hpa II and the Hha I digests. In the α-chorionic gonadotropin gene region, although both polyps had a similar Hpa II pattern, cancer 1 differed markedly and cancer 2 differed both from the polyps and from cancer 1 (Fig. 2).

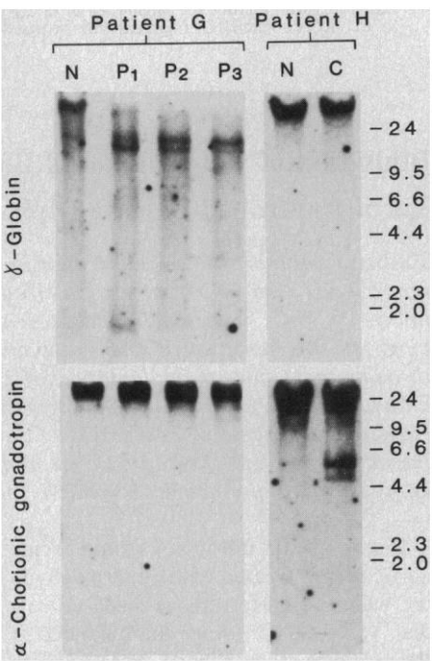

Fig. 3. Comparison between two patients of Hha I methylation patterns of the γ-globin and the α-chorionic gonadotropin genes. Samples for hybridization with γ-globin and with α-chorionic gonadotropin were separated on 0.7 and 0.8 percent gels, respectively. N, normal; P, benign polyp; C, malignant cancer.

There was also heterogeneity of DNA hypomethylation from patient to patient (Fig. 3). The γ-globin gene appeared to be substantially hypomethylated in all three polyps from patient G. In contrast, the α-chorionic gonadotropin gene showed little difference between the normal and neoplastic tissues. The opposite was true for patient H: the α-chorionic gonadotropin gene was clearly hypomethylated in the tumor compared to the normal tissue while the γ-globin gene was not affected (Fig. 3). Therefore, in addition to hypomethylation being an early event in neoplasia, substantial heterogeneity in the methylation patterns is apparently generated as a result of this event. The heterogeneity in methylation patterns reported in this study has obvious parallels to the heterogeneous biological properties often noted among tumors of the same type (*17*).

Patients G and E were of particular interest in that they had Gardner's syndrome. This disorder is characterized by the development of numerous benign colonic polyps, some of which eventually become malignant (*18*). In these patients even the smallest (2 mm) polyps showed substantial hypomethylation, whereas DNA from the "normal" colonic mucosa of these patients, which was quite hyperplastic, showed no significant hypomethylation compared to normal colonic mucosa from other patients (Table 1 and Fig. 3). These data suggest that methylation changes may appear specifically between the stages of hyperplasia and benign neoplasia.

In conclusion, these results demonstrate that DNA from both benign and malignant colonic neoplasms is hypomethylated and, while clearly not random, is heterogeneous where it occurs. That the methylation changes occur prior to malignancy suggests that an alteration in the DNA methylation pathway could be a key event in the initiation of neoplasia. Hypomethylation might influence tumor development in at least two ways. First, the hypomethylation of some genes might be enough to predispose them to expression. Consistent with this possibility is the fact that agents

that inhibit DNA methylation, such as 5-azacytidine, result in the hypomethylation and expression of some genes but not others (*19*). If hypomethylation leads to expression of genes important in neoplastic growth, then cells exhibiting a defect in the control of methylation may obtain a selective advantage (*20*). Second, hypomethylation might inhibit chromosome condensation which, in turn, might lead to problems in chromosome pairing and disjunction. Indeed, experimentally induced hypomethylation leads to areas of chromosome decondensation with resultant mitotic chromosomal abnormalities (*21*). In support of such a mechanism for tumorigenesis, it has been shown that 5-azacytidine induces transformation of CHEF/18 cells at high frequency. In these cells, the transformation event is in every case associated with DNA hypomethylation and a specific chromosomal translocation (*22*).

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15. The following gene fragment probes were used: (i) a 3.4-kb Hind III genomic fragment derived from the plasmid p5G1 containing human γ-crystallin gene sequences (S. Meakin *et al.*, in preparation); this probe hybridizes with several members of the crystallin gene family; (ii) a 0.8-kb Hind III complementary DNA (cDNA) fragment derived from the plasmid chGH800/pBR322 containing human growth hormone sequence [J. A. Martial, R. A. Hallewell, J. D. Baxter, H. M. Goodman, *Science* **205**, 602 (1979)]; (iii) a 0.6-kb Hind III cDNA fragment derived from the plasmid α-HCG/pBR322 containing the coding region for human α-chorionic gonadotropin [J. C. Fiddes, H. M. Goodman, *Nature (London)* **281**, 351 (1979)]; (iv) a 1.1-kb TaqI cDNA fragment derived from the plasmid JW151 containing human γ-globin sequences [J. T. Wilson *et al.*, *Nucleic Acids Res.* **5**, 562 (1978)]; (v) a 1.2-kb Hinf I genomic DNA fragment 5′ of the human insulin gene [P. S. Rotwein *et al.*, *N. Engl. J. Med.* **308**, 65 (1983)]; (vi) a 2.7-kb Eco RI genomic fragment derived from the plasmid pACYC401 containing human pro-opiomelanocortin sequences [A. C. Y.-C. Chang, M. Cochet, S. N. Cohen, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4890 (1980)]; (vii) a 0.6-kb Hind III cDNA fragment derived from the plasmid pPE-B containing human β-chorionic gonadotropin gene sequences [J. C. Fiddes and H. M. Goodman, *Nature (London)* **286**, 684 (1980)]; (viii) a 6-kb Eco RI v-*sis* DNA fragment derived from the plasmid SSV11C11/pBR322 containing sequences homologous to platelet-derived growth factor [K. C. Robbins, S. G. Devare, S. A. Aaronson, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2918 (1981)]; (ix) a 0.8-kb Pst I cDNA fragment derived from the plasmid pHAF2 containing the 3′ end of the human α-fetoprotein gene [T. Morinaga, M. Sakai, T. G. Wegmann, T. Tamaoki, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4604 (1983)]; and (x) a 0.8-kb Hpa II cDNA fragment derived from the plasmid pPTHm122 containing human parathyroid hormone sequences [G. N. Hendy, H. M. Kronenberg, J. T. Potts, A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7365 (1981)]. All DNA fragment probes were prepared by oligo-labeling [see (*24*)].
16. Repetitive DNA sequences were examined in 22 neoplasms with a probe for satellite DNA (a 0.3-kb Eco RI genomic DNA fragment derived from the plasmid pB(Eco RI-2°)6 [B. Shafit-Zagardo, J. J. Maio, F. L. Brown, *Nucleic Acids Res.* **10**, 3175 (1982)].
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23. Colonic tissue was frozen in liquid nitrogen and stored at −80°C until use. Approximately 50 mg of tissue was added to 10 ml of 20 m*M* EDTA containing 10 m*M* NaCl and 0.5*M* tris-HCl, *p*H 9.0, to which was added 1 ml of 10 percent sodium dodecyl sulfate containing proteinase K (4 mg/ml) warmed to 45°C. The sample was vortexed intermittently, and incubated at 45°C for 10 to 16 hours. It was then forced through an 18-gauge needle three times, extracted three to four times with phenol and chloroform, once with chloroform, and precipitated with ethanol. DNA concentrations were determined by the method of Burton [K. Burton, *Biochem J.* **62**, 315 (1956)]. Gels were washed for 30 minutes in 0.1 percent HCl, 30 minutes in 0.5*M* NaOH and 1.0*M* NaCl, and then 45 minutes in 3*M* NaCl with 1*M* tris-HCl, *p*H 7.5 before transfer.
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27. We thank the following investigators for probes: M. Breitman and L.-c. Tsui for the γ-crystallin gene prior to publication; H. Kazazian and C. Boehm for γ-globin; P. Rotwein for insulin; H. Kronenberg for parathyroid hormone; P. Seeburg for human growth hormone; H. Goodman for α-chorionic gonadotropin; J. Fiddes for β-chorionic gonadotropin; A. C. Y.-C. Chang for pro-opiomelanocortin; K. C. Robbins for platelet-derived growth factor; J. Maio for satellite DNA; and T. Tamaoki for α-fetoprotein. This work was supported by the Hartford Foundation (A.P.F.), the Clayton Fund, training grant CA09071 (S.E.G.), and grant CA35494 from the National Institutes of Health, Department of Health and Human Services.

23 November 1984; accepted 14 January 1985

Inhibition of Calcification of Bioprosthetic Heart Valves by Local Controlled-Release Diphosphonate

Abstract. *Bioprostheses fabricated from porcine aortic valves are widely used to replace diseased heart valves. Calcification is the principal cause of the clinical failure of these devices. In the present study, inhibition of the calcification of bioprosthetic heart valve cusps implanted subcutaneously in rats was achieved through the adjacent implantation of controlled-release matrices containing the anticalcification agent ethanehydroxydiphosphonate dispersed in a copolymer of ethylene-vinyl acetate. Prevention of calcification was virtually complete, without the adverse effects of retarded bone and somatic growth that accompany systemic administration of ethanehydroxydiphosphonate.*

Bioprostheses fabricated from porcine aortic valves treated with glutaraldehyde are widely used to replace diseased cardiac valves (*1–3*). More than 300,000 of these bioprostheses have been used in clinical implants since 1971 (*4–7*). Cuspal calcification is the most frequent cause of the clinical failure of these devices (*4–6*) and necessitates removal of the prostheses after 5 years in more than 50 percent of the children (*5*) and 5 to 10 percent of the adults receiving the valve implants (*2, 6, 7*). The pathogenesis of cuspal calcification is not completely understood (*7*), and there is no effective therapy for its prevention (*7*). Its pathophysiology can be reproduced with orthotopic valve replacements in sheep (*8*) or calves (*9*), or with subcutaneous cuspal implants in rabbits (*10*), mice (*11*), or rats (*12*). Subcutaneous implants are used because the time course of the mineral-