Fig. 2 have each been explicitly associated with the formation of cyclones in both hemispheres (*26*).

Identification of westerly wind bursts in the central equatorial Pacific before El Niño events of the past does not improve our capability to predict future El Niño events, beyond what can already be accomplished from monitoring the southern oscillation (*27, 28*). The strong zonal wind bursts near the date line are neither necessary (for example, there were no bursts before the 1963 El Niño, and very weak bursts occurred before the 1976 El Niño) nor sufficient (for example, strong bursts in 1974 and 1977 were not followed by significant El Niño events) for the existence of El Niño. Other factors, beyond the fluctuations of equatorial winds near the date line, must be influencing the onset and development of El Niño.

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Fluoride Directly Stimulates Proliferation and Alkaline Phosphatase Activity of Bone-Forming Cells

Abstract. *Fluoride is one of the most potent but least well understood stimulators of bone formation in vivo. Bone formation was shown to arise from direct effects on bone cells. Treatment with sodium fluoride increased proliferation and alkaline phosphatase activity of bone cells in vitro and increased bone formation in embryonic calvaria at concentrations that stimulate bone formation in vivo.*

Fluoride is essential in the diet and is thought to be required for normal dental and skeletal growth (*1*). The recommended allowance of fluoride is 1 to 4 mg per day. Doses of 20 to 100 mg per day, or more, usually cause abnormal increases in skeletal mass, even to the point of sclerosis (*2*). This may represent an exaggerated physiological response or a new and unrelated action, but because the effect of excess fluoride is selective for bone, it has therapeutic applications. Clinical studies have shown that NaF is the most potent agent for increasing bone volume in patients with osteoporosis (*3, 4*). Although the mechanism is unknown, the skeletal response to supplemental NaF is characterized by increases in (i) the rate of bone formation (*4, 5*); (ii) the number of osteoblasts, or bone-forming cells (*4, 6*); and (iii) the serum activity of skeletal alkaline phosphatase (ALP) (*7–9*), an osteoblastic isoenzyme.

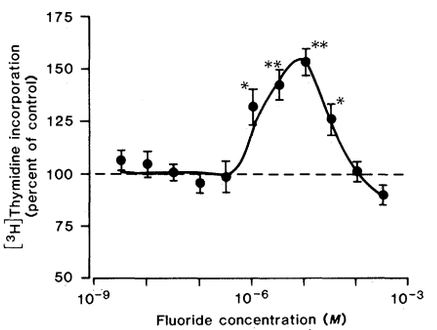

Fig. 1. Dose-response curve for fluoride. Calvarial cell proliferation estimated as [^{3}H]thymidine incorporation, shown as percent of control (no fluoride), versus NaF concentration. Values represent the mean ± S.E.M. of six replicates. Dashed line indicates the mean of the control values. A single asterisk indicates a significant difference from controls at $P < 0.05$; a double asterisk indicates $P < 0.005$.

To discover the mechanism whereby fluoride stimulates bone formation, we sought (i) to determine whether any of the characteristic skeletal responses to NaF could be attributed to direct effects on cells in the osteoblast line and (ii) to examine the interactions between NaF and two other bone cell mitogens—parathyroid hormone (PTH) and human skeletal growth factor (hSGF) (*10–13*). To these ends we have examined the effects of NaF on embryonic chick bone cells in vitro and on embryonic chick bone in organ culture (*14, 15*).

Bone cells for these studies were prepared from the calvaria of 15-day embryonic chicks by sequential collagenase digestion and were cultured in serum-free Fitton-Jackson modified BGJ_b medium (*16*). Histological analysis of the calvaria before digestion has shown that essentially all of the cells available for release were members of the osteoblast cell line. Even in monolayer culture these cells maintained the following characteristics of osteoblasts and osteoblast progenitors: expression of a skeletal-type ALP activity; response to PTH with an increased adenosine 3′,5′-monophosphate (cyclic AMP) production (*11*); conversion of 25-hydroxyvitamin D_3 to more polar metabolites (*17*); and response to a bone-derived mitogen (hSGF) that is specific for skeletal tissues (*12, 13*).

To estimate cell proliferation we incubated the calvarial cells overnight and then exposed them to effectors (such as NaF and hSGF) for an additional 18 to 24 hours [this corresponds to the cell cycle

time and allows for an optimal response to hSGF (*13*).] Cell proliferation was assessed by the incorporation of [^{3}H]thymidine into DNA during the final 2 hours of the incubation (*16*). [^{3}H]Thymidine incorporation was increased by exposure to NaF (Fig. 1). The pool size of [^{3}H]-thymidine was not affected by fluoride (*18, 19*). Cell number was increased to 162 ± 7 percent of control values after 36 hours of exposure to 10 μ*M* NaF ($P < 0.005$) (*20*). The increase in cell number, which was also observed after exposure to 2.5 μ*M* and 25 μ*M* NaF (data not shown), and the absence of effect of NaF on the pool size of [^{3}H]thymidine strongly suggest that the observed increase in [^{3}H]thymidine incorporation reflects, at least in part, an increased rate of cell proliferation. The effect of NaF on [^{3}H]thymidine incorporation was confined to a narrow concentration range (2 to 50 μ*M*), which corresponds closely to the serum levels of NaF recommended for increased bone formation in vivo (*21*). Half-maximal stimulation of [^{3}H]thymidine incorporation was seen with 3 to 4 μ*M* NaF. The mitogenic effect was specific for bone cells. Incorporation of [^{3}H]thymidine was not affected by NaF in identically treated cultures of embryonic chick skin, muscle, kidney, liver, or intestinal cells (*22*). The effect of NaF on calvarial cells, which was not observed with other halogens (NaBr or NaI), was apparently inductive. Cells exposed to 2.5 μ*M* NaF for the first 4 hours of an 18-hour incubation (they were rinsed twice and changed to NaF-free medium after a 4-hour exposure) showed an increase in [^{3}H]thymidine incorporation after 18 hours [162 ± 9.5 percent of control ($P < 0.005$)].

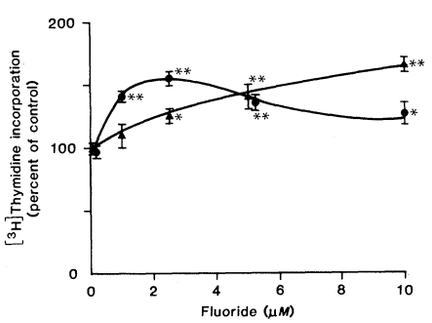

Fig. 2. Interaction of fluoride and PTH. Calvarial cell proliferation ([^{3}H]thymidine incorporation), shown as percent of control (no fluoride, no PTH), versus NaF concentration for cells incubated with (●) or without (▲) 0.1 n*M* PTH. Values represent the mean ± S.E.M. of six replicates. A single asterisk indicates a significant difference from controls at $P < 0.05$; a double asterisk indicates $P < 0.005$. Values for 1, 2.5, and 10 μ*M* NaF with added PTH are significantly different from values without added PTH ($P < 0.05$).

Table 1. Effects of NaF in vitro. Effects are shown as mean percent of control ± S.E.M., with $N = 6$ for calvarial cells and $N = 9$ to 16 for calvaria. Absolute values (mean ± S.E.M.) for control activities were as follows. Cells: [^{3}H]thymidine incorporation, 1223 ± 44 count/min per well; cell number, 7064 ± 351 per well; ALP activity, 0.107 ± 0.005 U per milligram of protein. Bones: ^{45}Ca deposition, 93,322 ± 5238 count/min per milligram of dry weight; [^{3}H]hydroxyproline incorporation, 1240 ± 103 count/min per milligram of dry weight. References for methodologies are given in the text.

Parameter tested	NaF		Effect (percent of control)	*P*
	Concentration (μ*M*)	Exposure (hours)		
	Cells			
[^{3}H]Thymidine incorporation	10	18	163 ± 14	< 0.005
Cell number	10	36	162 ± 17	< 0.005
ALP activity	10	144	435 ± 33	< 0.001
	Bones			
[^{3}H]Thymidine incorporation	25	72	146 ± 10	< 0.01
ALP activity	2.5	144	156 ± 14	< 0.01
^{45}Ca deposition	2.5	144	131 ± 6	< 0.002
[^{3}H]Hydroxyproline incorporation	2.5	144	152 ± 14	< 0.01

Exposure to NaF during the final 4 hours of the 18-hour incubation had no such effect. These results indicate that NaF can have direct effects on cells in the osteoblast line.

Exposure to NaF also increased ALP activity in the cultured calvarial cells and in the surrounding serum-free medium [see (*23*) for assay methodology]. After 144 hours of exposure to 10 μ*M* NaF, ALP activity in the medium was increased to 262 ± 11 percent of control ($P < 0.001$) and the ALP activity in the cells was increased to 435 ± 33 percent of control ($P < 0.005$). (All ALP values are adjusted for variation in protein concentration.) Since ALP activity is more abundant in populations of differentiated osteoblasts than in populations of osteoblast precursors, our results suggest that NaF stimulates both the proliferation and the differentiation of embryonic chick cells in the osteoblast cell line, and this occurs at doses that stimulate bone formation in vivo. We have not yet determined whether the number of ALP-positive cells or the amount of ALP per positive cell is increased by exposure to NaF.

The NaF-induced increase in [^{3}H]thymidine incorporation in calvarial cells in vitro was enhanced by coincubation with PTH or with partially purified hSGF. With an 18-hour incubation, 0.1 n*M* PTH did not affect [^{3}H]thymidine incorporation, but it did increase the stimulation seen with 2 μ*M* NaF, from 125 to 155 percent of control ($P < 0.01$ for the difference). A NaF dose-response curve obtained in the presence of 0.1 n*M* PTH (Fig. 2) confirmed an increased response at lower concentrations of NaF, but showed no increase in the maximum response (that is, the concentration of NaF required for half-maximal stimulation was decreased, but the maximum effect was unchanged). The fact that PTH also reduced the response to 10 μ*M* NaF suggests that PTH may amplify the activity of NaF, effectively shifting the biphasic dose-response curve to lower NaF concentration (compare Fig. 1 with the curve in Fig. 2 for NaF plus PTH.) There was also a positive interaction of hSGF with NaF. The maximal response of the calvarial cells to NaF was increased by hSGF (Fig. 3). The concentration of NaF required for half-maximal stimulation was not affected by hSGF. These data show that the mechanisms of NaF- and hSGF-stimulated cell proliferation do not share a common rate-determining step. Our results will not

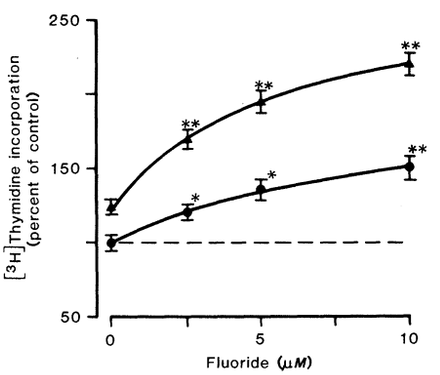

Fig. 3. Interaction of fluoride and hSGF. Calvarial cell proliferation ([^{3}H]thymidine incorporation), shown as percent of control (no fluoride, no hSGF), versus fluoride concentration for cells with (▲, upper curve) or without (●) added hSGF (2 μg/ml). (Bovine serum albumin has no effect at concentrations less than 20 μg/ml.) Values represent the mean ± S.E.M. of six replicates. Dashed line indicates the mean of the control values. A single asterisk indicates a significant difference from controls without fluoride at $P < 0.05$; a double asterisk indicates $P < 0.005$.

permit a similar distinction between the mechanism of NaF- and PTH-stimulated cell proliferation, since the effects of these agents were not additive.

To ensure that these apparently direct effects of NaF on embryonic chick calvarial cells were not peculiar to monolayer culture, we examined the responses of embryonic chick bone to NaF in an organ culture system. Half-calvaria (frontal and parietal bones) were dissected from 13-day embryonic chicks and cultured in serum-free BGJ_b medium as described (*24*). After overnight incubation the medium was changed and NaF was added. After an additional 18 hours, either [^{3}H]proline or ^{45}Ca was also added. The medium was changed at 36- to 48-hour intervals, but the exposure to NaF and the radioisotopes was continuous. After incubation, the bones were individually extracted with butanol to release membrane-bound ALP activity (*23*), and the ALP activity and protein concentration were measured in each extract. The extracted bones were dried, weighed, and hydrolyzed in acid (*24*), and radioactivity was determined in portions of the hydrolyzates. For bone labeled with [^{3}H]proline, [^{3}H]hydroxyproline (in counts per minute per milligram of dry weight) was determined as an index of collagen synthesis (*24*). Collagen production, Ca deposition, and ALP activity were all increased in the calvaria by exposure to NaF (Table 1). These results were consistent with our earlier results with calvarial cells. Additional studies revealed that the incorporation of [^{3}H]thymidine into DNA (*25*) was also increased in half-calvaria cultured for 72 hours in the presence of 25 μM NaF (146 ± 13 percent of control; $P < 0.01$); this is consistent with the interpretation that the increased bone formation was mediated, at least in part, by an increase in osteoblast number.

The effect of NaF on the ALP content of the bones was dose-dependent and saturable. Exposure to 10 μM NaF for 72 hours increased ALP activity to a maximum of 215 percent of control ($P < 0.001$), and half of this maximal effect was seen with 2 to 3 μM NaF. This dose of fluoride agrees with that required from half-maximal stimulation of calvarial cell proliferation (3 to 4 μM). Because, in other systems, most of the biochemical effects of NaF require millimolar concentrations (*26*), bone cells would appear to be unusually sensitive to this agent. Furthermore, our observations on cell specificity suggest that NaF is affecting activities that are unique to bone cells or are particularly sensitive in bone cells (*27*).

In summary, we have shown for the first time in vitro that NaF can (i) increase the proliferation rate of bone cells, as assessed by [^{3}H]thymidine incorporation, both in monolayer culture and in organ culture; (ii) increase the ALP content of bone cells and of embryonic bone; and (iii) enhance the growth and mineralization of embryonic bone. Furthermore, we have shown that the effects of NaF on [^{3}H]thymidine incorporation can be modulated by PTH and by hSGF. Since NaF increases osteoblast number and ALP activity in vivo, these data suggest that the increased bone formation seen with NaF results, at least in part, from direct effects on bone cells in the osteoblast cell line.

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25. Half-calvaria from 13-day embryos ($N = 16$) were exposed to 25 μM NaF for 90 hours, along with untreated controls. [^{3}H]Thymidine (0.2 μCi/ml) was present between 18 and 90 hours. The bones were demineralized in TCA, and insoluble [^{3}H]thymidine (that is, DNA) was washed and counts were determined.
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27. In preliminary studies we determined that NaF does not stimulate bone formation by directly inhibiting ALP activity or by affecting cyclic AMP production. Although NaF can clearly affect the level of ALP activity in bone, and although it can inhibit ALP activity (inhibition constant >0.5 mM), it does not inhibit ALP activity, either in extracts or in situ, at the micromolar concentrations that stimulate bone formation [see (*19*) for assay methodology]. Similarly, although NaF, at millimolar concentrations, can increase cyclic AMP production (*24*), and although bone cells have hormone-specific adenylate cyclase systems, NaF did not affect cyclic AMP production in our assay system at doses that increased bone formation. NaF was incubated with confluent cultures of calvarial cells at doses ranging from 1 μM to 1 mM for 15 minutes at 37°C, and cyclic AMP production was determined by a radioreceptor assay (*28*). PTH was included as a positive control.
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