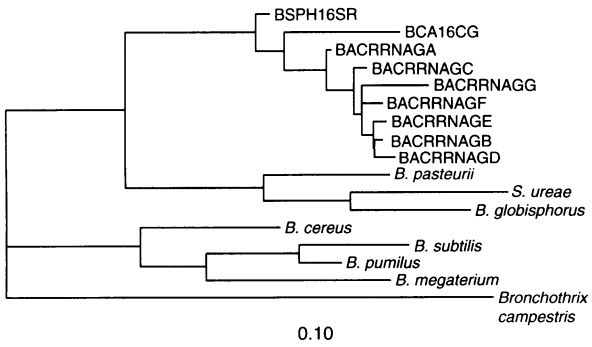

Fig. 1. Phylogenetic tree for *B. sphaericus*. GenBank accession numbers for *B. sphaericus* are as follows: BACRRNAGF (L14015); BACRRNAGD (L14013); BACRRNAGE (L14014); BACRRNAGB (L14011); BACRRNAGC (L14012); BACRRNAGA (L14010); BACRRNAGG (L14016); BSPH16SR (X60639); *S. ureae* (L38654); *B. pasteurii* (X60631); *B. globisporus* (X60644); *B. cereus* (X55063); *B. subtilis* (X60646); *B. pumilus* (X60637); *B. megaterium* (X60629); and BCA16CG, the putatively ancient *B. sphaericus* isolate, BCA16 (L38654). Sequences were aligned manually with the use of Genetic Data Environment (GDE 2.1) text editor. Trees were constructed by the maximum likelihood method with the use of the DNAML program of PHYLIP 3.5 (*4*) and a least squares algorithm for fitting additive trees to proximity data (*5*). *Bronchothrix campestris* (X56156) was used as the outgroup, with 2000 bootstrap replications, randomized data input, and global rearrangement of data. Six independent runs were evaluated. All resulting trees were identical. Branch lengths were drawn to scale, with the use of branch lengths obtained from maximum likelihood analysis and TreeTool (*6*).

cabinet in which the amber is processed, then from the laboratory at large, then from the building, and finally from the grounds surrounding the building. We have processed more than 80 amber specimens, both before and after the recovery of BCA16. During those recoveries we have sampled the sterilized amber itself before processing as well as representative sites of the safety cabinet, the laboratory, and the building itself (including the filters on air ducts). We have cataloged potential contaminants on the surface of Dominican and Mexican ambers; these include coliforms, diphtheroids, pseudomonads, endospore-forming rods, and other Gram-positive bacteria, but not *B. sphaericus*. Similarly, we have not recovered *B. sphaericus* from any of the sites tested to date. On the basis of these results, the only plausible source of BCA16, we have concluded, is from within the amber inclusion (*Proplebeia dominicana*) itself. We continue to sample the laboratory environment for *B. sphaericus*.

We and other colleagues have noted the sequence discrepancies pointed out by Beckenbach. The sequence published in figure 2 of our report is the correct sequence. We have updated our initial GenBank submission (GenBank accession number L38654) to reflect the actual sequence data, which now includes 1482 bases of the 16S rRNA gene of BCA16 for further scientific scrutiny. The discrepancies noted by Beckenbach between the sequences in figures 2 and 3 in our report, although disturbing, do not affect, in essence, the secondary structure of the rRNA molecule illustrated in figure 3 of our report. This is supported by the reconstruction of the secondary structure of the entire 16S rRNA molecule obtained from BCA16.

Beckenbach suggests that a better test of antiquity would be to compare the putatively ancient strain (BCA16) with the modern strains growing in our laboratory, presumably to eliminate the possibility that BCA16 was a modern contaminant from our laboratory. We had no strains of *B. sphaericus* growing in our laboratory before the recovery of BCA16 (*B. sphaericus*). It was not until much after we recovered and identified BCA16 as *B. sphaericus* that we endeavored to isolate *B. sphaericus* from bees (NM13, PJ23, and PJ18 in table 1 in our report) or obtain them from the American Type Culture Collection (ATCC 13805 and ATCC 17932). Even then, the cultivation and characterization of all six *B. sphaericus* isolates were conducted in another laboratory (in another floor of the building), eliminating the likelihood that one of them could serve as a source of contamination from which BCA16 could originate. We performed DNA sequence comparisons with sequences published in nucleic acid databases, primarily because we wished to introduce no extant DNA into our laboratory.

Our conclusion that BCA16 was an ancient isolate is not based solely on sequence comparisons, but on several lines of evidence that include phenetic and phylogenetic analysis. Furthermore, a compelling case can be made by comparing the putatively ancient *B. sphaericus* isolate (BCA16) with the sequence of the amplicon PD_Ex6 (GenBank accession number L38655) obtained from the abdominal tissue of bees in the same amber specimen. These sequences were essentially identical (figures 1 and 2 of our report), which suggests that the BCA16 DNA (or that of a similar organism) was present in the amber inclusion before the organism was cultivated in the laboratory.

Although further tests must be done, when all the evidence gathered thus far are evaluated and weighed, they appear to support our claim that BCA16 is indeed ancient.

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The Effect of Sodium Salicylate and Aspirin on NF-κB

Elizabeth Kopp and Sankar Ghosh find that activation of the transcription factor nuclear factor–κB (NF-κB) is inhibited by aspirin and salicylate, which suggests an explanation for the anti-inflammatory nature of these drugs (*1*). Because the conclusion has significant implications for the development of novel anti-inflammatory agents, we explored the phenomenon further. We found that at concentrations required for inhibition of NF-κB–dependent transcription, sodium salicylate inhibits activation of a variety of transcription factors. This appears to result from the ability of salicylate to nonspecifically inhibit cellular kinases.

Consistent with the previous report (*1*), we found that salicylate inhibited phorbol 12-myristate 13-acetate (PMA)/ionomycin-dependent induction of NF-κB DNA binding activity (not shown) and similarly induced transcription from an NF-κB–dependent enhancer (Fig. 1A). To ascertain the nature of this event, we examined the specificity of salicylate. Salicylate inhibited transcription from an AP-1–dependent enhancer induced by PMA/ionomycin (Fig. 1B). This effect is not secondary to the inhibition of NF-κB, as in these cells the immunosuppressive drug FK-506 also inhibits its induction of NF-κB–dependent promoter activity by PMA/ionomycin, but has no inhibitory effect on induction of AP-1–dependent activity (*2*). As activation of NF-κB and AP-1 share the same stimuli,

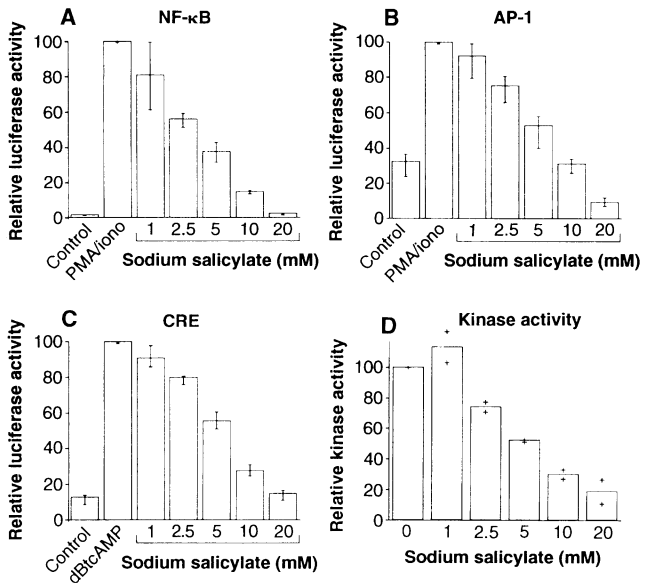

Fig. 1. Effects of salicylate on promoter activity and cellular kinase activity. Jurkat cells were transfected (5) with plasmids containing a luciferase gene (Promega) transcribed from artificial promoters dependent on six NF-κB binding sites (**A**), three AP-1 binding sites (**B**) (2), or three CRE binding sites (**C**). After 24 hours the cells were left untreated [Control, (A) to (C)], treated with 25 ng/ml PMA and 3.5 μg/ml ionomycin and 0 to 20 mM salicylate [(A) and (B)], or treated with 1 mM dibutyryl-cAMP (Sigma, St. Louis, Missouri) and 0 to 20 mM salicylate (C). After 3 hours the cells were harvested and assayed for luciferase activity as suggested by Promega (Madison, Wisconsin). Each bar indicates the average and standard deviation of assays from three independent transfections, normalized for protein concentration (D). Jurkat cells (5×10^7) were harvested at 5×10^5/ml, washed with cold PBS, lysed by 1× freeze-thaw in 100 μl of a buffer containing 25 mM Hepes (pH 7.7), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% NP-40, 0.5 mM dithiothreitol (DTT), 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg/ml leupeptin, and 1 mM PMSF. The kinase activity in 5 μl of lysate was assayed in 25 μl buffer containing 20 mM Hepes (pH 7.7), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 20 mM PNPP, 2 mM DTT, 20 μM ATP, 3.75 μCi [γ^{33}P]ATP (2000 Ci/mmol), and 0 to 20 mM salicylate. After 15 min at 30°C, the reaction was centrifuged through a Pierce (Rockford, Illinois) SpinZyme Basic Separation Unit according to the manufacturers instructions and phosphorylated material was quantitated by liquid scintillation counting. Each bar indicates the average of two assays; individual data values are indicated (+) in (D).

they might also share a common activation pathway that might contain a component uniquely sensitive to salicylate. We therefore examined the effect of salicylate on an independent signal transduction pathway, activation of a cAMP (adenosine 3′,5′-monophosphate) Responsive Element (CRE) (3).

Salicylate was as effective at inhibiting transcription from a CRE-dependent enhancer induced by dibutyryl-cAMP as it was from the other promoters tested (Fig. 1C). To our knowledge there is no specific component shared by the PMA/ionomycin- and cAMP-dependent signal transduction pathways. Salicylate affects neither transcription itself nor the translation or activity of the reporter enzyme (1). Thus, our observations suggested some general effect of salicylate on the activity of independent signal transduction pathways. These pathways are activated by numerous kinases that might all be targets of salicylate. To assess whether salicylate nonspecifically affects cellular kinases, we measured bulk transfer of phosphate from [γ^{33}P]ATP (adenosine triphospate) to basic material in lysates of nonstimulated, proliferating Jurkat cells. Salicylate inhibited 80% of all kinase activity detectable in these lysates; one half of its effective dose (ED_{50}) in this assay (2.5 to 5 mM) is identical to its ED_{50} on activation of DNA binding and induction of transcription (Fig. 1D).

From these results we conclude that the report by Kopp and Ghosh vastly underestimates the effects of high concentrations of salicylate on cellular physiology. The data they presented, while unquestionably accurate, do not support the contention that activation of NF-κB plays a unique role in inflammation or is a unique target of high- dose salicylate. Inhibition of NF-κB activation is not a unique effect of this anti-inflammatory agent, since other cellular events are equally sensitive to its action. Rather, salicylate given in high doses appears to exhibit nonspecific pharmacological effects on cellular kinases. Salicylate concentrations of 1 to 2 mM in serum are required to achieve an anti-inflammatory effect, and concentrations of 6.5 mM are extremely toxic (4). Because salicylate concentrations that cause nonspecific inhibition of kinase activity in vitro are close to concentrations reported to be required for both clinical effect and broad toxicity, we believe that the lack of specificity may explain both the clinical effectiveness and the toxicity of salicylate.

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Response: Frantz and O'Neill raise a concern that salicylates are not specific for the inhibition of NF-κB and instead suggest that they nonspecifically inhibit all cellular kinases. However, we find that in cells in tissue culture, 2 mM sodium salicylate (which inhibits NF-κB induction on average by 70%) has no effect on cellular viability and growth kinetics (1), indicating that even if salicylates inhibit cellular kinases, they do not affect general cellular metabolism or growth. Since NF-κB is an important transcription factor that is clearly involved in inflammation, it was reasonable to speculate that these drugs work in part by interfering with the pro-inflammatory activity of NF-κB. Our intent was not to suggest that salicylates are uniquely specific for NF-κB (it is well-known that aspirin inhibits prostaglandin production), we were merely demonstrating a possible relationship between a known inflammatory mediator, NF-κB, and a family of known anti-inflammatory drugs, the salicylates. Recently, two other groups have identified NF-κB as one of the cellular targets of another class of widely prescribed anti-inflammatory drugs, the corticosteroids (2). Like the salicylates, these drugs do not specifically target NF-κB, however, the inhibition of NF-κB is at present a persuasive explanation for their therapeutic effects in vivo.

As described in our report, the presence of salicylates blocked the degradation of IκB, which suggests that they were interfering with a component of the signaling pathway, most likely a serine-threonine kinase. Although we did not further examine the mechanism of this interference, we dispute the contention of Frantz and O'Neill that there is no precedence for the cAMP-dependent pathway and the PMA/ionomycin pathway converging with the activation of NF-κB. In *Drosophila*, the cAMP-dependent protein kinase activates the NF-κB homolog dorsal by phosphorylating it (3). Furthermore, the activation of NF-κB by cAMP analogs and forskolin have also been reported (4). Also, all members of the rel protein family share a conserved, canonical PKA site which has not yet been adequately investigated for function.

Finally, we hope that our results and the

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