theoretical fit than other least squares regressions (37). Therefore, we conducted regression analyses on the basis of both the OLS(y|x) and OLS_{BIS} methods.
20. From the data of Peters [appendix IIIb in (2)], we estimated the hypothetical energy use OLS_{BIS} slope of aquatic invertebrates to be 0.79 (median value; $n = 17$). Thus, abundance would have to scale with mass to the −0.8 power for energy equivalence to hold if we assume the slope of OLS_{BIS} of log body mass against log metabolic rate to be 0.8.
21. ANCOVA comparing regression lines gives statistically insignificant differences ($P > 0.5$) for both intercepts and slopes in the two contrasts performed (Mynach versus Seebach).
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38. We thank G. Bretschko from the Biological Station Lunz, Austria, for his hospitality and scientific contributions and two anonymous referees for helpful comments and advice. Funded by NERC grant GR3/09844 to M.T.

17 March 2000; accepted 22 June 2000

Prokaryotic Regulation of Epithelial Responses by Inhibition of IκB-α Ubiquitination

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Epithelia of the vertebrate intestinal tract characteristically maintain an inflammatory hyporesponsiveness toward the lumenal prokaryotic microflora. We report the identification of enteric organisms (nonvirulent *Salmonella* strains) whose direct interaction with model human epithelia attenuate synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli. This immunosuppressive effect involves inhibition of the inhibitor κB/nuclear factor κB (IκB/NF-κB) pathway by blockade of IκB-α degradation, which prevents subsequent nuclear translocation of active NF-κB dimer. Although phosphorylation of IκB-α occurs, subsequent polyubiquitination necessary for regulated IκB-α degradation is completely abrogated. These data suggest that prokaryotic determinants could be responsible for the unique tolerance of the gastrointestinal mucosa to proinflammatory stimuli.

In humans, the mucosal lining of the intestine coexists in intimate contact with a diverse prokaryotic microflora. The intestinal epithelial cells have necessarily evolved mechanisms to prevent or limit activation of cellular immuno-inflammatory stress responses in this microbe- and antigen-rich environment (*1*). Immune and inflammatory responses in the gut and other immunocompetent tissues often involves the transcription factor NF-κB. This DNA binding protein is the transcriptional effector of an evolutionarily conserved regulatory pathway that is activated by a myriad of proinflammatory stimuli and is required for the de novo synthesis of numerous proinflammatory cytokines, chemokines, adhesion proteins, and other molecules critical for normal immuno-inflammatory function (*2*). Proinflammatory stimuli activate NF-κB through tightly regulated phosphorylation, ubiquitination, and proteolysis of a physically associated class of inhibitor molecules, IκB's (*3*). IκB is inducibly phosphorylated by a multisubunit kinase complex (IκB kinase) and is subsequently ubiquitinated by a second multiprotein complex, recently isolated by several groups and designated E3-$SCF^{\beta\text{-TrCP}}$, with the β-TrCP subunit functioning as the IκB-specific ubiquitin ligase (E3) (*4–7*). Polyubiquitinated IκB is thus targeted for degradation by the 26*S* proteasome (*8*), allowing NF-κB to translocate to the nucleus, bind to its sequence recognition motif on target promoters, and activate transcription of effector genes, an example of which is the neutrophil chemokine interleukin-8 (IL-8). A variety of endogenous anti-inflammatory mediators, as well as clinically effective anti-inflammatory drugs, are known to exert their effects, at least in part, by blockade of various aspects of the NF-κB signal transduction pathway (*9*); however, there has been no description of interference with the ubiquitination step.

Although the vast majority of enteric organisms do not elicit intestinal inflammation, it is becoming increasingly recognized that enteropathogens that cause acute inflammatory colitis do activate the NF-κB pathway, resulting in secretion of chemokines including IL-8 (*10–12*). We hypothesized that if proinflammatory enteric pathogens activate NF-κB and subsequent events, nonpathogenic microorganisms may be able to selectively attenuate this pathway as a mechanism of intestinal immune tolerance. We observed that colonization of the apical aspect of polarized T84 model epithelia with nonpathogenic *Salmonella* strains, both laboratory-derived and naturally occurring (*S. typhimurium* $PhoP^c$ and *Salmonella pullorum*), attenuates basolateral IL-8 secretion characteristically elicited by apical infection with proinflammatory strains (wild-type *S. typhimurium* and Hil A mutant) (Fig. 1A) (*13–15*). Colonization with *S. typhimurium* $PhoP^c$ and *S. pullorum* was also capable of attenuating the IL-8 secretion elicited by a spectrum of proinflammatory stimuli [tumor necrosis factor-α (TNF-α), the calcium mobilizing agent carbachol, and the phorbol ester PMA (phorbol 12-myristate 13-acetate)] (Fig. 1B). This anti-inflammatory effect could be eliminated by nondenaturing heat killing of the organisms and was not present in bacterial lysates or conditioned media (Fig. 1C), suggesting the phenomenon is mediated through direct interactions of the epithelium with viable bacteria. The anti-inflammatory effects were maximal after a precolonization period of 30 min, though detectable suppressive effects were observed with colonization 10 min after addition of proinflammatory agonist (*16*). Colonization of T84 model epithelia with the anti-inflammatory organisms showed no strain-specific effects on transepithelial resistance or short-circuit currents elicited with forskolin. These indices of normal

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signaling and barrier function indicated cytotoxicity was not responsible for the anti-inflammatory effect (*17*).

To determine if the observed effects involved changes in IL-8 transcription or RNA turnover, Northern blotting was performed (Fig. 1D, top row). As expected, TNF-α treatment resulted in de novo appearance of IL-8 mRNA within 30 min, with levels increasing up to 2 hours (*18*). In contrast, colonization with *S. pullorum* resulted only in trace appearance of IL-8 mRNA at 30 min, with no detectable transcript at 1 or 2 hours. In epithelia colonized with *S. pullorum* and subsequently exposed to TNF-α, levels of IL-8 transcript were markedly reduced at all time points compared to cells treated with TNF-α alone, consistent with data obtained with secreted IL-8. Transcript levels of IκB-α, itself a rapidly inducible NF-κB–dependent gene (*2*), behaved in a very similar manner (Fig. 1D, middle row), consistent with a generalized inhibition of NF-κB dependent genes. Hybridization of a glyceraldehyde phosphate dehydrogenase (GAPDH) probe was unchanged in all experimental conditions, indicating that the repressive effect was not secondary to a global increase in mRNA turnover (Fig. 1D, bottom row).

Because IL-8 and IκB-α are NF-κB–dependent genes, we studied the effects of the anti-inflammatory organisms on the NF-κB activation pathway. Anti-inflammatory salmonellae, but not wild-type *S. typhimurium*, inhibited activation of reporter constructs controlled both by the native IL-8 promoter and synthetic NF-κB response elements (Fig. 2A). Immunolocalization of the p65 subunit of NF-κB in HeLa epithelial cells revealed that colonization with the anti-inflammatory organisms prevented nuclear translocation of p65 in response to TNF-α (Fig. 2B). Furthermore, the anti-inflammatory organisms caused the p65 to be redistributed in a granular, perinuclear localization even in the absence of proinflammatory stimulation. To determine if the observed blockade of NF-κB activation was occurring at the level of IκB-α degradation, Western blot analysis of whole-cell lysates was performed (Fig. 2, C through E). Colonization of T84 model epithelia with anti-inflammatory organisms prior to TNF-α or proinflammatory wild-type *S. typhimurium* challenge resulted in stabilization of IκB-α, in contrast to the nearly complete IκB-α degradation induced by TNF-α or wild-type *S. typhimurium* alone (Fig. 2, C and D). Identical results were obtained with two other human epithelial cell lines, HT-29 and HeLa, but not with monocytic U293 cells or human umbilical vein endothelial cells, suggesting that the anti-inflammatory effect may be a property of epithelial cells in general (*16*). The anti-inflammatory effects on IκB-α degradation required a minimal multiplicity of infection (MOI) of 10 organisms/cell and, consistent with the IL-8 secretion data, could not be mediated by heat-killed organisms or bacterial culture supernates (Fig. 2E). The anti-inflammatory effect on IκB-α degradation was seen maximally after 30 min of colonization and could not be inhibited with cycloheximide (20 μg/ml, added 30 min before experimental infection) (*16*).

Surprisingly, colonization of T84 model epithelia with anti-inflammatory organisms induced the phosphorylation of IκB-α and did so to an even greater extent than did wild-type *S. typhimurium*, *InvA*, or TNF-α (Fig. 3A). The presence of continuously phosphorylated IκB-α in the absence of degradation and subsequent NF-κB translocation is distinct from the reported mechanism of NF-κB blockade mediated by the enteroinvasive pathogen *Yersinia* sp. (*19, 20*). These organisms prevent IκB phosphorylation via the activity of the translocated prokaryotic effector molecule YopJ, which inhibits mitogen-activated protein kinase kinase (MAPKK) and subsequent IκB kinase activation. In contrast, all *Salmonella* strains studied, including the anti-inflammatory strains, activated the MAPKK/JNK pathway, which is the only other signaling pathway necessary for IL-8 transcription in response to *Salmonella* (Fig. 3B) (*11*).

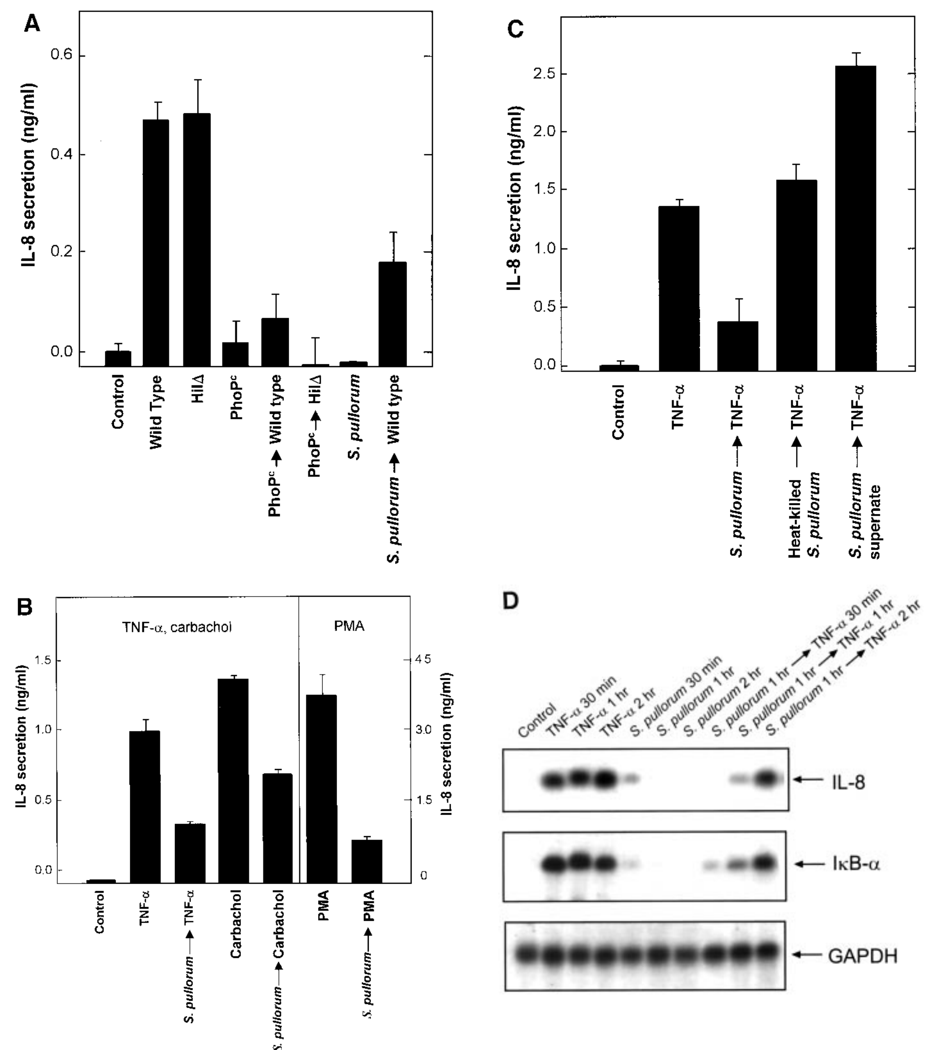

Fig. 1. Colonization with anti-inflammatory *Salmonella* inhibits induced epithelial responses. [(**A**) through (**C**)] Enzyme-linked immunosorbent assay (ELISA) of secreted IL-8 from model epithelia (*13, 14*). Data are the means ± SEM of three experiments. (A) Cells were colonized apically with the indicated bacteria for 1 hour and washed, after which the second strain of bacteria was added for an additional hour. (B) Cells were colonized apically as in (A), washed, and subsequently challenged with basolateral TNF-α (10 ng/ml), carbachol (100 μM), and PMA (1 μg/ml) for 30 min as indicated. (C) Cells were treated apically with live and heat-killed (80°C for 10 min) cultures and with conditioned media (full-density cultures equivalent to a MOI of 30 were incubated alone at 37°C for 1 hour and sterilized through a 0.2-μm filter) for 1 hour, washed and challenged with basolateral TNF-α. (**D**) Total RNA was analyzed by Northern hybridization as described (*12*). Blots were sequentially hybridized with ^{32}P-labeled cDNA probes encoding human IL-8, IκB-α, and GAPDH.

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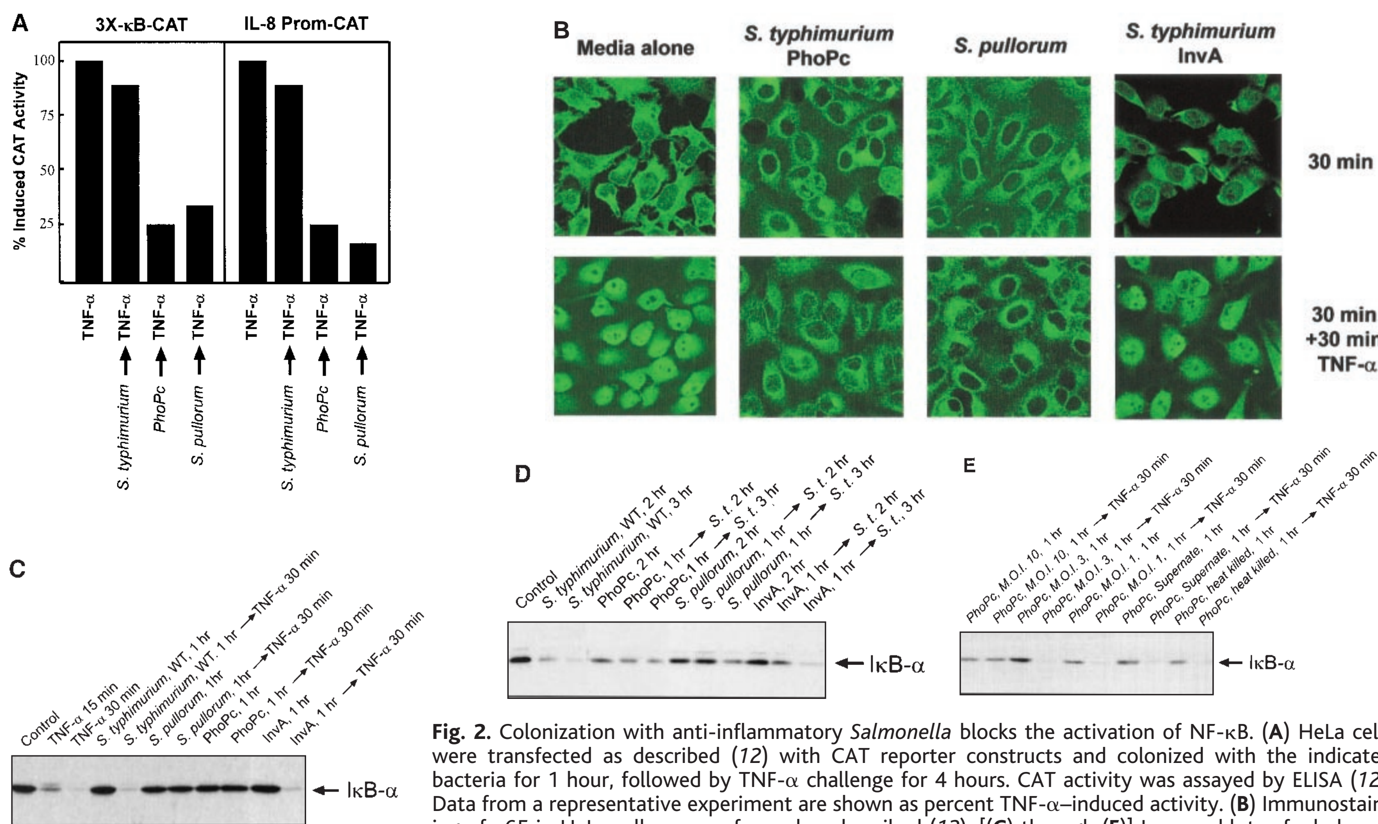

Fig. 2. Colonization with anti-inflammatory *Salmonella* blocks the activation of NF-κB. (**A**) HeLa cells were transfected as described (*12*) with CAT reporter constructs and colonized with the indicated bacteria for 1 hour, followed by TNF-α challenge for 4 hours. CAT activity was assayed by ELISA (*12*). Data from a representative experiment are shown as percent TNF-α–induced activity. (**B**) Immunostaining of p65 in HeLa cells was performed as described (*12*). [(**C**) through (**E**)] Immunoblots of whole-cell IκB-α levels in T84 cells (*12*). Lysates were prepared from cells colonized apically with the indicated organisms, and subsequently challenged by basolateral TNF-α (C) or apical *S. typhimurium* (D) as indicated. In (E), cells were treated on the apical surface with diluted and heat-killed cultures of *S. typhimurium* PhoP^c or with conditioned bacterial media for 1 hour before challenge with TNF-α.

To allow further study of modified IκB-α, T84 model epithelia were pretreated with the peptide aldehyde proteasomal inhibitor MG-132. Under these conditions, IκB-α degradation in response to TNF-α was abrogated and the labile phospho-IκB-α induced by proinflammatory signals such as TNF-α or wild-type *S. typhimurium* could be visualized, as previously reported (Fig. 3C, bottom row) (*12*). Additionally, very high molecular weight bands consistent with polyubiquitinated IκB-α were observed, as previously reported (Fig. 3C, top row) (*8, 21*). Notably, these higher molecular weight species were absent in whole-cell lysates derived from both PhoP^c- and *S. pullorum*–infected cells, whereas phosphorylated species were abundant. Furthermore, when cells colonized with the anti-inflammatory bacteria were subsequently challenged with TNF-α, no ubiquitinated IκB-α species were observed (Fig. 3D). The polyubiquitinated form of IκB-α was confirmed by immunoprecipitation (Fig. 3E) (*22*). Collectively, these data indicate that anti-inflammatory salmonellae induce phosphorylation of IκB-α, much like proinflammatory species, yet they attenuate subsequent ubiquitination of IκB-α, thus preventing its degradation.

Besides IκB-α, the only other known substrate of the E3-SCF$^{\beta-TrCP}$ complex is phospho-β-catenin (*4*). Under basal conditions, this protein is constitutively phosphorylated by GSK-3β, ubiquitinated, and degraded, thus preventing nuclear accumulation and consequent activation of β-catenin responsive genes, which have been implicated in epithelial growth control (*23, 24*). When T84 model epithelia were incubated in the presence of proteasomal inhibitors, accumulation of ubiquitinated adducts could be visualized over several hours (Fig. 4A) (*23*). When model epithelia were colonized by wild-type *S. typhimurium*, followed by addition of MG-132, ubiquitination of β-catenin could also be seen. However, colonization with the anti-inflammatory salmonellae, again followed by addition of MG-132, totally abrogated the appearance of ubiquitinated β-catenin over the time course studied. These results support the hypothesis that *S. typhimurium* PhoP^c and *S. pullorum* mediate an anti-inflammatory state by inhibition of ubiquitination, and could suggest that the effects of this class of bacteria may extend beyond reduction of inflammation and influence cellular proliferation and differentiation.

We investigated whether the observed inhibition of IκB-α ubiquitination reflected a global blockade of the cellular ubiquitination machinery. Ubiquitin-protein conjugates were considerably increased in MG-132–treated T84 model epithelia within 15 min (Fig. 4B, lanes 1 through 4). Epithelial monolayers colonized with all test bacteria in the absence of MG-132 did not induce accumulation of ubiquitinated protein species (Fig. 4B, lanes 5, 6, 10, 11, 15, 16, 20, and 21). Finally, model epithelia colonized with test bacteria for 60 min were followed with the addition of MG-132. The observed increase in abundance of ubiquitin-conjugated proteins was no different in T84 model epithelia colonized with *S. pullorum* or *S. typhimurium* PhoP^c than in epithelia colonized with control organisms (compare lanes 7 through 9 and 22 through 24 with 12 through 14 and 17 through 19). Under identical conditions, colonization with the anti-inflammatory organisms would prevent induced ubiquitination of IκB-α. This experiment suggests that the attenuation of ubiquitination is specific to the currently known E3-SCF$^{\beta-TrCP}$ substrates IκB-α and β-catenin.

Here, we describe inhibiting responses of model intestinal epithelia to diverse inflammatory stimuli, including pathogenic bacteria. This anti-inflammatory effect required the direct interaction of epithelial cells with viable nonpathogenic enteric prokaryotes. If this phenomenon can be extrapolated to intact vertebrate organisms, it would suggest that certain bacteria-host

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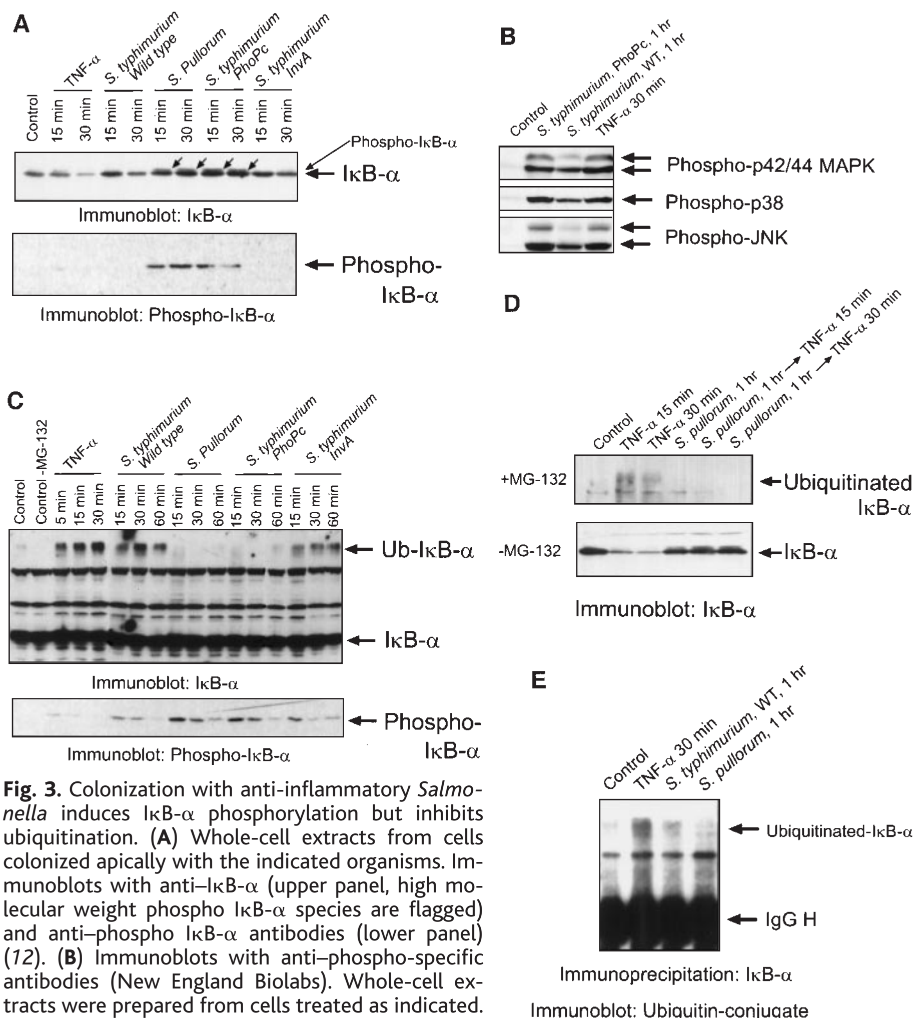

Fig. 3. Colonization with anti-inflammatory *Salmonella* induces IκB-α phosphorylation but inhibits ubiquitination. (**A**) Whole-cell extracts from cells colonized apically with the indicated organisms. Immunoblots with anti–IκB-α (upper panel, high molecular weight phospho IκB-α species are flagged) and anti–phospho IκB-α antibodies (lower panel) (*12*). (**B**) Immunoblots with anti–phospho-specific antibodies (New England Biolabs). Whole-cell extracts were prepared from cells treated as indicated. (**C** and **D**) Cells were pretreated with 50 μM MG-132 for 30 min and activated/colonized as indicated. Immunoblots with anti–IκB-α antibodies [(C), upper panel and (D)] and anti–phospho IκB-α antibodies [(C), lower panel]. (**E**) Immunoprecipitation with IκB-α followed by immunoblot with anti-ubiquitin conjugate antibody (*22*). HT-29 cells were pretreated with 50 mM MG-132 for 1 hour and activated/colonized as indicated.

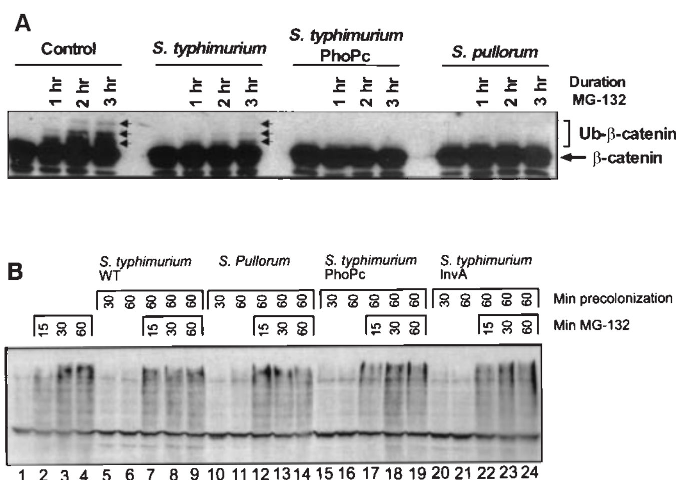

Fig. 4. (**A**) Immunoblots with anti–β-catenin antibodies (Transduction Laboratories). Model epithelia were colonized with the indicated strains for 30 min before addition of 50 μM MG-132. (**B**) Immunoblot with anti-ubiquitin conjugate antibodies (Affiniti Research). Model epithelia were treated as indicated with 50 μM MG-132, with test bacteria applied apically, or with test bacteria followed by wash and subsequent addition of MG-132.

interactions may induce local inhibition of acute inflammatory responses, utilizing either host- or bacteria-derived factors. Our observations suggest that the anti-inflammatory bacterial/epithelial interaction may provide a factor that either reduces activity of the IκB E3-SCF complex or increases a specific de-ubiquitinating activity. Such a hypothetical factor could be translocated from the bacterium into the host cell via a type III secretion apparatus (*25*), or alternatively, represents a eukaryotic-derived anti-inflammatory compound elicited by cellular interactions with a specific bacterial factor (*26*). At equilibrium, attenuation of acute inflammatory responses by certain prokaryotes could contribute to the characteristic tolerance of the mucosal immune system, whereas disruption of this equilibrium could contribute to the pathogenesis of enteric infections as well as idiopathic inflammatory bowel disease. In relation to this hypothesis, it is relevant that the use of nonpathogenic enteric organisms, termed "probiotics," are now being explored as therapeutic agents in inflammatory bowel disease. Although the mechanism of probiotic action is not understood, the beneficial effects are consistent with an anti-inflammatory state conferred by nonpathogenic organisms. It appears that the luminal microflora can send negative as well as positive signals to mucosal lining cells as a means of productive, symbiotic interactions with the host.

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22. Model epithelia were treated with 50 μM MG-132 for 30 min before apical infection of test organisms or basolateral addition of TNF-α. Cells were lysed in 200 μl of RIPA. We added 20 μl of agarose-conjugated antibodies to IκB-α (anti–IκB-α) (Santa Cruz) to 70 μl of clarified lysate and allowed it to bind for 4 hours. Beads were washed three times in 500 μl of lysis buffer, and bound proteins were released by boiling in denaturing loading buffer. Samples were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with anti-ubiquitin conjugate (Affiniti).
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27. Supported by NIH grants DK09800 to A.T.G., DK-47662 and DK-35932 to J.L.M., and HL60033 to A.S.N.

11 May 2000; accepted 5 July 2000

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(September 1, 2000)
Science **289** (5484), 1560-1563. [doi: 10.1126/science.289.5484.1560]

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