bone structure. There were thus nine individuals in that sex-age group and a total sample of 119. A femur and tibia from the right or left side was taken from each individual; equal numbers of each side were included in each sex-age group.
12. A digitizer (Talos Systems model RP622B) with an active surface area of 61 by 61 cm was used. The magnification of cross sections during tracing was approximately ×8.
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15. Percentage differences are between 20- to 39-year and 40+-year age groups (see Table 1). The results of regression analysis and analysis of variance for the smaller age group categories (*14*) also support these conclusions.
16. The quantities I_{max} and I_{min} are measures of the areal distribution of bone, which along with material properties determine the maximum and minimum bending rigidities of a section; J determines the torsional rigidity [only approximate for noncircular sections—see (*8*) and R. L. Piziali, T. K. Hight, D. A. Nagel, *J. Biomech.* **9**, 695 (1976)]. Second moments of area are calculated as the product of the unit areas and their squared distances from the neutral axis (I_{max}, I_{min}) or centroid (J) of the section (*13*).
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22 March 1982; revised 10 May 1982

Phagocyte Impotence Caused by an Invasive Bacterial Adenylate Cyclase

Abstract. *For unknown reasons, humans infected with the bacterium* Bordetella pertussis *are exceptionally vulnerable to secondary infections.* Bordetella *species elaborate a soluble, heat-stable, and highly active adenylate cyclase. This enzyme is internalized by phagocytic cells and catalyzes the unregulated formation of adenosine 3',5'-monophosphate (cyclic AMP), thereby disrupting normal cellular function. This unusual phenomenon may explain* Bordetella*-induced aphylaxis and may prove to be useful for investigating a variety of cyclic AMP–governed processes.*

Bacteria of the genus *Bordetella* cause acute and chronic respiratory diseases in many species of animals. In humans, *B. pertussis* produces whooping cough, a prolonged infection of the respiratory tree characterized by severe paroxysms of coughing interrupted by inspiratory whoops. Despite effective immunization programs in some countries, whooping cough remains a leading cause of childhood mortality (*1*).

Among the unusual features of *B. pertussis* infection are an absence of fever, a lack of neutrophilia, and a high incidence of secondary bacterial pneumonias (*2*). These features suggest impaired host defense. Indeed, alveolar macrophages from rabbits chronically infected with *B. bronchiseptica* do not produce superoxide in response to inflammatory stimuli (*3*).

Pathogenic *Bordetella* spp. elaborate a unique, heat-stable, and highly active adenylate cyclase that is associated with the bacterial cell envelope and is also present in the supernatant of suspension cultures (*4*). We hypothesized that this enzyme might enter phagocytic cells and generate adenosine 3',5'-monophosphate (cyclic AMP), a known inhibitor of phagocyte functions (*5*). The resulting cyclic AMP–mediated impairment of neutrophil and macrophage function might defend *Bordetella* against phagocytic attack and might also explain *Bordetella*-induced aphylaxis.

We initially tested this hypothesis by investigating the effects, on human peripheral blood neutrophils (*6*), of culture medium in which virulent *B. pertussis* had been grown (*7*). The supernatant fluid of *B. pertussis* cultures contains a labile factor that causes dose-dependent suppression of superoxide production by human neutrophils stimulated with opsonized zymosan (*8*) (Fig. 1A). The stability of this bacterial inhibitor is improved when whole *B. pertussis* organisms are placed in 4*M* urea and stored at −70°C. Extensive dialysis to eliminate the urea, followed by centrifugation to remove insoluble particulates, reproducibly yields potent inhibitory activity (*9*) (Fig. 1). All subsequent experiments were performed with such urea-extracted, dialyzed preparations.

The inhibitory effects of *Bordetella* products on superoxide production are not limited to neutrophils; human alveolar macrophages (*10*) exposed to *B. pertussis* extract also show marked suppression of superoxide production (*11*) (Fig. 1B). In addition, *Bordetella* products inhibit neutrophil chemotaxis and particle ingestion (results not shown). Perhaps most important, the *Bordetella* products induce a profound bactericidal defect in human neutrophils (Fig. 2) which resembles that found in chronic granulomatous disease (*12*). Our findings agree with earlier reports describing *B. pertussis*

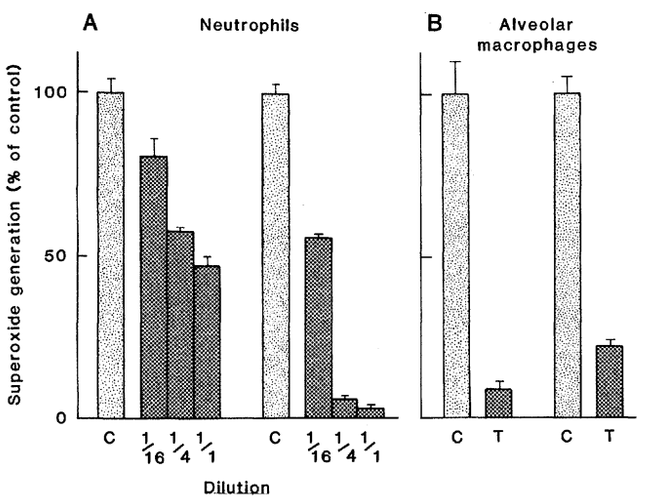

Fig. 1. Superoxide generation by stimulated human phagocytes and inhibition by *Bordetella* products. (A) Human neutrophils, 2 × 10^6, suspended in 200 μl of Hanks balanced salt solution, were incubated for 10 minutes at 37°C with 200 μl of the indicated dilution of the supernatant of 48-hour cultures of *B. pertussis* (protein content, 120 μg/ml) (left panel) and with dialyzed extract of *B. pertussis* organisms (protein content, 520 μg/ml) (right panel). Cytochrome *c* (1.2 mg) and opsonized zymosan (1 mg) were added (total volume, 1 ml) and the superoxide-dependent reduction of ferricytochrome *c* was determined after 10 additional minutes of incubation at 37°C as previously described (*8*). Results are expressed as percentages of control (untreated values), and bars represent the range of independent triplicate determinations. (B) Human alveolar macrophages (10^6) suspended in 100 μl of Hanks balanced salt solution were incubated with 100 μl of dialyzed extract of *B. pertussis* (*T*) or 100 μl of external dialysis fluid (*C*) as above. The cells were then stimulated by the addition of 1 mg of opsonized zymosan (left bars) or 0.1 μg of phorbol myristate acetate (right bars). Superoxide production was assessed by following luminol-enhanced chemiluminescence as described (*11*). Results represent the mean and range of triplicate determinations.

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Table 1. Heat- and protease-stable adenylate cyclase activity in human neutrophils (PMN) before and after incubation with *B. pertussis* extracts. After incubation for 20 minutes at 37°C or 0°C, cell pellets were obtained by centrifugation and the associated cyclicAMP was determined (*16*). Cells were then washed thrice, exposed to trypsin (200 μg/ml for 5 minutes at 37°C), again washed thrice and immediately homogenized under hypotonic conditions. After restoration of isotonicity, the homogenates were heated to 100°C for 5 minutes. The adenylate cyclase activity in the whole boiled homogenates was then determined (*15*).

Sample	Treatment	Cyclic AMP (pmole/ 10^7 PMN)	Adenylate cyclase (pmole/10^7 PMN-min)
Neutrophils	Incubated for 20 minutes at 37°C, washed, trypsinized, washed, homogenized	4.9	0, 0*
Neutrophils plus *B. pertussis* extract (540 μg/10^7 PMN)	Incubated for 20 minutes at 37°C, washed, trypsinized, washed, homogenized	1296	41.9, 28.0, 45.1
Neutrophils plus *B. pertussis* extract (540 μg/10^7 PMN)	Incubated for 20 minutes at 0°C, washed, trypsinized, washed, homogenized	6.7	4.2, 4.3, 4.8

*Limit of detection, < 1 pmole per 10^7 PMN per minute.

factors capable of inhibiting neutrophil chemotaxis and oxygen consumption (*13*) and certain aspects of macrophage motility (*14*).

Consistent with our hypothesis, dialyzed urea extracts of *B. pertussis* contain immense adenylate cyclase activity (*15*), typically > 1,000,000 pmole per milligram of protein per minute. Fresh culture supernatants, in accordance with their less potent effects on phagocytes, contain less adenylate cyclase activity. However, in both cases, these *Bordetella* adenylate cyclase activities greatly exceed those reported for mammalian enzymes.

To determine whether aberrations in phagocyte cyclic AMP content might be caused by this bacterial cyclase, we measured cyclic AMP (*16*) within neutrophils incubated with *Bordetella* extracts. After a brief incubation period, human neutrophils undergo a striking increase in intracellular cyclic AMP (Fig. 3). The accumulation of cyclic AMP is dose- and time-dependent, reaching 1500 to 2200 pmole per 10^7 neutrophils after 20 minutes of incubation. Such massive elevations in phagocyte cyclic AMP have not, to our knowledge, been reported previously. Indeed, earlier studies indicate that increases of intracellular cyclic AMP of only 10 to 20 pmole per 10^7 neutrophils are sufficient to impair many phagocyte functions (*5*).

Evidently, the bacterial adenylate cyclase reaches the cytoplasmic compartment of the phagocytes. Neutrophils incubated with *Bordetella* extract for 20 minutes at 37°C accumulate heat-stable adenylate cyclase activity which is cell-associated and protected against proteolytic digestion (*17*) (Table 1). Since mammalian adenylate cyclase is heat-labile, this activity is bacterial in origin. Protection of the bacterial enzyme against proteolytic attack supports the idea that it has been internalized, but not destroyed, by the neutrophils. Although the precise mechanism whereby the bacterial adenylate cyclase enters phagocytic cells is not known, we have found that neutrophils incubated at 0°C accumulate neither cyclic AMP nor protease-insensitive adenylate cyclase activity (Table 1). Therefore, a temperature-dependent process (possibly endocytosis) may be required for penetration. It should be noted that purified *Bordetella* adenylate cyclase does not enter phagocytes; even partial purification of this enzyme destroys its biological effectiveness although the specific activity may be enriched manyfold. Therefore, in its native state, *Bordetella* adenylate cyclase may well exist as part of a multimeric complex, all parts of which are required for cellular penetration and expression of intracellular effect.

Bordetella pertussis produces at least one other factor (so-called "pertussis toxin") which influences the cyclic AMP metabolism of mammalian cells (*18*). However, in contrast with the effects of *Bordetella* adenylate cyclase reported herein, the pertussis toxin requires > 12 hours to exert an effect, is readily separated from *Bordetella* adenylate cyclase, and affects exclusively the response of mammalian cells to usual agonists of adenylate cyclase.

Thus, an unusual and highly active adenylate cyclase produced by *Bordetella* species is internalized by mammali-

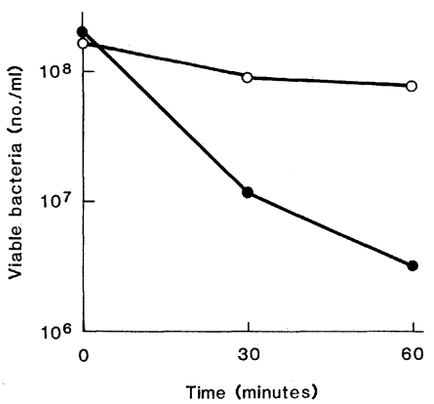

Fig. 2. Neutrophil killing defect induced by *Bordetella* extract. Human neutrophils (2 × 10^7 per milliliter) suspended in Hanks balanced salt solution were incubated for 5 minutes at 37°C with an equal volume of dialyzed *Bordetella* extract or dialysate control. The killing of *Staphylococcus aureus* 502A was assessed as described (*12*) by admixing 5 × 10^6 neutrophils, 2 × 10^8 bacteria, and 0.1 ml of pooled human serum in a total volume of 1 ml. Numbers of viable bacteria remaining were determined by plating dilutions of the incubation suspension removed at 0, 30, and 60 minutes. Each point represents the mean of quadruplicate determinations. Control tubes containing no neutrophils showed no change in bacterial count. Symbols: ○, *Bordetella*-treated neutrophils; ●, control neutrophils.

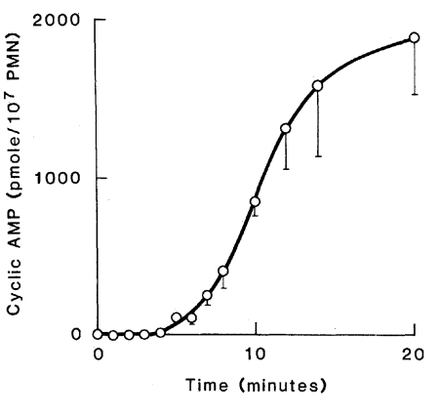

Fig. 3. Accumulation of cyclic AMP in human neutrophils (*PMN*) incubated with dialyzed *Bordetella* extract. Neutrophils, 10^7 per milliliter in Hanks balanced salt solution, were incubated at 37°C with equal volumes of dialyzed *Bordetella* extract (protein content, 520 μg/ml) for the times shown. Total cyclic AMP was determined as described (*16*). Values shown represent the means and standard error of seven separate (duplicate) determinations with neutrophils from four different donors. Normal neutrophils contain 2 to 5 pmole of cyclic AMP per 10^7 cells, and these amounts do not change during control incubations. Separate experiments (not shown), in which neutrophil pellets were obtained by brief centrifugation after incubation, indicated that > 90 percent of the total recoverable cyclic AMP is associated with the cell pellet.

an phagocytic cells and generates therein very large amounts of cyclic AMP. This, in turn, suppresses a variety of neutrophil and macrophage functions including superoxide production, chemotaxis, and bacterial killing. This phenomenon probably explains previous observations of alveolar macrophage dysfunction in *B. bronchiseptica*–infected rabbits and may explain the greatly increased susceptibility to secondary bacterial pneumonias seen in *B. pertussis*–infected humans. Our observations may also help elucidate the pathogenesis of atrophic infectious rhinitis in pigs, a chronic *Bordetella* infection in which there is agenesis and dissolution of the turbinate bones within the snout. Indeed, programmed elevations of cyclic AMP have been implicated in the atrophy and loss of certain cell types during normal morphogenesis (*19*).

The *Bordetella* adenylate cyclase probably represents an important adaptive strategy for the bacterium, in that suppression of host phagocytic response will permit the survival and replication of an otherwise vulnerable organism. *Bordetella* accomplishes this through the purposeful misuse of a control system that normally operates as a negative modulator of various cellular functions. We anticipate that this unusual bacterial adenylate cyclase will add to our understanding of the pathogenesis of *Bordetella* infections. This enzyme may be used to manipulate intracellular cyclic nucleotide metabolism in a variety of experimental and, perhaps eventually, clinical situations.

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9. *Bordetella pertussis* organisms (*7*) enriched to $\sim 10^{11}$ per milliliter by centrifugation, were admixed with urea to 4*M* while being stirred constantly, and then were stored at −70°C. Immediately before use, these extracts were dialyzed at 4°C against 2000 volumes of phosphate-buffered saline, *p*H 7.4, for 18 hours to remove the urea. Insoluble material was removed by centrifugation for 30 minutes at 20,000*g*. The possibility that residual urea or contaminants therein might account for some of the inhibitory effects was ruled out by incubating control cells with (i) Stainer-Scholte growth medium treated in parallel with the bacterial culture, (ii) equivalent volumes of the external dialysis fluid, and (iii) concentrations of urea up to 50 m*M*. None of these inhibited the neutrophil response to opsonized zymosan or phorbol myristate acetate.
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25 May 1982

Initiation of Endochondral Calcification Is Related to Changes in the Redox State of Hypertrophic Chondrocytes

Abstract. *The level of pyridine nucleotides (NADH and NAD^+) in the mineralizing growth plate of the chick was ascertained by high-resolution scanning microfluorimetry and biochemical analysis. Scanning electron microscopy and light microscopy were used to relate the concentrations of NADH and NAD^+ to stages of chondrocyte maturation. A dramatic increase was found in the relative concentration of reduced pyridine nucleotides in the hypertrophic zone. On either side of this zone, in proliferating and calcifying cartilage, there was a decrease in NADH fluorescence, and the NADH/NAD^+ ratio was depressed. The finding that NADH accumulated in the tissue zone associated with the earliest deposition of bone mineral supports the hypothesis that a change in the redox state initiates tissue mineralization.*

Studies of the epiphyseal growth plate have shown that, during the mineralization process, chondrocytes undergo sequential metabolic and developmental changes. In the premineralizing regions of the plate, cells concentrate inorganic ions in their mitochondria (*1*). At the calcification front, cellular Ca^{2+} and phosphate (P_i) efflux is related to the low tissue O_2 tension (*2*). Ion efflux may also result from the accumulation of phosphoenolpyruvate. This glycolytic intermediate is found in high levels in hypoxic cells and can induce Ca^{2+} release from isolated chondrocyte mitochondria (*3*). Thus, several lines of investigation suggest that cell ion discharge and mineral deposition is associated with hypoxia-related events.

Experimental support for the concept that redox conditions directly modulate endochondrial mineralization is largely inferential. While there have been measurements of normal respiratory activity as well as measurements of glycolytic and pentose shunt pathways in chondrocytes, there are neither calculated values nor direct determinations of redox-sensitive components (*4*). We have combined biochemical and morphological techniques to measure the major redox couple of cells in the growth plate. The

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