INFECTION AND IMMUNITY, Nov. 1974, p. 1146-1151

Vol. 10, No. 5
Printed in U.S.A.

Diphtheria Pathogenesis in Guinea Pig Tracheal Organ Culture

JOEL B. BASEMAN AND ALBERT M. COLLIER

Departments of Bacteriology and Immunology and Pediatrics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received for publication 15 July 1974

The effect of diphtheria toxin on guinea pig trachea in organ culture was examined to measure the susceptibility of respiratory epithelial cells to toxin action. Exposure of individual tracheal rings to toxin resulted in cessation of protein synthesis as well as the development of cytopathology within a few hours. Continued incubation led to further inhibition of protein synthesis and extensive disorganization of the epithelial layer. Other inhibitors of protein synthesis were monitored for their effect on the structural integrity of tracheal cells but were found incapable of eliciting similar histopathology. Early after its addition, toxin at minute concentrations possessed cytotoxic properties as well as the ability to inhibit protein synthesis. Interpretation of these data is correlated with current information on the structure and activity of diphtheria toxin.

Although the biochemistry of diphtheria intoxication has been explained in molecular terms (8, 10, 12), the manner in which diphtheria toxin alters host cell function in the intact animal leading to morphologic damage and possible death remains unclear. It is well documented that diphtheria toxin inhibits protein synthesis in a variety of sensitive tissues by inactivating elongation factor 2 (EF2) (3, 5, 6). However, respiratory epithelial cells have not been specifically examined although they would appear to be a most likely candidate for characterization of toxin action.

The technique of tracheal organ culture provides respiratory epithelial cells in a viable and differentiated state and enables studies on disease pathogenesis in specialized tissue of the respiratory tract. Reports describing the interaction of viruses (2) and mycoplasmas (7) with ciliated epithelium have demonstrated the usefulness of this technique. This study examines the influence of diphtheria toxin on protein synthesis and on the development of cytopathology in guinea pig tracheal organ cultures. Other chemical inhibitors of protein synthesis are examined for comparative purposes.

MATERIALS AND METHODS

Diphtheria toxin and antitoxin. Two lots of toxin both having approximately 50 guinea pig minimal lethal doses per Lf (2.4 μg of protein per Lf) were used. Crystalline toxin was obtained through the courtesy of the Wellcome Research Laboratory, Beckenham, England. Additional toxin and antitoxin (anti-CRM197, 240 U/ml) were a gift from A. M. Pappenheimer, Jr., Harvard University.

Other chemical inhibitors of protein synthesis. Cycloheximide was purchased from Schwarz-Mann and puromycin and tetracycline from ICN Nutritional Biochemicals Corp.

Organ culture. Guinea pigs weighing approximately 400 g were anesthetized with sodium pentobarbital, and blood was removed by cardiac puncture to prevent excessive bleeding at the time of surgery. Tracheal rings were prepared as earlier described (7). Rings of similar size were incubated in small sterile petri dishes (no. 1006, Falcon Plastics) containing 3.0 ml of 0.3× Eagle minimal essential medium in Hanks balanced salts. Diphtheria toxin, antitoxin, or other inhibitors of protein synthesis were then added to these cultures.

Protein synthesis in guinea pig tracheal rings. At selected intervals, individual tracheal rings were removed from petri dishes, transferred to microtiter wells (Cooke Engineering Co.) containing 50 μliters of the appropriate medium, and radiolabeled for 45 min with 1 μCi of ^{3}H-labeled amino acid mixture (ICN Radioisotope Division). Selected rings were then washed in cold minimal essential medium and placed in 0.25% sodium dodecyl sulfate buffer, followed by 0.1 N NaOH to facilitate solubilization of cell material. Albumin (0.05%) was added as carrier protein before the addition of trichloroacetic acid to a final concentration of 5%. After overnight storage in the cold, the precipitate was collected on 0.45-μm membrane filters (Millipore Corp.), washed in cold 5% trichloroacetic acid supplemented with unlabeled amino acids, dried, and counted in the scintillation spectrometer.

Histopathology and radioautography. Tracheal rings were removed from organ culture dishes and prepared as described by Collier and Baseman (7). After embedding and sectioning, tracheal rings were coated with Ilford L-4 nuclear-tract emulsion, stored in the dark for 3 weeks, and later developed with

Kodak D-19. Slides were stained with Richardson's blue before microscopic examination.

RESULTS

Effect of toxin on ^{3}H-labeled amino acid incorporation by tracheal rings. Low toxin concentrations prevented incorporation of radiolabeled amino acids into protein of sensitive cells or tissues after a lag period (3, 12). Figure 1 shows that protein synthesis in tracheal organ cultures is also sensitive to diphtheria toxin, the kinetics of inhibition being dependent upon toxin concentration. Levels of toxin equivalent to 0.075 Lf/ml (180 ng of protein per ml) inhibited protein synthesis by 80% within 24 h, whereas higher toxin concentrations had a more rapid and pronounced effect. The presence of equivalent amounts of antitoxin added simultaneously with toxin prevented the inhibition.

Tracheal ring histopathology and radioautography after exposure to toxin. Tracheal rings incubated with antitoxin or toxin plus antitoxin retained normal cellular organization with the maintenance of the pseudostratified columnar epithelium (Fig. 2A and B). The cilia were well preserved and the basement membrane and lamina propria remained intact. Metabolically active cells were readily located by radioautography, the heaviest grain concentration occurring over the respiratory epithelium. Nonciliated epithelial cells incorporated radiolabel to a greater extent than ciliated cells, suggesting a higher rate of protein synthesis. Exposure of tracheal rings to toxin, however, was accompanied by early histological changes in the epithelial layer (Fig. 2C). Although the epithelium maintained a resemblance to normal architecture after exposure to toxin for 8 h, necrotic cells were observed within the epithelial layer along with disruption of cell-to-cell contact. Radioautography demonstrated decreased grain localization over intoxicated respiratory epithelial cells when compared to toxin-antitoxin-treated controls, indicating inhibition of protein synthesis. After exposure to toxin for 24 h, there was considerable disorganization of the epithelium along with extensive loss of cells into the lumen (Fig. 2D). Cell nuclei

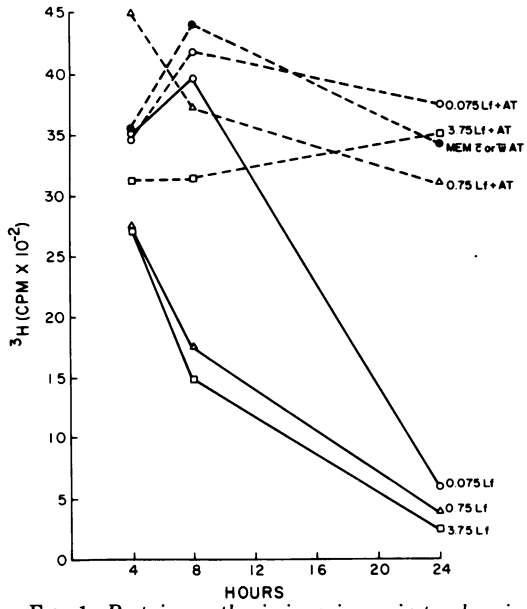

FIG. 1. *Protein synthesis in guinea pig trachea in organ culture exposed to diphtheria toxin with and without antitoxin (AT). Tracheal rings were radiolabeled with ^{3}H-labeled amino acids for 45 min before harvest. Each point represents the average corrected value of duplicate rings from three separate experiments.*

appeared swollen and chromatin margination was evident. Also, the respiratory epithelial layer was represented by small islands of injured epithelial cells (Fig. 2E) with complete loss of the epithelium on either side. Decreased cellularity of the lamina propria was also evident. A few cells in the population continued to incorporate radiolabel after incubation with toxin for 8 and 24 h. Similar cytopathology was observed in tissues exposed to other toxin concentrations including levels as low as 0.075 Lf/ml.

Effect of other inhibitors of protein synthesis on tracheal ring histology. Since cytopathology accompanied inhibition of protein synthesis by toxin, the influence of other drugs on the structural integrity of tracheal cells was monitored. These studies were performed to

FIG. 2. *Guinea pig trachea in organ culture. Radioautographs prepared by radiolabeling with ^{3}H-labeled amino acids for 45 min before staining with Richardson's blue. ×850. (A) Trachea incubated with 1 U of diphtheria antitoxin per ml for 24 h. (B) Trachea incubated with 1 Lf/ml of diphtheria toxin simultaneously with 1 U of antitoxin per ml for 24 h. (C) Trachea exposed to 1 Lf/ml of diphtheria toxin for 8 h. Note injured cells within the epithelium. (D) Trachea exposed to 1 Lf of diphtheria toxin per ml for 24 h. Disorganization of epithelial layer with sloughing of cells into lumen is evident. Note decreased grain concentration. (E) Trachea exposed to 1 Lf of diphtheria toxin per ml for 24 h. Small island of remaining, injured epithelium with decreased grain localization. (F) Trachea exposed to 150 μg of cycloheximide per ml for 24 h. Note retention of integrity of epithelium accompanied by decreased grain concentration.*

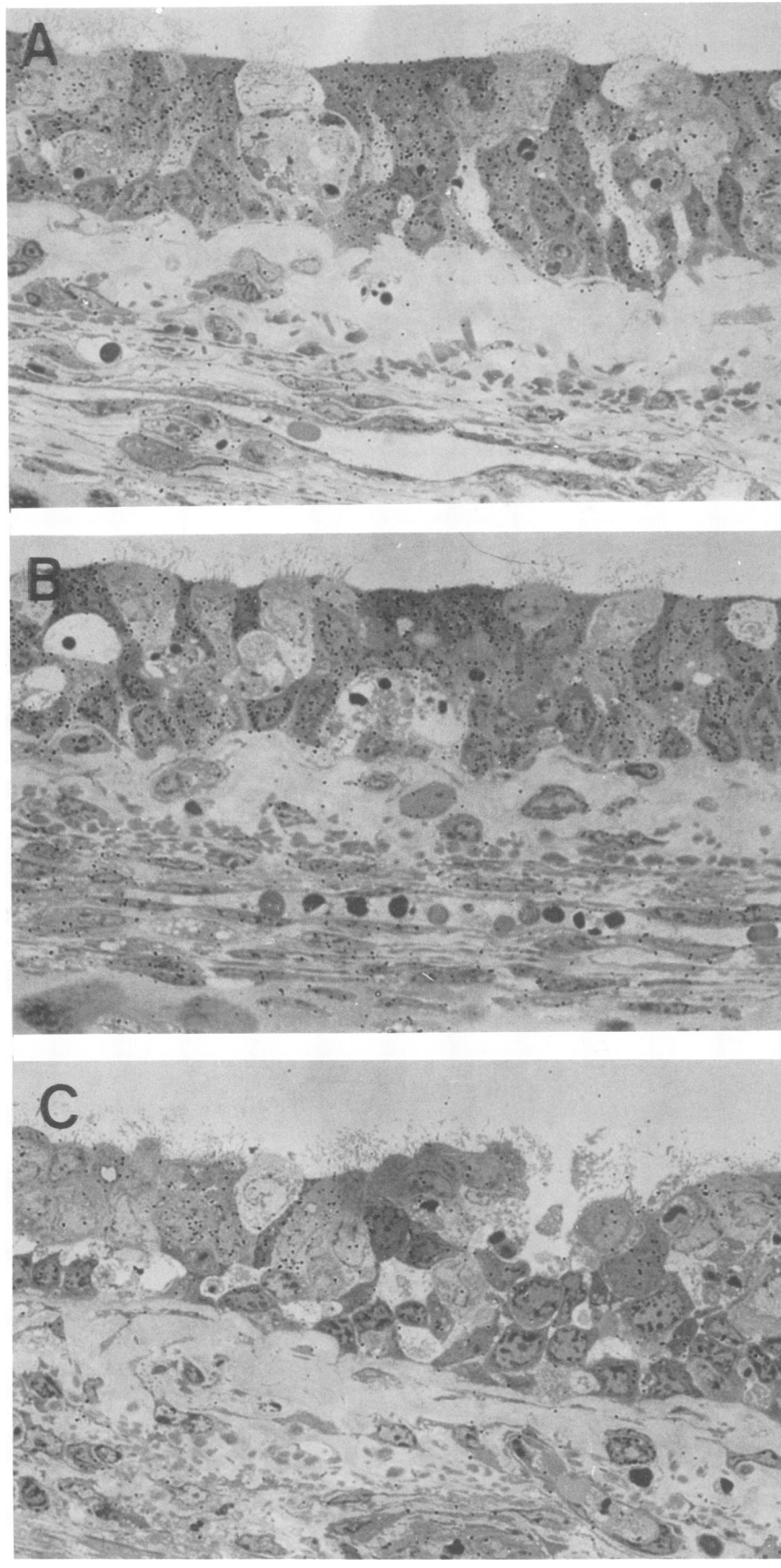

FIG. 2. *A–C*

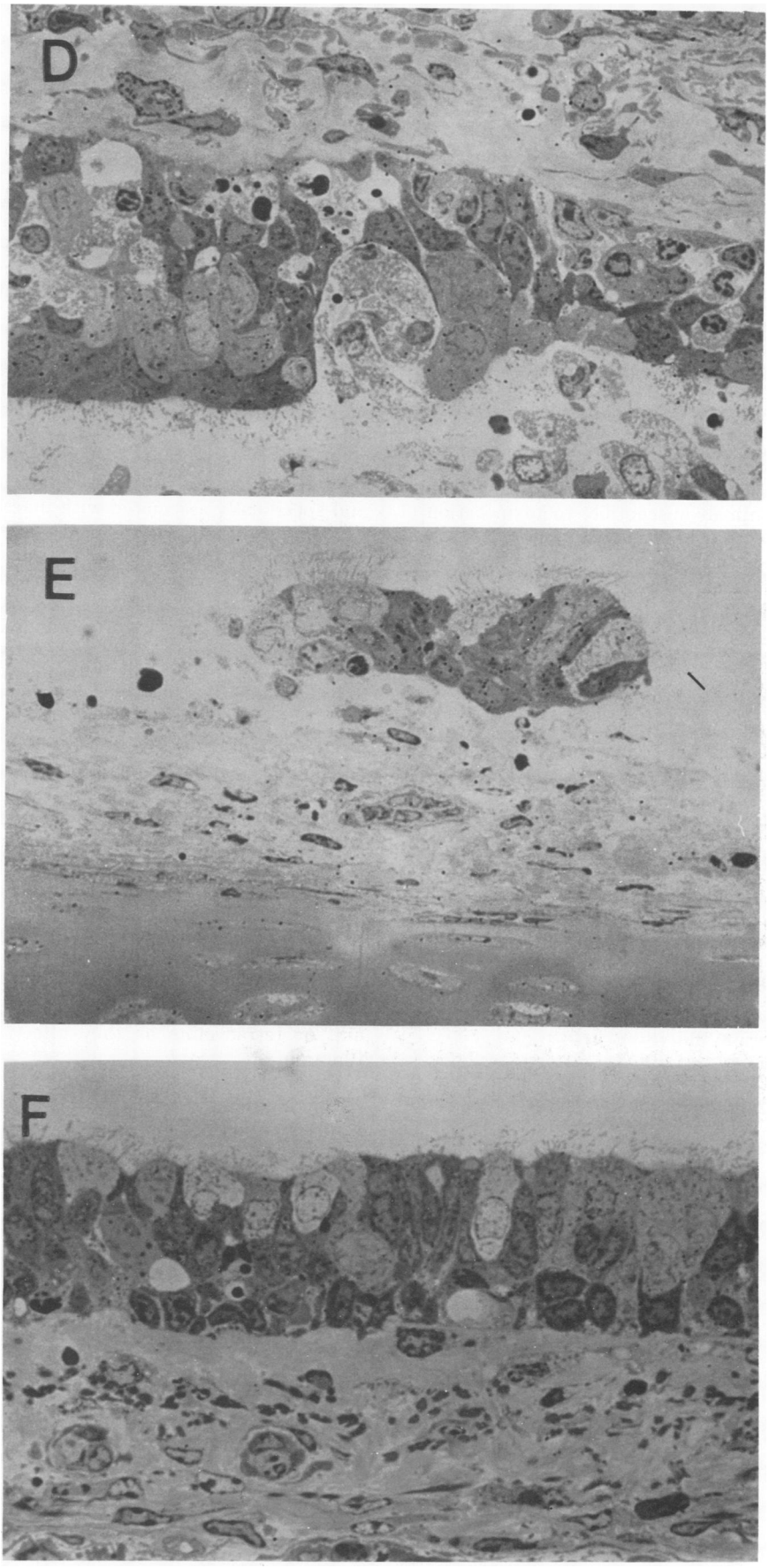

FIG. 2. *D–F*

demonstrate whether general inhibition of protein synthesis resulted in histological changes or whether cellular changes specifically accompanied the cell-toxin interaction. Although inhibition of protein synthesis followed exposure of tracheal rings to selected drugs (Table 1), histological changes were much reduced when compared to toxin-treated cultures (Fig. 2F). Some small necrotic foci could be observed in rings incubated for 24 h with tetracycline (150 μg/ml) (data not shown); however, in general, the ciliated epithelium remained intact.

DISCUSSION

It is well known that tissue pathology is common in human diphtheria and in experimental animals susceptible to toxin action (1, 16). A search of the literature, however, reveals no detailed studies which correlate tissue sensitivity to toxin with the development of histologic abnormalities. Such a study might help to explain the highly toxic nature of small levels of toxin in severe or fatal diphtheria.

After exposure of guinea pig tracheal organ cultures to diphtheria toxin, cytopathology accompanied inhibition of protein synthesis. When the rate of protein synthesis declined by about 50% within 8 h, obvious damage had already occurred in the epithelium. Further destruction of host cell integrity was apparent after longer incubation with toxin. It should be noted that particular cells on the surface and within the epithelial layer continued to incorporate radiolabel during prolonged incubation with toxin. This selective resistance within the cell population to inhibition of protein synthesis could be due to inaccessible receptor sites for toxin binding or to the lack of specific attachment sites on individual cells.

TABLE 1. *Effect of various drugs on inhibition of protein synthesis in guinea pig tracheal organ culture*

Inhibitor	Counts/min per ring[a]	
	Incubation (h)	
	8	24
None	2,783	2,652
Diphtheria toxin (1 Lf/ml)	1,353	493
Puromycin (150 μg/ml)	573	190
Cycloheximide (150 μg/ml)	483	330
Tetracycline (150 μg/ml)	672	523

[a] Tracheal rings were radiolabeled with 1 μCi of ^{3}H-labeled amino acids during the last 45 min of the incubation. The reported values are corrected for background and represent the average of duplicate samples from three separate experiments.

The finding that diphtheria toxin has a much increased cytotoxic effect on tracheal cells in contrast to other inhibitors of protein synthesis is of interest. It is known that intact diphtheria toxin is comprised of two components, fragments A and B covalently linked by a disulfide bond (9). As an intact molecule, toxin remains enzymically inactive. After treatment by proteases in the presence of sulfhydryl-reducing agents, the toxin molecule becomes activated with fragment A possessing all enzymic activity for inactivation of EF2 (11). Fragment B is required for attachment of toxin to cell membrane receptors, thus allowing entry of fragment A into the cytoplasm (15). Clearly, inhibition of protein synthesis in intoxicated cells is initially and specifically associated with inactivation of EF2. But why should toxin at rather minute concentrations bring about histological changes in tracheal cells when compared to other inhibitors of protein synthesis? Considering the unique nature of the toxin molecule as well as its mode of action, the difference between toxin and other drugs might be reflected in the mechanism by which sensitive cells respond to toxin, for example by activation of the molecule at the membrane surface, or association of fragment B with cell membrane receptors, or entrance of fragment A within the cell. Thus, activation of the intact toxin molecule (molecular weight, 62,000) at the membrane surface of a susceptible cell may play a distinct and separable role from that of inactivation of EF2 in the development of diphtheria pathogenesis. Furthermore, inactivation of EF2 by toxin may set off a series of cellular events such as the activation of proteases, nucleases, and lysosomes not elicited by cycloheximide, puromycin, or tetracycline as they affect ribosomal function.

Strauss had earlier suggested that the loss of ^{32}P by intoxicated HeLa cell monolayers might result from cell membrane damage by toxin (14). It should be pointed out, however, that sensitive HeLa cells growing in suspension and chick fibroblasts in confluent monolayers do not develop obvious, extensive cytopathology during similar incubations with toxin although protein synthesis is severely inhibited (unpublished data). Nevertheless, errors in diphtheria immunization in which toxin was administered subcutaneously to children resulted in the development of necrosis and pseudomembrane formation in the nasopharyngeal region (16), a most characteristic sign of classical diphtheria intoxication. These observations might be explained by selective tissue affinity and sensitiv-

ity to toxin and point out the relevance of using differentiated target cells such as the respiratory epithelium for studies of respiratory disease pathogenesis.

The mechanism by which diphtheria toxin leads to histopathology and death in humans and experimental animals is still uncertain, especially in light of recent work by Bonventre in which chronic diphtheritic toxemia in guinea pigs resulted in little protein synthesis impairment (4). In this case, guinea pigs were inoculated intraperitoneally with sublethal doses of toxin and tissues were monitored in vivo for the ability to incorporate [^{3}H]leucine into protein 24 h before death. It is difficult to evaluate these data when one considers the natural course of diphtheria intoxication which requires the location of toxinogenic *Corynebacterium diphtheriae* organisms in the pharyngeal region. Under these conditions, the bacteria are not invasive and toxin diffuses into surrounding tissue. It may be that exposure of trachea in organ culture to toxin is more representative of natural conditions of infection than methodology previously described (3, 5, 6). It does seem certain that inactivation of EF2 coupled with the apparent cytotoxicity of the toxin molecule could readily destroy specialized cells required for tissue maintenance or for the synthesis of critical regulatory factors necessary for normal host function, resulting in injury or death to the susceptible host.

ACKNOWLEDGMENTS

We thank W. A. Clyde for valuable advice and M. Wilson for technical assistance.

This work was supported by the Medical and Research Command, Department of the Army, under research contract DADA-17-73-C-3097.

LITERATURE CITED

1. Andrewes, F. W., W. Bullock, S. R. Douglas, G. Greyer, A. G. Gardner, P. Fildes, J. C. G. Ledingham, and C. G. L. Wolf. 1923. Diphtheria, its bacteriology, pathology and immunology. Medical Research Council, London.
2. Barski, G., R. Nourilsky, and F. Cornefert. 1957. Resistance of respiratory ciliated epithelium to action of polio and adeno viruses *in vitro*. Proc. Soc. Exp. Biol. Med. **96**:386-391.
3. Baseman, J. B., A. M. Pappenheimer, Jr., D. M. Gill, and A. A. Harper. 1970. Action of diphtheria toxin in the guinea pig. J. Exp. Med. **132**:1138-1152.
4. Bonventre, P. F. 1973. Studies on the mode of action of diphtheria toxin. V. Protein metabolism in a guinea pig model simulating chronic diphtheritic toxemia. Infect. Immunity **7**:556-560.
5. Bonventre, P. F., and J. G. Imhoff. 1966. Studies on the mode of action of diphtheria toxin. I. Protein synthesis in guinea pig tissues. J. Exp. Med. **124**:1107-1121.
6. Bonventre, P. F., and C. B. Saelinger. 1972. Inhibition of protein synthesis after intravenous or intramuscular challenge with diphtheria toxin. Infect. Immunity **6**:418-421.
7. Collier, A. M., and J. B. Baseman. 1973. Organ culture techniques with mycoplasmas. Ann. N. Y. Acad. Sci. **225**:277-289.
8. Collier, R. J. 1967. Effect of diphtheria toxin on protein synthesis: inactivation of one of the transfer factors. J. Mol. Biol. **25**:83-98.
9. Gill, D. M., and L. L. Dinius. 1971. Observations on the structure of diphtheria toxin. J. Biol. Chem. **246**:1485-1491.
10. Gill, D. M., A. M. Pappenheimer, Jr., and J. B. Baseman. 1969. Studies on transferase II using diphtheria toxin. Cold Spring Harbor Symp. Quant. Biol. **34**:595-602.
11. Gill, D. M., and A. M. Pappenheimer, Jr. 1971. Structure-activity relationships in diphtheria toxin. J. Biol. Chem. **246**:1492-1495.
12. Gill, D. M., A. M. Pappenheimer, Jr., and T. Uchida. 1973. Diphtheria toxin, protein synthesis and the cell. Fed. Proc. **32**:1508-1515.
13. Moehring, T. J., J. M. Moehring, R. J. Kuchler, and M. Solotorofsky. 1967. The response of cultured mammalian cells to diphtheria toxin. I. Amino acid transport, accumulation, and incorporation in normal and intoxicated sensitive cells. J. Exp. Med. **126**:407-422.
14. Strauss, N. 1960. The effect of diphtheria toxin on the metabolism of Hela cells. II. Effect on nucleic acid metabolism. J. Exp. Med. **112**:350-359.
15. Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper. 1973. Diphtheria toxin and related proteins. II. Kinetic studies on intoxication of Hela cells by diphtheria toxin and related proteins. J. Biol. Chem. **248**:3845-3850.
16. Wilson, G. S. 1967. The hazards of immunization. The Athlone Press, University of London, London.