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Serum Antibody Prevents Lethal Murine Influenza Pneumonitis But Not Tracheitis

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This paper reports studies showing the effects of serum antibody upon influenza infection at two different sites: the trachea and lung. Tracheal desquamation, pulmonary consolidation, death, and virus shedding were examined after infection of mice with a lethal A/Port Chalmers/1/73 (H3N2) influenza virus. Immune serum administered intraperitoneally before infection prevented death and pulmonary consolidation and also significantly lowered lung virus shedding as compared with controls receiving normal serum. However, this protection did not extend to the ciliated epithelium of the trachea because serum antibody did not prevent desquamation of the trachea or significantly decrease viral yield from the trachea. These results indicate that serum antibody is protective against severe pulmonary parenchymal disease but not for disease of the ciliated epithelium.

Experiments with animal models have given conflicting answers as to the role of serum antibody in protection against influenza. Loosli et al. (8), Schulman et al. (13), and Virelizier et al. (21) have shown protection against lethal influenza pneumonitis in mice with passively administered serum antibody. Small et al. (18) have shown that serum antibody does not protect ferrets from influenza infection of the upper respiratory tract. These apparently contradictory results suggest either that ferrets differ from mice in their host defense mechanisms against influenza or that the upper respiratory tract host defense mechanisms differ from those of the lung parenchyma. Although mice have been studied almost exclusively from the point of view of the lethal pneumonitis, they manifest tracheitis and bronchitis (11). In this report we show that passively administered serum antibody decreases both lung virus and lung consolidation and is life saving but has no significant protective effect on the ciliated epithelium of the trachea.

MATERIALS AND METHODS

Animals. Five- to six-week-old male, inbred Swiss white mice of the A/J strain were obtained from Jackson Laboratories, Bar Harbor, Maine. They were used for the experiments at 14 to 15 weeks of age. Mature ferrets were obtained from Marshall Research Animals, Inc., North Rose, N. Y. Mice and ferrets were maintained by the animal care facilities, University of Florida, until used in the experiments.

Virus. An egg grown, nonlethal strain of A/Port Chalmers/1/73 (H3N2) influenza virus was mouse adapted by passing twice in mice. The lung homogenate was used to infect a group of 5- to 6-week-old mice intranasally under light ether anesthesia. This virus suspension contained 10^4 50% egg infectious doses in 0.05 ml. This group of mice was used at 14 to 15 weeks of age as convalescent mice. The virus used in challenge studies was the same egg-grown strain of A/Port Chalmers/1/73 (H3N2) influenza virus passaged in A/J Swiss white mice six times to produce a strain lethal for this mouse in 8 to 10 days. The virus pool contained $10^{5.5}$ EID_{50} per ml. A 0.05-ml amount of this pool, contained 10^1 50% mouse lethal doses and 10^3 50% mouse infectious doses when inoculated intranasally into 12-week-old mice under light ether anesthesia. The virus pool was stored at −70°C in 1-ml portions.

Serum pools. Ferrets which were infected 6 months previously with the original egg-grown A/Port Chalmers/1/73 (H3N2) virus were vaccinated intramuscularly with a killed whole virus homologous vaccine and bled 1 week later for immune serum (IS). Normal serum (NS) was obtained from a group of virgin ferrets. Sera were heat inactivated and filter sterilized with 0.45-μm filters (Millipore Corp., Bedford, Mass.) before use. A volume of 1.25 ml of serum contained 512 hemagglutination inhibiting units (HAI) and could be given intraperitoneally in two divided doses over 2 days without death of any of the mice. Serum antibody levels achieved 24 h after the second dose of IS ranged from 32 to 256 HAI with a mean of 55 HAI. The antisera also undoubtedly contained antineuraminidase antibody. However, this was not measured since protection has been found to correlate best with HAI antibody (20).

Virus isolation. The tracheobronchial tree was removed in toto, and the trachea was separated from the lungs at the carina. A 1-mm segment was removed from the lower end of the trachea and was used for viral isolation; the remainder was used for scanning

electron microscope studies. The lungs and tracheal segments were homogenized separately in tissue grinders with 1 ml of L-15 medium (Microbiological Associates, Walkersville, Md.) containing antibiotics and stored at −70°C. Samples were assayed for virus by injecting 0.1 ml of 10-fold dilutions into 10-day-old embryonated eggs. Virus titers were calculated by the Reed-Muench method (12) and expressed as 50% egg infectious doses per milliliter (i.e., per 1 mm of trachea or per pair of lungs).

Serological testing. Sera were prepared by kaolin and chicken erythrocyte adsorption and heated at 56°C for 30 min before HAI assay by the microtitration method (14).

Pathology. Lung pathology was assessed by gross examination of the lung for hemorrhagic consolidation and tracheal desquamation was observed by scanning electron microscopy. Scoring of lesions was done on a 0 to 4 basis representing percentages of lung and tracheal surface involved (see footnote to Table 1).

Scanning electron microscopy. Tracheas for scanning electron microscopy studies were placed in a buffered fixative composed of 2.5% glutaraldehyde, 0.1 M sodium cacodylate, and 0.1% $CaCl_2$ (pH 7.4) and allowed to fix for at least 24 h before further preparation. The tracheas were then removed from the fixative and bisected longitudinally, and one part was dehydrated in graded concentrations of acetone (70 to 100%). Specimens were critical point dried in a Bomar SPC 900/Ex critical point drying machine (Bomar Corp., Tacoma, Wash.) coated with gold-palladium in a Hummer II shadowing machine (Technics, Alexandria, Va.) and examined with a Novascan 30 electron microscope (Semco, Ottawa, Canada).

Statistical analysis. Viral and antibody titers were compared by the *t* test (9), and mortality was compared by the Fisher exact test (17).

Experimental protocol. Three groups of animals designated convalescent, NS and IS were studied as outlined in Fig. 1. Convalescent animals were intranasally infected 2 months previously with the mouse adapted A/Port Chalmers/1/73 (H3N2) nonlethal strain and had HAI titers ranging from 32 to 128 HAI with a mean of 91 HAI. They did not receive further treatment. The NS and IS groups received 0.75 ml of NS and IS, respectively, at day −2 and 0.5 ml at day −1. The NS animals had HAI titers of less than 8 HAI, whereas the IS group mean titer was 55 HAI. On day 0 all animals in the three groups received 0.05 ml of the challenge virus intranasally after light ether anesthesia. At the times indicated on the protocol, animals were sacrificed by orbital plexus exsanguination, after sodium pentobarbital anesthesia (Nembutal, Abbott Laboratories) (0.06 mg/g intraperitoneally), and the tracheobronchial tree was removed. The remaining animals were observed for up to 2 weeks for mortality.

RESULTS

Table 1 is a compilation of the results obtained with samples from three groups of animals sacrificed at 3, 5, and 7 days postinfection. The convalescent animals were solidly immune to reinfection on the basis of lack of detectable lung and tracheal virus, and absence of gross lung lesions, tracheal desquamation, and mortality. Hence, it seemed that the viral inoculum was not so excessive that it overwhelmed the immune host defenses of the convalescent animals. In Table 1 when comparing the NS group and IS group, major differences were observed. Lung virus titers were between 100- and 1,000-fold less

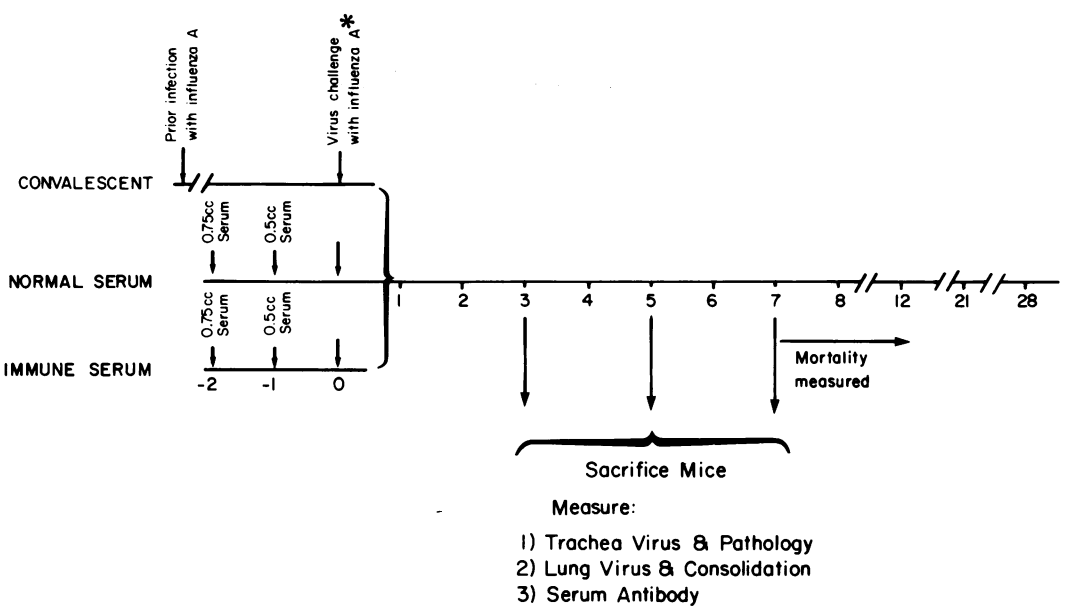

FIG. 1. *Experimental protocol. *, Sixth-passage mouse-adapted virus shown to be 100% lethal for A/J mice.*

TABLE 1. *Effects of administered serum antibody on viral titers, lung disease, and tracheal disease*[a]

Treatment groups	No. of animals	Lung virus titer ($EID_{50/lung}$)[b]	Lung score consolidation[c]	Tracheal virus titer ($EID_{50/mm}$)	Tracheal score desquamation[c]
Day 3					
NS	5	$10^{6.1 \pm 0.4}$ [d]	1.4	$10^{2.4 \pm 0.6}$ [e]	4
IS	5	$10^{3.4 \pm 0.9}$ [d]	0	$10^{1.8 \pm 1.0}$ [e]	2
Conval	4	<1	0	<1	0
Day 5					
NS	ND[f]	ND	ND	ND	ND
IS	5	$10^{3.8 \pm 0.9}$	0	$10^{2.2 \pm 01.7}$	4
Conval	ND	ND	ND	ND	ND
Day 7					
NS	5	$10^{5.3 \pm 0.9}$ [d]	3.2	$10^{2.1 \pm 01.7}$ [e]	Regeneration
IS	5	$10^{2.1 \pm 1.6}$ [d]	0	$10^{1.0 \pm 1.4}$ [e]	Regeneration
Conval	4	<1	0	<1	0

[a] Mortality days 8 to 14: NS, 8/8; IS, 0/8, Conval, 0/8 ($P = 0.0008$ Fisher's exact test).
[b] EID_{50}, 50% egg infectious doses.
[c] 0, None; 1 = 5 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; 4 = 75 to 100%.
[d] $P < 0.005$ (t test).
[e] P not significant.
[f] ND, Not done.

at both days 3 and 7 in the IS-treated group ($P < 0.005$). Lung consolidation was present in the NS-treated group on day 3 and involved over 75% of the lung on day 7, but was absent in the IS-treated group on both these days, as well as on day 5. All animals treated with NS died between days 8 and 10, whereas none of the IS-treated or convalescent animals died ($P = 0.0008$). Hence, serum antibody prevented death from pneumonitis and modified lung disease as evidenced by reduced pulmonary virus titers and the absence of gross consolidation in the IS-treated group.

The effects of serum antibody on tracheal mucosal infection (Table 1) were quite different from those observed on the lung. Viral titers were not significantly different in the NS- and IS-treated groups on days 3 or 7. Not only did serum antibody not prevent tracheal infection (as evidenced by recovery of virus), but it did not prevent disease as noted by scanning electron microscope examination.

Scanning electron micrographs, representative of the changes observed in the 33 animals sacrificed, are shown in Fig. 2A through F. Figure 2A is representative of the tracheas of the convalescent animals after challenge with the sixth passage virus. There was no desquamation on either day 3 or 7, and this picture is indistinguishable from that seen in uninfected male Swiss white A/J mice (data not shown). This absence of desquamation is consistent with the lack of virus in the tracheas and lungs of these animals. Figures 2B and C are representative micrographs from animals treated with NS and killed at 3 and 7 days, respectively. There is complete desquamation of the mucosa by day 3 (Fig. 2B), and regeneration is ongoing by day 7 (Fig. 2C). The micrograph on day 7 demonstrates regenerating ciliated and serous cells as well as a bronchial gland opening. These results are indistinguishable from what is seen in normal untreated mice infected with a nonlethal influenza virus (Ramphal et al., manuscript in preparation); namely, between 2 and 3 days postinfection, there is complete desquamation of ciliated and nonciliated cells with exposure of the basal layer and initiation of regeneration by day 7 with almost complete regeneration by 2 weeks.

In contrast to the uniform changes observed in the NS group, a spectrum of changes was noted in the IS group. On day 3 the tracheas of all the animals were desquamated. The degree of desquamation varied from about 50% (Fig. 2D) to almost 100%. On day 5 the IS group showed complete desquamation of the trachea with early differentiation of the basal cells (Fig. 2E). The changes observed on day 5 were similar in all five animals. On day 7 postinfection four of five IS animals had changes which were similar to those seen in NS animals, i.e., desquamation had occurred and regeneration had begun (Fig. 2F). One animal (not shown) had a desquamated trachea which had not begun to regenerate at this time. In summary, animals treated with NS behaved like virgin animals demonstrating complete desquamation by day 3 and regeneration ongoing at day 7, whereas IS-treated animals showed variability and delay in the time course of desquamation, but did des-

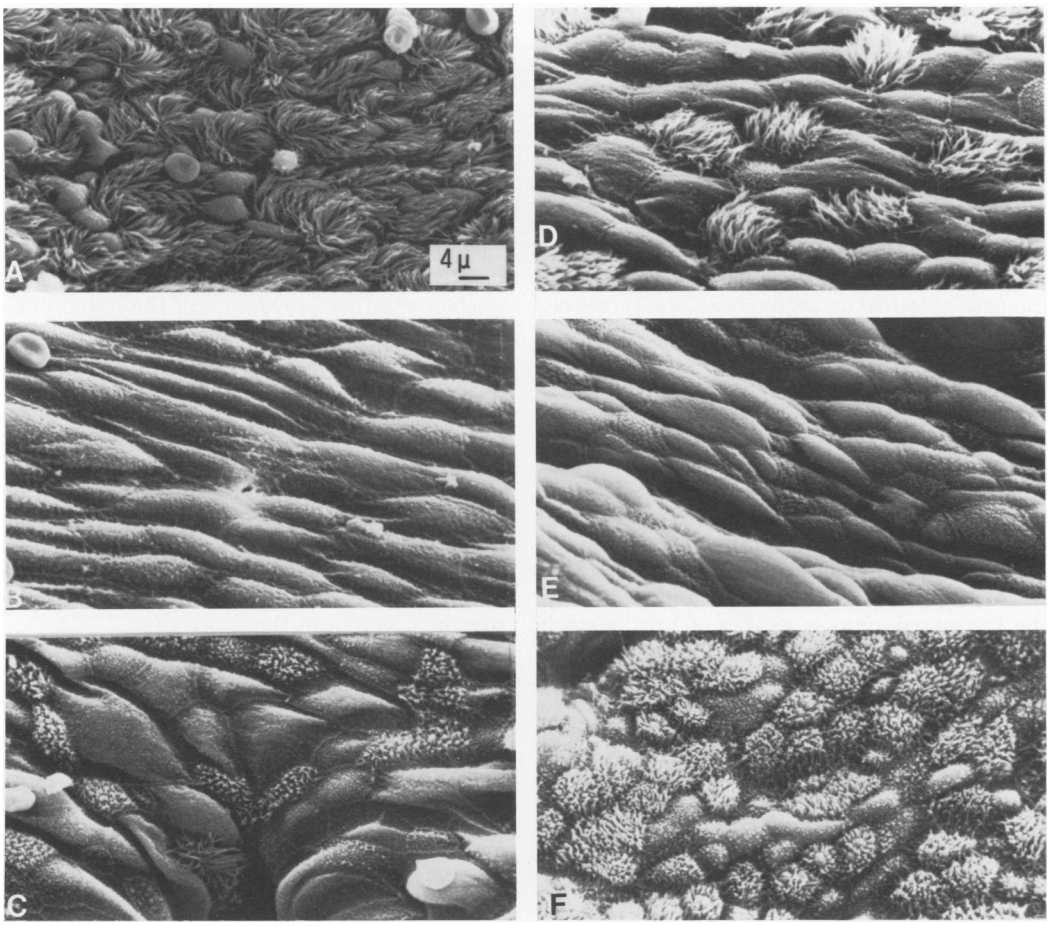

FIG. 2. *(A) Tracheal surface of a convalescent animal 3 or 7 days after viral challenge. The mucosa is intact. A few erythrocytes are seen on the surface. (B) Tracheal surface of a NS-treated animal 3 days after viral challenge. Both ciliated and serous cells have been desquamated, exposing the basal layer of cells. (C) Tracheal surface of a NS-treated animal 7 days after viral challenge. Desquamation has occurred, and regeneration is evident. Young ciliated and nonciliated cells are seen. The opening to a submucosal gland is also visible. (D) Tracheal surface of an IS-treated animal 3 days after viral challenge. There is loss of most ciliated cells. (E) Tracheal surface of an IS-treated animal 5 days after viral challenge. Complete desquamation of ciliated cells has occurred. The basal layer is regenerating; there are discernible differences between cell types as early as this day. (F) Tracheal surface of an IS-treated animal 7 days after viral challenge. Desquamation has occurred, and far advanced regeneration is visible.*

quamate entirely at some time between day 3 and 5.

DISCUSSION

Previous studies have shown protection of mice by pretreatment with serum antibody (8, 13, 21), but have not separated disease of the ciliated epithelium from disease at the alveolar level. Influenza in humans and ferrets is primarily a disease of the ciliated epithelium, although fulminant alveolar disease is seen from time to time (10, 15). The lesions of the ciliated epithelium described for the trachea of humans (23) and the nose of the ferret (4) have been seen in the mouse (6, 11); therefore, from the pathological point of view, there are no essential differences in the disease of the ciliated epithelium in these three species. By observing both the tracheal and alveolar lesions in the mouse, we have shown that the protective role of serum antibody is confined to the lung and that the serum antibody alone does not prevent desquamation of ciliated epithelium of the murine trachea. Antibody titers of convalescent animals were not

significantly different from the IS-treated group; however, the convalescent animals were protected from both tracheitis and pneumonitis, indicating that some factor other than serum antibody prevented infection. Serum antibody titers as low as 32 HAI were protective for the lung, whereas even high titers of 256 HAI failed to prevent tracheal disease. Serum antibody seemed to limit viral multiplication in the lung but had no significant effect on tracheal virus titers. These observations are consistent with the data obtained on antibody classes recovered from different levels of the respiratory tree. Immunoglobulin G was the predominant immunoglobulin class in the most distal regions of the respiratory tree and concentrations decreased as sampling was done in more proximal regions (7, 22). Therefore, under normal conditions, before any inflammatory response has taken place, there would be little immunoglobulin G on the ciliated epithelium.

However, serum antibody may have modified tracheal disease in some ways; for example, in the IS-treated animals a delay of 1 or 2 days in the desquamating process was noted, and regeneration was possibly more rapid, as inferred from the maturity of the cells noted on day 7. If the alveolar cell is the site of initial viral infection and multiplication, as postulated by some workers (1), and the infection then ascends, serum antibody could conceivably have delayed tracheal infection by an effect on virus multiplication in the lungs. We have no explanation for the morphological suggestion of more rapid regeneration in some IS-treated animals. If serum antibody was neutralizing virus and was responsible for the rapid regeneration, then a difference in tracheal virus titers between the IS and NS groups would have been expected. No significant difference was found.

Whether serum antibody plays a protective role against influenza infections in humans has been a subject of debate. Although data supporting such a protective role have been reported (5), these data correlate serum antibody with protection but do not demonstrate a cause and effect relationship. We suspect that humans are similar to mice in that the lung is protected by serum antibody but the ciliated epithelium is not. Since serum antibody seems to prevent the lethal pneumonitis in the mouse, it can be inferred that measures designed to maximize serum antibody, i.e., parenteral vaccination, will protect against the lethal pneumonitis, and this has been amply shown in both mice (3) and ferrets (16). However, the situation with regard to protection of ciliated epithelium by parenteral vaccination may be different. Studies in the ferret have shown that systemic factors are irrelevant to protection from nasal influenza infections (2); other studies have shown a lack of protection against nasal infections in ferrets after parenteral vaccination, although the lungs were protected (16, 19). We suspect from this study on serum antibody that parenteral vaccination would protect mouse lung but not the trachea.

In conclusion, our studies suggest that serum antibody does not prevent influenza infection of the ciliated respiratory epithelium of mice but does prevent the lethal pulmonary disease. It is unclear whether prevention of the lethal pneumonitis is a result of prevention of alveolar infection or promotion of recovery, because virus obtained from the lung may have come from intrapulmonary airways. Recovery of the tracheal surface seemed to be enhanced by the presence of serum antibody in some animals. We suggest that studies which examine the pathogenesis, immunity, and recovery from influenza need to address the disease of the ciliated epithelium and lung separately, because the pathogenetic, protective, and recovery mechanisms may be different at these two sites.

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LITERATURE CITED

1. **Albrecht, P., D. Blaskovic, B. Styk, and M. Koller.** 1963. Course of A2 influenza in intranasally infected mice examined by the fluorescent antibody technique. Acta Virol. **7**:405–413.
2. **Barber, W. H., and P. A. Small, Jr.** 1978. Local and systemic immunity to influenza infection in ferrets. Infect. Immun. **21**:221–228.
3. **Fazekas de St. Groth, S., and M. Donnelly.** 1950. Studies in experimental immunology of influenza. IV. The protective value of active immunization. Aust. J. Exp. Biol. Med. Sci. **28**:61–75.
4. **Francis, T., Jr., and C. H. Stuart-Harris.** 1938. Studies on the nasal histology of epidemic influenza virus infection in the ferret. J. Exp. Med. **68**:789–802.
5. **Hoskins, T. W., J. R. Davies, A. Allchin, C. L. Miller, and T. M. Pollock.** 1973. Controlled trial of inactivated influenza vaccine containing the A/Hong Kong strain during an outbreak of influenza due to the A/England/42/72 strain. Lancet **ii**:116–120.
6. **Iida, T., and F. B. Bang.** 1963. Infection of the upper respiratory tract of mice with influenza A virus. Am. J. Hyg. **77**:169–176.
7. **Kaltreider, H. B., and M. K. L. Chan.** 1976. The class specific immunoglobulin composition of fluids obtained from various levels of the canine respiratory tract. J. Immunol. **116**:423–429.
8. **Loosli, C. G., D. Hamre, and B. S. Berlin.** 1953. Airborne influenza virus A infections in immunized animals. Trans. Assoc. Am. Phys. **66**:222–230.
9. **Mendenhall, W.** 1975. Introduction to probability and statistics, p. 214. Duxbury Press, North Scituate, Mass.
10. **Mulder, J., and J. F. Ph. Hers.** 1972. Influenza. Wolters-Noordhoff Publishing, Groningen, The Netherlands.

11. **Nelson, A. A., and J. W. Oliphant.** 1939. Histopathological changes in mice inoculated with influenza virus. Public Health Rep. **54**:2044-2054.
12. **Reed, L. J., and H. Muench.** 1938. A simple method for estimating 50% end point. Am. J. Hyg. **27**:493-497.
13. **Schulman, J. L., M. Khakpour, and E. D. Kilbourne.** 1968. Protective effects of specific immunity to viral neuraminidase on influenza virus infection of mice. J. Virol. **2**:778-786.
14. **Sever, J. L.** 1962. Application of a microtechnique to viral serological investigations. J. Immunol. **88**:320-329.
15. **Shope, R. E.** 1934. The infection of ferrets with swine influenza virus. J. Exp. Med. **60**:49-61.
16. **Shope, R. E.** 1936. Immunization experiments with swine influenza virus. J. Exp. Med. **64**:47-61.
17. **Siegel, S.** 1956. Nonparametric statistics, p. 96. McGraw-Hill Book Co., New York.
18. **Small, P. A., Jr., R. H. Waldman, J. C. Bruno, and G. E. Gifford.** 1976. Influenza infection in ferrets: role of serum antibody in protection and recovery. Infect. Immun. **13**:417-424.
19. **Smith, W., C. H. Andrewes, and P. P. Laidlaw.** 1935. Influenza: experiments on the immunization of ferrets and mice. Br. J. Exp. Pathol. **16**:291-302.
20. **Stuart-Harris, C. H., and G. C. Schild.** 1976. Influenza—the virus and the disease, p. 150. Publishing Sciences Group, Inc., Littleton, Mass.
21. **Virelizier, J. L.** 1975. Host defenses against influenza virus: the role of antihemagglutinin antibody. J. Immunol. **115**:434-439.
22. **Waldman, R. H., P. F. Jurgensen, G. N. Olsen, R. Ganguly, and J. E. Johnson III.** 1973. Immune response of the human respiratory tract. I. Immunoglobulin levels and influenza virus vaccine response. J. Immunol. **111**:38-41.
23. **Walsh, J. J., L. F. Dietlein, F. N. Low, G. E. Burch, and W. J. Mogabgab.** 1961. Bronchotracheal response in human influenza. Arch. Intern. Med. **108**:376-388.