INFECTION AND IMMUNITY, Sept. 1972, p. 398-402
Copyright © 1972 American Society for Microbiology
Vol. 6, No. 3
Printed in U.S.A.

Murine Resistance to Inhaled *Neisseria meningitidis* After Infection with an Encephalomyocarditis Virus

E. GOLDSTEIN, W. C. BUHLES, T. G. AKERS, AND N. VEDROS

Section of Infectious and Immunologic Diseases, Department of Medicine, University of California, Davis, California 95616, and the Naval Biomedical Research Laboratory, University of California, Berkeley, California 94720

Received for publication 20 March 1972

A reduction in pulmonary anti-bacterial activity due to a preceding viral illness has been suggested as the mechanism responsible for some meningococcal infections of the lung. We investigated this proposed pathogenesis by infecting mice with airborne encephalomyocarditis virus (EMC) and then challenging them 1, 4, and 7 days later with aerosols of *Neisseria meningitidis*. Meningococcal clearance was assessed by comparing the numbers of bacteria present immediately after inhaling the aerosols with the numbers present 3 hr later. To insure that EMC virus adequately depressed murine defense mechanisms, we also determined staphylococcal killing rates at 4 hr by using radiophosphorus-labeled staphylococcal aerosols. Viral infection depressed murine pulmonary antimeningococcal activity at 1 and 4 days ($P < 0.01$) but not at 7 days. Intrapulmonary staphylococcal killing was impaired on day 4 ($P < 0.01$) but not on days 1 or 7. Pulmonary viral titers decreased rapidly from 10^7 to 10^3 plaque-forming units/ml of lung during the experimental period. According to these data viral disease transiently depresses resistance to meningococcal infection. This impairment in host resistance is present while the viral titer is decreasing and follows a relatively similar pattern to the transient decrease noted for staphylococci.

The significance of *Neisseria meningitidis* as a respiratory pathogen has recently been rediscovered (26, 27; J. A. Reinarz et al., Abstr. Intersci. Conf. Antimicrob. Ag. Chemother., 11th, p. 35, 1971). It has been suggested that some of these infections were due to a preceding viral illness (6, 26, 27). According to this hypothesis, pulmonary defense systems which ordinarily restrict the spread of *N. meningitidis* became impaired because of a viral infection and allowed the development of pneumonia. The present studies were designed to test this hypothesis in a murine model permitting alterations in pulmonary resistance to infection that corresponded to those which occurred in man (11, 12, 15, 18). Mice infected with airborne encephalomyocarditis virus (EMC) were challenged 1, 4, and 7 days later with aerosolized *N. meningitidis*. Separate groups of viral-infected mice were simultaneously challenged with aerosols of radiophosphorus-labeled staphylococci to confirm the ability of EMC virus to depress murine defense mechanisms.

MATERIALS AND METHODS

Female NAMRU mice weighing 16 to 20 g were used in all experiments (8). Animals were housed in plastic cages and fed mouse pellets and water ad libitum.

Microorganisms. Pools of mengovirus 37A (M-37A), an avirulent thermostable mengovirus mutant (5) of the EMC group of viruses, were prepared in L cells (*l*-929) and assayed as previously reported (1).

N. meningitidis, group C, strain SD 3C from the Neisseria Repository of the Naval Biomedical Research Laboratory at the University of California, Berkeley, was cultured in flasks containing 100 ml of Mueller-Hinton broth at 37 C for 4 hr. This strain of *N. meningitidis* was originally isolated from the cerebrospinal fluid of a patient with meningitis. The strain was used prior to its third passage from this source. When mixed with mucin and injected intraperitoneally in mice, the median lethal dose (LD_{50}) for this strain is 20 bacterial cells. Three milliliters of antifoam B from the Dow corning Corp. was added to 30 ml of the Mueller-Hinton culture. Twenty-nine milliliters of this suspension was used for aerosoliza-

tion and 1 ml was used to determine the bacterial concentration.

Staphylococcus aureus strain 566 was cultured and labeled with phosphorus-32 (^{32}P) by the method of Green and Kass (16). In this procedure, the test bacteria are inoculated into 25 ml of a phosphorus-free culture media containing 1.0 mCi of ^{32}P. After 16 hr of growth at 37 C in a shaker water bath, the labeled cells are removed by centrifugation, washed, and resuspended in 8.0 ml of saline. Five milliliters of this suspension was used for aerosolization.

Animal exposure. The aerosol apparatus and the experimental procedures used in the M-37A and meningococcal studies have been described (2, 21). One hundred and twenty mice were infected with aerosols of M-37A. One, 4, and 7 days after viral infection, infected and control mice were exposed to aerosols of *N. meningitidis*. Half of these animals were sacrificed by cervical dislocation immediately after exposure to the meningococcal aerosol, and the remainder at 3 hr later. The lungs of each mouse were removed aseptically and homogenized, and the bacterial concentration was determined by counting surface colonies on Mueller-Hinton chocolate agar.

Pulmonary bacterial clearance rates for *N. meningitidis* were determined by the previously described group mean clearance method (15). The mean bacterial count of each group of mice at each time period was calculated. Bacterial clearance was then expressed as the number of viable bacteria present immediately after the aerosol exposure (bacterial count 0 hr) minus the number present at three hours (bacterial count 3 hr) divided by the initial number of bacteria (bacterial count 0 hr):

$$\text{Percent bacterial clearance} = (\text{bacterial count 0 hr} - \text{bacterial count 3 hr}/\text{bacterial count 0 hr}) \times 100$$

These data were analyzed for significance of difference by the Theorem of Wilks (15).

Pulmonary bactericidal activity rates for *S. aureus* were also measured at 1, 4, and 7 days after viral infection by exposing groups of 10 viral-infected and 10 control mice to aerosols of radiolabeled (^{32}P) *S. aureus* in an aerosol apparatus designed to produce uniform inhalation dosages of infecting bacteria (24). Four hours after aerosol exposure, the animals were killed with ether, and measurements of pulmonary radioactivity and bacterial concentration were performed. Radioisotope concentrations were determined by liquid scintillation counting. Pour-plate methods were used for bacterial enumeration. To establish the number of bacteria represented by ^{32}P radioactivity, an Andersen sampler was attached to the aerosol chamber during the period of staphylococcal infection. Quantitative measurements of radioactivity and bacteria were made from a 1.0- to 2.0-μm sampling plate. A ratio of bacterial concentration to radioactivity was computed and designated the aerosol labeling ratio or aerosol constant (K). The mathematical expression of pulmonary bactericidal activity for an individual mouse could then be computed from the formula:

$$\text{Percent bactericidal activity} = (\text{bacterial count 4 hr}/{}^{32}P_{4\text{ hr}} \times K) \times 100$$

Percent bactericidal activity is the rate at which bacteria are killed within the lungs, where bacterial count 4 hr equals bacterial count at 4 hr and $^{32}P_{4\text{ hr}}$ equals radiophosphorus count at 4 hr (13). The analysis of variance method was used to test for significance of differences.

Pulmonary viral titers were determined in separate groups of animals at each time period using a plaque assay for M-37A (3). Lung samples were obtained at 1, 4, and 7 days after the viral infection from the various treatment groups for histological examination. The entire experiment was repeated twice.

The effect on murine antibacterial defense systems of the inhalation of tissue culture media was evaluated in a control series of experiments. 1, 4, and 7 days after exposure to aerosols of tissue culture media, bacterial killing rates for *N. meningitidis* and for *S. aureus* were determined in treated and control mice.

RESULTS

Infection with M-37A caused slight mortality and morbidity. Ninety-five percent or more of infected mice survived each of the three viral-infections. In each experimental series the viral infected and control mice showed a 3 to 4 g gain in weight during the 7-day experimental period.

Pulmonary viral titers decreased rapidly from an average of 10^7 plaque-forming units (PFU)/ml of lung homogenate at day 1, to 10^3 PFU/ml of lung homogenate at day 7. The values for the three experimental sets were combined and are presented in Table 1.

Table 2 summarizes the data for all of the meningococcal experiments at each of the time intervals studied. Meningococcal clearance rates were significantly decreased 1 and 4 days following viral infection ($P < 0.01$), and returned to normal at day 7. Equivalent numbers of bacteria were inhaled by infected and control mice at days 4 and 7. Fewer meningococci were inhaled by the infected mice on day 1, but the difference may not be significant ($P > 0.05$).

The data for the experiments in which the intrapulmonary rate of inactivation of *S. aureus* was determined are contained in Table 3. The values for the numbers of viable staphylococci present per milliliter of lung homogenate at 4 hr, the corresponding pulmonary radiophosphorus concentration, the aerosol constant, and the percent bactericidal activity are shown.

Staphylococcal bactericidal activity was decreased significantly at day 4 ($P < 0.01$). The small differences in bactericidal activity which

were present on days 1 and 7 were not significant ($P > 0.05$). The average radioactivity is an index of the numbers of bacteria deposited in the lungs (13). Although these concentrations tended to be less in the infected mice than the controls, the decreases were not statistically significant ($P > 0.05$).

Histological examination of pulmonary tissue specimens showed a mild degree of vascular hyperemia, interstitial edema and inflammation, and foci of peribronchial inflammation. Pneumonia and intra-alveolar edema were not found. The epithelial cells lining the bronchi were intact.

No difference in intrapulmonary bacterial clearance rates were found in the series of experiments in which mice inhaled tissue culture media. More than 95% of the inhaled meningococci were killed by the mice at 1, 4, and 7 days after exposure to the aerosol of tissue culture media, and similar meningococcal clearance rates were present in untreated controls. Equivalent staphylococcal bactericidal activity rates for the treated and control mice were also found (tissue culture day 1, 71.0 ± 4.1; day 4, 75.1 ± 3.6; day 7, 85.3 ± 1.7; untreated day 1, 82.6 ± 3.2; day 4, 73.1 ± 4.0; day 7, 82.4 ± 3.1).

DISCUSSION

The ability of mice to kill inhaled meningococci was decreased 1 and 4 days following a mild infection with an avirulent encephalomyocarditis virus. A similarly transient decrease in resistance to inhaled staphylococci was observed at 4 days. Since neither of these abnormalities was present in mice that inhaled tissue culture media, the impairment in bacterial resistance can be attributed to the infecting virus.

The finding that meningococcal clearance was decreased significantly on day 1, whereas staphylococcal clearance was not, may reflect either a quantitative difference in viral-induced impairment in host defense mechanisms between the two microorganisms or an experimental difference related to the methods used to measure murine antibacterial activity. The former is more likely as previous experiments with this model have shown that different bacterial species are killed at different rates (14, 17) and that inhibition of murine antibacterial activity can affect one bacterial species more than another (14, 17). Since, in most experiments, similar numbers of bacteria were inhaled by the viral-infected and control mice, the aforementioned differences in the rate of antibacterial activity cannot be attributed to variations in ventilation or distribution of inhaled microorganisms. The presence of the defect at day four when viral titers are decreasing rapidly, suggests either an earlier interference of virus with host function and delay in the expression of this underlying abnormality, or the development of a pathological process which continues to progress during the period of decreasing viral titers.

Similar observations have been reported in other previous investigations of viral-bacterial interaction (7, 9, 10, 20, 23, 29, 30). Infection with influenza A (7, 9, 10, 12, 20), reovirus (23), and pneumotropic viruses (30) have been shown to predispose to pulmonary superinfection with staphylococci (12, 10, 23, 29) *Hemophilus influenzae* (27), pneumococci (29, 20), and *Mycobacterium tuberculosis* (29).

The ability of different viruses to reduce host

TABLE 1. *Virus content of the lungs after exposure of mice to aerosols of mengovirus-37A*

Day	Titer in PFU/ml of lung homogenate[a]
1	2.2×10^7
4	7.0×10^3
7	1.6×10^3

[a] Mean titer for the three experiments performed at each time period. PFU, plaque-forming units.

TABLE 2. *Effect of an encephalomyocarditis virus infection on the pulmonary bacterial clearance rate for Neisseria meningitidis*

Day	Exptl group	Bacterial count in lungs[a]		Bacterial clearance (%)[b]	P
		0 Hr	3 Hr		
1	Control	14,025 ± 1,854 (20)[d]	266 ± 53 (20)	94.7 ± 1.8	<0.01
	Virus	10,190 ± 1,013 (20)	3,307 ± 796 (20)	69.3 ± 7.4	
4	Control	9,771 ± 1,242 (30)	119 ± 52 (30)	98.8 ± 0.2	<0.01
	Virus	9,853 ± 878 (30)	2,530 ± 745 (30)	71.0 ± 7.7	
7	Control	18,617 ± 1,705 (20)	852 ± 245 (20)	95.7 ± 1.0	>0.05
	Virus	15,994 ± 2,627 (20)	954 ± 521 (20)	92.1 ± 2.8	

[a] Mean ± standard error.
[b] Clearance in percent ± standard error.
[c] Probability.
[d] Number in parentheses is the number of animals in each group.

TABLE 3. *Effect of an encephalomyocarditis virus infection on the pulmonary bactericidal activity rate for Staphylococcus aureus*

Set	Day	Exptl group[a]	Bacterial count per ml of lung[b]	^{32}P count per ml of lung[b]	K[c]	Bactericidal activity (%)[b]
1	1	Control	3,505 ± 467	445 ± 31	20.0	58.7 ± 7.6
		Virus	2,748 ± 390	308 ± 22		54.7 ± 6.0
	4	Control	3,072 ± 364	539 ± 20	17.3	66.5 ± 4.0
		Virus	6,150 ± 724	464 ± 31		22.7 ± 7.9
	7	Control	3,609 ± 403	698 ± 39	19.2	73.3 ± 2.6
		Virus	3,891 ± 635	630 ± 53		69.1 ± 3.4
2	1	Control	14,280 ± 1,719	578 ± 35	97.0	73.8 ± 3.2
		Virus	23,890 ± 1,730	651 ± 23		61.8 ± 2.8
	4	Control	6,600 ± 760	705 ± 29	38.5	75.9 ± 2.3
		Virus	12,590 ± 1,663	743 ± 59		55.8 ± 4.8
	7	Control	11,540 ± 1,652	819 ± 35	50.2	72.2 ± 3.4
		Virus	7,750 ± 1,259	751 ± 38		79.4 ± 3.3
3	4	Control	2,680 ± 330	388 ± 38	25.7	70.8 ± 3.7
		Virus	5,630 ± 683	393 ± 27		43.3 ± 6.5

Overall mean data

Day	Exptl group	Bacterial count per ml of lung[b]	^{32}P count per ml of lung[b]	Bactericidal activity %[a]	p[d]
1	Control (20)[e]	8,893 ± 1,510	512 ± 27	66.3 ± 4.4	>0.05
	Virus (20)	13,319 ± 2,574	480 ± 42	58.2 ± 3.3	
4	Control (30)	4,117 ± 402	544 ± 21	71.1 ± 2.1	<0.01
	Virus (30)	8,123 ± 806	534 ± 32	40.8 ± 4.5	
7	Control (20)	7,575 ± 1,230	759 ± 29	72.7 ± 2.1	>0.05
	Virus (20)	7,521 ± 1,783	690 ± 34	77.7 ± 2.4	

[a] Ten mice were studied in each experimental group.
[b] Mean ± standard error.
[c] Aerosol constant.
[d] Probability.
[e] Number in parentheses is the total number of animals in each group.

resistance to many kinds of bacteria suggests a common pathogenesis for these bacterial superinfections. Viral infections have been shown to enhance pulmonary susceptibility to infection by (i) damaging tracheobronchial epithelium, and thereby causing local bacterial proliferation and mucociliary dysfunction (22, 31); (ii) impairing alveolar macrophage function by inducing intra-alveolar edema (19), or possibly exerting a toxic effect upon the macrophage itself (12, 23). In the present experiments damage to tracheobronchial epithelium or mucociliary dysfunction were probably not responsible for the defect in host resistance. Histological examination of the infected lungs showed that the relatively benign M-37A did not cause significant damage to tracheobronchial epithelium, and a viral-induced delay in the physical removal rate for bacteria is unlikely to account for the defect in host resistance, since in this experimental model only a small proportion of the pulmonary bacterial clearance rate is due to mucociliary function (13, 16).

A defect in phagocytosis by the alveolar macrophage system could account for the viral-induced impairment in antibacterial activity. Previous experiments have shown that in the mouse the alveolar macrophage system is the primary defense mechanism against inhaled bacteria (16). As, has been mentioned, viruses can interfere with phagocytic function indirectly by causing the development of edema within the lungs, and thus, prevent the macrophage from trapping suspended microorganisms, or the virus might inhibit cell function directly. Since edema and tissue damage were minimal, a cellular defect would seem to be the more significant deficit in these investigations.

The relevance of these investigations to patient situations is speculative. Clinically, it is possible that an antecedant viral infection may predispose to meningococcal infection of the lung (26, 27). This conclusion is consistent with data indicating that viral infections lead to other kinds of bacterial penumonias (25, 28). The rediscovery of *N. meningitidis* as a respiratory pathogen is recent and hence epidemiological investigations of the association are not yet available. However, two contemporary studies are of interest. Eickhov (6)

used an indirect hemagglutination test as an indicator of recent meningococcal infection and found a relationship between infection with influenza virus and the acquisition of group C or Y meningococci. Artenstein and co-workers (4) studied a similar population of military personnel and did not find a correlation between antecedant viral infection and meningococcal meningitis. From these observations, it would appear that minor infections, but not serious meningococcal disease, may occur following certain types of viral illness. It is conceivable, however, that in the inordinately susceptible individual a viral-induced depression of resistance to meningococcal infection, such as occurred in our experiments, prevents eradication of meningococci thereby enhancing the possibility for respiratory infection.

ACKNOWLEDGMENTS

We thank Catherine Prato, Henry Bray, M. Carroll Eagle, and Mark B. Taylor for their aid in these studies.

This research was supported by Public Health Service grants APO 0628 and 01239 APC from the National Institutes for Environmental Health and training grant 5 T01 AI 00384 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Akers, T. G., S. B. Bond, and L. J. Goldberg. 1966. Effect of temperature and relative humidity on survival of airborne Columbia SK group viruses. Appl. Microbiol. **14**:361–364.
2. Akers, T. G., S. B. Bond, C. Papke, and W. R. Leif. 1966. Virulence and immunogenicity in mice of airborne encephalomyocarditis viruses and their infectious nucleic acids. J. Immunol. **97**:379–385.
3. Akers, T. G., S. H. Madin, and F. L. Schaffer. 1968. The pathogenicity in mice of aerosols of encephalomyocarditis group viruses or their infectious nucleic acids. J. Immunol. **100**:120–127.
4. Artenstein, M. S., J. H. Rust, D. H. Hunter, T. H. Lemson, and E. L. Buesscher. 1967. Acute respiratory disease and meningococcal infection in Army recruits. J. Amer. Med. Ass. **201**:1004–1008.
5. Brownstein, B., and A. F. Graham. 1961. Interaction of Mengo virus with L cells. Virology **14**:303–311.
6. Eickhov, T. C. 1971. Sero-epidemiologic studies of meningococcal infection with the indirect hemagglutination test. J. Infect. Dis. **123**:519–526.
7. Francis, T., and M. C. DeTorregrossa. 1945. Combined infection in mice with *H. influenzae* and influenza virus by the intranasal route. J. Infect. Dis. **76**:70–77.
8. Garber, E. D., and F. C. Hauth. 1950. A new mutation with asymmetrical expression in the mouse. J. Hered. **41**:122–124.
9. Gerone, P. J., T. G. Ward, and W. A. Chappell. 1957. Combined infections in mice with influenza virus and *Diplococcus pneumoniae*. Amer. J. Hyg. **66**:331–341.
10. Glover, R. E. 1941. Spread of infection from the respiratory tract of the ferret. II. Association of influenza A virus and streptococcus C. Brit. J. Exp. Pathol. **22**:98–107.
11. Goldstein, E., and G. M. Green. 1967. Alterations in the pathogenicity of *Pasteurella pneumotropica* for the murine lung caused by changes in pulmonary antibacterial activity. J. Bacteriol **93**:1651–1656.
12. Green, G. M. 1966. Patterns of bacterial clearance in murine influenza. Antimicrob. Ag. Chemother. 1965, p. 26–29.
13. Green, G. M., and E. Goldstein. 1966. A method for quantitating intrapulmonary bacterial inactivation in individual animals. J. Lab. Clin. Med. **68**:669–677.
14. Green, L. H., and G. M. Green. 1968. Differential suppression of pulmonary antibacterial activity as the mechanism of selection of a pathogen in mixed bacterial infection of the lung. Amer. Rev. Resp. Dis. **98**:819–824.
15 Green, G. M., and E. H. Kass. 1964. Factors influencing the clearance of bacteria by the lung. J. Clin. Invest. **43**:769–776.
16. Green, G. M., and E. H. Kass. 1964. The role of the alveolar macrophage in the clearance of bacteria from the lung. J. Exp. Med. **119**:167–176.
17. Green, G. M., and E. H. Kass. 1965. The influence of bacterial species on pulmonary resistance to infection in mice subjected to hypoxia, cold stress, and ethanolic intoxication. Brit. J. Exp. Pathol. **46**:360–366.
18. Hahn, F., E. Goldstein, and D. L. Dungworth. 1971. Effect of whole-body x-irradiation on pulmonary bactericidal function. Radiat. Res. **47**:461–471.
19. Harford, C. G., and M. Hara. 1950. Pulmonary edema in influenzal pneumonia of the mouse and the relation of fluid in the lung to the inception of pneumococcal pneumonia. J. Exp. Med. **91**:245–259.
20. Harford, C. G., B. Leidler, and M. Hara. 1949. Effect of the lesion due to influenza virus on the resistance of mice to inhaled pneumococci. J. Exp. Med. **89**:53–67.
21. Hottle, G. A., M. H. Johnston, and A. Nelson. 1968. Infectivity of *Neisseria meningitidis* for animals, p. 29–34. Naval Biomedical Laboratory, 43rd Technical Progress Report.
22. Kilburn, K. H. 1967. Cilia and mucus transport as determinants of the response of lung to air pollutants. Arch. Environ. Health **14**:77–91.
23. Klein, J. O., G. M. Green, J. G. Tilles, E. H. Kass, and M. Finland. 1969. Effect of intranasal reovirus infection on antibacterial activity of mouse lung. J. Infect. Dis. **119**:43–50.
24. Laurenzi, G. A., L. Berman, M. First, and E. H. Kass. 1964. A quantitative study of the deposition and clearance of bacteria in the murine lung. J. Clin. Invest. **43**:759–768.
25. Louria, D. B., H. I. Blumenfeld, J. T. Ellis, E. D. Kilbourne, and D. E. Rogers. 1959. Studies on influenza in the pandemic of 1957–1958. II. Pulmonary complications of influenza. J. Clin. Invest. **38**:213–265.
26. Putsch, R. W., J. D. Hamilton, and E. Wolinsky. 1970. *Neisseria meningitidis*, a respiratory pathogen? J. Infect. Dis. **121**:48–54.
27. Reinarz, J. A., M. A. Sande, J. Silva, Jr., and J. R. Graybill. 1972. Meningococcal pneumonia: a frequent complication of adenoviral infection? Clin. Res. **20**:54.
28. Schwarzmann, S. W., J. L. Adler, R. J. Sullivan, and W. M. Marine. 1971. Bacterial pneumonia during the Hong Kong Influenza epidemic of 1968–1969. Arch. Int. Med. **127**: 1037–1041.
29. Sellers, T. F., J. Schulman, C. Bouvier, R. McCune, and E. D. Kilbourne. 1961. The influence of influenza virus infection on exogenous staphylococcal and endogenous murine bacterial infection of the bronchopulmonary viruses of mice. J. Exp. Med. **114**:237–256.
30. Volkert, M., C. Pierce, F. L. Horsfall, and R. J. Dubos. 1947. The enhancing effect of concurrent infection with pneumotropic viruses on pulmonary tuberculosis in mice. J. Exp. Med. **86**:203–213.
31. Walsh, J. J., L. F. Pietlein, F. N. Low, G. E. Burch, and W. S. Mogabgab. 1961. Bronchotracheal response in human influenza. Type A, Asian strain, as studied by light, and electron microscopic examination of bronchoscopic biopsies. Arch. Int. Med. **108**:376–388.