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Responses of Mice Immunized with Influenza Virus by Aerosol and Parenteral Routes

GEORGE H. SCOTT* AND ROBERT J. SYDISKIS[1]

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

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Antibody levels in sera and respiratory secretions and resistance to respiratory infections were examined in mice given live influenza virus in small-particle (2 μm) aerosols, large-particle (10 μm) aerosols, intraperitoneally, and subcutaneously. After parenteral administration antibody was found primarily in the serum, but small amounts were recovered in bronchoalveolar washings after 2 to 3 weeks. Specific antibody was present in both sera and bronchoalveolar washings from mice given virus in small-particle aerosols to achieve virus dissemination throughout the respiratory tract. Immunoglobulin A, immunoglobulin G, and trace amounts of immunoglobulin M, all specific for the infecting virus, were detected in bronchoalveolar washings of small-particle aerosol-infected mice. Virus administration in large-particle aerosols (for primary virus localization in upper respiratory tract) at doses greater than those required to initiate infection with small-particle aerosols failed to stimulate production of antibody in sera or bronchoalveolar washings. Small-particle aerosol-immunized mice were resistant to subsequent challenge with $10^{2.0}$ respiratory median lethal doses of virulent virus, whereas large-particle aerosol-immunized mice were not protected. Parenteral immunization modified the course of the disease in challenged mice and reduced mortality rates but did not prevent reinfection of the respiratory tract.

Resistance to respiratory viral influenza infections has been found to correlate with the level of antibody in bronchial secretions and not necessarily with the amount in serum (3, 15). In addition, active antibodies in nasopharyngeal secretions are reported to be associated primarily with locally synthesized 11S immunoglobulin A (IgA) (17, 19, 21). There is considerable evidence that maximum stimulation of these local antibodies requires deposition of antigen in the respiratory tract rather than in extrarespiratory areas (8, 20, 21). In this regard, Waldman et al. (22) demonstrated the feasibility of aerosol immunization against influenza. Their data suggest that the secretory system in the respiratory tract cannot be considered as a single unit and that the method employed for administration of antigen may determine the specific areas stimulated to produce antibody. Other investigators have amply demonstrated the potential for aerosol administration of antigen in mass immunization programs (E. W. Larson, J. W. Dominik, A. H. Rowberg, and G. A. Higbee, submitted for publication; 22, 23). In spite of an impressive amount of research, however, optimum methods for effecting immunogenic response with vaccines administered to the respiratory tract have not been determined. Therefore, in the present report various methods for stimulating active immunity to influenza by respiratory or parenteral administration of infective virus are compared and evaluated. Humoral and respiratory tract antibodies and their relation to induction of resistance in mice were examined after administration of type A influenza virus in small-particle aerosols (SPA), to achieve virus deposition throughout the entire respiratory tract, or in large-particle aerosols (LPA), to limit virus deposition predominantly to the upper respiratory tract, and by the intraperitoneal (i.p.) or subcutaneous (s.c.) routes.

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MATERIALS AND METHODS

Virus. (i) Preparation of working stock. Influenza virus, strain A/Aichi/2/68 (H3N2), had a history of 11 passages in embryonated eggs; adaptation

[1] Present address: Department of Microbiology, School of Dentistry, University of Maryland, Baltimore, Md. 21201.

to mice was accomplished in 9 serial passages by intranasal (i.n.) instillation of supernatant fluid from a suspension of homogenized lungs from mice infected 3 to 4 days previously. After the 9th passage, allantoic cavities of 10-day-old embryonated chicken eggs were inoculated with pooled supernatant fluids from infected lungs. After incubation for 48 h at 35 C, infected allantoic fluid was harvested and clarified by centrifugation at 1,200 × g at 4 C for 15 min. Antibiotics were added to the clarified fluid to achieve a final concentration of 250 units of penicillin/ml and 100 μg of streptomycin/ml; aliquots of the suspension were stored at −60 C. Each thawed aliquot contained $10^{8.7}$ egg median infectious doses (EID_{50}) per ml. Formalin-killed virus was obtained by treating this material with a 1:2,000 solution of formaldehyde by the method of Phillips et al. (16).

(ii) Assay procedures. Samples were diluted in heart infusion broth (HIB) containing antibiotics and titrated for virus in 9- to 11-day-old embryonated chicken eggs as described by Hirst (8). Virus titers, expressed as EID_{50}, were estimated by the Spearman-Kärber method as described by Dougherty (3).

(iii) Preparations for aerosol exposures. For SPA generated with the Collison spray apparatus the stock virus was diluted in HIB supplemented with antibiotics; 4% skim milk solids and 0.2% Tween 80 were added to the diluent for preparations disseminated with the spinning top generator.

Experimental animals. Six- or seven-week-old outbred Swiss mice, Crl:COBS^{R}CD^R-1(ICR)BR, were used without regard to sex. Groups of 20 to 30 mice per cage were provided food and water ad libitum.

Experimental outline. In dose response experiments, groups of 250 mice were exposed to virus doses of $10^{0.8}$ to $10^{4.6}$ EID_{50} in SPA (2 μm) or LPA (10 μm) or were injected s.c. or i.p. with doses of $10^{3.7}$ to $10^{7.7}$ EID_{50}. Random lots of 10 mice from each group were examined at selected intervals over a 28-day period after the primary virus exposure or inoculation. Lungs were examined for gross pathology, pooled, and assayed for virus. Serum and bronchoalveolar washings (BAW) were pooled and assayed for specific hemagglutination-inhibition (HI) antibody and immunoglobulin. Resistance of experimental mice was assessed by exposure (15 days postinoculation) to $10^{2.0}$ median respiratory lethal doses ($MRLD_{50}$) in SPA, a dose that usually killed untreated control mice within 8 days. The $MRLD_{50}$ for mice administered in SPA was estimated to be equivalent to $10^{3.0}$ EID_{50}. Mice surviving the rechallenge were sampled at intervals over a 16-day period and examined as described above.

Collection and processing of samples. (i) Sera. Mice were bled from the orbital venous plexus with glass capillary tubes. The blood was centrifuged after 30 min at 37 C, and the serum was removed. Unless otherwise indicated, sera from all mice within a group were pooled before storage at −20 C.

(ii) Gross lung lesion scores. Mice were killed by cervical dislocation, and the ventral wall of the thorax was removed to expose the intact lungs. The lungs were examined in situ for lesions and scored according to the degree of consolidation as outlined by Fazekas de St. Groth (4): 100% consolidation = 4; 50% = 2; 25% = 1; death from the virus on day 6 or earlier was scored as 5.

(iii) Collection of BAW. The respiratory organs were left in situ, and a small incision was made in the trachea within 5 mm of the larynx. Through this opening a blunt 19-gauge needle attached to a 1-ml syringe was inserted, and the lung was inflated by injecting 0.7 ml of phosphate-buffered saline (pH 7.2); approximately 0.5 ml of aspirate was drawn into the syringe. Washings from 10 to 15 mice were pooled, and cellular debris was removed by centrifugation at 2,000 × g for 15 min. Pooled samples containing visible erythrocytes were discarded. Supernatant fluids were concentrated 10-fold by filtration through a Pellicon membrane (Millipore Corp., Bedford, Mass.) with a nominal molecular weight cutoff of 25,000. The concentrated samples were stored at −20 C until assayed.

(iv) Lung, liver, and spleen suspensions. Both lungs were removed by resecting the main bronchi at the hilus. Lungs, livers, and spleens were weighed and ground in HIB with antibiotics to yield a 10% (wt/vol) suspension. The suspensions were centrifuged, and the resulting supernatant fluids were stored at −60 C.

Titration of HI activity. Samples of serum were treated with receptor-destroying enzyme (Microbiological Associates, Bethesda, Md.), adsorbed with guinea pig erythrocytes, and assayed for HI activity as described by Salk (18). Preliminary treatment with receptor-destroying enzyme was not required for BAW samples. Titrations were performed in microtiter plates with guinea pig erythrocytes.

Immunoglobulin assays. Immunoglobulin concentrations in BAW were estimated by radial immunodiffusion as described by Palmer and Woods (14). A reference standard with known concentrations of mouse immunoglobulins and monospecific goat antisera against mouse IgA, IgM, and IgG were obtained from Meloy Laboratories, Springfield, Va. A pool of normal murine sera containing 0.1% sodium azide was stored at −60 C in small amounts for use as a working reference antigen.

Fluorescent antibody procedures. The ability of immunoglobulins in BAW to bind specifically with influenza virus-infected cells was examined using a modification of precedures described by Geller and Pittman (5). Monolayers of influenza-infected Vero cells were layered with BAW obtained from infected mice at selected intervals after infection. Immunoglobulins bound to the infected cells were stained with fluorescein isothiocyanate-labeled antibodies to each class and examined microscopically using transmitted illumination (490 nm).

Aerosol procedures. A modified Henderson-type exposure tube (7) was fitted with either a Collison atomizer for SPA (14) or a May (12) spinning top aerosol generator for creating LPA as described by Young et al. (27). The exposure tube was modified to accommodate two wire cages suitable for whole body exposure of 30 to 40 mice. Particle size distribution of SPA was estimated in preliminary calibration trials in which uranine (soluble sodium fluorescein;

Fisher Scientific Co., Fairlawn, N.J.) was employed as a mass tracer. Dye concentrations in samples collected with a series of single-stage impactors were measured with a fluorophotometer. Particle sizes in each LPA were monitored with the Royco light-scatter multichannel scanner (programmable particle counting system, model 202, Royco Instruments, Inc., Menlo Park, Calif.). Particles in 14 size increments ranging from 0.9 to 30 μm were counted. The mass median diameters of the aerosols were calculated as described by Malligo and Idoine (11).

Aerosols for virus studies were sampled at a rate of 12.5 liters/min at the midpoint of each trial with all-glass impingers (1) that contained 20 ml of HIB supplemented with antibiotics. Impinger fluids were assayed for virus to provide estimates of aerosol concentration. A mean value of 25 ml/min was obtained using the general equation derived by Guyton (6) for computing minute-volume of mammals to estimate inhaled doses. In each 10-min aerosol exposure, the inhaled dose was estimated as the number of virus units in 250 ml of aerosol.

RESULTS

Particle size of experimental aerosols. In four replicate trials where SPA were generated with the Collison spray apparatus, the mean median diameter was 1.83 μm with an average geometric standard deviation of 1.92. Particle size distribution varied little among trials and was limited to sizes that would escape upper respiratory impingement and penetrate to the bronchioles and alveolar sacs; 94% of the aerosol mass was contained in particles with diameters of 5 μm or less, and 82% was contained in particles less than 3 μm in diameter. The average mean median diameter for nine aerosols generated with the spinning top disseminator was 10.4 μm with a mean geometric standard deviation of 1.22. These aerosols, however, were polydispersed; although 95% of the mass was in particles 7 to 15 μm in diameter, approximately 2% of the mass was distributed in small particles capable of deposition in the lungs.

Dose response experiments. Severity of lung infection was dependent on both the viral dose and the method of administration. Lung virus titers, pathology, and mortality after primary inoculations are given in Table 1. SPA doses of $10^{1.7}$ EID_{50} resulted in severe lung infections as evidenced by more than 50% lung consolidation and a mortality rate of 14%. However, the lungs of surviving mice were free of detectable virus by day 12. Although virus doses of $10^{2.2}$ EID_{50} in LPA did not infect the lungs of most of the mice, a dose of $10^{4.6}$ EID_{50} produced lung infection, virus replication, and lung lesions not greatly different from those seen after infection with much smaller doses administered in SPA. Both lesion formation and virus titers in the lungs were highly variable when virus was administered by i.p. or s.c. inoculations; small amounts of virus were isolated from a number of pooled lung samples from mice infected with $10^{5.7}$ or $10^{7.7}$ EID_{50}, but persistence of virus was erratic, suggesting that if virus replication occurred in the lungs, it was limited to a few individuals in the test population.

Virus administered by all routes of inoculation was capable of inducing development of serum HI activity, but the response was dose dependent (Table 2). An approximately 10^4-fold or 10^6-fold increase in active virus given by the i.p. or s.c. route, respectively, was required to achieve serum HI titers comparable to those resulting from SPA challenge. Antibody was detected in serum 4 days after parenteral injection and 8 to 12 days after aerosol administration of sublethal virus doses. It should be noted that concentrated aerosols ($10^{4.6}$ EID_{50}) of large particles contained enough virus in small satellite particles to account for the observed lung infections and antibody production.

In general, humoral antibody was detectable prior to development of demonstrable levels in BAW, and HI titers for serum were markedly higher than titers for BAW. However, HI activity in BAW from mice exposed to $10^{1.7}$ EID_{50} in SPA was observed by 12 days postexposure, at the same time as circulating antibody, and by 16 days HI titers for serum and BAW concentrates were essentially the same. Following i.p. or s.c. inoculation, HI antibody in BAW appeared to develop more slowly, with only small amounts detectable at days 16 to 28.

Significant amounts of IgA and IgG and trace amounts of IgM were observed in BAW from aerosol-inoculated mice by 8 to 12 days postinoculation (Table 3). These immunoglobulins were detectable earlier than specific HI antibody and during developmental stages of lung lesion formation. Although mice inoculated by the i.p. route had no visible lung lesions, small amounts of IgG and no IgA nor IgM were present in their 8- and 10-day BAW. Immunoglobulins were not detected in washings from s.c. inoculated mice. All classes of immunoglobulin in BAW from aerosol-infected mice were capable of binding specifically with the infecting virus. Immunofluorescent staining of influenza-infected cells overlayed with BAW revealed the presence of viral-specific IgM and IgG at 7 days, and of all classes of immunoglobulin at 13 and 16 days postinfection. Positive staining of infected cells pretreated with BAW from immune mice was blocked when appropriate concentrations of unlabeled anti-IgG, -IgM, or -IgA were applied prior to the homologous fluorescein iso-

TABLE 1. *Virus titers and lesions in the lungs of mice exposed to influenza virus by four methods*

Route	Dose (log_{10} EID_{50})	Mean lesion score (day 8)	Estimated virus titer/lung (log_{10} EID_{50} by days)						% Mortality
			0	2	4	8	12	16	
SPA exposure	0.8	0	0[a]	2.8	0	0	0	0	0
	1.7	2.5	0	7.0	7.3	6.4	0	0	14
	2.7	4.3	2.5	7.5	8.2	6.5	0	ND[b]	44
LPA exposure	1.7	0	0	0	3.5	0	0	0	1
	2.2	0.1	0	0	6.1	0	0	0	1
	4.6	2.7	0	6.8	7.5	5.5	0	0	9
i.p. inoculation	3.7	0	0	1.5	0	0	0	0	0
	5.7	0	0	2.5	0	0	3.2	0	0
	7.7	0	3.3	2.0	2.3	0	2.5	0	0
s.c. inoculation	3.7	0	0	1.5	0	0	0	0	0
	5.7	0	0	2.0	0	0	2.5	0	0
	7.7	0	0	1.5	1.8	0	0	0	0

[a] No virus detected; 1.5 log_{10} EID_{50}/lung was the lowest level of assay sensitivity.
[b] ND, Not done; all mice were dead.

TABLE 2. *HI titers in serum and concentrated BAW from mice after exposure to influenza virus*

Source	Days postinoculation	Reciprocal titer by exposure method and dose (log_{10} EID_{50})											
		SPA			LPA			i.p.			s.c.		
		0.8	1.7	2.7	1.7	2.2	4.6	3.7	5.7	7.7	3.7	5.7	7.7
Serum[a]	2	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
	4	<10	<10	<10	<10	<10	<10	<10	40	80	<10	<10	<10
	8	<10	<10	20	<10	<10	80	<10	40	80	<10	<10	10
	12	10	20	40	10	<10	80	<10	40	80	<10	<10	20
	16	40	80	ND[b]	10	<10	160	<10	40	80	<10	<10	40
	28	40	ND	ND	10	ND	ND	<10	80	80	<10	10	40
BAW[c]	8	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
	12	<2	2	16	<2	<2	16	<2	2	<2	<2	<2	<2
	16	<2	64	ND	<2	<2	64	<2	2	<2	<2	<2	<2
	28	<2	ND	ND	2	ND	ND	<2	8	4	<2	<2	2

[a] Sera from 10 mice at each point were pooled for titration.
[b] ND, Not done.
[c] Washings from 10 mice were pooled and concentrated 10-fold prior to titration.

thiocyanate-labeled antibody. Fluorescing cells were not observed when infected cells were pretreated with BAW from normal mice or when noninfected cells were pretreated with BAW from immune mice prior to staining.

Protection against the lethal effect of respiratory challenge was afforded mice vaccinated with an adequate dose of live virus, regardless of the route of exposure. Administration by aerosol, however, was markedly more effective than parenteral inoculation. Estimates of peak virus titers in the lungs, lung lesion scores, and percentage survival rates for mice challenged with 10^2 $MRLD_{50}$ in SPA 14 days after primary exposure are presented in Table 4. After challenge of mice that had been immunized with only $10^{1.7}$ EID_{50} in SPA, no evidence of virus replication was noted in the lungs, and all mice survived. Similar survival rates were achieved in groups immunized with $10^{4.6}$ EID_{50} in LPA and in groups injected i.p. with $10^{5.7}$ EID_{50} or s.c. with $10^{7.7}$ EID_{50}. Virus titers in pooled lung samples from parenterally immunized mice were similar to those in nonimmune controls.

TABLE 3. *Immunoglobulins IgA, IgG, and IgM in concentrated BAW from mice exposed to live influenza virus*

Days postexposure	Concentration[a] (mg/100 ml) by exposure method and dose (EID_{50})								
	SPA ($10^{1.7}$)			LPA ($10^{4.6}$)			i.p. ($10^{5.7}$)		
	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM
0	0	<9	0	0	<9	0	0	<9	0
2	0	<9	0	0	<9	0	0	<9	0
4	0	<9	0	0	<9	0	0	<9	0
8	29	164	8	0	63	4	0	16	0
12	29	18	2	29	310	8	0	41	0
16	20	82	8	29	164	8	0	<9	0
28	ND[b]	ND	ND	ND	ND	ND	0	0	0

[a] Washings were concentrated 10-fold prior to assay.
[b] ND, Not done.

TABLE 4. *Resistance to influenza infection[a] of mice previously inoculated by different routes with virulent influenza virus*

Primary exposure		Lung infection (maximum values)		% Survival
Exposure route	Dose ($\log_{10}$ EID_{50})	Lesion score	Virus titer[b] ($\log_{10}$ EID_{50})	
SPA	Control[c]	4.0	7.7	22
	0.8	3.3	8.3	36
	1.7	0.2	Neg[d]	100
	2.7	1.9	Neg	100
LPA	Control	4.5	8.5	10
	1.7	4.5	9.5	7
	2.2	4.3	9.0	24
	4.6	1.2	5.0	100
i.p.	Control	4.4	8.7	19
	3.7	4.5	8.5	19
	5.7	0.2	7.3	100
	7.7	0	3.8	100
s.c.	Control	4.4	7.5	9
	3.7	4.2	8.1	24
	5.7	3.1	7.7	40
	7.7	1.8	7.4	83

[a] All mice received 10^2 respiratory mean lethal doses in SPA (2-μm mass median diameter) 14 days after primary exposure.
[b] Lungs from 10 mice were pooled; values represent estimates of virus particles per lung.
[c] Nonvaccinated control mice.
[d] Neg, Negative.

The possibility that high virus levels in the pooled lung samples may represent undue influence by the contribution from lungs of only one or two mice was examined with groups of mice immunized by the most effective aerosol or parenteral route. Groups of 150 mice were exposed to live virus at a dose of $10^{1.5}$ EID_{50} in SPA or $10^{5.7}$ EID_{50} by the i.p route. A third group was injected i.p. with $10^{5.7}$ EID_{50} of formalin-killed virus. Serum and lung samples from individual mice and pooled BAW concentrates from each group were assayed for HI activity or virus content at selected intervals for 14 days. The lungs of all mice exposed to SPA became infected; virus titers reached at least $10^{7.5}$ EID_{50} in individual lungs, and a maximum mean titer of $10^{8.1}$ was achieved on day 4 (Table 5). Antibody activity was demonstrated within 8 days in serum from every mouse and in the BAW pool.

Mice from the i.p. live virus group were examined for the presence of virus in blood, liver, and spleen as well as in the lungs. Viremia was not detected in any mice. Infectious virus was demonstrable in 63% of the lungs and spleens and 75% of the livers from mice necropsied within 15 min after inoculation, but after 2 h lungs and livers from only 37% of the group contained virus, and no virus was recovered from spleens. All organs were free of virus by 8 h postinoculation. Pooled BAW from mice in the i.p. live virus group contained no HI activity, but 90% of the mice developed serum HI antibodies by day 4 after inoculation.

Mice from each experimental group and an equal number of nonvaccinated control mice were challenged 14 days later with 10^2 $MRLD_{50}$ of virus. Four days after challenge, no virus was detected in the lungs of aerosol-immunized mice, and all mice in the group survived a respiratory dose that killed 100% of nonvaccinated control mice (Table 6). In contrast, although 60% of i.p. live virus-vaccinated mice survived, influenza virus was recovered on day 4 from lungs of 90% of the group, and virus titers ranged from $10^{3.0}$ to $10^{7.4}$ EID_{50} per lung. Intraperitoneal vaccination with the formalin-killed virus was incapable of inducing either a

TABLE 5. *Mean[a] lung virus titers and antibody responses in mice after primary exposure to influenza virus*

Days post-exposure	Virus and HI titers by primary exposure route and dose					
	SPA ($10^{1.5}$ EID_{50})			i.p. ($10^{5.7}$ EID_{50})		
	Virus titer (log_{10} EID_{50})	Reciprocal HI titers: Serum	Reciprocal HI titers: BAW	Virus titer (log_{10} EID_{50})	Reciprocal HI titers: Serum	Reciprocal HI titers: BAW
0	0 (0/5)[b]	<10 (0/5)	<2	<2	<10 (0/10)	<2
1	5.2 (5/5)	<10 (0/5)	<2	0	<10 (0/10)	<2
4	8.1 (5/5)	<10 (0/5)	<2	0	12 (9/10)	<2
8	2.5 (5/5)	43 (5/5)	4	0	18 (8/10)	<2
14	0 (0/5)	139 (5/5)	8	0	34 (9/10)	<2

[a] BAW values represent single assays of a pool from five mice; all other values represent geometric means.
[b] Values in parentheses are number responding/number examined.

TABLE 6. *Resistance of individual mice to influenza reinfection when challenged with $10^{2.0}$ $MRLD_{50}$ as SPA at 14 days after primary infection*

Primary infection	Response to reinfection			
	Virus in 4-day lungs: Isolations	Virus in 4-day lungs: Titer[a] (log_{10} EID_{50})	10-day survival: No./total	10-day survival: %
SPA, $10^{1.5}$ EID_{50}, live	0/5		32/32	100
i.p., $10^{5.7}$ EID_{50}, live	9/10	5.7 (3.0–7.4)[b]	9/15	60
i.p., $10^{5.7}$ EID_{50}, killed	NT[c]	NT	0/15	0
None	5/5	8.0 (7.5–8.5)	0/32	0

[a] Geometric mean from infected mice.
[b] Values in parentheses represent range.
[c] NT, Not tested.

serological response or resistance; the vaccinated mice responded in every respect like nonvaccinated control mice.

Passive transfer of protection. The protective efficacy of antibody within the respiratory tract was investigated by comparing the respiratory resistance of mice pretreated by i.p. or i.n. administration of mouse anti-influenza serum. Undiluted antiserum or antiserum diluted 1:50 or 1:100 was administered to mice in dosages of 0.1 ml i.n. or 0.2 ml i.p. Two hours later the mice were challenged with 10^3 EID_{50} of virulent virus in SPA. Mice treated with undiluted antiserum were killed, and their lungs were assayed for virus at selected intervals for 13 days. Percentage of survival was determined at day 16. The maximum virus titers ($10^{8.5}$ EID_{50}) in lungs of mice given undiluted antiserum by the i.p. route was similar to that in control mice. However, maximum virus titers in the lungs of mice that received antibody by the i.n. route were about 2 logs lower. The degree of protection afforded mice was also influenced by the amount of antiserum employed. An i.n. instillation of a 1:50 antiserum dilution resulted in a 20% survival rate for mice challenged with a respiratory dose that killed all mice given a 1:5 antiserum dilution i.p. and all untreated mice.

DISCUSSION

Our data demonstrate that exposure to live influenza virus in SPA stimulates higher levels of immunity against subsequent influenza infection than exposure to virus in LPA or by parenteral inoculation. Bronchoalveolar doses of less than 50 EID_{50} of virus were capable of initiating virus replication in the lungs of all mice. The resulting infection stimulated production of circulating HI antibody and increased the levels of IgA, IgG, and HI titers in the lungs. More importantly, the mice were completely protected against subsequent respiratory challenges with virulent virus. Efforts to evaluate antibody responses to virus deposited in the upper respiratory tract from monodispersed LPA were not completely successful. In all probability, responses to high doses disseminated by the spinning top were caused by virus-laden satellite particles that were small enough to penetrate to the lungs. At low dose levels ($10^{2.2}$ EID_{50}) the concentration of these satellite

particles was minimal; most of the exposure dose was deposited in the upper respiratory tract. The failure to detect either seroconversion or antibody in BAW from these mice suggests that higher concentrations of virus are required to infect upper respiratory tract tissues than lung tissue. Larson et al. (submitted for publication), using an improved version of the spinning top, observed substantial virus replication in the upper respiratory tissues of mice administered high doses in large particles. Their data indicate that nasopharyngeal infection spreads down the respiratory tract to the tracheal tissues and subsequently to the lungs.

Live virus administered by parenteral routes failed to initiate detectable infection, but with adequate virus doses high levels of circulating HI antibody and small amounts of antibody in BAW were present after several weeks. Nevertheless, parenterally inoculated mice were only partially resistant to challenge with aerosolized virus. The aerosolized virus often replicated to high titers in the lungs of representative mice from groups that evidenced 100% survival. Although parenteral immunization may reduce the severity of clinical illness resulting from influenza, sufficient antibodies are not present at the site of infection to prevent virus replication. Thus, parenteral vaccination may not be as effective as immunization with SPA for curtailing spread of disease through a population.

Since parenteral inoculation stimulated rapid serum antibody development to high titer, the greater protection afforded by aerosol immunization cannot be attributed to serum antibody. This observation confirms the report of Fazekas de St. Groth and Donnelley (4) that resistance to reinfection had better correlation with the presence of antibody in the lungs than with antibody in the serum.

Many authors have reported on the importance of antibody in the respiratory tract in promoting protection against respiratory disease (9, 15, 19). Nevertheless, local antibody may not be the only factor that plays a significant role in the immune response of the lung. Cell-mediated factors may also be required for control of viral infections. Waldman et al. (25) studied responses to influenza vaccines and reported that alveolar lymphocytes from guinea pigs vaccinated with nose drops produced macrophage inhibitory factor, whereas splenic lymphocytes were essentially nonresponsive. In contrast, when antigen was administered parenterally, splenic lymphocytes were more active. Similar findings were reported by Hetsko et al. (Am. Rev. Respir. Dis. **109**:722, 1974), who demonstrated that lung cells, but not spleen cells, from mice produced macrophage inhibitory factor after infection with a cold-adapted influenza virus vaccine which primarily infected the upper respiratory tract. Virulent virus administered by the respiratory route produced evidence of both pulmonary and systemic stimulation of cellular immune mechanisms.

Whatever should subsequently prove to be the actual determinant of protection against respiratory infection, the accumulated evidence indicates that the immunogenic response can be stimulated most optimally by direct application of a replicating antigen in aerosols with a particle size range compatible with deposition throughout the respiratory tract.

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