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Intramuscular Inoculation of Live Respiratory Syncytial Virus Induces Immunity in Cotton Rats

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Intramuscular inoculation of infant or weanling cotton rats with $10^{2.2}$ to 10^4 plaque-forming units of respiratory syncytial virus induced significant or complete resistance to infection in both the upper and lower portions of the respiratory tract. This resistance did not appear to be the result of in vitro neutralization of virus during homogenization of tissue. Virus was not recovered from the local site of inoculation after 5 min and was never detected in the respiratory tract of intramuscularly inoculated rats. Attempts to detect viral antigens at the site of inoculation by using indirect immunofluorescence were unsuccessful. However, inactivation of infectivity of three different strains of respiratory syncytial virus markedly reduced or completely ablated antigenicity and protective efficacy by the intramuscular route. This suggests that these viruses underwent limited replication, perhaps restricted to an abortive cycle, at the local site of inoculation. An immunosuppressive effect of passive maternally derived immunity was observed. Only 50% of weanling rats possessing passive maternal serum antibody were successfully immunized by intramuscular vaccination with live virus.

A high priority has been assigned to the development of an effective vaccine for respiratory syncytial (RS) virus, since this agent is the major viral respiratory tract pathogen of infancy and early childhood (5). Initially, an inactivated vaccine, prepared in monkey kidney culture and adjuvanted with alum, was shown to be antigenic, but it was quickly found to be unsuitable (6, 8-10). The vaccine did not induce resistance to infection; it did, however, induce a state of altered reactivity that potentiated disease when vaccinees underwent natural infection (6, 8-10). As a result of this sobering experience, the development of an inactivated vaccine was abandoned and efforts were initiated to develop a live attenuated vaccine that would stimulate immunity without inducing a state of altered reactivity to infection. To date this approach has been only partially successful; however, several highly attenuated temperature-sensitive (*ts*) mutants are currently being readied for evaluation in humans (1, 2, 12, 13).

Recently, Buynak and his colleagues at the Merck Institute for Therapeutic Research reported that parenteral administration of wild-type RS virus, grown in human diploid cells, induced the development of serum neutralizing antibody in young children without causing any objective signs or symptoms of disease (4). These exciting findings clearly required amplification and extension, because evidence that virus replicated following intramuscular (IM) inoculation was not provided in the original report, nor was the possible immunosuppressive effect of maternally derived passive immunity on parenteral immunization addressed (4). The former issue requires resolution because a virus preparation that failed to replicate and served only as an inactivated antigen might induce potentiation of disease. Since an RS virus vaccine is most urgently needed during the first few months of life, the issue of immunosuppression by maternally derived serum antibody represents a potential obstacle to success of the parenteral vaccine approach in this age group.

These two issues were examined using cotton rats as the experimental model of infection. This rodent is permissive for RS virus and supports a moderately high level of viral replication in the upper and lower portions of the respiratory tract (11).

MATERIALS AND METHODS

Animals. Cotton rats (*Sigmodon hispidus*) were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health. The National Institutes of Health nucleus colony was maintained behind a germfree barrier. Animals were housed in large polycarbonate rat cages, with a bedding of hardwood chips, and fed a diet of standard rat chow and apples.

Randomly selected animals were test bled on arrival in the laboratory, and uniformly lacked serum neutralizing activity against RS virus.

Virus. Three strains of RS virus were used. The prototype Long strain was obtained from the American Type Culture Collection. Virus suspensions were prepared in HEp-2 cells as described elsewhere (11), and contained $10^{5.3}$ to $10^{5.6}$ plaque-forming units (PFU) per ml. A lyophilized live RS virus vaccine (lot 594), containing $10^{3.5}$ PFU/ml, was obtained from the Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa. Details of the preparation of this vaccine, grown in WI38 human diploid fibroblast cells, have recently been published (4). The Bernett strain of RS virus was obtained from Flow Laboratories, Rockville, Md. A suspension of this virus grown in African green monkey cells, maintained with Eagle minimum essential medium and 1% SPG(3), contained $10^{4.7}$ PFU/ml.

Mode of immunization. Cotton rats were inoculated with virus by the IM route at 7 or 42 days of age. Each animal was inoculated with virus in the left gluteal muscle; 7-day-old animals received 0.05 ml of virus suspension (Long strain), and adults received 0.05 ml (Long and Merck strains) or 0.10 ml (Bernett strain).

Three weeks after inoculation, animals were anesthetized with ether and challenged intranasally (IN) with 0.2 ml of virus suspension, diluted in Hanks balanced salt solution. Cotton rats inoculated with Long or Merck strain were challenged with Long strain (10^4 PFU). Those inoculated with the Bernett strain were challenged with homologous virus ($10^{3.5}$ PFU). Age-matched, previously uninoculated cotton rats were challenged IN at the same time as the immunized animals. Immediately before challenge, animals were bled from the retroorbital venous plexus using nonheparinized Natelson tubes, and serum was stored at −20°C. Four days after IN challenge, animals were anesthetized with pentobarbital and exsanguinated by cardiac puncture. Lungs, from which heart, thymus, and esophagus were removed, and nasal tissue, including nasal passages and turbinates, were quick-frozen separately and stored at −70°C until assayed.

Virus assay. Virus titer of infected tissue was determined by plaque assay on HEp-2 cells, as described previously (11).

Serum antibody assay. Serum neutralizing antibody to RS virus was assayed by measuring 60% plaque reduction on HEp-2 cell monolayers (7).

Immunofluorescence microscopy. Muscle tissue at the site of immunization was removed from rats, quick-frozen on dry ice, and stored at −70°C until sectioned. Cryostat sections were cut at 10 μm, fixed in acetone, and stained without delay. The indirect immunofluorescence technique was utilized in an attempt to detect and localize viral antigen (11). RS virus-infected and uninfected HEp-2 cell cover slips served as controls for the specificity of the procedure.

RESULTS

Resistance induced by IM inoculation of weanling rats with live RS virus. A single IM inoculation of 10^4 PFU of the Long strain of RS virus induced the development of a moderately high level of serum neutralizing antibody in weanling rats (Table 1). When these immunized animals were challenged IN with 10^4 PFU of the Long strain, infectious virus was not detected in lungs or nasal turbinates at a time when infection is ordinarily at its peak, i.e., 4 days postchallenge (11). This degree of resistance was equivalent to that induced by prior infection of the respiratory tract with RS virus (Table 1).

Conceivably, failure to detect virus in the lungs of IN-challenged rats could result from in vitro neutralization during homogenization of tissue. This possibility was investigated by thoroughly perfusing lungs of IN-challenged animals before grinding (Table 2). Despite thorough perfusion, the lungs of IM-immunized rats failed to yield virus following IN challenge. Furthermore, mixture of such lung homogenates with those of previously unimmunized animals challenged IN did not reduce the titer of virus in the latter suspensions. This indicates that the perfused homogenates from IM-immunized, IN-challenged rats lacked neutralizing activity, and presumably immune cellular activity, capable of effecting in vitro neutralization. Hence, failure to detect virus in the homogenates of perfused lungs from these animals indicates that IM immunization induced resistance to viral replication in the lower respiratory tract.

The efficacy of a commercially prepared live-virus vaccine (Merck strain) administered IM was also evaluated (Table 1). Since the titer of the reconstituted vaccine was nearly 100-fold lower than that of the Long strain, a direct comparison of immunogenicity of 10^4 PFU was not possible. However, one IM dose of $10^{2.2}$ PFU of the Merck strain induced substantial resistance to IN challenge with the Long strain (Table 1). Furthermore, a second IM inoculation, 2 weeks after the first and a week before IN challenge, induced as effective resistance as the higher-titered Long strain (Table 1). Animals that received either one or two inoculations of the Merck vaccine developed approximately the same titer of neutralizing antibody, a level lower than that induced by the Long strain.

The possibility that IM immunization might merely delay rather than prevent infection was examined by sacrificing animals vaccinated IM with the Long strain (10^4 PFU) at various times postchallenge, namely, 1, 3, 5, 8, 11, 14, 20, 35, and 49 days. Virus was not recovered from either the nose or lungs of any of these animals.

Efficacy of IM immunization in infant cotton rats. Seven-day-old cotton rats were inoculated IM with 10^4 PFU of RS virus (Long strain). Three weeks later these animals, and

TABLE 1. *Resistance of weanling cotton rats to IN challenge with RS virus induced by prior exposure to live RS virus*

Prior exposure to RS virus	Time of exposure prechallenge (weeks)	No. of rats tested	Serum neutralizing antibody at challenge[a]	Virus replication 4 days after challenge[b]			
				No. of rats with virus not detectable		GM of virus in tissue (PFU/g)	
				Nasal turbinates	Lungs	Nasal turbinates	Lungs
None	—	12	<20	0	0	$10^{4.6}$	$10^{4.7}$
10^4 PFU Long strain IN	3	11	1,209	11	11	$<10^{2.0}$	$<10^{2.0}$
10^4 PFU Long strain IM	3	12	442	10	12	$<10^{2.0}$	$<10^{2.0}$
$10^{2.2}$ PFU Merck strain IM	3	18	255	4	8	$10^{2.5}$	$10^{2.1}$
$10^{2.2}$ PFU Merck strain IM 2×	3 and 1	10	170	6	10	$10^{2.1}$	$<10^{2.0}$

[a] Measured by plaque reduction against Long strain and expressed as reciprocal of the geometric mean (GM). Serum was collected 3 weeks after initial inoculation of virus.

[b] Rats were challenged IN with 10^4 PFU of Long strain virus.

TABLE 2. *Evidence that in vitro neutralization is not responsible for pulmonary resistance observed in rats previously immunized with RS virus IM*

Lungs[a]	No. of rats tested	Virus in lung (PFU/g)	
		Geometric mean	Range
(A) Inoculated	6	$<10^2$	$<10^2$
(B) Not inoculated	6	$10^{3.8}$	$10^{3.2}$-$10^{4.8}$
(C) (A) + (B)	12	$10^{3.8}$	$10^{3.1}$-$10^{5.0}$

[a] Perfused lungs from rats challenged IN with 10^4 PFU of Long strain RS virus were sampled 4 days postchallenge. Rats in group (A) had been inoculated IM with 10^4 PFU of RS virus 3 weeks before; rats in group (B) were not inoculated. Group (C) combined equal quantities of lung from a group (A) rat and a group (B) rat.

untreated, age-matched controls, were challenged IN with 10^4 PFU of homologous virus. IM immunization was as effective in infant rats as in weanling animals (data not shown).

Does RS virus replicate at the local site or in the respiratory tract after IM inoculation? Two approaches were used to determine whether viral replication occurred at the site of IM inoculation or in the respiratory tract. Initially, direct evidence of viral replication after IM inoculation was sought. Animals were inoculated IM with 10^4 PFU of the Long strain of RS virus, and two rats were sacrificed at each of the following intervals: 5 min, 2 h, and 1, 2, 3, 4, 5, and 7 days. In each instance, muscle at the injection site (gluteal region), nose, and lungs were removed, homogenized immediately, and assayed for infectious virus. Virus was recovered from the injection site shortly after inoculation (5 min) but not thereafter. In no instance was virus recovered from the other organs. Muscle from the injection site of other animals was removed, quick-frozen, sectioned with a cryostat, and stained with fluorescein-labeled antibody in an attempt to detect viral antigen. These tissues were taken 2 h and 1, 2, 3, and 4 days after inoculation of 10^4 PFU of the Long strain. Appropriate positive and negative controls, described earlier, assured the validity of the staining procedure. In no instance was viral antigen detected.

Evidence of viral replication after IM inoculation was also sought by comparing antigenicity and immunogenicity of inactivated virus with that of live virus from the same suspension. Two methods of inactivation were used. Three strains of RS virus (Long, Merck, and Bernett) were exposed to ultraviolet (UV) light (germicidal lamp, short wavelength), at an intensity of 3,000 ergs/cm^2·s for 40 s, a time just sufficient to inactivate virus. The exposure time was determined by plotting an inactivation curve. Also the Bernett strain was inactivated by the addition of Formalin to a concentration of 1:4,000, followed by incubation at 37°C for 72 h. Both UV- and Formalin-inactivated virus were assayed on HEp-2 cells at the time of inoculation to ensure the absence of detectable live virus. The limit of sensitivity of this assay, performed with multiple samples, was 10 PFU/ml.

Randomized weanling cotton rats were inoculated IM with either live or inactivated virus derived from the same suspension. Three weeks later the animals were challenged IN with live virus and sacrificed 4 days later. Animals immunized with Long and Merck strains of RS virus were challenged with 10^4 PFU of Long strain; those immunized with the Bernett strain were challenged with $10^{3.5}$ PFU of homologous virus.

The three strains of RS virus induced effective resistance when administered live IM; however,

UV or Formalin inactivation markedly reduced antigenicity and protective efficacy of virus (Table 3).

Does maternally derived passive immunity exert an immunosuppressive effect on parenteral immunization with RS virus? RS virus has its greatest impact as a cause of serious lower respiratory tract disease during the first 3 months of life, a time when infants possess a moderately high titer of maternally derived serum antibody (5). This might pose a serious obstacle to parenteral immunization with RS virus at this critical time, since an immunosuppressive effect of passive antibody would be anticipated. To investigate this possibility we studied the response to IM immunization of weanling rats born to mothers that had been infected IN with RS virus 28 days before parturition. Weanlings from these mothers were inoculated IM with 10^4 PFU of the Long strain of RS virus at 20 or 37 days of age and then challenged IN with the same quantity of this virus 21 days later (Table 4). An immunosuppressive effect of passive immunity was observed: only 1 of 14 rats born to an immune mother developed an increase in serum neutralizing antibody. In contrast, each of 18 weanlings born to a nonimmune mother developed a moderate to high titer of serum antibody in response to IM inoculation of the same dose of virus (Tables 1 and 3).

Only 50% of the 14 weanling rats born to an immune mother were rendered resistant to IN challenge by IM immunization. These rats (no. 8 to 14, Table 4) exhibited partial or complete resistance to pulmonary virus replication. In contrast, the remaining rats (no. 1 to 7, Table 4) were not successfully immunized by parenteral inoculation of virus.

Rats are protected by passive immunity acquired from their mother for at least 30 days after birth (data not shown), whereas by 34 to 58 days this protective effect is only partially operative or has disappeared (Table 4). Thus, the resistance of IM-immunized rats (no. 8–14) cannot be ascribed to an effect of passive maternal immunity.

The titer of serum neutralizing antibody at the time weanling rats were challenged IN was not predictive of resistance of the lungs to viral replication. Thus, rats no. 6 and 7, which had a low titer or lacked detectable antibody, were not resistant. In contrast, rats no. 8, 10, 11, and 12, which had a comparable level of serum antibody at the time of challenge, were partially or completely resistant.

DISCUSSION

IM inoculation of infant or weanling cotton rats with $10^{2.2}$ to 10^4 PFU of RS virus induced significant or complete resistance to infection in both the upper and lower portions of the respiratory tract. This resistance could not be explained by in vitro neutralization of virus during homogenization of tissue and thus appeared to represent a bona fide manifestation of immunity.

Previously, Buynak and colleagues demonstrated that IM inoculation of $10^{3.5}$ PFU of the

TABLE 3. *Effect of UV or Formalin upon antigenicity and immunogenicity of RS virus administered IM*

Virus strain	Treatment[a]	No. of rats	Serum neutralizing antibody at challenge[b]	Virus replication 4 days after IN challenge			
				No. of rats with virus not detectable		GM of virus in tissue (PFU/g)[c]	
				Nasal turbinates	Lungs	Nasal turbinates	Lungs
Long	None (10^4 PFU)	6	649	2	6	$10^{2.1}$*	$<10^{2.0}$*
	UV	7	38	0	0	$10^{3.9}$*	$10^{3.7}$*
Merck	None ($10^{2.2}$ PFU)	5	172	2	3	$10^{2.6}$*	$10^{2.2}$*
	UV	5	<20	0	0	$10^{3.8}$*	$10^{4.0}$*
Bernett	None ($10^{3.7}$ PFU(	11	72	3	7	$10^{2.9}$**	$10^{2.4}$**
	UV	10	21	0	2	$10^{4.6}$**	$10^{4.0}$**
	Formalin	9	23	0	0	$10^{4.3}$**	$10^{4.2}$**

[a] UV: Virus suspension subjected to 120,000 ergs/cm^2 delivered as 3,000 ergs/cm^2·s for 40 s. Formalin: 1:4,000 dilution of 37% formaldehyde, incubation at 37°C for 72 h. Live virus could not be detected after this treatment.

[b] Measured by plaque reduction against Long strain and expressed as reciprocal of geometric mean (GM). Serum was collected 3 weeks after IM inoculation of virus.

[c] *Cotton rats in this group were challenged with Long strain, 10^4 PFU; **, cotton rats in this group were challenged with Bernett strain, $10^{3.5}$ PFU.

TABLE 4. *Effect of maternally derived passive immunity on immunization with parenterally administered live virus*

Mother	Immunization	Age (days) when:		Litter no.	Rat no.	Serum neutralizing antibody[a] at:		Virus recovered (log_{10} PFU/g)[b]	
		Immunized	Challenged			Immunization (GM)	Challenge (GM)	Nasal turbinates (GM)	Lungs (GM)
Infected[c]	10^4 PFU of RS virus (Long) IM	20	41	1	1–5	82–146 (124)	33–48 (39)	3.6–5.0 (4.4)	3.0–5.3 (4.0)
				2	6	88	21	4.8	4.0
					7	146	<20	4.3	4.0
					8	106	<20	2.3	2.7
					9	134	37	3.8	2.0
					10	143	21	3.8	<2.0
		37	58	3	11	95	<20	4.0	<2.0
					12	48	<20	2.5	<2.0
					13[d]	58	225	2.0	<2.0
					14	23	28	2.7	<2.0
Infected[c]	None		34	4[e]	15–17		27–37 (33)	3.6–3.8 (3.7)	3.4–4.3 (3.8)
					18–19		<20	3.5, 4.3	4.2, 4.8
			50	5	20–24		<20	<2.0–3.9 (3.3)	4.3–5.0 (4.6)
			58	6[f]	25–27		<20	3.7–5.0 (4.1)	5.0–5.2 (5.1)
Not infected	None		40–50	7–8	28–40		<20	3.6–5.1 (4.5)	4.1–5.4 (4.6)

[a] Reciprocal of plaque reduction titer. GM, Geometric mean.
[b] Rats were challenged IN with 10^4 PFU of Long strain virus and sacrificed 4 days later.
[c] Infected with RS virus IN before pregnancy.
[d] Only rat no. 13 developed an increase in serum antibody following immunization.
[e] Mother had serum neutralizing antibody titer of 1:724 at the time litter was challenged.
[f] Mother had serum neutralizing antibody of 1:2,850 at the time litter was challenged.

Merck strain of RS virus, grown in human diploid fibroblast cells, induced the development of serum neutralizing antibody in most infants and young children who initially lacked antibody (4). However, the vaccine was less antigenic when given to individuals who had pre-existing serum antibody. These observations raised two issues which we attempted to resolve using cotton rats as a model for experimental immunoprophylaxis.

First, the mechanism of induction of immunity was examined in an attempt to determine whether viral replication at the site of inoculation or within the respiratory tract was responsible for the resistance observed after IM inoculation of $10^{2.2}$ to 10^4 PFU of live virus. Virus was not recovered from the local site of inoculation after 5 min and was never detected in the nose or lungs at any time after IM inoculation. Furthermore, attempts to detect viral antigens at the site of IM inoculation were unsuccessful. However, inactivation of infectivity of three strains of virus by the minimal UV dose required for inactivation markedly reduced or completely ablated their antigenicity and protective efficacy. Although this observation does not constitute unequivocal evidence for the occurrence of viral replication upon IM inoculation, it suggests that limited replication, perhaps restricted to an abortive cycle, was responsible for stimulation of immunity by the small quantities of virus employed.

The issue of viral replication is of more than academic interest, because an inactivated, antigenic RS virus vaccine used previously in a series of trials did not stimulate immunity but did induce a state of altered reactivity such that disease was enhanced when vaccinees underwent natural infection (6, 8–10). This disease potentiation effect could not be ascribed to tissue culture or medium constituents in the vaccine, since a comparison group of vaccinees who received a parainfluenza virus vaccine prepared in the same manner as the RS virus vaccine did not exhibit potentiation of disease when infected naturally with RS virus. Hence the mechanism by which a small quantity of live RS virus stimulated an immunological response must be resolved to be certain that it differs from that of inactivated vaccine. As cited above, this seems

to be the case, since it appears that live virus undergoes limited replication following parenteral inoculation.

Second, the possibility that passive immunity might interfere with the effectiveness of parenteral immunization with live RS virus was examined because immunosuppression could pose a serious obstacle to this approach. Thus, the greatest need for an RS virus vaccine is in the first few months of life, a time when infants possess a moderately high level of maternally derived RS virus serum antibody. We attempted to simulate these conditions by administering live RS virus IM to weanling rats possessing passive serum antibody derived from their immune mothers. In this situation an immunosuppressive effect of passive immunity was observed. Only 50% of inoculated weanling rats were rendered resistant to subsequent IN challenge with RS virus. This suggests that parenteral immunization with live virus may not be effective in protecting human infants against RS virus during their period of greatest vulnerability to serious RS virus disease, i.e., the first 3 months of life. If this be the case, the usefulness of live IM virus vaccine may be limited to individuals over 6 months of age who have escaped natural infection and who have lost most or all of their passive maternally derived serum antibody. The outlook for IM vaccination of individuals who have undergone prior infection is not encouraging; Buynak's study indicated that seropositive children respond poorly to vaccine (4).

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