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Mechanisms of Immunity to Respiratory Syncytial Virus in Cotton Rats

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Active immunity and maternally transmitted passive immunity to respiratory syncytial virus (RSV) were studied in cotton rats. Animals infected with respiratory syncytial virus developed complete resistance to pulmonary reinfection, which lasted at least 18 months. Nasal resistance was of shorter duration and began to diminish in 8 months. Pulmonary resistance was transferred by parabiosis, but nasal resistance was not. Adoptive transfer studies with fractionated convalescent blood showed that serum antibody, but not circulating lymphocytes, conferred pulmonary resistance. Immune females conferred antibody to their young prenatally and postnatally, with most of the antibody being transferred via colostrum and milk. Maternally transmitted immunity was more effective in the lungs than in the nose and was transient in both organs. Foster nursing experiments showed colostrum and milk to be the most important routes of immune transfer. Although resistance in infants generally correlated with serum neutralizing antibody levels, several exceptions to this correlation suggested that immune factors other than neutralizing antibody may also play an important role in maternal passive immunity.

More than a quarter of a century has passed since the discovery of respiratory syncytial virus (RSV) in 1956. Since that time, RSV has assumed a position of importance as a human pathogen, accounting for more cases of infantile bronchiolitis and pneumonia than any other virus. Despite many epidemiological and clinical studies (1), much remains to be learned concerning the immunological mechanisms involved in resistance to these diseases. Studies of humans have not defined which portions of the immune system are responsible for recovery from infection and resistance to reinfection, nor have they shed light on the unusual ability of RSV to reinfect the host a short time after initial infection.

In addition to these problems, additional questions have arisen concerning the role of maternally transmitted immunological factors in resistance during the early months of life. For several decades, there have been reports suggesting that breastfeeding might have a protective effect against respiratory infections of infancy, and in 1976 Gardner and co-workers described a correlation between breastfeeding and resistance to RSV disease (2).

Subsequent work in our laboratory demonstrated that infant ferrets which nursed on immune mothers acquired temporary immunity to RSV (8). However, the relevance of these observations to humans is not clear because the transfer of immunity in ferrets differs in that antibody does not cross the placenta (9).

The cotton rat does allow transfer of antibody across the placenta, and for this reason we used it as a model to examine the mechanisms of maternally transmitted passive immunity to RSV. Since this animal is also a highly permissive host for RSV replication we used it to study active immunity.

MATERIALS AND METHODS

Animals. Cotton rats (*Sigmodon hispidus*) were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health. A small nucleus colony, maintained behind a germfree barrier for the past 10 years, provided animals for the production colony. All adult animals were inoculated with inactivated Sendai virus vaccine (Microbiological Associates) at least 3 weeks before use in any experiment.

Virus. In early experiments, the Long strain of RSV grown in HEp-2 cells was used. In later experiments, the A-2 strain of the virus was used, so that comparisons might be made with vaccine-candidate mutants of the A-2 strain. In each experiment, animals previously tested with one strain of virus were challenged with the homologous strain. No differences were seen in results with the two strains.

Virus assay. Animals were sacrificed by carbon dioxide asphyxiation. Lungs and nasal tissues (including nasal turbinates) were homogenized in 10 parts (wt/vol) of Hanks balanced salt solution supplemented with 0.218 M sucrose, 4.4 mM glutamate, 3.8 mM KH_2PO_4, and 7.2 mM K_2HPO_4 and stored at −70°C until assayed. Viral titer was determined by plaque assay on HEp-2 cell monolayers, as previously described (5).

Antibody assay. Neutralizing antibody was measured by a plaque-reduction neutralization assay as previously described, using a 60% plaque-reduction endpoint (5).

RESULTS

Duration of active immunity. Clinical studies have shown that active immunity to RSV is of limited duration. Information is not available, however, to estimate the duration of immunity in the lower respiratory tract.

An 18-month experiment was conducted to examine the duration of immunity in the upper and lower portions of the respiratory tract of cotton rats. Young adult animals were first test-bled to verify the absence of preexisting RSV neutralizing antibody. The animals were then anesthetized with methoxyflurane and inoculated intranasally with approximately 10^4 PFU of virus, in a volume sufficient to reach the lungs (0.1 ml for a 100-g animal). Age-matched untreated control animals were included in the study and housed on the same racks as immune animals. There was no evidence of nosocomial infection during the experiment, either by a rise in antibody titer of immune animals or by the appearance of antiviral antibody in the control animals. At intervals over the next 18 months, previously infected and control animals were bled and then challenged intranasally with homologous virus. Four days after challenge (the time of maximum viral replication in cotton rats), the animals were sacrificed, and the viral titer in pulmonary and nasal tissues was determined. Nasal tissues were completely resistant to reinfection for approximately 8 months after initial infection and then gradually became permissive to viral replication (Table 1). By the 18th month, the level of replication in nasal tissue had continued to increase, but had not reached the level seen in control animals.

A different pattern of immunity was seen in the lungs, which were completely resistant to reinfection throughout the experiment. Antibody titer gradually decreased with time, in a pattern which suggests an inverse relationship with permissiveness of nasal tissues. However, virus replicated in the nasal tissues of some rats despite a relatively high level of serum antibody, suggesting that serum antibody does not play a major role in resistance at this site.

Effectors of active immunity. Active immunity was then examined by using the technique of parabiosis, in which two animals, surgically linked to each other, develop cross-circulation of whole blood across a capillary network which forms at the common wound site (7). Animals were anesthetized with methoxyflurane and shaved on one side. A skin incision, reaching from the hip to the shoulder, was made with scissors. The skin on each side of the wound was reflected from the underlying tissues. A second incision was made through the abdominal musculature, beginning at the posterior border of the rib cage and extending posteriorly for 1 cm. The four wound edges of musculature (two from

TABLE 1. Duration of immunity to RSV in cotton rats

Time after initial infection (months)	Previously infected rats				Uninfected control rats			
	No. of rats	Reciprocal of prechallenge neutralizing antibody titer [range (geometric mean)]	Titer of virus (log_{10} PFU/g) 4 days after challenge with 10^4 PFU of RSV [range (geometric mean)]		No. of rats	Reciprocal of prechallenge neutralizing antibody titer [range (geometric mean)]	Titer of virus (log_{10} PFU/g) 4 days after challenge with 10^4 PFU of RSV [range (geometric mean)]	
			Nose	Lungs			Nose	Lungs
1	6	217–3,151 (827)	<2.0	<2.0	3	<20	4.2–4.6 (4.7)	4.0–4.7 (4.3)
2	5	64–424 (174)	<2.0	<2.0	3	<20	4.3–5.0 (4.7)	4.2–5.3 (4.7)
3	1	2,850	<2.0	<2.0				
4	4	82–618 (195)	<2.0	<2.0	3	<20	4.7–5.2 (5.0)	4.3–4.9 (4.7)
5	1	361	<2.0	<2.0				
6	2	65–216	<2.0	<2.0				
7	1	139	<2.0	<2.0				
8	1	48	2.5	<2.0	3	<20	4.6–4.9 (4.7)	4.5–5.2 (4.8)
11	3	113–559 (214)	2.0–3.4 (2.8)	<2.0				
12	3	20–87 (39)	2.0–3.3 (2.7)	<2.0	3	<20	4.8–5.9 (5.2)	5.3–5.5 (5.4)
18	2	48–53	2.8–3.8	<2.0	3	<20	4.5–5.1 (4.9)	4.1–5.3 (4.9)

each animal) were then approximated and secured by a series of interrupted sutures. After closure of these wounds, the skin of the two animals was connected by a continuous suture, such that the closed skin wound completely encircled the abdominal anastomosis. To prevent tearing of the sutured wounds, it was necessary to fashion a girdle about the animals. This consisted of several layers of 1-in (2.54-cm) adhesive tape into which had been inserted an aluminum shim (from a soft-drink can). This shim prevented the animals from chewing through the girdle. The posterior border of the girdle was secured to the abdominal skin by a series of interrupted sutures, to prevent dislocation of the girdle by the hind feet. Each pair of surgically linked animals was placed in a separate cage and given a diet of rat chow and apples.

Three types of sex-matched pairings were studied. In the first, two control animals were linked. In the second, an immune animal, inoculated intranasally with virus 21 days earlier, was linked to another immune animal. In the third, an immune animal was connected to an uninfected control animal. Initially, littermates were used for each type of pairing. In the case of control-to-immune pairings, half of a litter was removed from its own mother shortly after birth, inoculated with virus, and then placed with a foster mother. In later experiments, sex-matched nonlittermates yielded the same results as paired littermates.

Seven days after surgery the capillary cross-circulation was well established. RSV antibody passed freely from an immune to a control partner, and ^{51}Cr-labeled cotton rat erythrocytes injected into one partner were quickly redistributed into the circulation of the other. (In each of three experiments the radioactive count in the washed erythrocytes of the acceptor animal exceeded 60% of the count of the donor animal within 1 h.)

On postsurgical day 7, each animal was bled from the eye and then challenged intranasally with 10^4 PFU of virus. Four days later the animals were sacrificed, and the pulmonary and nasal tissues were prepared for viral assay. Seven control-to-control pairs were studied to determine base-line values of pulmonary and nasal viral replication. The geometric mean viral titers of these seronegative rats were $10^{4.2}$ PFU/g for nasal tissues and $10^{4.5}$ PFU/g for the lungs (Table 2). In contrast, the five immune-to-immune pairs were immune to nasal and pulmonary infection after parabiosis.

Sixteen immune-to-control pairings were studied. Control members of these pairs acquired a moderately high level of serum neutralizing antibody after parabiosis (mean titer, 1:399). The antibody titers of immune and control partners were equivalent by day 7 postsurgery, resulting in a twofold diminution of titer of the immune partner. The 16 immune partners of immune-to-control pairings were completely resistant to nasal and pulmonary infection, whereas their control partners exhibited pulmonary but not nasal resistance.

Although these data indicate that immunity to pulmonary infection was transferred via the parabiotic linkage, it was possible that failure to detect virus might be due to in vitro viral neutralization by immune factors present in the lungs when animals were sacrificed. To rule out this possibility, we combined lung tissue from passively immunized parabiotic animals with infected lung tissue of control animals. Homogenates of combined tissues yielded the same amount of virus as those of lung suspensions from infected rats that were not mixed with lung suspension from passively immunized animals (data not shown), indicating that the protection seen in immune-to-control pairings was due to bona fide passive immunity rather than in vitro virus neutralization.

Since parabiosis showed that pulmonary im-

TABLE 2. Transfer of immunity by parabiosis[a]

Pairing	Partner (no.)	Reciprocal of serum neutralizing antibody at time of challenge 7 days postparabiosis (geometric mean)	Titer of virus 4 days after challenge with 10^4 PFU of RSV (geometric mean, $\log_{10}$ PFU/g)[b]	
			Nose	Lungs
Immune-to-Immune	Immune (10)	766	<2.0[c]	<2.0[c]
Immune-to-Control	Immune (16)	366	<2.0[c]	<2.0[c]
	Control (16)	399	4.1[d]	2.1[c]
Control-to-Control	Control (14)	<20	4.2	4.5

[a] Rats were bled 7 days after parabiosis, challenged at that time, and assayed for level of virus replication 4 days later.

[b] Significance values compared with control-to-control pairings: c, $P < 0.001$; d, not significant ($P > 0.05$).

munity can be transferred via whole blood, further studies were conducted to determine which portions of the blood account for such immunity. In the first study, immune serum (neutralizing antibody titer, 1:1,250), obtained from cotton rats inoculated intranasally 21 days previously with wild-type RSV, was administered intraperitoneally to infant animals at a dosage of 0.5 ml/10 g of body weight. Control infants received an equal volume of normal cotton rat serum. Twenty-four hours later, both groups of animals were challenged intranasally with RSV and then sacrificed 4 days later. Complete to near-complete pulmonary resistance was seen in immune serum recipients, whereas only minimal resistance was detected in the nose (Table 3).

The effect of immune lymphocytes was next examined. Whole blood, obtained from animals inoculated intranasally with RSV 21 days earlier, was fractionated on a Percoll-balanced salt solution gradient, yielding a preparation of greater than 90% viable lymphocytes. Approximately 2×10^7 such cells were injected intracardially (right ventricle) into 19 weanling cotton rats. An additional 10 animals received cells plus antiserum (the antiserum being given intraperitoneally) or antiserum alone. Twenty-four hours later, animals were challenged intranasally with RSV. A protective effect attributable to lymphocytes was not observed (data not shown). That is, cells alone provided neither nasal nor pulmonary resistance, and cells given with serum did not provide greater resistance than serum alone. Thus, it appears that the population of immune lymphocytes circulating 21 days after infection did not have a direct role in immunity.

Maternally transmitted passive immunity. A preliminary study showed that immune cotton rats transferred resistance to RSV infection to their offspring (data not shown). Foster-nursing experiments were then conducted to determine the route of immune transfer. Pregnant females were placed in nesting boxes equipped with wire mesh false bottoms, and newborn animals dropped through the mesh without suckling on their mothers. Infants were then placed with their own or foster mothers for 4 days, at which time they were bled from the eye and challenged intranasally with RSV.

Infants born to and suckled by immune mothers acquired a moderately high titer of neutralizing antibody, were partially resistant to nasal infection, and were almost totally resistant to pulmonary infection (Table 4). Infants born to immune mothers and suckled by control mothers acquired less than 10% of the antibody of the first group; they did not exhibit significant nasal resistance but did show slight, though significant ($P < 0.001$), pulmonary resistance. Infants born to control mothers and nursed on immune mothers acquired 65% of the antibody detected in the first group, but exhibited the same degree of nasal and pulmonary resistance.

The duration of maternal passive immunity was examined by challenging offspring of immune mothers at various ages up to 6 weeks (Table 5). Passive immunity was transient, detectable for only 1 week in the nose and 4 weeks in the lungs. Antibody levels diminished in relation to decrease of resistance.

A final experiment was conducted to determine how long after infection mothers were able to transfer immunity to their offspring. Females were inoculated intranasally as in previous experiments. Infants born to these mothers were allowed to suckle for 3 to 4 days and were then transferred to control foster mothers. At that time, the infants were bled from the eye and challenged intranasally with RSV. Since many of the immune mothers subsequently gave birth to more litters, this protocol allowed us to compare the degree of passive immunity conferred upon each litter.

Six mothers produced serial litters in this experiment (Fig. 1). Within each group of litters the titer of serum neutralizing antibody either remained level or gradually decreased in later litters. This was expected, as previous experiments had shown antibody levels in adult animals to decrease gradually after infection. However, comparison of antibody levels in each litter

TABLE 3. Effect of passive transfer of immune serum

Serum inoculated intraperitoneally	No. of rats	Serum neutralizing antibody at time of challenge (geometric mean, reciprocal)	Titer of virus 4 days after challenge with 10^4 PFU of RSV (geometric mean, $\log_{10}$ PFU/g)	
			Nose	Lungs
Immune serum (neutralizing antibody titer, 1:1,250)	9	270	5.2[a]	2.2[a]
Control serum (neutralizing antibody titer, <1:20)	10	<20	6.0	4.6

[a] Significance value compared with animals receiving control serum, $P < 0.001$.

TABLE 4. Passive transfer of maternal immunity

Group	No. of rats	Serum neutralizing antibodies at time of challenge, 4 days postnatal (geometric mean, reciprocal)	Titer of virus 4 days after challenge with 10^4 PFU of RSV (geometric mean, $\log_{10}$ PFU/g)	
			Nose	Lungs
Infant rats				
Born to and suckled by immune mothers	31	1,151	3.7[a]	2.1[a]
Born to immune mother and suckled by control mothers	16	103	5.3[b]	3.9[a]
Born to control mother and suckled by immune mothers	9	750	4.0[a]	<2.0[a]
Born to and suckled by control mothers	34	<20	5.7	4.5
Immune mothers	14	944	<2.0	<2.0
Control mothers	16	<20	5.0	4.4

[a,b] Significance values compared with infants born to and suckled by control mother: *a*, $P < 0.001$; *b*, not significant ($P > 0.05$).

with pulmonary viral titers yielded unexpected results. Litters of mothers no. 2 and 5 had a high level of antibody prechallenge and low pulmonary virus titers postchallenge, suggesting a correlation between antibody and resistance to infection. The first litter of mother no. 1 had high antibody prechallenge and a low titer of virus recovered from the lungs postchallenge. The second litter had lower antibody and higher pulmonary virus titers, also suggesting a link between antibody and resistance. Patterns exhibited by litters from the other three mothers, however, varied from the first three. The second litter of mother no. 4 showed a slightly higher antibody titer than the first, yet a higher pulmonary viral titer—not what one would expect if neutralizing antibody were the effector of pulmonary resistance. Even more surprising were the litters of mothers no. 3 and 6. The second litter of these mothers had nearly the same or a slightly lower antibody level as the first, but a significantly reduced titer of virus was detected in the lungs.

Taken together, these observations suggest that serum neutralizing antibody may not be the sole effector of passive pulmonary immunity. Further evidence for this interpretation was found when the data from all litters of immune mothers, in this and other experiments, were combined (Fig. 2). Although there is a weak inverse correlation betwen antibody level and pulmonary viral titer ($P = 0.05$) among infants of

TABLE 5. Duration of maternal passive immunity

Infant rats	Day challenged with 10^4 PFU of RSV	No. of rats	Serum neutralizing antibody at time of challenge (geometric mean, reciprocal)	Titer of virus 4 days after challenge (geometric mean, $\log_{10}$ PFU/g)	
				Nose	Lungs
Born to and suckled by immune mothers	4	21	905	4.5[b]	2.3[a]
	7	22	468	5.4[c]	3.0[a]
	14	16	349	5.2[c]	3.2[a]
	28	25	90	5.6[c]	4.2[a]
	42	16	29	5.1[c]	4.0[c]
Born to and suckled by control mothers	4	23	<20	5.7	4.6
	7	11	<20	5.8	4.5
	14	9	<20	5.6	4.8
	28	15	<20	5.6	4.8
	42	15	<20	5.3	4.2

[a,b,c] Significance values compared with age-matched infants born to and suckled by control mothers: *a*, $P < 0.001$; *b*, $P < 0.005$; *c*, not significant ($P < 0.05$).

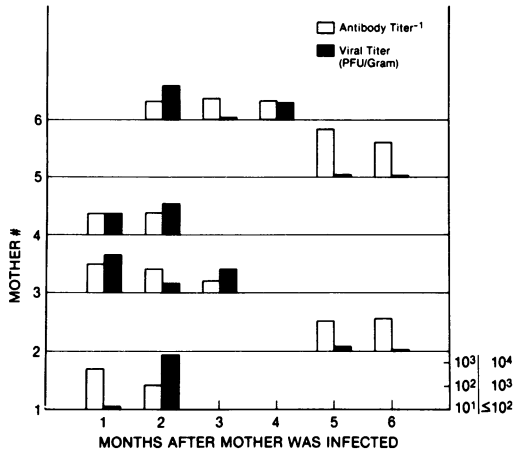

FIG. 1. Viral and neutralizing antibody titers in infant cotton rats born to mothers previously infected with RSV. Each bar represents the geometric mean antibody titer (open bars) or viral titer (solid bars) of one litter. Titers are expressed as reciprocal 60% plaque-reduction dilution, or PFU of virus per gram of wet tissue.

immune mothers, some infants showed complete pulmonary immunity despite a very low or undetectable level of antibody. In addition, some susceptible infants had a high level of antibody.

DISCUSSION

One of the unusual features of RSV is the high frequency of reinfection that occurs during childhood. Clearly, immunity to RSV is not permanent. What is not clear is the temporal pattern of immunity in the upper and lower portions of the respiratory tract. Is immunity in these two regions synchronous as regards duration or is immunity in one region more durable and more effective than in the other region? In an attempt to answer one of these questions experimentally, we studied duration of immunity in the nose and lungs of the cotton rat. The lungs remained completely resistant over an 18-month interval, whereas the nose became partially permissive at 8 months. These findings are consistent with observations in young children that second and third infections are more often associated with less severe disease than is primary infection and that reinfection is less likely to involve the lower respiratory tract. A corollary of our observations is that the level of immunity to RSV in the upper respiratory tract does not necessarily reflect resistance in the lungs.

We next attempted to identify the effectors of resistance to RSV in cotton rats. Immunity transferred through a parabiotic linkage protected the lungs but not the nose of cotton rats. The component of blood responsible for this protective effect appeared to be serum neutralizing antibody since cotton rats given convalescent-phase serum parenterally exhibited the same type and degree of resistance as parabiosed rats. In contrast, lymphocytes fractionated from the same blood did not provide protection.

The effect of RSV serum neutralizing antibodies on resistance in humans is less clear. Young infants hospitalized with RSV bronchiolitis or pneumonia usually possess a moderately high level of neutralizing antibodies in their acute-phase serum, and the mean titer of antibodies is only twofold lower than that observed in infants without RSV disease (4). Furthermore, there was considerable overlap of antibody levels in patients with RSV disease and those without such illness. Evidence for an effect of serum antibodies was recently observed during a prospective study of infants whose cord sera contained various levels of neutralizing antibodies. Those infants who later developed RSV disease had a mean twofold-lower level of antibody at birth than did infants who did not have a later RSV illness. However, as in the earlier study,

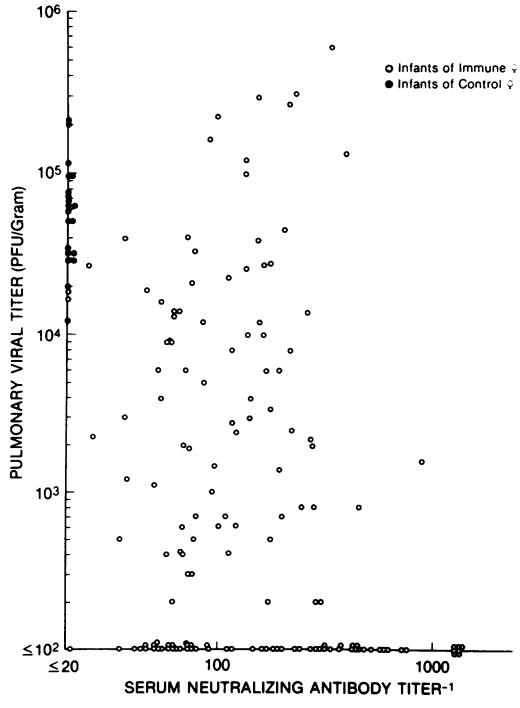

FIG. 2. Viral and neutralizing antibody titers in infant cotton rats born to nonimmune mothers (●) or to mothers previously infected with RSV (○). Each circle represents one animal.

the difference was only twofold, and there was considerable overlap in antibody titers in the two comparison groups (3).

Perhaps the greater effectiveness of serum neutralizing antibodies seen in cotton rats may be quantitative. The mean antibody titer of adoptively immunized cotton rats was 1:270, whereas the mean level of antibody observed in the serum of acutely ill patients with RSV disease was significantly lower i.e., 1:40 to 1:100 for infants 2 to 5 months of age.

In the past few years, there has been considerable interest in the transmission of immunity to RSV from mother to infant via colostrum and milk. Workers in the United Kingdom have described an apparent beneficial effect of breast-feeding upon serious RSV disease during infancy. This phenomenon was investigated in cotton rats, and it was found that immune mothers transferred resistance to RSV to their infants. Maternally transferred immunity was more effective in the lungs than in the nose. This form of immunity was transferred primarily by colostrum and generally correlated with serum neutralizing antibody. However, there were exceptions to this correlation, suggesting that immune factors other than neutralizing antibody may also play an important role in maternal passive immunity. For example, transfer of RSV-specific lymphocytes may be involved. The occurrence of such transfer in humans is suggested by the observation that tuberculin sensitivity can be transferred from a nursing mother to her newborn infant (6).

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