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Parenteral Immunization with Live Respiratory Syncytial Virus Is Blocked in Seropositive Cotton Rats

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Seropositive cotton rats cannot be immunized by intramuscular inoculation of live respiratory syncytial virus. Passively administered antiserum was shown to effect the immunosuppression. By contrast, the presence of serum antibody did not block immunization by the intranasal route.

The importance of respiratory syncytial (RS) virus as the major viral respiratory tract pathogen of infancy (6) has led to intensive efforts to develop an effective vaccine. The first such effort resulted in a Formalin-inactivated vaccine with alum as its adjuvant. Although antigenic, it did not induce resistance to infection. Furthermore, it produced a state of altered reactivity that potentiated disease when vaccinees underwent natural infection (9–11). As a result of this experience, the development of an inactivated vaccine was abandoned, and efforts focused on the attenuation of RS virus in the hope that a live attenuated vaccine could stimulate immunity without altering reactivity to natural infection. Although this approach has been only partially successful, several attenuated temperature-sensitive mutants are currently being readied for evaluation in humans (1, 2, 14–15).

A novel approach to RS virus vaccination, the parenteral inoculation of live wild-type virus, was investigated in our laboratory. Using cotton rats, we found that intramuscular (i.m.) inoculation of live virus induced immunity to nasal and pulmonary infections (13). It appeared likely that the immunogenicity of the vaccine was dependent upon viral replication, as inactivation of the virus abolished immunogenicity. However, the amount of replication required for an effective immune response appeared to be quite low, perhaps restricted to a single abortive cycle, as infectious virus could not be recovered from the injection site more than 5 min after inoculation.

The success of this vaccine protocol in humans would depend, in part, on the immunogenicity of the vaccine in the presence of serum antibodies, since most potential human vaccinees (infants under 6 months of age) possess maternally transmitted antibodies. Studies in a small number of cotton rats which had passive maternal antibodies at the time of i.m. inoculation showed that none of these animals developed complete nasal immunity, and only 5 of 14 developed complete pulmonary immunity (13). Further evidence of an immunosuppressive effect of serum antibodies on i.m. inoculation was provided by the observation that only 1 of the 14 cotton rats developed an increase in serum-neutralizing antibodies after i.m. inoculation.

Using the cotton rat model, we have amplified these observations on the immunosuppressive effect of antibodies. We have confirmed that passively administered serum antibody is immunosuppressive and that even minute quantities of antibodies are sufficient to block the immune response.

MATERIALS AND METHODS

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

Animals were test bled from the eye before being included in the study to rule out the possibility of preexisting antibody against RS virus.

Virus. The A-2 strain of RS virus was prepared by Flow Laboratories, Inc., McLean, Va. A suspension of this virus (lot F-521) grown in African green monkey kidney cells and maintained with Eagle minimum essential medium–1% SPG (3) contained $10^{4.7}$ PFU per ml.

Mode of immunization. Seronegative mothers were used for all experiments. Infant cotton rats approximately 1 week old were inoculated intraperitoneally (i.p.) with 0.2 ml of pooled cotton rat serum. Sera were collected by exsanguination of animals which had not been inoculated with RS virus (control pool) or which had received approximately 10^4 PFU of A-2 strain RS virus intranasally (i.n.) 28 days before the bleeding (anti-RS pool).

At 24 h after i.p. administration of pooled serum, infant cotton rats were ear-tagged, and a small amount of blood was withdrawn from the retro-orbital venous plexus. The animals were then inoculated i.m. with $10^{3.4}$ PFU of virus in a volume of 0.05 ml.

At 3 weeks after i.m. inoculation, animals were anesthetized with methoxyflurane (Penthrane, Abbott Laboratories, North Chicago, Ill.), bled from the retro-orbital plexus, and challenged i.n. with $10^{3.7}$ PFU of virus in a volume of 0.1 ml.

At 4 days after the i.n. challenge, animals were sacrificed with carbon dioxide. Lungs and nasal tissue, including nasal passages and turbinates, were homogenized separately in 10 volumes of Hanks balanced salt solution with 1% sucrose potassium gultamate, quick-frozen on dry ice, and stored at −70°C until assayed.

Virus assay. The virus titer of infected tissue was determined by plaque assay on HEp-2 cells as described previously (12).

Serum antibody assay. Serum specimens obtained by eye bleeding were stored at −20°C before assay. Serum-neutralizing antibody to RS virus was assayed by measuring 60% plaque reduction on HEp-2 cell monolayers (8).

RESULTS

Effect of serum antibody to RS virus on subsequent parenteral immunization. Cotton rats inoculated with either control serum (group A) or anti-RS virus antiserum (group B) were inoculated i.m. with live RS virus 24 h later and challenged after 3 weeks to determine their immune status (Table 1). Of 16 animals receiving control serum, none had serum-neutralizing activity 24 h later. By contrast, all of the 13 animals which received anti-RS virus antiserum had demonstrable antiviral activity in their serum at that time, the geometric mean titer being 1:86.

At 3 weeks after i.m. vaccination and immediately before i.n. challenge, the opposite pattern was seen. Each of the animals which had initially received control serum (group A) developed a serological response to the i.m. vaccine the geometric mean titer for the group being 1:93. Conversely, none of the rats which had initially received anti-RS virus antiserum (group B) had measurable amounts of serum antibodies to the virus at the time of i.n. challenge.

The effect of passively acquired antibodies on the effectiveness of i.m. vaccination with RS virus was seen when nasal and pulmonary tissues were assayed for infectious virus after i.n. challenge (Table 1). Each of the animals primed with control serum before i.m. vaccination with live RS virus was completely resistant to growth of the challenge virus in the lungs. Nasal immunity was less striking, with 31% of the animals (5 of 16) showing complete resistance to infection. However, each of the 16 animals showed at least a 10-fold reduction in virus titer compared with the geometric mean titer seen in group B animals (data not shown). Comparison of geometric mean virus titers of the two groups showed a 100-fold or greater reduction in virus titers in group A animals in both organs ($P < 0.001$, Students t test). Group B animals were further compared with animals which had received neither i.p. serum nor i.m. virus (group C) (Table 1). Viral titers in the lungs and noses of the two groups after i.n. challenge did not differ significantly, indicating that the immunosuppressive effect of the antibodies was complete.

Dosage effect of antiserum. The striking immunosuppressive effect of anti-RS virus antiserum upon i.m. immunization with live RS virus prompted further study to determine the amount of antiviral antibodies required for blocking immunization. Seronegative cotton rats, approximately 1 week of age, were divided into five groups. Group 1 received 0.2 ml of pooled cotton rat anti-RS virus antiserum i.p. (antibody titer, 1:970). Group 2 received 0.2 ml of a mixture of 1 part antiserum and 3 parts control serum, group 3 received 1 part antiserum to 63 parts control serum, and group 4 received 1 part antiserum to 255 parts control serum. Group 5 received 0.2 ml of control serum i.p. After 24 h each animal was eye bled, ear-tagged, and inoculated i.m. with live RS virus in the same manner as in experiment 1. After 3 weeks, the animals were again eye bled and challenged i.n. as in experiment 1.

The effect of graded i.p. doses of antiserum upon postvaccination antibody production is shown in Fig. 1. As expected, animals receiving the largest dose of antiserum showed the highest serum titer when eye bled 24 h later. Of eight animals receiving 1 part antiserum to 255 parts control serum, only one had a measurable quantity of antiviral antibodies at the time of i.m. inoculation, and that at a low titer (1:32). Animals receiving only control serum lacked detectable amounts of RS virus antibodies at the time of i.m. virus inoculation.

The antibody titers 3 weeks later showed a pattern similar to that seen in experiment 1. Animals receiving antiserum before i.m. inoculation exhibited an immunosuppressive effect (Fig. 1), with the exception of the group receiving the smallest dose of RS virus antibodies. Five of the eight animals in this group showed a slight increase in antibodies after i.m. inoculation (data not shown). Animals primed only with control serum generally showed the greatest antibody response (Fig. 1), although three of the eight animals in this group did not develop a postvaccination antibody response (data not shown).

The immune status of the animals was determined by viral assay of nasal and pulmonary tissues after i.n. challenge (Fig. 2). Each of the animals which had been primed with control

TABLE 1. Resistance of weanling cotton rats to i.n. challenge with RS virus induced by i.m. inoculation of live RS virus

Treatment before i.m. virus inoculation	No. of rats tested	Serum-neutralizing antibody amt at time of i.m. virus inoculation[a]	Serum-neutralizing antibody amt at time of i.n. virus inoculation[a]	Virus replication 4 days after challenge[b]			
				No. of rats from which virus was not recovered		Geometric mean of virus in tissue (PFU/g)[c]	
				Nasal turbinates	Lungs	Nasal turbinates	Lungs
Control serum (group A)[d]	16	<20	93	5 (31)	16 (100)	$10^{2.3}$	$10^{2.3}$
RS antiserum (group B)	13	86	<20	0	0	$10^{4.3}$	$10^{4.1}$
None (group C)[e]	19		<20	0	0	$10^{4.6}$	$10^{4.5}$

[a] Expressed as reciprocal of geometric mean.
[b] Rats were challenged i.n. with $10^{3.7}$ PFU of A-2 strain virus 3 weeks after i.m. inoculation with $10^{3.4}$ PFU of live A-2 strain virus. Numbers in parentheses are percentages.
[c] For differences between groups A and B, $P < 0.001$; differences between groups B and C were not significant.
[d] Cotton rat serum (0.2 ml) was administered i.p. to each animal 24 h before i.m. inoculation with live RS virus. The neutralizing titer of anti-RS antiserum was 1:1,250. Control serum showed no neutralizing activity at 1:20.
[e] Control animals receiving neither i.p. serum nor i.m. virus.

serum developed resistance after i.m. inoculation. This resistance was manifest by the complete suppression of virus replication in the noses and lungs, even in the three animals which had not developed a serological response to i.m. inoculated virus.

By contrast, none of the animals which had been primed with immune serum, even at a dilution of 1:256, exhibited resistance in the lungs or nose after vaccination. The amount of immune serum required to completely suppress the immune response to i.m. vaccination was very small, for seven of eight animals receiving a 1:256 dilution of immune serum were rendered refractory to the i.m. vaccine despite having a serum titer of antibodies to RS virus at the time of vaccination which was below the level of detectability of the neutralizing antibody assay.

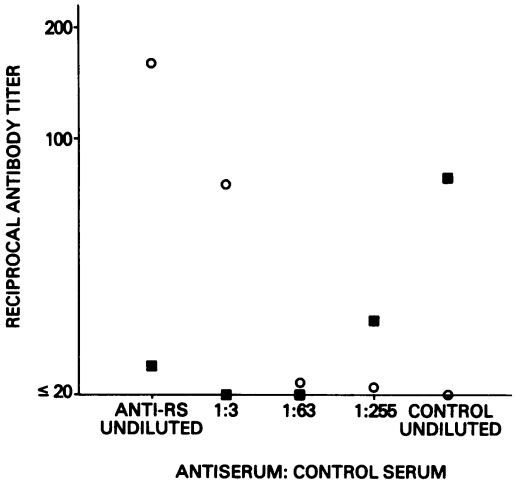

FIG. 1. Geometric mean reciprocal neutralizing antibody titers of cotton rats. Prevaccine titer (○) was obtained from serum of animals 24 h after i.p. injection of serum; postvaccine titer (■) was obtained from serum of the same animals 3 weeks after i.m. inoculation of live RS virus. Number of animals in each group: anti-RS undiluted, 9; 1:3, 10; 1:63, 3; 1:255, 8; control undiluted, 8.

DISCUSSION

Inoculation of weanling cotton rats i.m. with $10^{2.2}$ to 10^4 PFU of live RS virus was previously shown to induce significant or complete resistance to infection in both the upper and the lower portions of the respiratory tract (13). The possibility that passive immunity might interfere with the effectiveness of parenteral immunization with live RS virus was first raised by Buynak and colleagues, who observed a marked reduction in antigenicity of such a vaccine in children with preexisting serum antibodies (5). Their data, however, were restricted to measurements of antibodies. Our initial studies in cotton rats confirmed their observation that serological response to vaccination with live RS virus is suppressed in the presence of preexisting serum antibodies and provided additional data to show that immunity to subsequent challenge with live virus, a more meaningful measure of vaccine efficacy, was also blocked in animals with preexisting antibodies (13).

Because our data were based on studies of infant cotton rats born to RS virus-immune mothers, we could only suggest that serum factors accounted for immunosuppression, since it is not known which immune factors besides serum antibodies are transferred from mothers to offspring in cotton rats. The present study attempted to show whether serum factors alone (presumably antibodies) were capable of block-

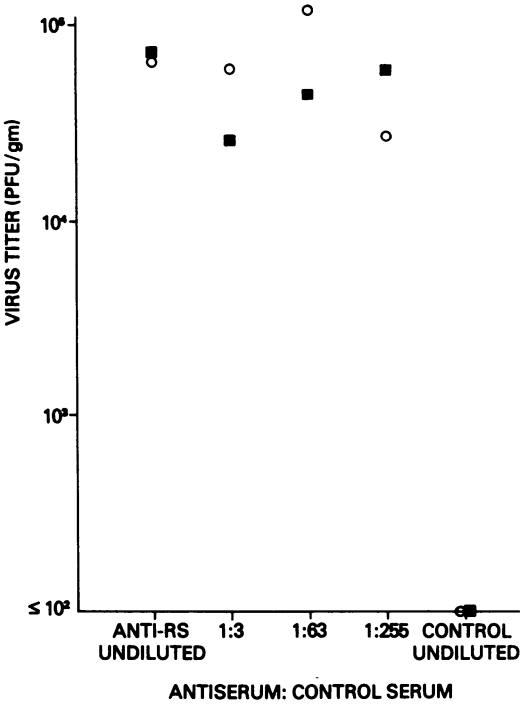

FIG. 2. Geometric mean viral titers in lungs (○) and noses (■) of cotton rats 4 days after i.n. challenge with wild-type virus (same animals as in Fig. 1). Animals were primed by injecting serum i.p. 24 h before immunization with live RS virus. At 3 weeks after i.m. immunization, animals were challenged i.n. with homologous virus.

ing the efficacy of i.m. vaccination. In addition, we sought to determine the relative amount of antibodies (or concomitant serum immune factors) capable of blocking live virus vaccine given i.m.

In experiment 1 (Table 1), it appeared that factors in immune serum were capable of blocking completely the immune response to i.m. vaccination. The observation that cotton rats receiving an equivalent volume of normal serum were effectively immunized by i.m. inoculation showed that the blocking factors in immune serum were specific immunological substances. This experiment, however, did not allow further definition of the blocking components of immune serum.

In experiment 2, we attempted to determine the dilution endpoint of serum blocking factor(s) required for immunosuppression. Surprisingly, antiserum diluted 1:256 was sufficient to block completely the efficacy of the i.m. vaccination, although this dilution of antiserum did not yield detectable amounts of neutralizing antibodies in the serum of recipient rats.

The initial report of a live, parenterally administered RS virus vaccine (5) raised hopes that efforts spanning nearly two decades to develop an effective vaccine might have succeeded. The concomitant observation that the vaccine might be less antigenic in seropositive vaccines, however, tempered those hopes. A follow-up report by the same group (4) showing a serological response in 97% (113 of 116) of seronegative vaccinees but only in 8% (4 of 52) of seropositive vaccinees cast further doubt as to the practicability of such a vaccine.

The observation that serum antibodies (or a concomitant) at a level below that detectable by neutralizing antibody assay blocked both a serological response and resistance to infection suggests that such a vaccine may not be useful to those most in need of immunoprophylaxis. The greatest impact of RS virus occurs during the first 6 months of life, when most infants possess maternally derived serum-neutralizing antibodies for RS virus.

The immunosuppressive effect of antiserum on parenteral RS virus vaccination raises a further question of a similar effect on i.n. administered vaccines. This is a particularly important issue since initial clinical trials of an attenuated live virus vaccine administered i.n. are in progress. Using the same pools of cotton rat serum described in this paper, we inoculated infant cotton rats i.p. and administered a live attenuated RS virus NG 16 (*ts*-1) i.n. the following day. The attenuated virus had previously been shown to induce complete immunity to wild-type RS virus infection in unprimed cotton rats (unpublished data). At 3 weeks after i.n. inoculation of vaccine, rats were challenged i.n. with wild-type RS virus. A total of 32 animals were used, 19 receiving immune serum and 13 receiving control serum. Titration of virus in the lungs and noses of animals 4 days after challenge showed that all animals were protected from challenge whether they had received immune or control serum before vaccination (data not shown). Hence, it would appear that the immunosuppressive effect seen with i.m. vaccination is not seen with i.n. vaccination and that topically applied RS virus vaccines may offer effective prophylaxis regardless of the serological status of the vaccinee.

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