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Effectiveness of Topically Administered Neutralizing Antibodies in Experimental Immunotherapy of Respiratory Syncytial Virus Infection in Cotton Rats

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Initial studies of the prophylactic effect of parenterally administered respiratory syncytial virus (RSV)-neutralizing antibodies in cotton rats indicated that virus replication in lung tissues was restricted when animals with preexisting antibody titers in serum of 1:100 or more (as measured by plaque reduction) were challenged intranasally with 10^4 PFU of virus. Subsequently, a therapeutic effect of parenterally administered RSV antibodies (present in human gamma globulin) was demonstrated in both cotton rats and owl monkeys. Parenteral inoculation of RSV-infected cotton rats or owl monkeys with purified human immunoglobulin licensed for intravenous administration in humans (IVIG) effected a $10^{-1.7}$ to $10^{-2.7}$ reduction in the level of pulmonary virus at the height of infection. Because of these encouraging results, we examined topical administration of IVIG to determine whether it was also effective and whether it offered an advantage over the parenteral route with regard to simplicity and the dose required for full therapeutic effect. IVIG (0.025 g/kg) administered topically by the intranasal route to anesthetized cotton rats at the height of RSV infection effected a $10^{2.2}$-fold reduction in viral titers of pulmonary tissues and a complete clearance of detectable virus in 92% of the animals within 24 h. In contrast, 4 g of IVIG per kg was required to produce a comparable therapeutic effect when the material was administered parenterally. Thus, the therapeutic effect of IVIG was 160 times greater by the topical route than by parenteral inoculation.

Initial studies of the prophylactic effect of parenterally administered respiratory syncytial virus (RSV)-neutralizing antibodies in cotton rats indicated that virus replication in lung tissues was restricted when animals with antibody titers in serum of 1:100 or more (as measured by plaque reduction) were challenged intranasally with 10^4 PFU of virus. Furthermore, the lung tissues of cotton rats with titers of passively acquired neutralizing antibodies in serum of 1:380 or greater were completely or almost completely resistant to infection by RSV. The prophylactic effect of passively administered RSV antibodies was observed with both cotton rat convalescent serum and purified human immunoglobulin licensed for intravenous administration in humans (IVIG).

Subsequently, a therapeutic effect of parenterally administered RSV antibodies was demonstrated both in cotton rats and owl monkeys. Parenteral inoculation of cotton rats or owl monkeys with IVIG (8 or 3 g/kg, respectively) at the height of RSV infection effected a $10^{-1.7}$ to $10^{-2.7}$ reduction in the level of virus in pulmonary tissues. Approximately twice the level of neutralizing antibodies in serum was required to achieve a therapeutic effect compared with that required to achieve a prophylactic effect. A significant decrease in virus titers of pulmonary tissues was evident in cotton rats within 3 h after inoculation of IVIG, and a full therapeutic effect was achieved by 24 h without the development of discernible pulmonary immunopathology.

Because of these encouraging results we examined topical administration of IVIG to determine whether it was also effective and whether it offered an advantage over the parenteral route with regard to simplicity and the dose required for full therapeutic effect.

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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 germ-free barrier for the past 10 years, was the source of animals for the production colony. Adult animals were immunized with inactivated Sendai virus vaccine (Microbiological Associates, Bethesda, Md.) at least 3 weeks before study. *Sigmodon fulviventer* were obtained from our own colony which had been initiated with wild-trapped animals.

IVIG administration. A single lot of purified human IVIG (lot 2.370.069.0, Sandoglobulin; Sandoz, Inc., East Hanover, N.J.) was used for all experiments. This lot had an RSV-neutralizing antibody titer of 1:2905. Cotton rats infected 3 days earlier by intranasal (i.n.) instillation of the A2 strain of RSV ($10^{4.0}$ PFU per animal) were anesthetized with methoxyflurane and were inoculated intraperitoneally (i.p.) or i.n. with IVIG. The volume of IVIG inoculated i.n. was 0.1 ml per 100 g of body weight; the animals ranged in weight from 100 to 120 g. Administration (i.n.) of IVIG to fully anesthetized cotton rats resulted in rapid delivery of the inoculum to the lungs.

Virus assay. Animals were sacrificed by carbon dioxide asphyxiation 24 h after administration of IVIG. Lung 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

TABLE 1. Therapeutic effect of human IVIG administered i.p. or i.n. at 3 days post infection

Route of IVIG administration[a]	IVIG dose (g/kg of body wt)	No. of animals	Level in serum 1 day after IVIG treatment		Viral titer for[b]:	
			Titer of RSV-neutralizing antibodies (reciprocal)	IVIG (μg/ml)	Lung tissues	Nasal tissues
Untreated control		24	<20	0	4.26 ± 0.08 (0)	4.80 ± 0.17 (0)
i.p.	8.0	13	692	16,829	2.15 ± 0.10[c] (85)	2.82 ± 0.36[c] (62)
	4.0	10	518	12,379	2.04 ± 0.05[c] (90)	3.57 ± 0.36[d] (10)
	2.0	10	302	4,769	2.26 ± 0.19[c] (80)	3.61 ± 0.44[d] (20)
	1.0	8	142	3,760	3.27 ± 0.27[c] (13)	4.85 ± 0.14[e] (0)
	0.25	4	59	1,670	4.13 ± 0.03[e] (0)	4.81 ± 0.07[e] (0)
i.n.	0.1	13	<20	200	<2.0[c] (100)	4.96 ± 0.14[e] (0)
	0.05	11	<20	140	<2.0[c] (100)	4.74 ± 0.21[e] (0)
	0.025	12	<20	61	2.06 ± 0.06[c] (92)	4.85 ± 0.15[e] (0)
	0.0125	10	NT[f]	NT	2.78 ± 0.19[c] (30)	4.36 ± 0.26[e] (0)
	0.00625	10	NT	NT	3.50 ± 0.14[c] (0)	4.30 ± 0.25[e] (0)
	0.003125	10	NT	NT	4.15 ± 0.14[e] (0)	4.86 ± 0.27[e] (0)
	0.0015625	12	NT	NT	4.86 ± 0.12[e] (0)	5.39 ± 0.17[e] (0)

[a] IVIG was administered 3 days after infection ($10^{4.0}$ PFU of RSV).
[b] Viral titers ($\log_{10}$ PFU/g [geometric mean ± standard error]) were determined 4 days after infection; numbers in parentheses represent the percentage of animals that were free of detectable virus.
[c] Significance of reduction of viral titers of IVIG-treated infected animals compared with those of untreated controls ($P < 0.001$).
[d] $P < 0.005$.
[e] Not significant.
[f] NT, Not tested.

KH_2PO_4–3.2 mM K_2HPO_4, and the resulting suspension was stored at −70°C until assayed. Virus titers were determined by plaque assay on HEp-2 cell monolayers as previously described and were expressed as PFU per gram of tissue (6).

Antibody and immunoglobulin assays. Neutralizing antibody was measured by a plaque-reduction neutralization assay as previously described by using a 60% plaque-reduction endpoint (6). Human immunoglobulin G in cotton rat serum was measured by radial immunodiffusion (Meloy Laboratories, Springfield, Va.), and low levels (<10 mg/dl) were confirmed by a competitive inhibition enzyme-linked immunosorbent assay (1).

Histology. The lungs of the animals were removed from the thorax and were inflated through the trachea with neutral buffered Formalin. Histologic sections were stained with hematoxylin and eosin.

RESULTS

Comparison of therapeutic efficacy of IVIG administered parenterally (i.p.) or topically (i.n.). Administration of IVIG by the i.p. route in a dose of 2 g/kg or more 3 days after infection of the respiratory tract with RSV reduced the titer of RSV in the lungs 10^{-2} or more within 24 h. As a consequence, virus could not be detected on day 4 postinfection in pulmonary tissues of 80 to 90% of the animals treated in this manner (Table 1). A significant reduction in the quantity of virus in nasal tissues was also observed, but this effect was not as dramatic as that observed for lung tissues.

IVIG administered by the i.n. route appeared to be more effective than IVIG inoculated parenterally. Thus, topically administered IVIG cleared RSV in pulmonary tissues of all animals when a dose of 0.05 g/kg or greater was used. However, IVIG administered in this way had no effect on the quantity of RSV present in nasal tissues (Table 1). The therapeutic effect of i.n. administered IVIG on pulmonary viral titer diminished with decreasing dose, and the minimum effective dose was observed to be 0.00625 g/kg ($P < 0.001$). Cotton rats inoculated topically with the largest dose of IVIG (0.1 g/kg) had near peak levels of this material in serum within 5 h, but RSV antibodies did not reach a concentration which allowed detection by the neutralization assay. The concentration of IVIG in serum 24 h after topical administration ranged from 61 to 140 μg/ml for cotton rats which received 0.025 to 0.05 g/kg of body weight, a dose which cleared or almost completely cleared RSV from pulmonary tissues. The concentration of IVIG in the serum of cotton rats which exhibited a similar therapeutic effect when IVIG was administered parenterally (4 to 8 g/kg of body weight) was 120 to 202 times higher, i.e., 12,379 to 16,829 μg/ml (Table 1).

Therapeutic effect of i.n. administered IVIG not due to neutralization in vitro. The possibility that the observed reduction in viral titers of pulmonary tissues of animals treated by topical administration of IVIG was caused by neutralization in vitro was evaluated by mixing an equal amount of infected lung tissue from a treated cotton rat (human IVIG administered i.n. at a dose of 0.1 g/kg 3 days postinfection) with infected lung tissue from a cotton rat which had not received IVIG. Mixed homogenates yielded the same amount of virus as lung suspensions from infected cotton rats that had not received IVIG (Table 2). This observation indicated that the therapeutic effect observed for IVIG recipients was due to bona fide passive immunity rather than to neutralization of virus in vitro after homogenization of lung tissue.

Topical IVIG therapy did not prolong infection. We also examined the possibility that topical administration of IVIG (0.1 g/kg) might reduce viral titer on day 4 postchallenge but still prolong the course of infection. This question was addressed by comparing the titer of RSV in tissues of infected IVIG-treated and control animals at intervals after treatment (Table 3). In each instance, tissues from IVIG-

TABLE 2. Therapeutic effect of IVIG administered topically after infection was not attributable to in vitro neutralization during homogenization of lung tissues

Lung homogenates from infected rats (no. tested)[a]	Viral titer[b]
RSV antiserum at 3 days postinfection (4)	2.76 ± 0.16[c]
No antiserum (4)	5.57 ± 0.23
Mixture of RSV antiserum (4) with no antiserum (4)	5.39 ± 0.13[d]

[a] Lungs from RSV-infected rats, treated or not treated i.n. with IVIG (0.1 g/kg) on day 3 postinfection, homogenized and titrated for virus on day 4 postinfection.
[b] Viral titers ($\log_{10}$ PFU/g [geometric mean ± standard error]) were determined 4 days after RSV challenge (10^4 PFU).
[c] Significant reduction of viral titers ($P < 0.001$).
[d] No significant reduction of viral titers.

treated animals yielded significantly smaller amounts of virus than did tissues from control animals, and there was no evidence of a rebound in virus replication.

Topical IVIG therapy not associated with increased pulmonary immunopathology. Recently, we observed that cotton rats previously vaccinated with Formalin-inactivated RSV developed enhanced pulmonary pathology resembling an Arthus reaction when they were infected with RSV (5). To determine whether immunotherapy might initiate immunopathology, we harvested lung tissues from infected cotton rats which had been treated i.n. with IVIG (0.1 g/kg) on day 3 postinfection and examined the material histologically. Tissues taken 24 and 96 h after i.n. administration of IVIG showed no evidence of pathology.

Prophylactic effect of topical IVIG. The possibility that topically administered IVIG might prove useful during annual RSV epidemics for short-term prophylaxis in high-risk situations (such as for infants in an intensive care nursery or for patients with congenital heart or pulmonary disease) was examined by administering IVIG i.n. (0.1 g/kg) before infection and then challenging the animals i.n. with RSV (10^4 PFU per animal) at intervals up to 7 days after IVIG administration. At 4 days after challenge, the animals were sacrificed, and the titers of virus in pulmonary tissues were assayed (Table 4). Although the degree of protection was inversely proportional to the interval between IVIG inoculation and virus challenge, significant protection ($P < 0.005$) was detected for as long as 7 days after the immunoglobulin preparation was administered.

DISCUSSION

Although a protective role of RSV-neutralizing antibodies in serum was originally doubted, more recent evidence from experimental studies with animals and epidemiologic investigations with humans indicates that high levels of such RSV antibodies have a significant prophylactic or therapeutic effect (3, 4). Indeed, on the basis of observations made with cotton rats and monkeys, a clinical trial of the therapeutic efficacy of intravenously administered IVIG is now in progress for infants hospitalized with RSV pneumonia or bronchiolitis. Generic Sandoglobulin (Sandoz) is being used in this trial. This material consistently contains a high level of RSV-neutralizing antibodies, and thus it is not necessary to identify specific lots or to concentrate and purify RSV antibodies from IVIG. On the other hand, it has been necessary to inoculate a large amount of IVIG to achieve the desired therapeutic level of RSV antibodies in serum. For example, as much as 2 to 4 g of IVIG per kg was necessary to achieve a maximal therapeutic level of circulating antibodies in cotton rats (Table 1).

One alternative to such a high-dose regimen would be the use of an antibody preparation with a greater concentration of RSV antibodies, and efforts are underway to produce such a preparation. A second approach is to administer IVIG by a different, more effective, route than that previously used. This approach has the practical advantage of permitting the use of a material which is already licensed for parenteral use in the United States and in other countries.

The second approach was investigated in this study by comparing the therapeutic efficacy of IVIG administered topically or parenterally. IVIG (0.025 g/kg) administered topically by the i.n. route to anesthetized cotton rats effected a $10^{2.2}$-fold reduction in viral titers of pulmonary tissues and a complete clearance of detectable virus in 92% of the animals. In contrast, 4 g of IVIG per kg was required to produce a comparable therapeutic effect when the material was administered parenterally. Thus, the therapeutic effect of IVIG was 160 times greater by the topical route than by parenteral inoculation. The technique used to deliver IVIG to the lungs involved i.n. inoculation of anesthetized animals. Although this method is not suitable for use in humans, it did establish the greater therapeutic effect of topical immunoglobulin. However, it should be possible to achieve the same enhanced therapeutic effect in humans by using a small-particle aerosol.

As was the case with systemically administered antibodies, the therapeutic effect of i.n. IVIG stemmed from an in vivo neutralization of RSV infection, rather than being an artefact of neutralization in vitro during homogenization of

TABLE 3. Reduction in level of RSV in pulmonary tissues after topical administration of IVIG was not followed by a rebound in virus multiplication

Cotton rat species (treatment)	Viral titer on indicated day after treatment with IVIG i.n.[a]:					
	1	2	3	4	5	7
S. hispidus (IVIG treated)	<2.0 (4)	NT	2.41 ± 0.32 (4)	NT	NT	<2.0 (4)
S. hispidus (untreated)	4.69 ± 0.30 (4)	NT	4.57 ± 0.16 (4)	NT	NT	<2.0 (4)
S. fulviventer (IVIG treated)	2.61 ± 0.62 (4)	2.73 ± 0.63 (4)	2.61 ± 0.42 (4)	<2.0 (4)	<2.0 (4)	<2.0 (5)
S. fulviventer (untreated)	5.05 ± 0.13 (4)	4.96 ± 0.11 (4)	3.75 ± 0.19 (4)	3.05 ± 0.36 (4)	<2.0 (4)	<2.0 (2)

[a] Rats were infected with 10^4 PFU of RSV and either treated or not treated with IVIG on day 3 postinfection. Viral titers ($\log_{10}$ PFU/g; geometric mean ± standard error) were measured on indicated day after treatment. Numbers in parentheses indicate the number of animals in the group. NT, Not tested.

TABLE 4. Topically administered IVIG retains antiviral activity in lung tissues for up to 7 days

Cotton rat species	Viral titer for untreated cotton rats and for those pretreated with IVIG i.n. on indicated day before infection[a]:					
	Untreated	1 h	1 day	2 days	4 days	7 days
S. hispidus	5.85 ± 0.10 (8)[b]	<2.0[c] (4)	3.24 ± 0.43[c] (5)	4.26 ± 0.43[c] (5)	4.99 ± 0.06[c] (5)	5.39 ± 0.09[d] (6)
S. fulviventer	5.83 ± 0.17 (9)	2.05 ± 0.06[c] (5)	3.32 ± 0.33[c] (6)	3.29 ± 0.51[c] (5)	3.40 ± 0.33[c] (9)	4.73 ± 0.21[d] (7)

[a] Viral titers ($\log_{10}$ PFU/g [geometric mean ± standard error]) were determined 4 days after infection (10^4 PFU of RSV i.n.).
[b] Numbers in parentheses indicate the number of animals tested.
[c] Significance of reduction of viral titers of IVIG-treated infected animals compared with those of untreated controls ($P < 0.001$).
[d] $P < 0.005$.

lung tissues (4). Also, the therapeutic effect of IVIG was permanent because rebound of virus replication in the lungs was not detected. Furthermore, as observed during previous studies with parenterally administered antibodies, histologic evidence of immunopathology was not seen in lung tissues of infected animals which were treated topically with IVIG.

The therapeutic effect of topically administered IVIG appears to be mediated within the lumen of the lungs rather than within the parenchyma. The quantity of IVIG present in the serum of topically treated cotton rats which received the minimum dose required for the almost complete clearance of RSV from lung tissues (i.e., 0.025 g/kg) was 61 μg/ml. This level was 200 times lower than the 12,379 μg of IVIG per ml which was present in the serum of animals treated parenterally with the minimum dose required for the almost complete clearance of RSV from lung tissues (i.e., 4 g/kg). Thus, the amount of IVIG in the serum of infected cotton rats successfully treated topically with immunoglobulin was considerably below the concentration of IVIG associated with a full therapeutic effect in parenterally treated animals. This implies that IVIG exerts its therapeutic effect within the air passages of the lungs.

The most surprising finding to emerge from this study was the duration of activity of topically administered antibodies, wherein a single i.n. inoculation of IVIG provided significant resistance for up to 7 days. This suggests the presence of a receptor for immunoglobulin G in pulmonary tissues which is perhaps specific for the Fc portion of the antibody molecule. If IVIG behaves the same in the lungs of primates, it may be feasible to administer RSV antibodies prophylactically by aerosol to high-risk infants hospitalized during RSV epidemics, perhaps no more often than twice a week, and thereby substantially reduce the risk of nosocomial infection and disease. Also, consideration should be given to a clinical evaluation of the administration of single doses of IVIG, rapidly delivered by a small-particle aerosol, to determine the usefulness of this regimen in the therapy of serious RSV-induced lower respiratory tract disease in infants. It should be noted that the currently licensed therapy for RSV requires the delivery of ribavirin by small-particle aerosol for 12 to 20 h a day for at least 3 days (2, 7).

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