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Enhancement of Respiratory Syncytial Virus Pulmonary Pathology in Cotton Rats by Prior Intramuscular Inoculation of Formalin-Inactivated Virus

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Cotton rats previously inoculated with Formalin-inactivated respiratory syncytial virus (RSV) were challenged intranasally with live RSV to induce an enhancement of RSV disease similar to that observed after the administration of Formalin-inactivated RSV vaccine to human infants 20 years ago. Within 24 h after infection with RSV, cotton rats developed pulmonary lesions that reached a maximum by day 4. Histologically, the lesions resembled an experimental pulmonary Arthus reaction. An action of Formalin on RSV appears to be responsible for this effect, because live virus or virus heated in the absence of Formalin did not induce enhanced immunopathology. Selected epitopes on the fusion (F) or attachment (G) or both RSV surface glycoproteins that are involved in inducing neutralizing antibodies were modified to reduce or ablate their antigenicity. However, other epitopes on the F or G or both glycoproteins were not ablated by Formalin, because cotton rats inoculated parenterally with a Formalin-inactivated virus developed a high level of F and G antibodies measurable by an enzyme-linked immunosorbent assay. At this time, the effect of Formalin on RSV cannot be localized to either the F or G glycoprotein of RSV.

Approximately 20 years ago an inactivated respiratory syncytial virus (RSV) vaccine was prepared from a virus grown in African green monkey kidney cell culture, inactivated by a Formalin solution (1:4,000), and concentrated 100 times by centrifugation and alum precipitation. Although this vaccine induced a serum neutralizing antibody response, it did not protect young vaccinates against infection by RSV during subsequent epidemics (2, 5, 7, 8), when RSV vaccinates developed severe RSV lower respiratory tract disease significantly more often than did infants and young children who had received an inactivated parainfluenza type 1 virus vaccine prepared in a manner identical to that of the RSV vaccine. An analogous enhancement of illness caused by the rubeola virus was observed concurrently in children who had received a Formalin-inactivated measles vaccine prepared in a manner similar to that of the inactivated RSV vaccine (4). Potentiation of the measles infection was not observed until several years after vaccination, when vaccinates had lost their immunity. In contrast, potentiation of the RSV infection occurred within several months after vaccination.

A study of RSV vaccinates who had not yet been infected revealed that the vaccine induces a heightened cell-mediated immune response to RSV antigens as assayed in vitro by lymphocyte transformation (9). This response is significantly greater than that which occurs after infection. Surveillance of RSV vaccinates has revealed that potentiation of the disease occurs primarily during the initial RSV infection and that RSV vaccinates are not at increased risk during subsequent RSV infections (H. W. Kim, personal communication).

Retrospective analysis of serum specimens from vaccinates who had received inactivated measles vaccine indicates that Formalin inactivation of the virus does not alter antigenicity of the hemagglutinin glycoprotein (11, 12). However, the fusion (F) glycoprotein is altered so that antibodies that inhibit hemolysin (fusion) activity are not induced. It has been suggested that selective Formalin alteration of the fusion glycoprotein prevents stimulation of antibodies that inhibit the function of this glycoprotein and is thus responsible for the potentiation of measles infections which occurs when vaccinates lose their hemagglutination-inhibiting antibodies (11).

Until recently a similar analysis of the effect of Formalin on RSV glycoproteins was not possible because reagents required for assay of these antigens were not available. Within the past few years, monoclonal antibodies have been developed that are specific for the attachment (G) glycoprotein (analogous to the hemagglutinin of measles virus) and F glycoprotein of RSV, and these antibodies have been adapted to effect immunoaffinity purification of RSV glycoproteins from lysates of infected cells (16, 18). Such purified glycoproteins can now be used to measure the F or G glycoprotein-specific antibody response to a Formalin-treated virus. A functional test for F antibodies is also now available (17). Making use of these newly available reagents, we have investigated the phenomenon of disease potentiation by Formalin-inactivated RSV, with the cotton rat as a model of experimental RSV infection. The cotton rat was chosen for this purpose because it is the most permissive small laboratory animal known (14).

MATERIALS AND METHODS

Vaccines. Three preparations of Formalin-inactivated RSV were studied in cotton rats. The first was the original lot 100 RSV vaccine prepared and evaluated in clinical trials from

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1965 to 1966. This vaccine was prepared from primary African green monkey kidney cells infected with Bernett strain of RSV (11657) that is closely related to the prototype Long strain by cross-neutralization. The virus was inactivated by Formalin (1:4,000) during a 72-h incubation at 37°C and then concentrated 25 times by centrifugation in a Sharples rotor. A further fourfold concentration was achieved by alum precipitation. The lot 100 vaccine was stored at 4°C until it was used in this study; 0.1 ml was inoculated intramuscularly at three weekly intervals.

Two other experimental vaccines were prepared during this study to test the effect of Formalin on antigenicity in the absence of alum. HEp-2 cell cultures infected with the A2 strain of RSV (similar to the Long strain by cross-neutralization) were harvested, and clarified supernatant fluid was treated with Formalin at a concentration of 1:4,000 at 37°C for 72 h to inactivate infectivity. This material was tested either unconcentrated or after concentration 100 times by centrifugation at 8,000 × *g* for 14 h; 0.1 ml was inoculated intramuscularly into cotton rats at three weekly intervals.

Three suspensions containing the live virus (A2 strain) were also evaluated in cotton rats. This allowed us to compare the response to the inactivated virus with that induced by infection. Infected HEp-2 cells were harvested, and clarified supernatant fluid was used to infect cotton rats. The virus was instilled into the nose ($10^{4.5}$ PFU in 0.2 ml) or introduced by three weekly intramuscular inoculations ($10^{4.5}$ PFU per inoculation). The live virus preparation was also concentrated 100 times by centrifugation (8,000 × *g*) or incubated at 37°C for 72 h; 0.1 ml was inoculated intramuscularly at three weekly intervals. The live virus preparation ($10^{5.1}$ PFU) served as a control for the concentrated (100 times), inactivated virus preparation, while the incubated suspension ($10^{3.2}$ PFU) served as a control for heat inactivation of the virus during Formalin inactivation.

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

Virus. The A-2 strain of RSV, propagated in the HEp-2 cells, was used in all experiments.

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, and 3.8 mM KH_2HPO_4 and stored at −70°C until assayed. The virus titer was determined by a plaque assay on HEp-2 cell monolayers as previously described and was expressed in PFU per gram of tissue (14).

Antibody assays. Neutralizing antibodies were measured by a plaque reduction neutralization assay as previously described, with a 60% plaque reduction endpoint (14).

Antibodies to F and G glycoproteins were measured with an enzyme-linked immunosorbent assay (ELISA) (10). Purified F and G proteins which were prepared by immunoaffinity purification of RSV glycoproteins from lysates of infected cells (16, 18) were used at a concentration of 100 ng/ml to coat Immulin 1 polystyrene U-bottom microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). A sequence of cotton rat serum, rabbit anti-cotton rat immunoglobulin G (IgG; prepared in our laboratory), and alkaline phosphatase-conjugated goat anti-rabbit IgG (Miles Scientific Div. Miles Laboratories, Inc., Naperville, Ill.) was added to microtiter plates coated with F and G glycoprotein.

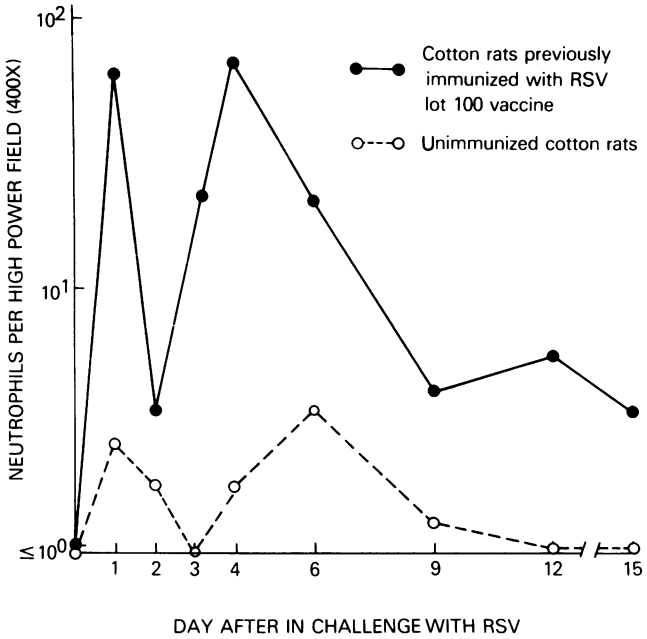

FIG. 1. Pulmonary infiltration by neutrophils in lot 100-vaccinated cotton rats and untreated cotton rats after intranasal challenge with RSV. Twenty high-power (×400) fields were counted for each animal, with data from 4 to 10 animals combined for each point.

Fusion-inhibiting antibodies were measured as previously described (17) by using HEp-2 cells.

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

RESULTS

Enhanced pathologic changes produced by Formalin-inactivated vaccine. RSV (A2 strain) induced minimal pathologic changes during the infection of cotton rats. A mild proliferative bronchiolitis appeared from 2 to 9 days after infection. Peribronchial infiltration of neutrophils and accumulation of neutrophils in bronchiolar epithelium and interalveolar spaces was minimal and usually peaked on day 6 of the infection. An attempt was made to quantitate this response (Fig. 1). Twenty high-power (×400) fields of lungs were examined, and the average number of neutrophils per field at various intervals after infection was determined (Fig. 1). A similar survey was made for lymphocytes (Fig. 2).

Cotton rats which had previously received three weekly intramuscular inoculations of 0.1 ml of lot 100 Formalin-inactivated RSV vaccine developed an enhanced pathologic response during infection with the virus 1 week after the final inoculation. Infiltration of neutrophils was more extensive than that seen after infection of unvaccinated cotton rats, although changes in bronchial and bronchiolar epithelium were milder (Fig. 3 to 5). The number of neutrophils in the lungs of infected, previously vaccinated cotton rats was approximately 20 times greater than that in the lungs of infected, previously unvaccinated animals (Fig. 1). The two peaks of infiltration were on days 1 and 4. Neutrophilic infiltrates were seen in clumps on day 1 (Fig. 6) but were

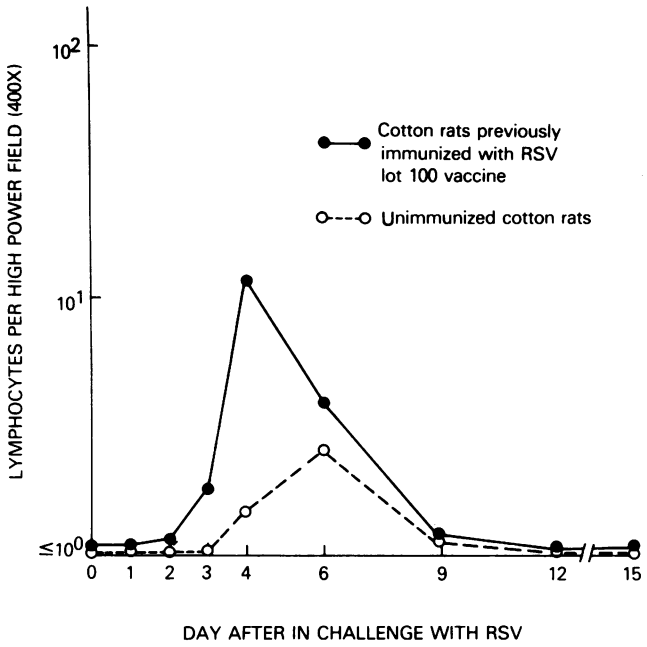

FIG. 2. Pulmonary infiltration by lymphocytes in lot 100-vaccinated cotton rats and untreated cotton rats after intranasal challenge with RSV. Data were tabulated as for Fig. 1.

dispersed on day 4 (Fig. 7 and 8). Neutrophilic infiltration was not accompanied by fluid, fibrin, or macrophages, and involved alveoli did not appear to be infected by bacteria or damaged by the presence of neutrophils. There was also an increase in infiltration of lymphocytes in the lungs of infected, previously vaccinated animals, but the increment was less than that seen for neutrophils (Fig. 2 and 5).

A similar enhancement of pulmonary pathology was also observed during the infection of cotton rats previously inoculated at three weekly intervals with 0.1 ml of either unconcentrated or concentrated (100 times) Formalin-inactivated RSV. (Animals challenged 5 weeks after the final inoculation of vaccine showed the same pattern of disease as those challenged at 1 week.) Cotton rats which were previously infected with RSV by three intramuscular inoculations of 0.1 ml of unconcentrated or concentrated (100 times) live virus or partially heat-inactivated virus did not develop discernible pulmonary pathology after the intranasal challenge with RSV.

Induced measurable resistance by Formalin-inactivated vaccine to RSV replication. The effect of vaccination with the lot 100 preparation on virus replication in the lungs was evaluated by inoculating unvaccinated or previously vaccinated cotton rats with $10^{4.3}$ PFU of RSV intranasally. Five animals in each group were sacrificed on the day of infection and 2, 4, and 7 days thereafter, and the lungs were titrated for the quantity of RSV by the plaque technique. Virus replication was suppressed approximately $10^{-1.3}$ in the lungs of cotton rats previously vaccinated with lot 100 vaccine compared with previously unvaccinated cotton rats (Fig. 9). Although not extensive, this degree of suppression of virus replication was nonetheless statistically significant ($P < 0.01$).

Serum antibody response to intramuscular inoculation of Formalin-inactivated RSV. The immunologic response of cotton rats to intramuscular inoculation of the three Formalin-inactivated RSV preparations was compared with that of cotton rats infected with RSV by intranasal or intramuscular inoculation (Table 1). Cotton rats which were inoculated with a Formalin-inactivated virus developed significantly fewer neutralizing antibodies than animals infected with a live virus by the intranasal or intramuscular route. Particularly striking was the failure of cotton rats vaccinated with the lot 100 preparation to develop detectable serum neutralizing antibodies. Despite failure to develop detectable neutralizing antibodies, these animals exhibited partial resistance to RSV in their lungs (Fig. 9). Cotton rats which were immunized with unconcentrated or concentrated (100×) Formalin-inactivated virus (not alum precipitated) did develop neutralizing antibodies, but the response was approximately 1/20 that of animals infected by a live virus administered intranasally or intramuscularly.

Four groups of cotton rats were tested by ELISA for RSV G and F glycoprotein-specific antibody responses in serum. Cotton rats inoculated with Formalin-inactivated RSV (lot 100 or concentrated [100 times] virus that is not alum precipitated) developed the same level of RSV F and G glycoprotein antibodies by the time of the virus challenge (21 or 28 days following the initial inoculation of vaccine) as did animals infected with RSV intranasally or intramuscularly. However, it should be noted that the cotton rats receiving the lot 100 vaccine had a slower antibody response to the G

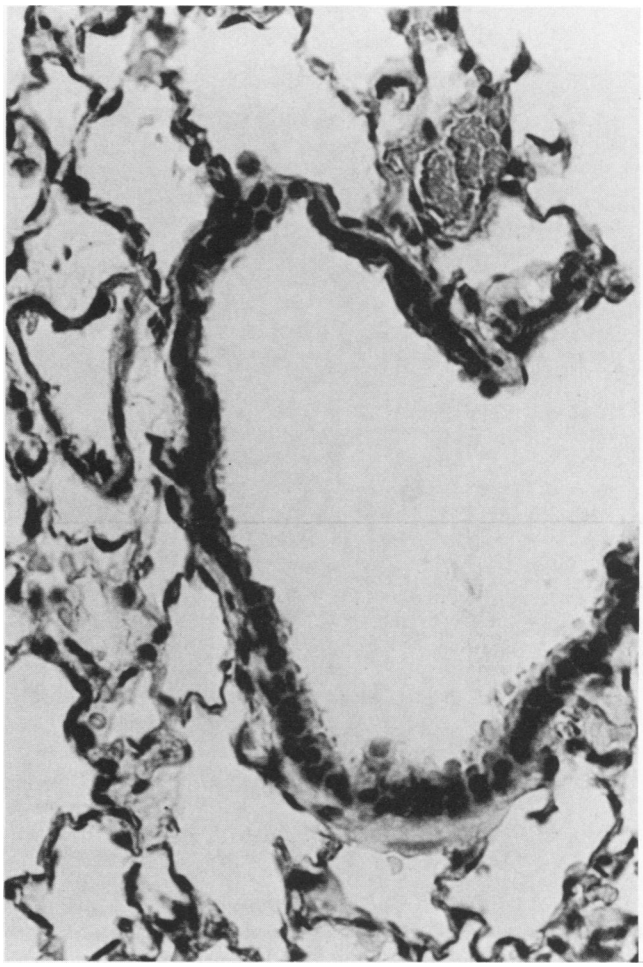

FIG. 3. Normal cotton rat lung. This and all subsequent sections were prepared by inflating lungs with Formalin and staining paraffin-embedded tissues with hematoxylin and eosin. Magnification, ×400.

glycoprotein than did infected cotton rats or cotton rats inoculated with the concentrated (100 times) Formalin-inactivated preparation that was not alum precipitated.

Cotton rats which were infected intranasally or intramuscularly with the live virus or were inoculated with the concentrated Formalin-inactivated virus developed comparable levels of serum antibodies to the F glycoprotein (as measured by ELISA) but only the animals which were infected via the respiratory route developed serum antibodies with anti-fusion activity. A dissociation was also observed with regard to serum neutralizing and anti-fusion antibodies. Cotton rats infected by the respiratory or intramuscular route developed the same level of serum-neutralizing antibodies, whereas only the former group of animals developed serum antibodies that had anti-fusion activity.

DISCUSSION

For the first time it was possible to induce a state of altered reactivity to RSV infection in a small laboratory animal by prior parenteral administration of Formalin-inactivated RSV. The enhancement of pulmonary pathology that occurred during the infection of vaccinated cotton rats was manifest primarily by increased infiltration of neutrophils in the alveoli and in the intraalveolar and peribronchial areas. This degree of enhancement was easily detected by microscopic examination of lung tissues, because pathologic changes observed during the infection of unvaccinated animals were minimal, consisting primarily of infiltration of small numbers of neutrophils and lymphocytes. In contrast, approximately 20 times as many neutrophils were observed in the lungs of infected, previously vaccinated cotton rats. Pulmonary changes in the vaccinated group were not extensive enough, however, to be detected by gross inspection of the lungs. Also, cotton rats that manifested enhanced pulmonary pathology that was detectable microscopically did not exhibit discernible signs of respiratory distress. Thus, enhancement of RSV pulmonary pathology can be reproduced in the laboratory, but the degree of enhancement does not approach that observed during the clinical evaluation of the lot 100 Formalin-inactivated RSV vaccine in infants.

Previous attempts to reproduce enhancement of RSV pathology in other small laboratory animals—hamsters, ferrets, and mice—met with failure. One explanation for these failures and our current success is that the cotton rat is a more permissive host for pulmonary infection with RSV than were the animals previously tested. This may not be the entire answer, however, because RSV replicates permissively in cotton rats, with attendant mild bronchiolitis, but the Formalin-treated virus does not enhance bronchiolitis in

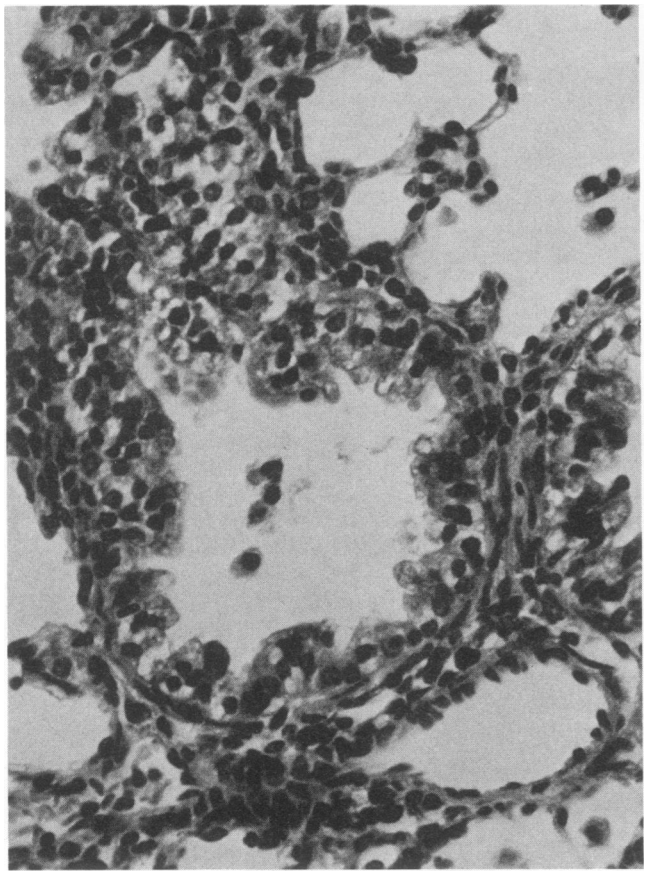

FIG. 4. Unimmunized cotton rat challenged intranasally with RSV and sacrificed 5 days later. Bronchiolar epithelial cells show sequential proliferation and desquamation of infected cells into the lumen. Magnification ×400.

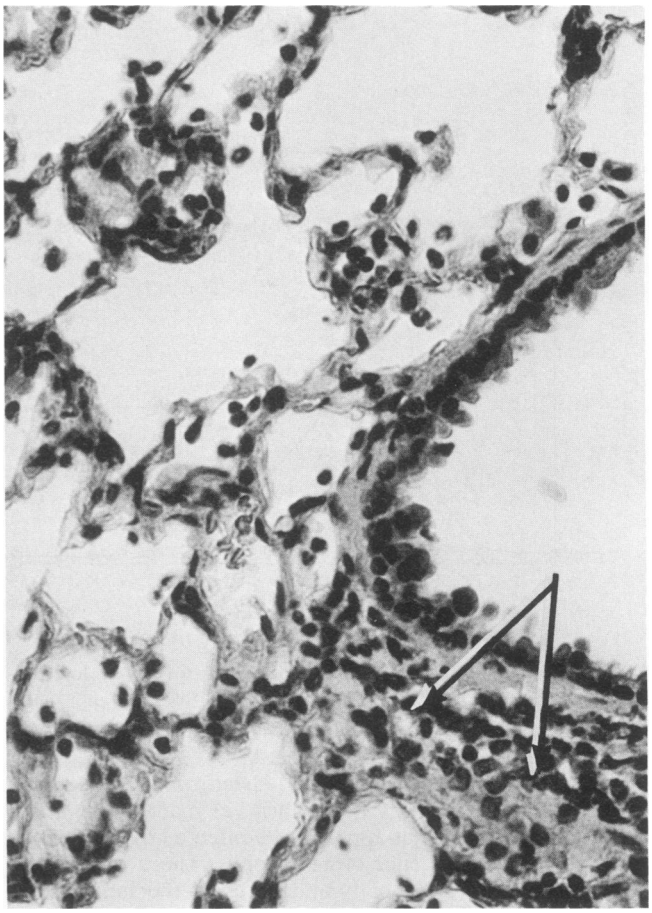

FIG. 5. Cotton rat immunized with three weekly intramuscular injections of lot 100 vaccine, then challenged intranasally with RSV, and sacrificed 5 days later. Mild bronchiolar changes—the focal loss of cilia, the extrusion of pyknotic nuclei into the lumen, and pseudostratification—are seen with the associated focal peribronchial, mixed lymphocytic infiltrate (arrows). Alveolar neutrophils are also present. Magnification, ×400.

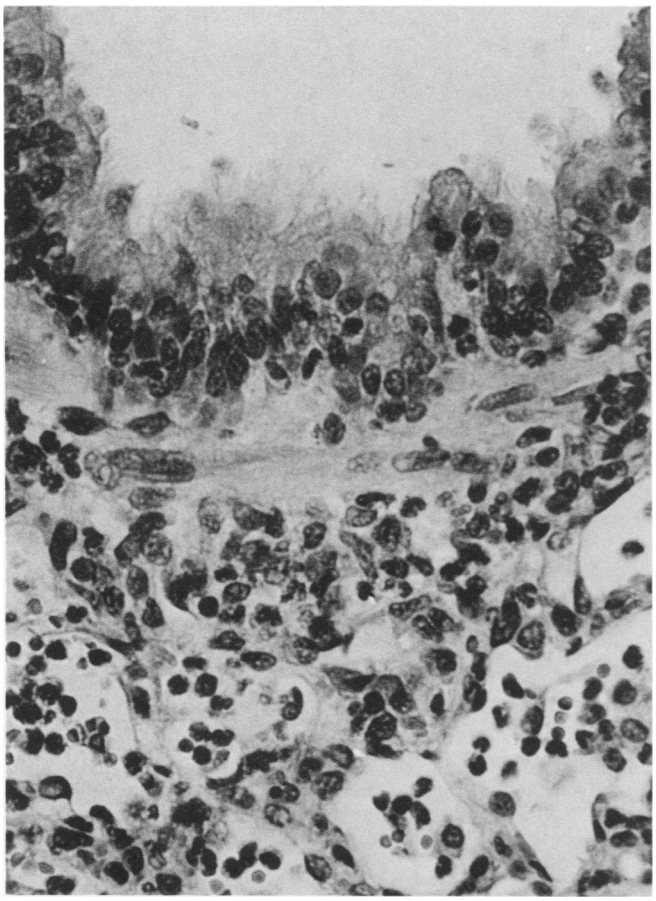

FIG. 6. Cotton rat treated as in Fig. 4 but sacrificed 1 day after challenge. Bronchiolar epithelium is normally ciliated with underlying cells thrown into folds, giving the appearance of hypercellularity. Neutrophils, predominantly in clumps, are seen in adjacent alveoli. Magnification, ×400.

these animals. Instead, disease potentiation is manifested by pneumonia.

The enhancement of pulmonary pathology was observed with three separate Formalin-inactivated virus preparations. (i) The lot 100 vaccine, which was concentrated 100 times and absorbed to alum, was prepared and tested clinically approximately 20 years ago. Potentiation of RSV disease was observed in infants who were given this vaccine. (ii) The next suspension contained a virus that was also concentrated 100-fold. (iii) The final suspension contained an unconcentrated virus. The last two virus suspensions were not absorbed to alum and were prepared just before evaluation in the current study of cotton rats. Despite these differences in preparation and storage, the three Formalin-inactivated virus suspensions each induced the same degree of enhancement of pulmonary pathology. It appears that neither alum nor concentration of the virus is required for the enhancement effect.

Infection with RSV by the respiratory or parenteral route did not lead to the enhancement of pulmonary pathology when cotton rats were subsequently challenged with RSV by the respiratory route. The opposite effect was observed. Prior infection induced solid resistance in the lungs to subsequent RSV challenge by the intranasal route. When the initial infection is induced by intranasal instillation of RSV, the virus replicates to a moderately high titer throughout the respiratory tract (14). When inoculated intramuscularly, RSV appears to undergo a single cycle of abortive infection which does not spread contiguously or systemically (15). Nonetheless, parenteral inoculation of relatively modest amounts of the virus (10^2 to 10^4 PFU) induces significant or complete resistance in the lungs to subsequent virus challenge by the respiratory route (15). Previously we had observed that Formalin inactivation of unconcentrated RSV abolished its capacity to induce pulmonary resistance to this virus. In the present study, Formalin inactivation of the lot 100 concentrated (100 times) virus preparation did not completely abrogate its protective effect; however, the degree of resistance induced in the lungs was considerably less than that provided by prior infection of the respiratory tract or the site of intramuscular inoculation (13, 15).

In the current study, the enhancement of pulmonary pathology was not observed if cotton rats were initially inoculated intramuscularly with live, unconcentrated, or concentrated (100 times) RSV. These preparations represented aliquots of the virus suspensions used for Formalin inactivation. This observation suggests that the enhancement of pulmonary pathology cannot be attributed to sensitization by cell culture or medium constituents.

The histopathologic changes seen in the lungs of infected

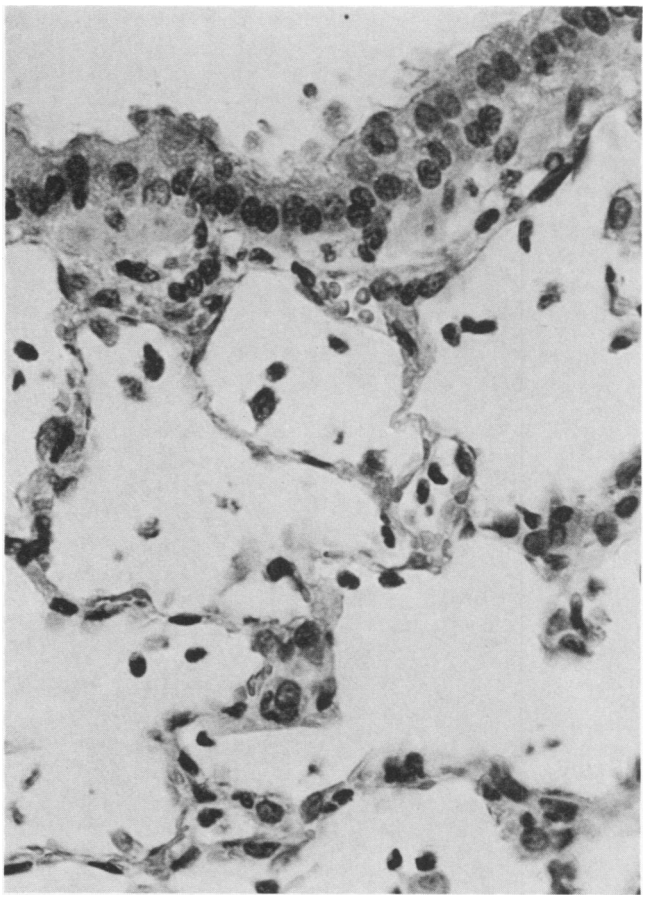

FIG. 7. Cotton rat treated as in Fig. 4 but sacrificed 4 days after challenge. Mild bronchiolar changes consist of cilia loss and pseudostratification, with adjacent alveoli containing dispersed neutrophils. Magnification, ×400.

cotton rats previously inoculated with a Formalin-inactivated virus provide several clues to the mechanisms responsible for vaccine-mediated enhancement. The initial appearance of a large number of clumped neutrophils in the lungs within 24 h of infection suggests that a type III (Arthus) immunopathologic process has occurred. Proof of this interpretation awaits the demonstration of immune complexes in the affected lungs and the failure of neutrophils to accumulate in the lungs of complement-depleted cotton rats.

The next wave of neutrophils, which appeared on day 4 of infection, does not fit the usual Arthus reaction, which is maximal within hours after the appearance of antigens, whether in the skin or the lungs (3, 6). Also, the typical Arthus reaction disappears within 3 days. The infiltration of lymphocytes and another peak of neutrophils occurred later at a time which is typical for delayed-type hypersensitivity (type IV immunopathologic process). This suggests that this phase of the process is mediated by sensitized lymphocytes. Indeed, a study of vaccinates who had received the lot 100 Formalin-inactivated RSV preparation during infancy and who had not yet been naturally infected with RSV revealed that the vaccine induces an enhanced cell-mediated immune response to RSV antigens as assayed by in vitro lymphocyte transformation (9).

Inactivated influenza A virus vaccine preferentially stimulates certain T cells that mediate delayed hypersensitivity

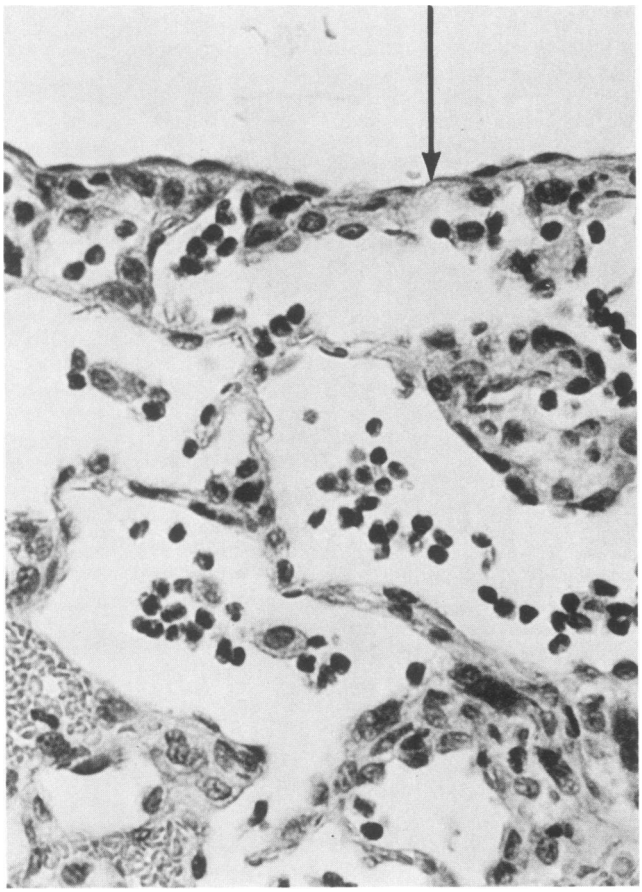

FIG. 8. Same lung section as Fig. 7, but showing infiltration by neutrophils to extend throughout the lung to the pleura (arrow). All alveoli contain either partially clumped or single, dispersed neutrophils. Magnification ×400.

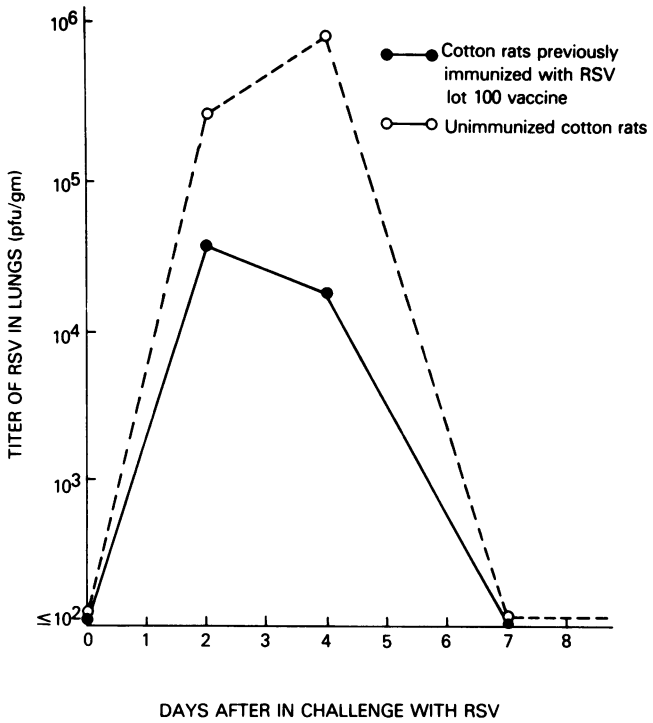

FIG. 9. Titer of virus in lungs of lot 100-vaccinated cotton rats or untreated cotton rats after intranasal challenge with RSV. Virus titers were determined by applying clarified lung homogenates to HEp-2 cells and calculating the number of PFU of virus per gram of tissue. Data from five animals were combined for each point.

and that have limited cytotoxic activity (1). Passive transfer of these cells increases the mortality rate of mice infected with influenza A virus. In contrast, passive transfer of T cells with more cytotoxicity facilitates recovery from infection. Thus, specific subsets of T cells can mediate an immunopathologic process. Unfortunately the lungs of animals which received the T cells with limited cytotoxicity were not studied histologically, so the character of the pulmonary cellular infiltrate stimulated by these cells remains unknown. The development of an inbred strain of cotton rats is imminent, and when such animals become available, we will be able to perform the appropriate adoptive transfer experiments in an effort to demonstrate an effector role for subsets of sensitized lymphocytes in the RSV potentiation effect.

The action of Formalin that is responsible for the disease enhancement of RSV requires additional study. For example, it is clear that selected epitopes on the F or G or both glycoproteins that are involved in inducing and reacting with neutralizing antibodies are modified to reduce or ablate their antigenicity. A neutralizing antibody response from the lot 100 vaccine was not detected in cotton rats. When the vaccine was prepared 20 years ago, however, it did induce moderate levels of serum-neutralizing antibodies in infants (7). The two Formalin-inactivated virus suspensions prepared during the present study induced serum-neutralizing antibodies in cotton rats, but the response was approximately 1/20 that of animals infected with RSV by intranasal or intramuscular inoculation. However, not all of the epitopes on the F or G or both glycoproteins were ablated by Formalin, because cotton rats inoculated parenterally with Formalin-inactivated virus developed a high level of F and G antibodies as measured by ELISA.

TABLE 1. Serum antibody response of cotton rats to intranasal and intramuscular inoculation with RSV

Route of inoculation	Virus inoculated	Titer of inoculum (log_{10} PFU)	Antibody assay	Reciprocal of serum antibody titer (log_{10}) (geometric mean ± SE)[a]			
				Preinoculation	14 days postinoculation	21 days postinoculation	28 days postinoculation
Intranasal	Live	4.5	Neutralization[b]	<1.30	2.95 ± 0.07		3.42 ± 0.12
			ELISA gp70	1.93 ± 0.20	4.02 ± 0.22		4.26 ± 0.15
			ELISA gp90	1.63 ± 0.20	3.66 ± 0.19		4.08 ± 0.15
			Fusion inhibition[c]	<1.30	1.30		1.90
Intramuscular[d]	Live	4.5	Neutralization	<1.30	3.00 ± 0.08	3.45 ± 0.07	
	Partially heat inactivated	3.2	Neutralization	<1.30	2.53 ± 0.07	2.91 ± 0.09	
	Formalin and heat inactivated	<0.0	Neutralization	<1.30	2.13 ± 0.13	2.28 ± 0.09	
	Live 100×	5.1	Neutralization	<1.30	3.04 ± 0.06	3.54 ± 0.09	
			ELISA gp70	1.15 ± 0.10	4.39 ± 0.11	4.84 ± 0.11	
			ELISA gp90	<1.00	3.56 ± 0.19	4.39 ± 0.16	
			Fusion inhibition	<1.30	<1.30	<1.30	
	100× Formalin and heat inactivated	<0.0	Neutralization	<1.30	1.75 ± 0.13	2.11 ± 0.19	
			ELISA gp70	1.15 ± 0.15	4.39 ± 0.11	4.76 ± 0.15	
			ELISA gp90	1.23 ± 0.16	3.78 ± 0.11	4.39 ± 0.23	
			Fusion inhibition	<1.30	<1.30	<1.30	
	Lot 100 (100×)	<0.0	Neutralization	<1.30	<1.30		<1.30
	Formalin and heat inactivated and alum precipitated		ELISA gp70	1.74 ± 0.19	3.91 ± 0.16		4.14 ± 0.21
			ELISA gp90	1.51 ± 0.16	2.17 ± 0.29		3.78 ± 0.18
			Fusion inhibition	<1.30	<1.30		<1.30

[a] 8 to 10 animals in each group.
[b] Plaque reduction neutralization assay.
[c] Titer was determined on a pool of sera from 8 to 10 animals.
[d] Inoculated on days 0, 7, and 14; all inactivated virus concentrations were incubated at 37°C for 72 h.

At this point the effect of Formalin cannot be localized to either the F or G glycoprotein. The results of the assay for fusion-inhibiting antibodies were not helpful in resolving this issue because anti-fusion antibodies were not detected in the serum of cotton rats which had been inoculated intramuscularly with live virus. Thus, the failure of cotton rats inoculated with Formalin-inactivated virus to develop anti-fusion antibodies could reflect the action of Formalin on the virus, the route of inoculation, or possibly both. Although the site(s) at which Formalin acts to produce its disease-enhancing effect has not been identified, it is clear that Formalin-treated RSV stimulates an unbalanced immune response, in which an unusually large proportion of the induced antibodies are directed against nonprotective epitopes on the viral surface glycoproteins. Consequently, effective resistance is not provided, but a high concentration of antibodies that bind to RSV proteins, such as the F and G glycoproteins, is present in the serum and is available to form complexes with viral antigens that are produced during infection. Also, the host may be primed for an accelerated immune response to nonprotective antigenic sites when infection occurs. Whether these phenomena involving nonprotective epitopes play a major role in enhancement remains to be determined. If this is proven, it will then be necessary to dissect the relative role of antibodies and cellular components in the enhancement phenomenon.

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