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NOTES

Respiratory Syncytial Virus Infection in Cyclophosphamide-Treated Cotton Rats

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Cotton rats infected intranasally with respiratory syncytial virus and immunosuppressed with cyclophosphamide shed virus for at least 7 weeks. Dissemination of virus beyond the respiratory tract was observed. In contrast, virus was recovered from infected, non-immunosuppressed rats for only 1 week, and only from the respiratory tract.

Human respiratory syncytial virus (RSV) infection displays unique interactions with the immune system. The disease occurs in its most severe form during the 1st year of life, including that period when maternally derived antibody is present in high titer (5). Fatal disease occurs most often in children with other debilitating illnesses (2, 3, 7). Prior infection confers some degree of protection, yet vaccination with Formalin-inactivated, alum-precipitated virus potentiates disease (4). To explore some of these host-virus interactions further, the cotton rat (*Sigmodon hispidus*) model was chosen to examine the effect of relative immunodeficiency on RSV infection (6).

Pilot experiments were conducted to determine the optimal dose of cyclophosphamide (Mead Johnson & Co.). A dose of 50 mg/kg, given intraperitoneally three times a week, was found to be sufficient to induce and maintain leukopenia (reducing the normal leukocyte count of $9.8 \times 10^3/mm^3$ to an average of $3.2 \times 10^3/mm^3$) without causing significant mortality. The total inability of RSV-infected animals treated with this dose of cyclophosphamide to produce serum-neutralizing antibody to the virus was further evidence of immunosuppression.

Animals were weighed and the drug dose was recalculated every week. Leukocyte counts fell over a 3-week period and then stabilized. At that point, animals were inoculated intranasally under methoxyflurane anesthesia with $10^{5.0}$ PFU of RSV strain A-2.

Animals were sacrificed weekly with pentobarbital. The nasal turbinates, liver, lung, and kidney were removed. A portion of each organ was preserved in 10% neutral buffered Formalin for histology. The remainder of each organ was weighed, diluted 1:10 in Hanks balanced salt solution, homogenized, and centrifuged for 5 min at $450 \times g$. The supernatant was frozen in dry ice and stored for later plaque assay as previously described (6). Formalin-fixed tissues were processed in a routine manner for light microscopy with a low-temperature (55°C) paraffin-based embedding compound. All ossified tissues were decalcified using EDTA at pH 7. Consecutive 4- or 5-μm sections were stained with hematoxylin and eosin or with a glucose oxidase-linked antibody to RSV (8).

Figure 1 shows the course of infection in 26 treated and 15 control animals. Virus was not recovered from control (non-immunosuppressed) rats after 7 days. In contrast, virus was recovered from cyclophosphamide-treated rats throughout the duration of the experiment. The final animal sacrificed (7 weeks postinfection) had more than 10^5 PFU of virus per g of lung tissue and 10^4 PFU/g of nasal tissue. Animals sacrificed earlier had peak lung titers approaching 10^7 PFU/g (about 100 times greater than the maximum titer measured in control animals). Nasal titers were about 10^5 PFU/g, also somewhat higher than in controls. RSV was isolated from the kidneys of two cyclophosphamide-treated animals (days 13 and 29) and from the liver of one of these animals (day 29). In no instance was virus detected outside the respiratory tract of control animals.

The results of histological examination are displayed in Table 1. In the control group, 2 of 12 animals displayed nasal pathology and 2 of 14 displayed pulmonary pathology. (In some instances insufficient tissue was available.) The

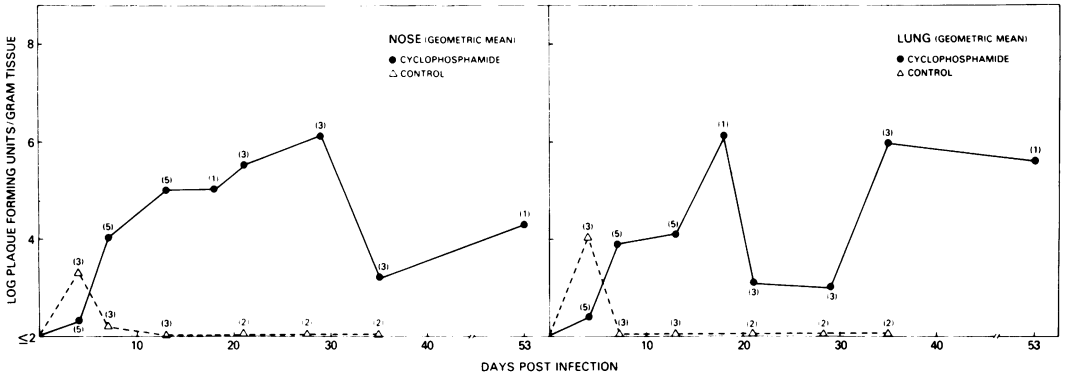

FIG. 1. Viral infectivity in cotton rat nasal and pulmonary tissues as a function of time after intranasal inoculation with RSV. Viral titers are expressed in PFU of virus per gram of fresh tissue. Numerals in parentheses show the number of animals sacrificed.

nasal pathology was a focal rhinitis, and pulmonary pathology consisted of a mild bronchiolitis, as had been seen in previous experiments (6).

Cyclophosphamide-treated animals showed a greater frequency of pathology and more severe lesions. Of 18 animals examined, 13 showed rhinitis, with an increasing frequency of mucosal necrosis, ulcerations, and desquamation from days 4 to 21 postinfection. Syncytial giant cells and cytoplasmic inclusion bodies were not seen in the nasal mucosa. Pulmonary pathology from days 4 to 7 in the treated animals consisted of interstitial thickening (one rat), mild to moderate lymphoplasmacytic peribronchiolitis (two rats), and mild perivasculitis (two rats). Of 16 treated animals examined from day 13 onward, 13 had interstitial pneumonia, and 14 had pulmonary parenchymal giant cells. Several rats showed evidence of secondary infection with microabscess formation. Both the treated and the untreated animals frequently displayed endobronchial papillary mucosal prominence, which increased over the course of the experiment. Changes were not seen in the remainder of the thoracic organs. Cotton rats which received cyclophosphamide but not RSV did not display significant pathology.

Examination of glucose oxidase-stained tissues of the cyclophosphamide-treated animals showed large amounts of RSV antigen in the nasal mucosa (Fig. 2). Affected areas had nearly confluent black deposits of stain similar to the maximal staining seen in untreated animals. The pattern of staining coincided with the pathological changes seen in the sections stained with hematoxylin and eosin. RSV antigen was detected in the lung tissue of all animals from which virus was recovered up to 50 days after infection. Antigen was distributed in a diffuse pattern in the pulmonary parenchyma. Endobronchial antigen was confined to individual columnar cells (Fig. 3 and 4). Control animals showed deposits of antigen only on day 4. The antigen was present in smaller amounts, primarily in the bronchial epithelium.

TABLE 1. Histological examination of cotton rats

Tissue examined	No. with pathological changes/no. examined on day:							
	4	7	13	18	21	29	35	38
Nose								
Control	0/3	0/3	0/3		2/2	0/1		
Cyclophosphamide	3/4	2/5	4/4	1/1	3/3			0/1
Lung								
Control	0/3	1/3	1/3		0/1	0/2	0/2	
Cyclophosphamide	4/5	3/5	3/5	1/1	2/3	3/3	3/3	1/1

One of the two kidneys from which virus was isolated showed specific stain deposited only in the renal papilla. The kidney of the other animal was negative, but by chance the specimen did not include the papilla. Histological examination of the virus-positive liver revealed focal necrotic lesions. Scattered cells within these lesions contained viral antigen.

Another investigator has studied the effect of cyclophosphamide on viral infection (1) and found that the immunosuppressive effect of the drug prolongs infection, though the mechanism of its effect is not known. Our protocol differed from earlier ones in that we maintained immunosuppression by repeated small doses of cyclophosphamide rather than by a single larger dose. Animals treated with multiple doses of the drug were unable to overcome RSV infection throughout the course of the experiment (7 weeks). Presumably, RSV infection could be prolonged indefinitely in cotton rats, for no

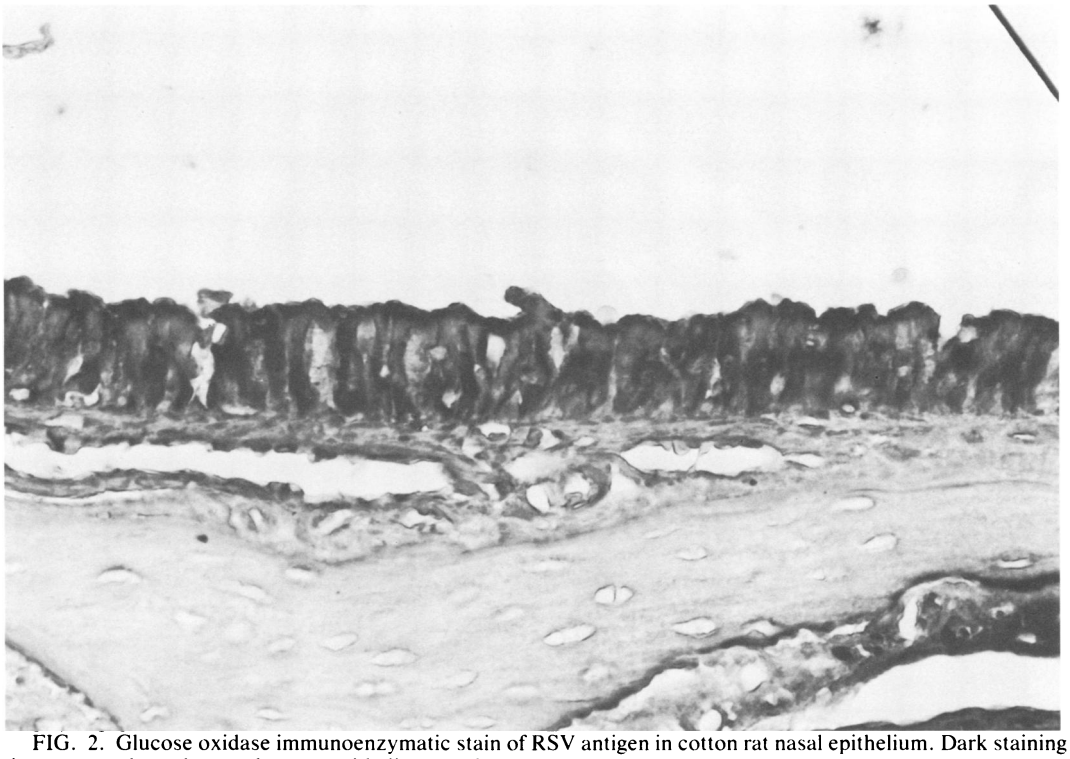

FIG. 2. Glucose oxidase immunoenzymatic stain of RSV antigen in cotton rat nasal epithelium. Dark staining is apparent throughout columnar epithelium. ×680.

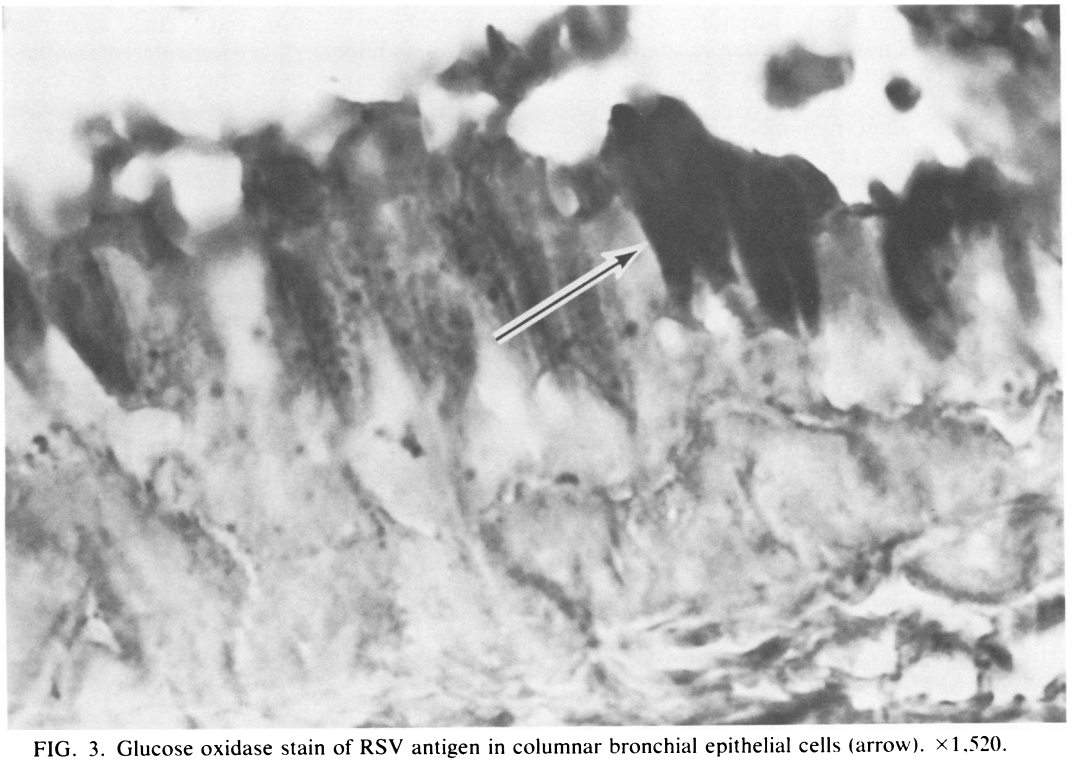

FIG. 3. Glucose oxidase stain of RSV antigen in columnar bronchial epithelial cells (arrow). ×1,520.

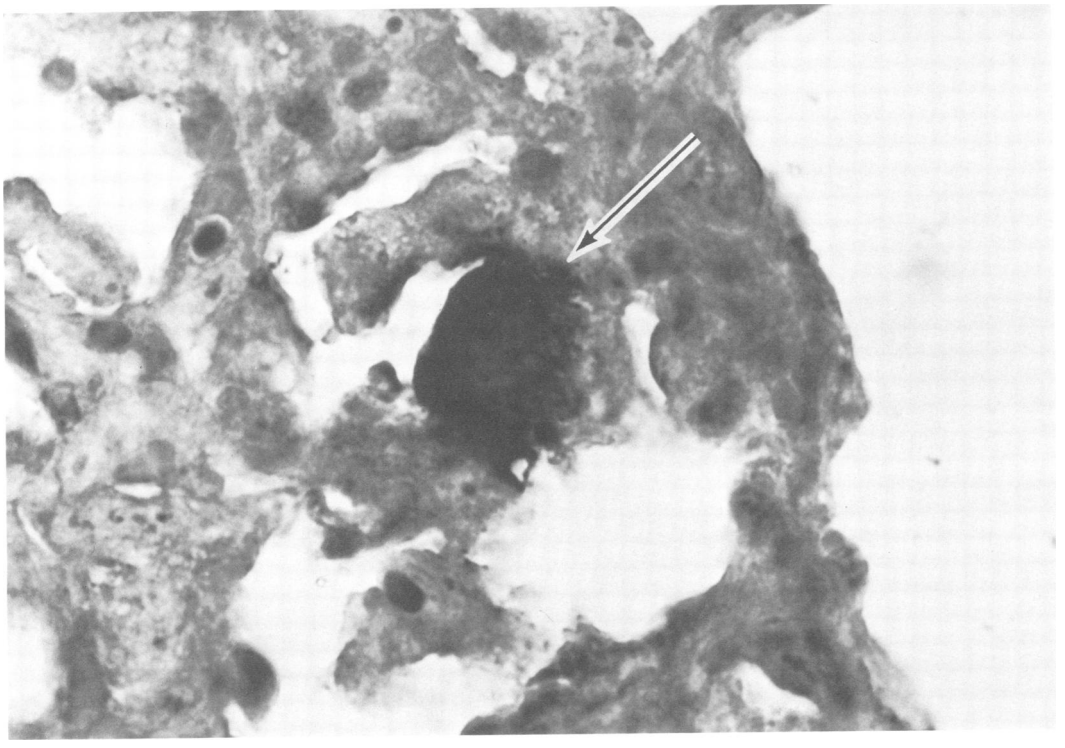

FIG. 4. Glucose oxidase stain of RSV antigen in pulmonary alveolar cells (arrow). ×1,520.

animals died from RSV infection despite high viral titers throughout the respiratory tract.

Although it is not known what portions of the cotton rat immune system are suppressed by cyclophosphamide, this model of persistent infection might allow immuno-reconstitution experiments (such as parabiosis or cell transfer) to determine the factors responsible for recovery from infection. Inbred cotton rats will soon be available for such experiments. An additional use of this model might be the in vivo testing of genetic stability of vaccine candidate attenuated viruses.

An unexpected finding of our study was the recovery of RSV from non-respiratory tissues of immunosuppressed animals. This suggests that under normal circumstances, immunological mechanisms play a role in restricting RSV infection to the respiratory tract. Coincident with this study, we have examined tissues from two cases of fatal RSV infection in humans in which the immunological function of the patients was suppressed (unpublished data). In one case, the patient was a child with a combined immunodeficiency; the other involved an elderly woman with leukemia who had been treated with immunosuppressive drugs. In both cases we found large quantities of RSV antigen in nearly all organs examined, including kidney and liver, the organs from which RSV was recovered in immunosuppressed cotton rats. Thus, although RSV has traditionally been considered a pathogen of infancy, we have preliminary evidence suggesting that it may have an important role as a pathogen of immunosuppressed hosts, regardless of age. We anticipate that the cotton rat model might allow further study of that role.

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