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Lymphocyte Transformation in Response to Antigens of Respiratory Syncytial Virus (40597)[1]

V. SCHAUF, C. PURCELL, M. MIZEN, AND S. MIZEN

Department of Pediatrics, University of Illinois College of Medicine, Chicago, Illinois 60612

The immunologic events which mediate, prevent, or cure respiratory syncytial virus (RSV) disease in the lower respiratory tract remain to be elucidated. Serum antibody, either maternally derived or vaccine induced, does not prevent RSV infection (1, 2). The peak incidence of pneumonia and bronchiolitis occurs in infancy when maternal serum antibody is present. RSV infection produced a greater incidence of lower respiratory tract infection in children with high antibody titers induced by immunization with an inactivated RSV vaccine than in unimmunized children. Although maternal neutralizing antibody does not prevent infection with RSV, the severity of pneumonia was inversely related to the infant's RSV antibody titer. The titer of serum neutralizing antibody did not correlate with the severity of bronchiolitis (3).

Secretory IgA to RSV may confer protection against infection (4). The development of secretory IgA antibodies to RSV is temporally related to the disappearance of the virus from the respiratory tract, indicating a possible role for this antibody in the cure of this infection (5). Nevertheless, some infants in McIntosh's study recovered with developing a secretory IgA response. It is also possible that the secretory IgA response may parallel the development of significant immunity without being primarily responsible.

Cell-mediated immune responses are important in limiting some viral infections. Lymphocyte transformation to RSV, a measure of cellular immunity, was found in recipients of inactivated RSV vaccine, in unimmunized adults, and in unimmunized children who had previously experienced natural RSV infection (6, 7). Lymphocyte transformation specific for RSV has also been described in the majority of infants under 6 months of age with lower respiratory tract RSV infection, and in some infants over 6 months of age (8). The role of RSV specific cellular immunity in the prevention, cure, or pathogenesis of RSV disease is still unclear. The purpose of the present study was to further delineate the occurrence of cell-mediated immunity to RSV as measured by lymphocyte transformation in infants, children, and adults.

Material and methods. Lymphocyte transformation to RSV antigens was studied after obtaining informed consent in 12 healthy newborn infants, in 48 babies and children from 1 month to 15 years of age, and in 44 adult hospital personnel without respiratory disease with an average age of 28 years. Samples were obtained from 1976–1978. The babies and children, most of whom were hospitalized, were categorized as to whether or not respiratory tract disease occurred. Respiratory tract diseases included upper respiratory tract infection, laryngotracheobronchitis, bronchiolitis, pneumonia, and status asthmaticus. Patients without respiratory tract disease had diagnoses including cellulitis, septic arthritis, urinary tract infection, and congenital intrauterine infection. Samples from patients with acute respiratory tract illness were obtained within 1–17 days (median 3 days) of the onset of the illness. Serial samples were obtained in 14 individuals.

A modification of the whole-blood culture technique of Pauly *et al.* was used (9). Heparinized blood was diluted 1:20 with medium RPMI 1640 (International Scientific Industries, Cary, Ill.) containing penicillin 100 units/ml and streptomycin 100 mcg/ml and glutamine 2 m*M*/ml. Three milliliters of diluted blood were placed in 16 × 150-mm sterile plastic tubes (Falcon Plastics, Oxnard, Calif.) for incubation with antigens or mitogens.

Complement-fixation (CF) antigens from uninfected Vero cells (control antigen) and

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from Vero cells infected with Long strain of RSV (RSV antigen) were purchased from Microbiological Associates, Bethesda, Maryland. Aliquots of single lots of antigen were stored at −90°C. The CF titer of the RSV antigen is 1:8. This material contained no detectable live RSV since inoculation did not result in cytopathic effects in suspectible HEP-2 cells. The undiluted control antigen contained 215 μg protein/ml and the RSV antigen contained 485 μg protein/ml (Lowrey method). Phytohemagglutinin (PHA-P) was purchased from Difco, Detroit, Michigan. Dilutions of RSV antigen, control antigen, or 10 μg PHA were immediately added to the diluted whole blood. Samples were incubated with 0.4 ml of antigen dilutions of 1:4, 1:40, 1:400, and 1:4,000. All determinations were performed in triplicate. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 5 days incubation, cultures were labeled with 2.0 μCi [^{3}H]thymidine (specific activity 2.0 Ci/mmole; Amersham Searle, Arlington Heights, Ill.). For the kinetic study, labeling was performed after 4, 5, 6, 7, or 8 days incubation.

The cultures were harvested 18–20 hr after labeling by washing three times with cold 3% glacial acetic acid. One drop of 30% H_2O_2 was added for 10–15 min to the remaining cell pellet. The pellet was solubilized with NCS (Amersham Searle, Arlington Heights, Ill.) and placed in 9 ml of a solution made with 4 g of Omniflor (New England Nuclear, Boston, Mass.) per liter of toluene. The samples were counted for 10 min in a Packard Model 3880 scintillation counter. Counts per minute were converted to disintegrations per minute (dpm) with the aid of a standard quench curve.

In addition, lymphocytes prepared from Ficoll–Hypaque gradients were also studied in some cases. Less than 5% polymorphonuclear leukocyte contamination was observed. Cells were adjusted to a final concentration of 2×10^6 lymphocytes/ml in culture medium consisting of 100 units/ml penicillin, 100 mcg/ml streptomycin, and 20% human serum type AB (ISI, Cary, Ill.). The final dilutions of RSV and control antigens were 1:50, 1:100, 1:500, and 1:1,000.

Triplicate cultures were performed in wells of flat-bottom microtiter plates (Costar, Cambridge, Mass.) containing 2×10^5 lymphocytes in 100 μl of culture medium and 100 μl of diluted RSV antigen or control antigen. Cultures were incubated in a humidified atmosphere containing 5% CO_2. After 6 days each well was labeled with 0.2 μCi [^{3}H]thymidine and harvested 16 hr later with an automatic cell harvester.

Titers of neutralizing antibodies in serum were determined by the plaque reduction assay (4) by Dr. Linda Richardson at the National Institutes of Health. Pharyngeal swabs obtained from 17 children hospitalized for respiratory illness were inoculated on WI-38, HEK, and HEP-2 cells. RSV isolates were confirmed by indirect immunofluorescence using rabbit antiserum to RSV.

A stimulation index (SI) was calculated by dividing mean dpm in RSV antigen cultures by mean dpm in control antigen cultures. An SI over 3.0 was considered positive, since the mean dpm in RSV antigen cultures was significantly greater than in control cultures by Student's *t* test. Possible correlations between [^{3}H]thymidine incorporation by RSV-stimulated lymphocytes and by PHA-stimulated lymphocytes were examined by regression analysis. Possible correlations between RSV-stimulated [^{3}H]thymidine uptake and neutralization antibody titers were similarly examined.

Results. Lymphocyte transformation to RSV antigens occurred in 17 of 44 (39%) healthy adults tested using unseparated lymphocytes (Table I). RSV antibody was present in 23 of 23 adults tested. The antigen dilutions producing SI ≥ 3.0 varied from individual to individual. The greatest percentage of positive responses occurred at a 1:40 antigen dilution (29%). The stimulation

TABLE I. PERCENTAGE OF INDIVIDUALS WITH LYMPHOCYTE REACTIVITY TO RSV ON FIRST ASSAY[a]

Subjects	Total	SI < 3.0	SI ≥ 3.0	%
Adults	44	27	17	39
Babies and children	48			
Respiratory disease	27	15	12	44
No respiratory disease	21	21	0	0
Newborn infants	12	12[b]	0	0

[a] SI ≧ 3.0 at any RSV antigen dilution.
[b] Eight determinations with whole blood culture and four with Ficoll/Hypaque-separated lymphocytes.

indices were as high as 16.3. The kinetics of the response of lymphocytes from one normal adult are shown in Figure 1. Reactivity was detectable after 5 days incubation, was maximal after 6 days, and persisted up to 8 days.

Sequential lymphocyte cultures were performed on two to nine occasions for 12 healthy adults. Lymphocyte reactivity was detected on the first analysis in 7 of the 12 individuals. Only 1 adult failed to show lymphocyte reactivity to RSV when more than one analysis was performed. This individual had an RSV neutralizing antibody titer greater than 1:1280.

Leucocytes obtained by Ficoll–Hypaque gradient centrifugation of peripheral blood from 11 healthy adults contained at least 95% mononuclear cells. Lymphocyte reactivity to RSV was present in 6 of these individuals. Stimulation indices were as high as 21.6.

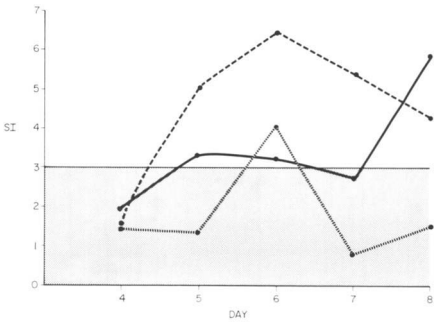

FIG. 1. The kinetics of a healthy adult's lymphocyte response to RSV antigens diluted 1:4 (●——●), 1:40 (●– – –●), 1:400 (●···●). SI is stimulation index.

Babies and children with respiratory diseases also manifested cellular immunity to RSV when tested using unseparated lymphocytes. An SI ≧ 3.0 was found in 12 of 27 of these patients (44%) (Table I). Respiratory diseases in patients with lymphocyte reactivity to RSV included URI, bronchiolitis, pneumonia, and asthma (Table II). These patients were studied from 3 to 14 days after the onset of illness (median 12 days). They ranged in age from 2 months to 15 years (Table II). As in adults, the optimal antigen dilution varied among individuals. Stimulation indices were as high as 11.8. Neutralizing antibody titers ranged from 1:156 to >1:1280. In contrast, none of 21 babies and children without respiratory diseases had lymphocyte reactivity to RSV demonstrable by the whole blood culture technique (Table I). None of 12 newborn infants tested using separated or unseparated lymphocytes had detectable reactivity to RSV (Table I).

Neutralizing antibody to RSV was found in 19 of the infants and children lacking demonstrable lymphocyte reactivity to RSV (Table III). Three additional infants lacking

TABLE II. MAXIMAL LYMPHOCYTE REACTIVITY TO RSV IN BABIES AND CHILDREN WITH RESPIRATORY TRACT DISEASE

Patient No.	Age	Diagnosis	No. days after onset	Antigen dilution	[^{3}H]Thymidine (dpm) Control antigen Mean	Control antigen SE	RSV antigen Mean	RSV antigen SE	SI	Reciprocal neutralizing antibody titer
1	1½ y	Asthma[a]	14	1:40	10134	458	35316	1940	3.5	NT[b]
2	2 y	URI[c]	14	1:40	11408	1653	47770	10353	4.2	NT
3	4 y	URI[c]	14	1:40	16918	1800	54455	6323	3.2	NT
4	9 y	URI[c]	14	1:40	11539	709	40807	9877	3.5	NT
5	1 y	Bronchiolitis	3	1:40	3340	477	17481	2596	5.2	174
6	2 mo	Pneumonia	4	1:4	3143	634	16750	6462	5.3	156
7	4 y	Pneumonia, bullous myringitis		1:40	3769	570	30591	2005	8.1	>1280
8	1½ y	URI		1:4	9294	2085	30099	3808	3.2	801
9	8 y	URI		1:4	3935	63	15061	935	3.8	NT
10	3 y	Asthma	14	1:4	4311	1004	50749	9768	11.8	NT
10	3¼ y	Asthma	10	1:40	4112	347	20485	3144	5.0	>1280
11	4½ y	Asthma	9	1:4	4023	849	23705	1700	5.9	NT
12	15 y	Asthma	6	1:400	2177	1258	7939	1517	3.6	>1280

[a] RSV isolated.
[b] Not tested.
[c] RSV isolated from household contact.

TABLE III. RSV SEROPOSITIVITY IN INFANTS AND CHILDREN WITH SI < 3.0

Patient No.	Age	Diagnosis	[^{3}H]Thymidine (dpm) Control antigen Mean	Control antigen SE	RSV antigen Mean	RSV antigen SE	SI	Reciprocal neutralizing antibody titer
			No respiratory disease					
13		Newborn	24015	1465	24682	384	1.0	>1280
14		Newborn	12168	3762	17694	1159	1.5	441
15		Newborn	8476	1573	18578	1142	2.2	>1280
16	1 mo.	Trisomy 21	4460	437	3391	227	0.8	35
17	2 mo.	CMV[a] infection	5018	1585	3844	273	0.8	326
18	1 y	CMV infection	8031	695	12524	1059	1.6	932
19	1 y	Healthy twin of 18	8780	194	16303	1317	1.9	484
20	6 y	Cellulitis	3924	375	8183	3239	2.1	973
21	8 y	Cellulitis	4530	152	6918	54	1.5	448
			With respiratory disease					
22	2 mo	Bronchiolitis[b]	7878	1057	16125	2084	2.0[c]	91[d]
23	2 mo	Bronchiolitis	8087	883	9306	557	1.2	240
24	2 mo	Bronchiolitis	5282	331	9050	1843	1.7	271
25	6 mo	Bronchiolitis	3271	552	3455	813	1.1	34
26	6 mo	Bronchiolitis	5430	283	12052	1612	2.2	>1280
27	2 mo	Pneumonia	6725	357	6399	752	1.0	304
28	2 mo	Pneumonia	8690	637	13217	2535	1.5	49
29	1½ y	Pneumonia, CMV infection	6303	1077	7950	2486	1.3	>1280
30	4½ y	Pneumonia	6615	4515	14263	6663	2.2	>1280
31	2 y	URI	3818	925	3456	744	1.0	377

[a] Cytomegalovirus.
[b] RSV isolated.
[c] Highest SI obtained from four samples in 2 ½ months.
[d] 3-weeks convalescent.

TABLE IV. LYMPHOCYTE REACTIVITY TO RSV IN PATIENTS WITH RESPIRATORY DISEASES BY AGE

Age	SI ≧3.0	SI <3.0
≦6 mo.	1[a]	10
>6 mo.	11	9

[a] χ^2 with Yates correction 4.61, $P < 0.05$.

lymphocyte reactivity to RSV were seronegative (titer <1:20). There was no correlation between RSV neutralizing antibody titer and SI in adults or in babies and children.

Among patients with respiratory diseases, lymphocyte reactivity to RSV occurred significantly less often in babies 6 months of age or less (1/11) than in patients over 6 months (11/20). χ^2 with Yates correction was 4.61, $P < 0.05$ (Table IV).

The PHA response in all age groups was highly variable. There was no correlation between lymphocyte incorporation of thymidine stimulated by PHA and that stimulated by RSV antigens. The lymphocyte response to RSV was not dependent on the season of the year.

RSV was isolated in only two patients, Nos. 1 and 22 (Tables II and III). Patient 1 had demonstrable reactivity to RSV as did her household contacts with URI. However, four assays over 2.5 months failed to reveal lymphocyte reactivity to RSV in patient 22, who was less than 6 months of age.

Discussion. It has been shown that immunization of children with inactivated RSV vaccine and that previous natural RSV infection of the upper respiratory tract are associated with cellular immunity to RSV (7). Lymphocyte transformation to RSV also occurs in infants after hospitalization for lower respiratory tract infection (8). The evidence from the present study is that cellular immunity to RSV is also frequently present in apparently healthy adults and in babies and children with respiratory tract illnesses (Table I).

Reactivity to RSV was detected in adults using both whole blood cultures containing autologous plasma and cultures consisting of 95% mononuclear cells lacking autologous plasma. Therefore, our assays most likely measures lymphocyte transformation to RSV.

Detection of lymphocyte transformation is facilitated by studying several antigen dilutions, as the optimal dilution varies from individual to individual (Table II). When sequential studies were performed in adults, lymphocyte reactivity could eventually be demonstrated in 11 of 12 individuals tested.

Recurrent asymptomatic RSV reinfection in adults may explain the occurrence of cellular immunity in apparently healthy adults. Conversely, the presence of cellular immunity to RSV in adults may explain the mild nature of RSV disease in adulthood before old age. Unfortunately, it is not possible to test these hypotheses using data from the present study.

The presence of cellular immunity to RSV in only those babies and children with respiratory tract disease suggests that these patients had recent RSV infection. This conclusion is tentative since in our study, RSV infection was poorly documented possibly due to inadequacies of specimen collection and processing. However, lymphocyte transformation to RSV did occur in children with documented RSV infection and bronchiolitis, pneumonia, or asthma (8).

It is unlikely that cellular immunity to RSV antedated lower respiratory tract disease in babies and children since none of 12 healthy neonates and none of 21 babies or children without respiratory disease had lymphocyte reactivity to RSV (Table I). Therefore it is unlikely that preexisting cellular immunity to RSV plays a role in the pathogenesis of lower respiratory tract disease in infants with RSV infection as postulated by Kim *et al.* (7).

Our finding of cellular immunity to RSV 3–14 days (median 12 days) after the onset of respiratory tract disease is consistent with the observation of Scott that cellular immunity develops during convalescence from RSV infection (8). The absence of cellular immunity to RSV in 15 of 27 babies and children with respiratory tract diseases may indicate that some of these patients had never been exposed to RSV. Alternatively, some of these patients were evaluated as early as 1 day after the onset of illness, probably before immunity had developed.

The absence of cellular immunity to RSV on four occasions over 2.5 months in an infant under 6 months of age with bronchiolitis due to RSV infection (patient 22) is noteworthy. Apparently, a single RSV infection does not always confer enough cellular immunity for that immunity to be demonstrable in our assay. Some of the patients reported by Scott also failed to develop lymphocyte transformation to RSV during convalescence from RSV infection (8).

Our findings of a significantly decreased frequency of cellular immunity to RSV in infants 6 months of age or less is in sharp contrast to the data of Scott (8). However, since our patients did not have documentation of RSV infection, it is possible that the patient groups are not comparable. Our infants had demonstrable PHA reactivity. Therefore it is unlikely that age-related difference in the intrinsic transformability of lymphocytes explain the differences observed in the RSV transformation response. Further studies using larger groups of patients with careful documentation of the etiology of the respiratory tract disease should settle this discrepancy. This distinction is of great importance in determining if cellular immunity mediates, prevents, or cures RSV lower respiratory tract disease.

Many individuals with neutralizing antibody to RSV lacked demonstrable lymphocyte reactivity (Table III). In infants, this antibody may have been maternally derived. However, after 1 year of age, it is likely that neutralizing antibody resulted from past RSV infection. Therefore the absence of lymphocyte reactivity in these individuals indicates that lasting cellular immunity to RSV as demonstrated in our assay requires more than one RSV infection. The lack of an inverse correlation between RSV SI and neutralizing antibody titer indicates that RSV antibodies probably do not generally block RSV-specific lymphocyte transformation. Further investigation is required to determine if blocking antibodies are present in individuals who do not manifest lymphocyte reactivity to RSV.

Specific cellular immunity to RSV may play a role in limiting the extent and severity of RSV infection. The absence of RSV lymphocyte transformation in infants at risk for bronchiolitis and pneumonia may explain their vulnerability. Conversely, the presence of RSV lymphocyte transformation in adults at low risk for severe RSV infection may explain their resistance. The presence of cel-

lular immunity to RSV in only those babies and children with respiratory disease may indicate that several RSV infections are necessary before immunity is as readily demonstrated as in adults. Moreover, the absence of transplacental transfer of cellular immunity to RSV in newborn infants and those without respiratory disease makes it unlikely that preexisting cell-mediated sensitization contributes to the pathogenesis of bronchiolitis or pneumonia. Sequential studies of specific cellular immunity in recipients of experimental RSV vaccines would help to delineate the role of this response in RSV infection. The interactions of specific immune cells and IgG and IgA both locally and systemically require further evaluation to determine the immunologic events which prevent, provoke, and cure RSV disease.

Summary. Cellular immunity to RSV was measured by lymphocyte transformation in infants, children, and adults. Lymphocyte transformation to RSV antigens occurred in 17 of 44 (39%) healthy adults. When sequential lymphocyte cultures were performed with healthy adults, 11/12 individuals had an SI $\geqq$ 3.0 on at least one occasion. In 12 of 27 babies and children with respiratory diseases, cellular immunity to RSV was detected. In contrast, none of 21 babies and children without respiratory diseases and none of 12 neonates had detectable lymphocyte reactivity to RSV. Among patients with respiratory diseases, lymphocyte reactivity to RSV occurred significantly less often in babies 6 months of age or less (1/11) than in patients over 6 months (11/20). Since cellular immunity to RSV was not found in babies and children without ongoing respiratory illness, it is unlikely that preexisting cellular immunity to RSV plays a role in the pathogenesis of lower respiratory tract disease in infants with RSV infection.

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