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Priming with Live Respiratory Syncytial Virus (RSV) Prevents the Enhanced Pulmonary Inflammatory Response Seen after RSV Challenge in BALB/c Mice Immunized with Formalin-Inactivated RSV

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To investigate enhanced disease associated with a formalin-inactivated (FI) respiratory syncytial virus (RSV) vaccine, we studied the pulmonary inflammatory response to RSV in BALB/c mice immunized with live RSV, FI-RSV, or combinations of the two. After RSV challenge, the number of granular cells, the ratio of $CD4^+/CD8^+$ lymphocytes, and the level of Th2-like cytokine mRNAs in the bronchoalveolar lavage specimens in mice immunized first with live RSV and then with FI-RSV were lower than that in FI-RSV-immunized mice and close to that in live RSV-immunized mice. These data suggest that prior live RSV infection prevents most of the enhanced inflammatory response seen in FI-RSV-immunized mice and might explain lack of enhanced disease in older FI-RSV-immunized children. A live RSV vaccine might similarly decrease the risk of enhanced disease with non-live RSV vaccines.

As the single most important cause of serious lower respiratory tract disease in infants and young children, respiratory syncytial virus (RSV) is a high priority for vaccine development (7). Developing a safe and effective RSV vaccine, however, has proven to be a formidable task with major obstacles being the young age at which children need to be vaccinated, the difficulty in inducing any protective immune response, and concern that a non-live virus vaccine might cause enhanced disease (10). The latter concern is based on clinical trials in which children immunized with a formalin-inactivated (FI) RSV vaccine suffered more severe disease with later natural infection than did unvaccinated children (3, 8).

Despite concerns about enhanced disease, non-live RSV vaccines are being pursued as candidate vaccines and might be key to boosting the weak protective immune response associated with both natural RSV infection and live virus vaccination. One key to the development of non-live virus vaccines is a clearer understanding of the pathogenesis of FI-RSV-induced enhanced disease. Studies with the cotton rat and the BALB/c mouse have begun to provide clues to the pathogenesis of FI-RSV enhanced disease. These studies have demonstrated increased histopathologic changes in lung tissue, increased number and different types of bronchoalveolar lavage (BAL) cells, and an increase in mRNA for Th2 cytokines (6, 13–15) in FI-RSV- compared to live RSV-immunized animals after RSV challenge. These findings plus studies of depletion of $CD4^+$ cells and neutralization of interleukin-4 (IL-4) and IL-10 (4, 5) suggest that induction of a Th2-like immune response is likely to be important to the pathogenesis of enhanced disease.

One aspect of the human FI-RSV vaccine clinical trials that has not been evaluated in animals is the lack of enhanced disease in older children. The leading hypothesis for this phenomenon is that prior infection in the older children primed their RSV immune response and prevented the disease-enhancing immune response associated with FI-RSV vaccination (1). In this report, we describe investigations of this hypothesis in which we looked at the effect that prior live virus infection has on the inflammatory response to RSV challenge in FI-RSV-immunized BALB/c mice.

(This work was presented in part at a meeting of the American Society for Virology, London, Ontario, Canada, 13 to 17 July 1996 [16].)

MATERIALS AND METHODS

Mice, virus, and immunization antigens. Female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, Ind.) were maintained under specific-pathogen-free conditions and used when 8 to 16 weeks old. Animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 86-23). Stocks of RSV strain A2 were propagated in HEp-2 or Vero cells. Live virus for immunization, FI-RSV in alum for immunization, and live virus for challenge or the corresponding control antigens were prepared as previously described (15).

Diagnostic assays. The infectivity titer of RSV in lung tissue was determined in an 18-h infectious focus assay using immunoperoxidase staining, and RSV-specific serum immunoglobulin G antibody titers were determined by an enzyme-linked immunosorbent assay as described earlier (15).

Flow cytometry. Freshly collected BAL cells were kept on ice; stained with fluorescein- or phycoerythrin-conjugated rat antibodies to mouse CD3, CD4, CD8, CD11b, or the respective isotype control (PharMingen, San Diego, Calif.); and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Forward-side scatter plot was used to define the lymphocyte and granulocyte populations. The total number of cells in BAL specimens was determined by hemocytometry.

mRNA extraction and RT-PCR. RNA was extracted from the cells in 0.5 ml of the BAL specimen with 1 ml of RNAzol B (Biotecx Laboratories, Houston, Tex.); cDNA was made by the reverse transcriptase (RT) reaction with poly(dT) primers; and cDNA was amplified by a 22- to 35-cycle PCR, with primers specific to mouse IL-2, IL-4, IL-5, IL-13, gamma interferon, and β-actin as described

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earlier (15), except that the following sequence was used as the antisense primer for IL-13: 5′-TTATTGAGGAGCTGAGCAACAT-3′. All specimens gave an appropriately sized band after β-actin PCR and ethidium bromide-stained agarose gel electrophoresis. The cytokine RT-PCR products were quantitated with the Perkin-Elmer QPCR system (Branchburg, N.J.) by measuring electrochemiluminescence generated from ruthenium chelate-labelled hybridization probes (15). Values of three times the negative control luminosity for IL-4 and IL-5 and of five times the negative control luminosity for IL-13 were considered to be positive for the respective cytokine mRNA.

Experimental design. All mice were (i) immunized with live RSV, FI-RSV, combinations of both, control antigens, or nothing; (ii) challenged with live RSV intranasally 6 or 10 weeks after the last immunization; and (iii) euthanized 4 or 8 days after challenge, after which BAL and serum specimens were collected. Second immunizations were given 4 weeks after the first immunization. Antigens for the immunizations included (i) alum-adsorbed FI-RSV containing 10^5 PFU equivalents of RSV or a comparable volume of FI control preparation in 0.05 ml of phosphate-buffered saline or (ii) live RSV containing 10^5 PFU of RSV in 0.05 ml of minimal essential medium without serum or similar dilution of control antigen. As previously described (15), FI-RSV and FI control were given intramuscularly, RSV and control immunizations were given intranasally, and RSV challenge was given intranasally (10^6 PFU of virus in a 0.1-ml volume). The challenge dose produced a titer of $10^{4.3}$ to $10^{4.8}$ PFU/g of lung tissue in unimmunized mice at day 4 after challenge.

To decrease responses to cellular antigens in the FI-RSV preparation, we used FI-RSV prepared from RSV grown in Vero cells for experiment 3. To control for cellular antigens induced by virus infection, we used human parainfluenza virus type 3 (HPIV-3) grown in Vero cells as the FI control for experiment 3.

RESULTS

In the first experiment, no virus could be isolated from lungs on day 4 after challenge from mice immunized twice with any combination of vaccines, and therefore, virus isolation was not performed in later experiments. All RSV-immunized mice had a response to RSV as indicated by the presence of RSV-specific immunoglobulin G antibodies detected in day 4 serum specimens (data not shown).

The characteristics of BAL cells by type of immunization are summarized in Table 1. In all three experiments, the number of total cells, lymphocytes, $CD4^+$ lymphocytes, and $CD8^+$ lymphocytes was lowest for mice immunized with control antigens, i.e., HEp-2 in experiments 1 and 3 and none in experiment 2 (Table 1). Compared to untreated-control antigen immunization, there were two patterns of increased responses to RSV challenge, one after live RSV and one after FI-RSV immunization. Live RSV immunization was associated with no increase in number of granular cells but a modest increase in number of lymphocytes with no change in relative numbers of $CD4^+$ and $CD8^+$ cells. An increase in the inflammatory response after a second exposure to RSV has been noted previously (9). FI-RSV immunization, on the other hand, was associated with a marked increase in number of granular cells and an increase in lymphocytes that was similar to that with live RSV but with an increase in $CD4^+$ cells and/or a decrease in $CD8^+$ cells (Table 1). This shift in type of lymphocytes is best illustrated by the ratio of $CD4^+/CD8^+$ cells. The granular cells were defined by forward and side scatter patterns indicative of granularity and were medium in size. These cells were $CD3^-$ and CD11b(dim), consistent with them being the eosinophils and neutrophils previously identified by light microscopy (reference 15 and data not shown).

The live RSV and FI-RSV patterns of inflammatory response could most simply be differentiated by number of granular cells and ratio of $CD4^+/CD8^+$ cells. We focused the remainder of the analysis on these two values. The differences in the two patterns were most prominent on day 8.

Consistent with previous results (15), the number of granular cells and ratio of $CD4^+/CD8^+$ cells was significantly higher (Table 1) for FI-RSV-immunized mice compared to live RSV-immunized mice on day 8. FI control-immunized mice also showed an increase in granular cells and ratio of $CD4^+/CD8^+$ cells that was qualitatively similar but of lower magnitude than that associated with FI-RSV (Table 1). The ratio of $CD4^+/CD8^+$ cells in FI control-immunized mice was higher ($P < 0.05$) than that in live RSV-immunized mice and lower ($P < 0.05$) than that in FI-RSV-immunized mice.

Prior immunization with live virus (RSV–FI-RSV [Table 1]) shifted the immune response to FI-RSV immunization toward that seen after live RSV. On day 8, RSV–FI-RSV-immunized mice had a number of granular cells similar to that seen in RSV-immunized mice and lower ($P < 0.05$) than that seen in FI-RSV-immunized mice. The ratio of $CD4^+/CD8^+$ cells in RSV–FI-RSV-immunized mice was intermediate between that seen in FI-RSV- and that in RSV-immunized mice, i.e., higher than that after live RSV immunization ($P < 0.05$) for experiments 1 and 2 and lower than that after FI-RSV immunization ($P < 0.05$) for all three experiments. When FI-RSV was given before live RSV (FI-RSV–RSV in experiment 1 [Table 1]), the number of granular cells and ratio of $CD4^+/CD8^+$ cells were higher ($P < 0.05$) than that seen in mice immunized with RSV before FI-RSV. This finding suggests that the first experience with RSV is especially important in patterning later responses.

The presence of IL-4, IL-5, and IL-13 cytokine mRNA in BAL specimens clearly distinguished between live virus- and FI-RSV-immunized mice (Table 2) while IL-2 and gamma interferon were detected in similar amounts in all immunization groups (data not shown). Live RSV-immunized mice had little detectable IL-4, IL-5, and IL-13 mRNA (Table 2). All mice immunized with FI-RSV had detectable mRNA for at least one of the three cytokines. The percentage of FI-RSV-immunized mice positive for the three cytokine mRNAs was higher ($P < 0.001$ or $P < 0.01$) than that of RSV-immunized mice. Mice immunized with FI–HEp-2 had rates of positivity for IL-4, IL-5, and IL-13 similar to that for FI-RSV-immunized mice while FI–HPIV-3-immunized mice had a lower rate ($P < 0.05$ [Fisher's exact test]) for IL-4 and IL-5 at day 4 and for IL-5 at day 8. We suspect these values were lower because the FI–HPIV-3 antigens were grown in Vero cells while the RSV challenge preparation was grown in HEp-2 cells. As noted in Table 2, prior immunization with RSV (RSV–FI-RSV) decreased the rate of cytokine mRNA positivity otherwise seen after FI-RSV immunization. This decrease in rate of positivity was significant ($P < 0.05$) for all cytokines except IL-5 at day 4. The rate in RSV–FI-RSV-immunized mice, however, remained higher than that in live RSV-immunized mice and significantly so ($P < 0.05$) for IL-5 at day 8. In experiment 3, when FI-RSV was prepared from Vero cell-grown virus, none of the RSV–FI-RSV-immunized animals were positive for these three cytokines. If RSV was given after FI-RSV (FI-RSV–RSV [Table 2]), the rate of positivity for these three cytokines was similar to that for FI-RSV.

DISCUSSION

This study is the first to address, in an animal model, the mechanism by which FI-RSV-vaccinated older children were protected from enhanced RSV disease. Previous studies have suggested that induction of a Th2-like instead of a Th1-like immune response by FI-RSV immunization is associated with and might explain enhanced disease (4–6, 13, 15). The data from this study suggest that lack of enhanced disease in older children might be explained by prior live virus infection preventing the Th2-like response induced by FI-RSV. This and a previous study demonstrated a marked increase in number of granular cells, ratio of $CD4^+/CD8^+$ cells, and presence of Th2 cytokine mRNA in FI-RSV- compared to live RSV-immunized mice (15). When live RSV was given before FI-RSV, the num-

TABLE 1. Priming with live RSV ameliorates FI-RSV immunization-associated cellular infiltration into the BAL fluid in challenged BALB/c mice[a]

Immunization(s)	4 days after challenge (n = 3 to 5)						8 days after challenge (n = 4 to 7)					
	Mean no. of indicated cells (10^5) ± SD in the BAL fluid					Ratio of $CD4^+/CD8^+$	Mean no. of indicated cells (10^5) ± SD in the BAL fluid					Ratio of $CD4^+/CD8^+$
	Total	Granular cells	Lymphocytes	$CD4^+$	$CD8^+$		Total	Granular cells	Lymphocytes	$CD4^+$	$CD8^+$	
Expt 1												
RSV (twice)	2.0 ± 0.3	0.1	0.6	0.2	0.1	1.7	8.0 ± 1.5	0.3 ± 0.1	5.7 ± 1.2	1.2 ± 0.3	3.8 ± 0.8	0.3 ± 0.0
RSV–FI-RSV	2.7 ± 0.4[d]	0.1	0.8	0.2	0.1	1.9	8.3 ± 1.9	0.5 ± 0.3	5.1 ± 0.9	1.8 ± 0.5	2.2 ± 0.6[d]	0.9 ± 0.5[d]
FI-RSV–RSV	5.8 ± 1.5[c]	1.0	1.9	0.9	0.2	4.2	10.0 ± 3.6	2.0 ± 1.0[c]	4.8 ± 1.5	2.6 ± 0.8[c]	1.0 ± 0.4[c]	2.8 ± 0.7[c]
FI-RSV (twice)	9.7 ± 1.7[c]	3.0	2.3	1.2	0.3	4.2	22.6 ± 9.4[c]	8.7 ± 4.0[c]	8.6 ± 3.9	5.7 ± 2.7[c]	0.8 ± 0.5[c]	7.4 ± 1.0[c]
FI-HEp-2 (twice)	4.0 ± 1.6[d]	0.8	1.5	1.1	0.2	6.3	4.5 ± 2.6[d]	0.9 ± 0.5	1.9 ± 1.3[c]	0.9 ± 0.6	0.3 ± 0.2[d]	2.4 ± 0.7[d]
HEp-2 (twice)	1.4 ± 0.5	0.1	0.4	0.1	0.1	1.4	3.6 ± 0.4[c]	0.2 ± 0.0[d]	2.2 ± 0.1[c]	0.6 ± 0.1[c]	1.0 ± 0.2[c]	0.6 ± 0.1[c]
Expt 2												
RSV[b] or RSV	5.4 ± 1.5	0.5 ± 0.2	2.3 ± 0.5	0.6 ± 0.0	0.4 ± 0.0	1.4 ± 0.1	8.6 ± 2.3	0.2 ± 0.1	5.8 ± 1.8	2.0 ± 0.5	2.6 ± 1.0	0.8 ± 0.2
RSV[b]–FI-RSV	ND	ND	ND	ND	ND	ND	10.8 ± 3.2	0.5 ± 0.2[d]	7.3 ± 2.5	3.9 ± 1.6[d]	1.5 ± 0.6[d]	2.7 ± 1.2[c]
FI-RSV[b] or FI-RSV	7.0 ± 4.3	1.0 ± 0.6	2.2 ± 1.5	1.1 ± 0.9	0.2 ± 0.1[d]	5.4 ± 2.9[d]	17.6 ± 5.4[c]	6.1 ± 3.3[c]	5.9 ± 1.3	3.6 ± 0.9[c]	0.7 ± 0.1[c]	5.1 ± 1.1[c]
None	1.8 ± 0.4[d]	0.7 ± 0.2	0.2 ± 0.1[d]	0.1 ± 0.0[d]	0.0 ± 0.0[d]	2.6 ± 1.3	5.5 ± 2.0[c]	0.3 ± 0.2	1.9 ± 1.1[c]	0.4 ± 0.2[c]	1.0 ± 0.6[c]	0.5 ± 0.1[c]
Expt 3												
RSV	4.1 ± 1.6	0.2 ± 0.1	1.9 ± 0.8	0.5 ± 0.3	0.4 ± 0.2	1.4 ± 0.4	5.7 ± 1.6	0.2 ± 0.1	4.0 ± 1.1	1.6 ± 0.5	1.5 ± 0.4	1.0 ± 0.1
RSV–FI-HPIV-3[b]	3.3 ± 1.3	0.2 ± 0.1	1.4 ± 0.6	0.5 ± 0.1	0.3 ± 0.1	1.7 ± 0.4	7.5 ± 0.9	0.3 ± 0.1	5.4 ± 0.7	2.4 ± 0.3	1.6 ± 0.4	1.5 ± 0.4
RSV–FI-RSV[b]	3.8 ± 0.8	0.2 ± 0.0	1.8 ± 0.3	0.6 ± 0.1	0.2 ± 0.1	2.8 ± 0.9[d]	7.8 ± 1.5	0.3 ± 0.1	5.5 ± 1.2	2.4 ± 0.6	2.0 ± 0.6	1.2 ± 0.3
HEp-2–FI-RSV[b]	2.9 ± 0.8	0.4 ± 0.1[d]	0.9 ± 0.2	0.5 ± 0.1	0.1 ± 0.0[d]	5.7 ± 1.9[d]	10.5 ± 2.2[d]	3.1 ± 1.7[d]	4.4 ± 0.7	2.3 ± 0.5	0.6 ± 0.1[d]	4.3 ± 1.3[d]
HEp-2–FI-HPIV-3[b]	2.7 ± 0.8	0.3 ± 0.3	1.0 ± 0.4	0.5 ± 0.2	0.1 ± 0.1	3.5 ± 1.4[d]	6.6 ± 2.4	1.1 ± 1.2	3.1 ± 1.0	1.5 ± 0.6	0.7 ± 0.2[d]	2.1 ± 0.6[d]
HEp-2	1.0 ± 0.5[d]	0.1 ± 0.0[d]	0.3 ± 0.2[d]	0.1 ± 0.1[d]	0.0 ± 0.0[d]	3.1 ± 1.1[d]	4.6 ± 1.1	0.2 ± 0.0	2.4 ± 0.5[d]	0.7 ± 0.1[d]	0.7 ± 0.2[d]	1.0 ± 0.3

[a] Mice were immunized with 10^5 PFU of live RSV or an equivalent dilution of indicated antigen with a 4-week interval between immunizations and challenged at week 10 with 10^6 PFU of RSV. Mice immunized only once were challenged at week 6 or 10 and are included in the same entries. RSV and HEp-2 cell control were administered intranasally under anesthesia, and FI antigens were administered intramuscularly. Total cell numbers were determined by hemocytometer; granular cell and lymphocyte subpopulations were determined by flow cytometric analysis. Results without standard deviations are from pooled BAL fluids of five mice. ND, not done.

[b] Virus was propagated in Vero cells (otherwise in HEp-2 cells).

[c] $P < 0.01$ versus mice immunized with live RSV by Mann-Whitney U test. For other statistical comparisons, see the text.

[d] $P < 0.05$ versus mice immunized with live RSV by Mann-Whitney U test.

TABLE 2. Priming with live RSV reduces Th2-like responses associated with FI-RSV immunization in BALB/c mice[a]

Immunization(s)	4 days after challenge				8 days after challenge			
	n	% Mice with BAL cells positive for:			*n*	% Mice with BAL cells positive for:		
		IL-4 mRNA	IL-5 mRNA	IL-13 mRNA		IL-4 mRNA	IL-5 mRNA	IL-13 mRNA
RSV (once or twice)	12	0	0	0	16	0	0	5
RSV–FI-HPIV-3	4	0	0	0	4	0	0	0
RSV–FI-RSV	9	33	22	11	11	0	27	18
FI-RSV–RSV	5	80[b]	100[b]	100[b]	5	40[c]	80[b]	20
FI-RSV (once or twice)	12	83[b]	67[b]	92[b]	16	56[b]	75[b]	100[b]
FI-HEp-2 (twice)	5	80[b]	100[b]	60[b]	5	40[c]	100[b]	40
FI-HPIV-3	4	0	0	50[c]	4	0	0	50
HEp-2 or none	12	0	0	0	16	0	0	0

[a] Mice were immunized with 10^5 PFU of live RSV or an equivalent dilution of indicated antigen with a 4-week interval between immunizations and challenged at week 10 with 10^6 PFU of RSV. Mice immunized only once were challenged at week 6 or 10. RSV and HEp-2 cell control were administered intranasally under anesthesia, and FI antigens were administered intramuscularly. Cytokine mRNA levels in cells obtained by BAL were determined by RT-PCR.

[b] $P < 0.01$ versus mice immunized with live RSV (Fisher's exact test).

[c] $P < 0.05$ versus mice immunized with live RSV (Fisher's exact test).

ber of granular cells, the ratio of $CD4^+/CD8^+$ cells, and production of Th2-like cytokine mRNA were decreased relative to that after FI-RSV. The number of granular cells was consistently decreased to levels comparable to that seen after live virus immunization while the ratio of $CD4^+/CD8^+$ cells and presence of cytokine mRNA decreased only to levels between those seen after live RSV and those after FI-RSV for experiments 1 and 2.

One possible explanation for the residual FI-RSV-like increase in $CD4^+/CD8^+$ ratio and Th2 cytokines is that priming with live RSV does not completely block the disease-enhancing immune response. The data from experiment 3 suggest an alternate explanation, i.e., live RSV does not effectively prime the memory immune response to FI cellular antigens. In this experiment, the ratio of $CD4^+/CD8^+$ cells and presence of Th2 cytokine mRNA in RSV–FI-RSV-immunized mice were similar to that in live RSV-immunized mice. We suspect that these values were lower because FI-RSV was prepared in a cell line (Vero cells of African green monkey origin) different from that for the challenge virus (HEp-2 cells of human origin), and memory cells induced by cellular antigens in FI-RSV contributed much less to the inflammatory response to RSV challenge. A response to foreign cellular antigens is not likely to be important to human disease since virus replicating in the lungs during natural infection would be associated only with the host's cellular antigens. Although the immune response to cellular antigens in FI-RSV does not overshadow the RSV-specific response, it is probably best to use different cell lines for immunizing antigens and challenge virus and thus minimize any response to cellular antigens and simplify interpretation of results.

One concern in animal model studies is their relevance to human disease. A number of similarities between the RSV and FI-RSV immune response in rodents and humans suggest that rodent models are appropriate for the study of FI-RSV enhanced disease. Both children and mice vaccinated with FI-RSV and then infected with RSV had an increase in eosinophils after RSV infection. The vaccinated children were noted to have a peripheral eosinophilia, and the two children who died had pulmonary eosinophilia (3, 8). Eosinophils and/or granular cells were seen in BAL specimens in the present and previous studies of RSV-challenged, FI-RSV-immunized BALB/c mice (15). Similarly, both humans and BALB/c mice developed a Th1-like cytokine response to RSV after live virus infection (2, 6, 15), and both humans and cotton rats developed an altered ratio of binding to neutralizing antibodies after FI-RSV vaccination (11, 12).

In summary, our data support the hypothesis that older children were protected from FI-RSV-induced enhanced disease because prior live virus infection primed their immune system and prevented the disease-enhancing memory immune response. Although we used RSV A2, a wild-type virus, in these studies, it is likely that attenuation affects the level and location of replication but not the type of immune response. If further study demonstrates that live virus vaccines prime RSV immunity similar to that associated with wild-type virus, then live virus vaccines can probably be used to prime and ensure the safety of the immune response to some subunit vaccines in RSV-naive children. The BALB/c mouse appears to be a good model to study this approach to RSV vaccination.

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