JOURNAL OF VIROLOGY, Sept. 2010, p. 9632–9636 Vol. 84, No. 18
0022-538X/10/$12.00 doi:10.1128/JVI.00451-10

Prophylactic Treatment with a G Glycoprotein Monoclonal Antibody Reduces Pulmonary Inflammation in Respiratory Syncytial Virus (RSV)-Challenged Naïve and Formalin-Inactivated RSV-Immunized BALB/c Mice▽

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Received 1 March 2010/Accepted 21 June 2010

We examined whether prophylactically administered anti-respiratory syncytial virus (anti-RSV) G monoclonal antibody (MAb) would decrease the pulmonary inflammation associated with primary RSV infection and formalin-inactivated RSV (FI-RSV)-enhanced disease in mice. MAb 131-2G administration 1 day prior to primary infection reduced the pulmonary inflammatory response and the level of RSV replication. Further, intact or $F(ab')_2$ forms of MAb 131-2G administered 1 day prior to infection in FI-RSV-vaccinated mice reduced enhanced inflammation and disease. This study shows that an anti-RSV G protein MAb might provide prophylaxis against both primary infection and FI-RSV-associated enhanced disease. It is possible that antibodies with similar reactivities might prevent enhanced disease and improve the safety of nonlive virus vaccines.

Respiratory syncytial virus (RSV) infection in infants and young children causes substantial bronchiolitis and pneumonia (11, 27, 28, 40) resulting in 40,000 to 125,000 hospitalizations in the United States each year (27). RSV is also a prominent cause of respiratory illness in older children; those of any age with compromised cardiac, pulmonary, or immune systems; and the elderly (6, 7, 11, 17, 18, 39). Despite extensive efforts toward vaccine development (3, 5, 8, 20, 30, 38), none is yet available. Currently, only preventive measures are available that focus on infection control to decrease transmission and prophylactic administration of a humanized IgG monoclonal antibody (MAb) directed against the F protein of RSV (palivizumab) that is recommended for high-risk infants and young children (4, 7, 17). To date, no treatment has been highly effective for active RSV infection (17, 21).

The first candidate vaccine, a formalin-inactivated RSV (FI-RSV) vaccine developed in the 1960s, not only failed to protect against disease but led to severe RSV-associated lower respiratory tract infection in young vaccine recipients upon subsequent natural infection (8, 16). The experience with FI-RSV has limited nonlive RSV vaccine development for the RSV-naïve infant and young child. Understanding the factors contributing to disease pathogenesis and FI-RSV vaccine-enhanced disease may identify ways to prevent such a response and to help achieve a safe and effective vaccine.

The RSV G, or attachment, protein has been implicated in the pathogenesis of disease after primary infection and FI-RSV-enhanced disease (2, 26, 31). The central conserved region of the G protein contains four evolutionarily conserved cysteines in a cysteine noose structure, within which lies a CX3C chemokine motif (9, 29, 34). The G protein CX3C motif is also immunoactive, as suggested by studies with the mouse model that show that G protein CX3C motif interaction with CX3CR1 alters pulmonary inflammation (41), RSV-specific T-cell responses (12), FI-RSV vaccine-enhanced disease, and expression of the neurokinin substance P (14) and also depresses respiratory rates (32). Recent studies demonstrated that therapeutic treatment with a murine anti-RSV G protein monoclonal antibody (MAb 131-2G) which blocks binding to CX3CR1 can reduce pulmonary inflammation associated with primary infection (13, 23). These findings led us to hypothesize that prophylactic administration of this anti-RSV G monoclonal antibody may also diminish pulmonary inflammation associated with RSV infection in naïve and in FI-RSV-vaccinated mice. In this study, we evaluate the impact of prophylactic administration of MAb 131-2G on the pulmonary inflammatory response to primary infection and to RSV challenge following FI-RSV immunization in mice.

Prophylactic anti-RSV G MAb treatment decreases pulmonary cell infiltrates and RSV replication in naïve mice. In accordance with institutional guidelines, 8- to 10-week-old, specific-pathogen-free, female BALB/c mice (The Jackson Laboratories) were intraperitoneally treated with 300 μg anti-RSV G MAb, 131-2G, or normal mouse Ig (Thermo Scientific)

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▽ Published ahead of print on 30 June 2010.

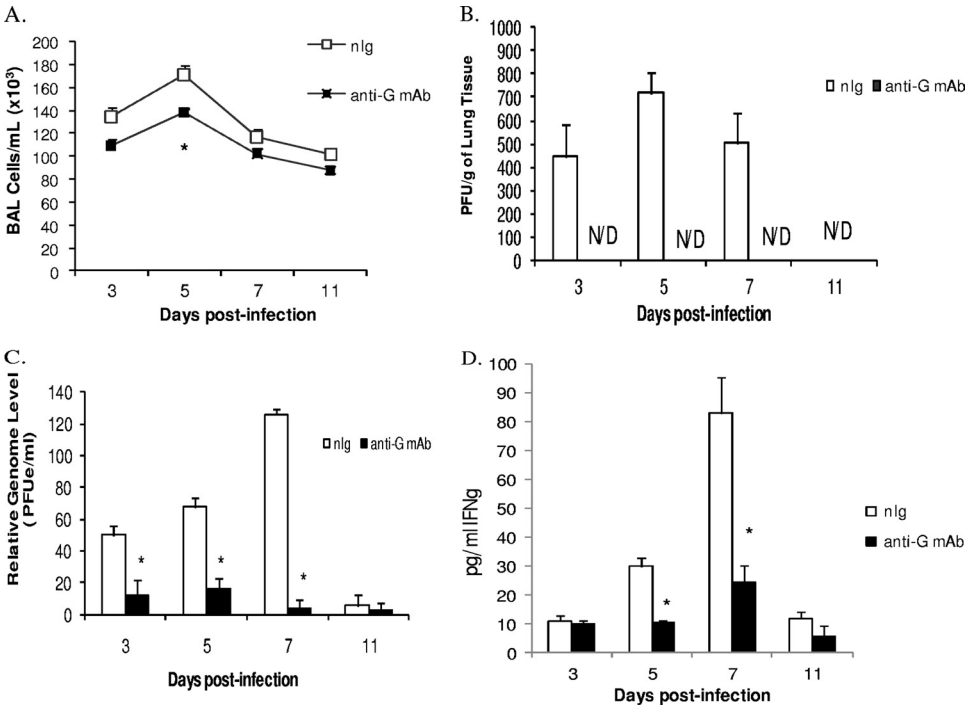

FIG. 1. Effect of MAb 131-2G prophylaxis on pulmonary leukocyte trafficking and RSV titers after primary RSV infection. (A) The mean BAL fluid cell numbers (±standard errors) in the lungs of antibody-treated (nIg or anti-G MAb) RSV-infected mice. (B) Virus titers (PFU/g of tissue; ±standard errors) in the lungs of RSV-infected mice. (C) Real-time RT-PCR M gene expression in the lungs of antibody-treated mice. e, equivalent. (D) IFN-γ levels (±standard errors) in cell-free BAL fluid. Results are representative of three independent experiments with no fewer than three mice per time point. Asterisks indicate a significant difference ($P < 0.05$) between nIg-treated and antibody-treated mice. N/D indicates virus titers below the level of detection.

1 day prior to intranasal challenge with 10^6 PFU of RSV strain A2 (35). Prophylactic treatment with MAb 131-2G resulted in an ~30% reduction in total bronchoalveolar lavage (BAL) fluid cell infiltration (Fig. 1A) compared to control antibody-treated mice. The level of pulmonary infiltration was significantly ($P < 0.05$) reduced at day 5 postinfection (p.i.), the time point corresponding to the peak of viral replication and pulmonary inflammation in the absence of prophylactic treatment (Fig. 1A). This decrease in cell number was associated with a decrease in most cell types in the BAL fluid with marked reductions early after infection, such as at day 3 p.i., for RB6-8C5$^+$ polymorphonuclear cells (PMNs) (73% reduction), DX5$^+$ natural killer (NK) cells (68% reduction), and CD4$^+$ and CD8$^+$ cells (67% and 55% reduction, respectively) as determined by flow cytometric analysis (35). Similar levels of reduction of these cell types were also observed at day 5 p.i., as were modest decreases in CD45R/B220$^+$ cells and CD11b$^+$ cells (data not shown). Cell-free BAL fluid supernatants were assayed for gamma interferon (IFN-γ) and interleukin-4 (IL-4) levels by enzyme-linked immunosorbent assay (ELISA) (eBioscience). The level of IFN-γ production in BAL fluids was significantly ($P < 0.05$) reduced at days 5 p.i. (64%) and 7 p.i. (71%) after the antibody prophylaxis (Fig. 1D), but the already low level of IL-4 at day 5 p.i. (6.2 ± 1.8 pg/ml) or day 7 p.i. (2.0 ± 0.4 pg/ml) was not substantially affected.

Prophylactic administration of MAb 131-2G significantly ($P < 0.05$) inhibited RSV replication at all time points examined compared to normal Ig (nIg) control antibody treatment. Infectious virus was not detectable by an immunostaining plaque assay (15) in the lungs of mice treated prophylactically with MAb 131-2G (Fig. 1B). Total RNA was extracted from homogenized lung tissue, and expression of the RSV matrix (M) gene was determined by real-time quantitative reverse transcription-PCR (RT-PCR). Transcripts of the RSV M gene were detected in both normal Ig-treated and MAb 131-2G-prophylactically treated mice through day 11 p.i., but message levels were significantly lower ($P < 0.05$) for the MAb 131-2G-prophylactically treated mice (Fig. 1B and C). As previously demonstrated, in the absence of detectable infectious virus, low levels of RSV RNA are detected in the lungs of infected mice by real-time RT-PCR (1, 22).

Prophylactic antibody treatment prevents FI-RSV vaccine-enhanced disease. Mice were immunized with 10^6 PFU equivalents of formalin-inactivated RSV/A2 (FI-A2) in the superficial gluteal muscle and then rested a minimum of 4 weeks prior to RSV challenge (14). Several studies have established that pronounced weight loss, pulmonary eosinophilia, and type 2 cytokine dominance are associated with enhanced FI-RSV vaccine disease in mice (19, 24, 37). We examined these and other pulmonary inflammatory markers following prophylactic MAb treatment in FI-RSV-vaccinated mice challenged with RSV (Fig. 2). No infectious virus or viral RNA was detected in the lungs of mice prophylactically treated with either MAb 131-2G or normal Ig (data not shown). However, treatment with MAb 131-2G was associated with a marked decrease in weight loss (Fig. 2B) and infiltration by pulmonary cells (Fig. 2A), in-

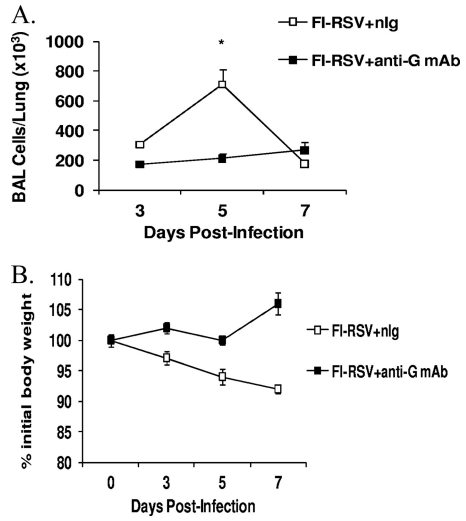

FIG. 2. Effect of MAb 131-2G prophylaxis on illness in FI-RSV-vaccinated mice. (A) Mean BAL cell numbers (±standard errors) in the lungs of FI-RSV-vaccinated mice treated with either nIg or MAb 131-2G. The data are representative of three independent experiments examining four mice per treatment. The asterisk indicates a significant difference ($P < 0.05$) between nIg-treated and antibody-treated mice. (B) Percent initial body weight (±standard error) of nIg- or MAb 131-2G (anti-G MAb)-treated FI-RSV-vaccinated mice after RSV challenge.

cluding eosinophils, i.e., <11% eosinophilia compared to normal Ig control-treated mice (23 to 42% eosinophilia) (data not shown). FI-A2- or FI-Vero-cell-immunized, mock-challenged mice had a minimal pulmonary inflammatory response that was unaffected by prophylactic administration of MAb 131-2G (data not shown). Prophylactic administration of MAb 131-2G F(ab′)$_2$ was associated with a similar decrease in total BAL fluid cell infiltration (Fig. 3A) and pulmonary eosinophilia (Fig. 3B) compared to normal Ig (nIg) treatment. Overall, both intact MAb 131-2G- and MAb 131-2G F(ab′)$_2$-treated mice had a dramatic reduction in all cell types and at all time points examined compared to nIg-treated mice (Table 1). These findings are consistent with previous studies showing that RSV G protein modifies trafficking to the lung of many cells, including CD11b$^+$, PMN, B220$^+$, and DX5$^+$ NK cells (33, 35, 36). Interestingly, at days 3 and 5 p.i. MAb 131-2G F(ab′)$_2$ was associated with a greater percent reduction for most cell types than was intact MAb 131-2G (Table 1). The similarity in results with the F(ab′)$_2$ and intact forms of MAb 131-2G demonstrates that the MAb effect is not associated with decreased replication of RSV. MAb 131-2G F(ab′)$_2$ does not increase viral clearance (data not shown). The levels of IL-4 in the cell-free BAL fluid were diminished as early as day 3 postchallenge and significantly reduced by days 5 and 7 postchallenge in mice treated with intact and F(ab′)$_2$ MAb 131-2G (Fig. 3C).

This study shows that prophylactic treatment with an anti-RSV G protein monoclonal antibody that blocks RSV G binding to CX3CR1 can both decrease the pulmonary inflammatory response to RSV infection and increase viral clearance. These findings are similar to those seen when this antibody was given as treatment after RSV infection of mice (13, 23). The ability of prophylactic administration of MAb 131-2G to down-

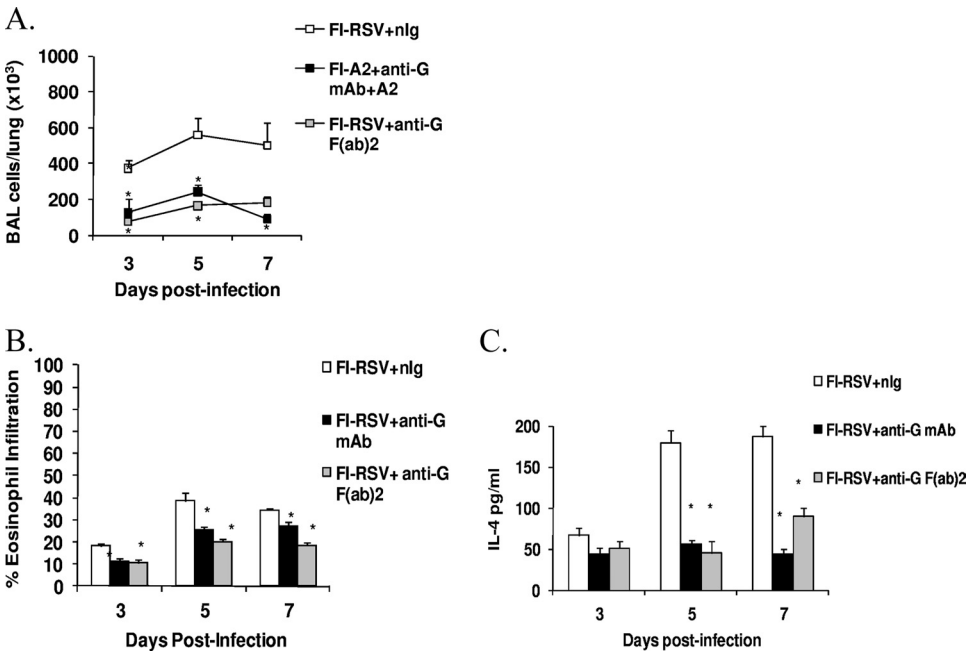

FIG. 3. Effect of MAb 131-2G F(ab′)$_2$ prophylaxis on illness in FI-RSV-vaccinated mice. (A) Mean BAL fluid cell numbers (±standard errors) in the lungs of nIg-, MAb 131-2G (anti-G MAb)-, or 131-2G F(ab′)$_2$ fragment [anti-G F(ab′)$_2$]-treated FI-RSV-immunized mice after RSV challenge. (B) Percentages of eosinophils, as determined by hematoxylin and eosin staining and microscopic analysis (±standard errors), in the lungs of treated FI-RSV-vaccinated mice after RSV challenge. (C) IL-4 levels (±standard errors) in cell-free BAL fluid supernatants from mice on days 3, 5, and 7 after RSV challenge. Representative data from three independent experiments examining four mice per treatment are shown. Asterisks indicate a significant difference ($P < 0.05$) in comparison to nIg-treated mice as determined by Student's t test.

TABLE 1. Pulmonary leukocyte infiltration following MAb 131-2G or 131-2G F(ab′)$_2$ antibody treatment 1 day prior to RSV infection in FI-RSV-immunized mice[a]

Day p.i.	Phenotype	nIg (mean total cells [10^3] ± standard error)	131-2G		131-2G F(ab′)$_2$	
			Mean total cells (10^3) ± standard error	% Reduction from nIg value	Mean total cells (10^3) ± standard error	% Reduction from nIg value
3	CD8	47.4 ± 5.0	7.3 ± 10.0	64	9.3 ± 7.0	**81**
	CD4	135.9 ± 16.0	37.1 ± 21.0	**73**	29.5 ± 23.0	**79**
	B220	56.4 ± 7.0	27.4 ± 16.0	52	11.3 ± 8.0	**80**
	PMN	123.2 ± 15.0	54.6 ± 32.0	56	22.4 ± 17.0	**82**
	CD11b	95.2 ± 11.0	31.7 ± 18.0	**67**	13.4 ± 10.0	**86**
	DX5	36.6 ± 4.0	12.4 ± 7.0	**67**	5.0 ± 3.0	**87**
5	CD8	64.7 ± 11.0	29.0 ± 4.0	**56**	17.0 ± 1.0	**74**
	CD4	305.5 ± 53.0	131.7 ± 21.0	**57**	87.1 ± 7.0	**72**
	B220	88.1 ± 15.0	32.2 ± 5.0	**64**	20.5 ± 2.0	**77**
	PMN	131.6 ± 23.0	38.6 ± 6.0	**71**	25.4 ± 2.0	**81**
	CD11b	113.2 ± 20.0	48.7 ± 8.0	**57**	21.9 ± 2.0	**81**
	DX5	20.6 ± 4.0	8.9 ± 1.0	**57**	8.2 ± 0.7	**61**
7	CD8	102.1 ± 27.0	9.1 ± 3.0	**92**	23.7 ± 4.0	**77**
	CD4	250.2 ± 65.0	24.9 ± 8.0	**91**	99.0 ± 18.0	61
	B220	59.8 ± 15.0	7.1 ± 2.0	**89**	26.2 ± 4.0	57
	PMN	123.1 ± 32.0	9.8 ± 3.0	**93**	27.9 ± 5.0	**78**
	CD11b	70.8 ± 18.0	12.4 ± 4.0	**83**	18.5 ± 3.0	**73**
	DX5	17.9 ± 5.0	2.9 ± 0.9	**84**	6.2 ± 1.0	66

[a] Data represent the mean total BAL fluid cells expressing CD8, CD4, CD45R/B220, RB6-8C5 (PMN), CD11b, or DX5 (NK) from nIg-, anti-RSV G MAb 131-2G-, or 131-2G F(ab′)$_2$-treated FI-RSV-immunized, RSV A2-infected mice. The total number of BAL fluid cells expressing a particular phenotype was determined by multiplying the mean total number of BAL fluid cells by the mean percentage of cells expressing that phenotype. Boldface values indicate a significant difference ($P < 0.05$) in comparison to nIg-treated mice. Data are representative of three independent experiments.

regulate the pulmonary inflammatory response is broad and involves many cell types, including those associated with inflammation and the innate and adaptive immune responses.

Although prophylactic administration with anti-G MAb (131-2G) resulted in significantly diminished viral load in the lung, the MAb did not totally block viral replication, as indicated by detection of viral RNA by real-time PCR. Studies that have analyzed RSV infection of epithelial cells and macrophages found that addition of anti-F MAbs inhibited productive infection but did not block all viral gene expression (1), suggesting that intracellular infection is at least in part refractory to these antibodies.

Numerous studies have implicated the G protein in pathogenesis of RSV disease (2, 26, 31), including several that show that RSV G protein CX3C-CX3CR1 interaction is important for the enhanced-disease phenotype-associated RSV challenge of FI-RSV-immunized mice (33, 35). In one study, FI-RSV-immunized mice challenged with viruses lacking a functional CX3C motif or pretreated with anti-CX3CR1 antibodies did not develop enhanced disease (14). Our data are consistent with and support these earlier observations. In the present study, we show that MAb 131-2G given prophylactically as an intact antibody or F(ab′)$_2$ fragments markedly downregulated the Th2-type inflammatory response to RSV challenge in FI-RSV-immunized mice as exemplified by the marked decrease in pulmonary eosinophilia, a hallmark of FI-RSV vaccine-enhanced disease (10, 25). The marked reduction in weight loss, an indicator of severity of illness in the mouse, is another clear indication of the ability of this MAb to alter the course of enhanced disease after RSV challenge in FI-RSV-immunized mice.

Taken together, the results suggest that MAb 131-2G given as a prophylactic (Fig. 1) or for treatment of infection in naïve or FI-RSV-immunized mice can substantially impact the virus-induced inflammatory response and likely decrease RSV disease (13). The decrease in pulmonary inflammation involves many cell types and is independent of the Fc portion of the antibody and viral clearance (23). We hypothesize that vaccination to achieve an anti-G protein antibody response with activities similar to that of MAb 131-2G might have a similar impact on the pulmonary inflammation and virus clearance and improve both the safety and efficacy of an RSV vaccine. Our recently reported study showing that vaccination with a polypeptide encompassing the central conserved region of the G protein (the binding site for MAb 131-2G) prevented body weight loss and lung histopathology and decreased lung virus titers after challenge with RSV strain A2 is consistent with this hypothesis (41). Moreover, serum antibodies from these vaccinated mice demonstrated G protein CX3C-CX3CR1 blocking activities *in vitro* (41). Thus, it is possible that a vaccine that includes an appropriately constructed component that induces antibodies that block G protein binding to CX3CR1 might eliminate the risk of enhanced disease that is otherwise present for a subunit or inactivated RSV vaccine in RSV-naïve infants.

This research was supported in part by an appointment to the Research Participation Program at CDC administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy and CDC. G.U.R. is an ORISE Fellow. This research was also supported in part by the National Institutes of Health (5RO1AI06275-03) and through the Georgia Research Alliance to R.A.T.

We thank Christopher Paddock (CDC) for technical assistance and Jennifer Harcourt for manuscript review. The RSV M gene primers were a gift from Dean Erdman (CDC).

R.A.T. and L.J.A. have an invention related to the study. No conflicts of interest exist among the authors based on federal regulations. G.U.R., H.C., C.M., and L.M.H. have no conflicts.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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