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Passively Transferred Monoclonal Antibody to the M2 Protein Inhibits Influenza A Virus Replication in Mice

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The M2 protein of influenza A virus is expressed on the surfaces of infected cells, and a monoclonal antibody to this protein inhibits plaque enlargement of sensitive influenza A viruses without reducing plaque titer (S. L. Zebedee and R. A. Lamb, J. Virol. 62:2762–2772, 1988). In the current study, passively transferred monoclonal antibody to M2 reduced the level of replication of influenza A virus but not of influenza B virus in the lungs of mice. These experiments demonstrated that antibody to a protein conserved among influenza A virus subtypes inhibits virus growth in vivo.

Antibodies directed against the external glycoproteins of influenza A virus, the hemagglutinin and neuraminidase (NA), are clearly an important component of protective immunity to influenza virus (1). In addition to the hemagglutinin and NA, a third viral protein, the M2 protein, is abundantly expressed on the surface of influenza A virus-infected cells (8, 20). Small quantities of the M2 protein were also recently detected in preparations of purified virions with a monoclonal antibody specific for the M2 protein (18). When added to the agarose overlay of a standard plaque titration, this monoclonal antibody also inhibited plaque enlargement of several influenza A viruses, including A/FW/50, A/Singapore/57, A/HK/68, A/Udorn/72, and A/USSR/77, without neutralizing virus infectivity (18). Thus, the monoclonal antibody to the M2 protein has an activity similar to that of antibodies to the influenza virus NA, which also inhibit plaque size enlargement without neutralizing infectivity (7). This inhibitory effect of M2 antibody is of considerable interest, since unlike the hemagglutinin and NA, the M2 protein is relatively conserved among influenza A virus subtypes (R. A. Lamb, *in* R. M. Krug, ed., *The Influenza Viruses*, in press) and thus represents a protein to which a cross-protective antibody response could be directed. Therefore, we evaluated the potential role of humoral immunity to the M2 protein in resistance to influenza A virus infection by passive transfer of monoclonal antibodies to mice.

The isolation and characterization of the monoclonal antibody to M2, designated 14C2, has been described previously (18). As control preparations, we used a mixture of two monoclonal antibodies to the hemagglutinin-neuraminidase protein of parainfluenza virus type 3, designated 170/439 (3), and a monoclonal antibody to the NA of the influenza A/Udorn/72(H3N2) virus, designated B40/5, also generously provided by K. Coelingh. All monoclonal antibodies were administered as crude ascitic fluid preparations. Before being used, ascitic fluids were tested for NA inhibition of a reassortant virus bearing the H7N2-72 virus surface antigens by using standard techniques (11) and for plaque size inhibition (PSI) of the A/Udorn/72 virus as previously described (18). The PSI titer was defined as the highest dilution of ascitic fluid resulting in a 50% or greater reduction in plaque size. The ascitic fluid containing the anti-NA monoclonal antibody B40/5 had an NA inhibition titer of 1:2,390 and a PSI titer of 1:25,600. NA-inhibiting activity was not detected (i.e., titer ≤1:5) in ascitic fluid containing either the anti-M2 monoclonal antibody 14C2 or the anti-HN mixture 170/439. The 14C2 ascitic fluid had a PSI titer of 1:640 against the A/Udorn/72 virus, while the 170/439 ascitic fluid had a PSI titer of 1:40. The low, nonspecific PSI titer of the 170/439 ascitic fluid likely represents inhibition by the ascitic fluid proteins of the trypsin required for plaque formation by the A/Udorn/72 virus in this system. The PSI titer of 14C2 ascitic fluid against the influenza B/Ann Arbor/1/86 virus (which does not require trypsin for plaque formation) was ≤1:10.

Four-week-old BALB/c mice received 2 ml of ascitic fluid, either undiluted or diluted in phosphate-buffered saline, pH 7.4, by intraperitoneal injection. One day later, mice were challenged with $10^{4.0}$ 50% tissue culture infective doses of either influenza A/Udorn/307/72(H3N2) virus or influenza B/Ann Arbor/1/86 virus. In the first study, animals received either antibody 14C2, 170/439, or B40/5 and were challenged with the A/Udorn/72 virus. In the second study, animals received either antibody 14C2 or 170/439 and were challenged with either the A/Udorn/72 virus or the B/Ann Arbor/86 virus. Animals were sacrificed on day 3 or 4 after infection, and virus titers in the lungs and nasal turbinates were determined as described previously (15).

The results of the first study are shown in Table 1. The level of virus replication in the lungs of animals receiving M2-specific antibody 14C2 was approximately 100-fold less than that in animals receiving PIV-3-specific antibody 170/439, and this difference was highly significant ($P < 0.01$). Antibody 14C2 inhibited virus replication at all dilutions tested. In addition, levels of influenza A virus replication in the nasal turbinates were somewhat reduced on day 4 in animals receiving 14C2. Despite a high NA inhibition titer and a marked effect on plaque enlargement of the A/Udorn/72 virus in vitro, the influenza A/Udorn/72 virus NA-specific antibody B40/5 had a minimal effect on virus replication in vivo. It has recently been shown that some influenza A virus NA-inhibiting monoclonal antibodies do not provide protection from infection in vivo (17). Presumably, antibody B40/5

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TABLE 1. Effect of passively transferred monoclonal antibodies on the replication of influenza A/Udorn/307/72 (H3N2) virus in BALB/c mice

Monoclonal antibody administered	Influenza A virus protein specificity	Reciprocal PSI titer against A/Udorn/72 virus	Dilution of antibody administered	No. of mice[a]	Virus titer (mean $\log_{10}$ $TCID_{50}$[b]/g ± SE)			
					Lung		Nasal turbinate	
					Day 3	Day 4	Day 3	Day 4
170/439	None	40	Undiluted	20	6.7 ± 0.1	6.3 ± 0.2	4.6 ± 0.1	4.9 ± 0.2
14C2	M2	640	Undiluted	20	4.7 ± 0.2[c]	4.6 ± 0.1[c]	4.6 ± 0.3	3.3 ± 0.3[c]
			1:3	20	5.4 ± 0.2[c]	5.0 ± 0.3[c]	4.3 ± 0.1	3.4 ± 0.2[c]
			1:9	20	5.0 ± 0.2[c]	5.0 ± 0.2[c]	4.6 ± 0.1	4.2 ± 0.1[c]
B 40/5	NA	25,600	Undiluted	10	6.0 ± 0.3[d]	5.9 ± 0.2	4.5 ± 0.4	4.5 ± 0.1[d]
			1:5	10	6.6 ± 0.2	5.8 ± 0.7	4.9 ± 0.3	4.7 ± 0.2
			1:25	10	6.1 ± 0.3	6.0 ± 0.3	4.0 ± 0.6	3.9 ± 0.9

[a] One-half of the mice in each group were sacrificed on day 3, and the remainder were sacrificed on day 4.
[b] $TCID_{50}$, 50% tissue culture infective dose.
[c] $P < 0.01$ compared with animals receiving antibody 170/439 (two-tailed t test).
[d] $0.5 < P < 0.10$ compared with animals receiving antibody 170/439.

belongs in this class of anti-NA monoclonal antibodies. In order to test the virus specificity of the effect of 14C2, a second study was performed in which mice were given either antibody 14C2 or 170/439 and challenged with either the A/Udorn/72 virus (sensitive to 14C2) or the B/AA/86 virus (not sensitive). The results of this experiment are shown in Table 2. The level of replication of the A/Udorn/72 virus in the lungs of animals receiving 14C2 was significantly reduced compared with that in animals receiving 170/439 ($P < 0.01$), while inhibition of virus replication was not seen in animals challenged with the B/AA/86 virus.

In this study, a monoclonal antibody to the M2 protein of influenza A virus specifically inhibited the replication of influenza A virus but not influenza B virus in the lungs of mice. Thus, the M2 antibody represents a nonneutralizing monoclonal antibody which inhibits virus replication in vivo. The mechanism by which influenza virus replication was restricted in vivo by the M2 antibody remains to be defined. The relationship between the in vivo and in vitro effects of monoclonal antibodies on virus growth and pathogenesis is complex, and both protection by nonneutralizing monoclonal antibodies and lack of protection by neutralizing monoclonal antibodies have been described (2, 4, 6, 9, 12, 13). Potential mechanisms of the in vivo effect of antibody 14C2 might include interference with the interaction of the M1 and M2 proteins during virus assembly (19) or interference with the release of virus from the cell by simultaneous binding to M2 proteins on the cell surface and on virions. Alternatively, since the M2 protein is abundantly expressed on the surfaces of infected cells, destruction of such cells before the release of virus by antibody-dependent complement-mediated cytolysis or antibody-dependent cellular cytotoxicity could also account for restriction of virus replication.

This study suggests that humoral immunity to the M2 protein could play a role in protection from naturally acquired influenza A virus infection. In previous studies, parenteral administration of polyclonal immune serum or of monoclonal antibodies directed against the hemagglutinin or NA, but not the nucleoprotein or matrix M1 protein, has protected mice from influenza A virus challenge (1, 10, 16). In these studies, as in the present one, a greater effect has been seen on virus replication in the lungs than in the upper respiratory tract, possibly reflecting a differential accessibility of serum antibody to the mucosa at these two sites. It is important to emphasize that the 100-fold level of reduction of virus replication seen in this study is much less than that mediated by passively transferred anti-hemagglutinin and anti-NA antibodies in mice (1). However, if M2 antibody were protective in humans, this could provide a partial explanation for the observation that adults are more resistant to infection and illness caused by influenza A virus than are children, even for subjects with comparable levels of serum hemagglutination-inhibiting antibody (5), since adults might have higher levels of antibody to the more conserved M2 protein. Natural protection mediated by antibody to the M2 protein could at best be partial, because severe disease and rapid spread of infection with influenza A virus occurred after antigenic shifts of the hemagglutinin and NA in 1957 and 1968, despite apparent conservation of RNA segment 7, which encodes the M1 and M2 proteins (13). Quantitative studies to assess the levels of M2 antibodies in human sera and in passively immunized mice associated with resistance to infection or illness will help to examine this question. In addition, further studies to determine the immune response to the M2 protein in humans after natural infection or immunization with influenza virus vaccine will help to define the importance of the M2 protein in humoral immunity to influenza A virus.

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TABLE 2. Effect of monoclonal antibodies on the replication of influenza A and B viruses in the lungs of BALB/c mice

Monoclonal antibody administered	Influenza A virus protein specificity	Dilution of antibody administered	No. of mice	Virus titer (mean $\log_{10}$ $TCID_{50}$[a]/ml ± SE) 4 days after challenge with:	
				A/Udorn/72 virus	B/Ann Arbor/86 virus
170/439	None	1:2	10	6.1 ± 0.1	6.4 ± 0.1
14C2	M2	1:2	10	4.7 ± 0.1[b]	6.2 ± 0.1

[a] $TCID_{50}$, 50% tissue culture infective dose.
[b] $P < 0.01$ compared with animals receiving 170/439 (two-tailed t test).

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