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Serum and Nasal Wash Antibodies Associated with Resistance to Experimental Challenge with Influenza A Wild-Type Virus

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To identify immunological predictors of resistance to influenza A infection and illness, the immunological status of live and inactivated virus vaccinees subsequently challenged with H1N1 or H3N2 wild-type virus was examined. We refer to prechallenge antibodies of vaccinees receiving live attenuated virus as infection induced and those receiving inactivated virus as inactivated vaccine induced. Inactivated vaccine-induced protection against wild-type virus infection or illness correlated with the level of neuraminidase-inhibiting antibody in serum, local hemagglutinin immunoglobulin G (IgG) (but not IgA) enzyme-linked immunosorbent assay antibody, and hemagglutination-inhibiting antibody in serum. In contrast, infection-induced resistance to wild-type virus infection correlated with local hemagglutinin IgA antibody and neuraminidase-inhibiting antibody in serum, but not with hemagglutination-inhibiting antibody in serum. These observations suggest that live vaccine virus infection-induced and inactivated vaccine-induced immunity may involve different compartments of the immune system; sufficient antibody in either serum or nasal secretions is capable of conferring resistance.

Antibodies in serum, secretory antibodies, cell-mediated immunity, and nonspecific innate factors have all been considered important in the prevention of or recovery from influenza infection and illness (1, 7, 10, 22, 23, 26). However, the exact roles and interrelationships of these diverse factors have not been clearly defined. The presence of hemagglutination-inhibiting (HAI) antibody in serum has long been associated with resistance to influenza virus infection (9, 11–13, 17, 22, 24). Results of other studies have suggested that neuraminidase- (NA) inhibiting (NI) antibody in serum contributes to immunity to influenza (2, 8, 15, 19, 25). This antibody has been associated both with protection against illness and resistance to wild-type virus replication in humans (8, 19). Evidence suggests that local antibody may also play a significant role in immunity to influenza (1, 3, 6, 16, 18; P. R. Johnson, S. Feldman, J. M. Thompson, J. D. Mahoney, and P. F. Wright, J. Infect. Dis., in press). Quantitatively different antibody responses are induced by live, attenuated, cold-adapted (*ca*) reassortant virus vaccines and inactivated virus vaccines (3, 5), yet both vaccines provide protection against influenza (3, 4). Which antibodies elicited by the two different forms of vaccine play a major role in preventing infection and illness? In an attempt to address this question, we examined the immunological status of vaccinated and unvaccinated volunteers before challenge with wild-type virus.

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The study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases, the Human Volunteer Research Committee at the University of Maryland, and the Committee on Human Investigation at the University of Rochester. Details of the clinical studies have been described elsewhere (3, 4, 5). Briefly, the volunteers were healthy college students, lacked a history of influenza vaccination, had an HAI antibody titer in serum of 1:8 or less to A/Washington/897/80 (H3N2) or A/Cali-fornia/10/78 (H1N1) virus, and gave written, informed consent. Some of these volunteers were administered live attenuated virus vaccine intranasally or inactivated virus vaccine parenterally. The live virus vaccine given was A/Washington/897/80 (H3N2) or A/California/10/78 (H1N1) *ca* reassortant virus. The commercial inactivated virus vaccine was ether extracted and contained 15 μg each of A/Brazil/11/78 (H1N1), A/Bangkok/1/79 (H3N2), and B/Singapore/222/79 hemagglutinin (HA) per 0.5-ml dose (Fluogen; Parke, Davis & Co., Morris Plains, N.J.).

To determine whether a vaccine induced resistance to wild-type virus, vaccinees were inoculated intranasally with homologous wild-type virus. Unvaccinated volunteers who had an HAI titer in serum of 1:8 or less to A/Washington/897/80 (H3N2) or A/California/10/78 (H1N1) virus served as controls. In one study, controls and volunteers vaccinated with A/Washington/897/80 (H3N2) *ca* virus ($10^{5.5}$ to $10^{7.5}$ 50% tissue culture infective doses [$TCID_{50}$]) or inactivated virus were challenged with $10^{6.0}$ $TCID_{50}$ of A/Washington/897/80 wild-type virus 1 to 2 months after vaccination (3). In a similar study, controls and vaccinees who received A/Washington/80 or A/California/78 *ca* virus or inactivated virus vaccine 7 months previously were challenged with $10^{6.0}$ $TCID_{50}$ of A/Washington/80 wild-type virus or $10^{4.0}$ $TCID_{50}$ of A/California/78 wild-type virus (4). The same suspension of wild-type virus was used in both studies to ensure comparability of the challenge inoculum. Influenza A/Washington/897/80 (H3N2) and influenza A/California/10/78 (H1N1) wild-type viruses were antigenically

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TABLE 1. Relation between levels of prechallenge antibodies in nasal wash or serum induced by naturally acquired influenza A virus infection, live attenuated vaccine virus infection, or immunization with inactivated virus vaccine and resistance to shedding or illness[a] caused by wild-type challenge virus

Immunity induced by:	Response to challenge	No. of volunteers challenged	Mean prechallenge antibody titer (reciprocal) in[b]:				
			Serum			Nasal wash	
			NI	HAI	HA Fab	HA Fab	HA IgA
Naturally acquired infection (controls)	Did not shed virus	9	1.5 ± 1.6	1.6 ± 1.1	10.0 ± 1.4	6.1 ± 1.3	5.0 ± 1.2
	Shed virus	33	0.5 ± 1.4	1.2 ± 0.4	9.9 ± 1.1	4.8 ± 2.4	3.8 ± 2.5
	Not ill	24	1.1 ± 1.6	1.3 ± 0.7	9.9 ± 1.2	4.4 ± 2.0	5.3 ± 1.9
	Ill	18	0.3 ± 1.3	1.2 ± 0.4	9.9 ± 1.1	3.6 ± 2.7	4.7 ± 2.6
Inactivated virus vaccine	Did not shed virus	17	3.4 ± 1.9	6.4 ± 1.1[c]	14.0 ± 1.7[c]	7.7 ± 2.2[d]	6.8 ± 1.6
	Shed virus	33	2.5 ± 2.4	3.7 ± 1.6[c]	12.0 ± 1.5[c]	5.8 ± 2.2[d]	6.0 ± 2.0
	Not ill	41	3.3 ± 2.1[c]	4.9 ± 1.8[d]	12.7 ± 1.9	6.8 ± 2.3[d]	6.4 ± 1.9
	Ill	9	0.5 ± 1.3[c]	3.3 ± 1.8[d]	12.6 ± 1.6	5.1 ± 2.1[d]	5.6 ± 1.8
Infection with live virus vaccine	Did not shed virus	37	3.3 ± 1.8[d]	2.6 ± 1.3[c]	11.0 ± 1.6[c]	7.0 ± 2.4[c]	6.9 ± 2.2[d]
	Shed virus	34	2.2 ± 2.2[d]	2.0 ± 1.0[c]	9.9 ± 1.6[c]	5.3 ± 2.3[c]	5.8 ± 2.3[d]
	Not ill	57	3.0 ± 2.8[d]	2.4 ± 1.2	10.5 ± 2.2	6.5 ± 2.3[d]	6.7 ± 2.2[d]
	Ill	14	1.7 ± 1.8[d]	1.8 ± 1.2	9.6 ± 1.7	4.8 ± 2.6[d]	5.2 ± 2.2[d]

[a] Includes both systemic and local illnesses.
[b] Titers are expressed as $\log_2$ values ± standard deviation.
[c] Differences between group means are statistically significant; $P < 0.01$ by two-tailed Student t test.
[d] Differences between group means are statistically significant; $P \leq 0.05$ by two-tailed Student t test.

similar to influenza A/Bangkok/1/79 (H3N2) and A/Brazil/11/78 (H1N1) viruses, respectively.

The challenged volunteers were considered ill if they developed any of the following symptoms: fever (>37.8°C); systemic symptoms (myalgia or chills or sweats); rhinorrhea, pharyngitis, or a constant cough for 2 consecutive days. An illness score that reflected severity of illness was calculated for each volunteer by assigning a specified number of points to these influenza-associated symptoms: fever (2), systemic illness (2), rhinorrhea (1), pharyngitis (1) cough (1). The maximum achievable score was 7.

Nasal-wash specimens for isolation of wild-type virus were collected before challenge and daily for 7 to 10 days afterward, as described previously (3, 18). The lowest titer ($\log_{10}$) of virus detected by our tissue culture assay was 0.75 $TCID_{50}$/ml; an undetectable level was assigned a titer ($\log_{10}$) of 0.5 $TCID_{50}$/ml. Because the duration and peak mean titers of wild-type virus shedding in challenged volunteers exhibited significant correlation ($r = 0.8405$; $P < 0.0001$), a composite of both parameters (i.e., a virus index) was calculated that reflected cumulative virus shedding for each volunteer that was challenged. This index was calculated by adding the peak titer of virus recovered to the total number of days of virus shedding. For example, a volunteer who shed virus for 3 days and had a peak mean titer ($\log_{10}$) of virus recovered of 4.5 $TCID_{50}$/ml would have a virus index of 7.5. The virus index for infected volunteers ranged from 0.5 to 13.8.

Prechallenge serum from all volunteers challenged in the two studies were tested by HAI and NI assays, and their prechallenge nasal washes were tested by enzyme-linked immunosorbent assay (ELISA) for HA immunoglobulin A (IgA) antibodies (20,21). In addition, the serum and nasal wash specimens were tested by ELISA for HA antibodies with a rabbit anti-human Fab serum that recognizes both heavy- and light-chain antibodies and detects total immunoglobulin (IgA, IgG, IgM) (21). The antigen used in the HAI tests was the A/Washington/897/80 (H3N2) or the A/California/10/78 (H1N1) wild-type virus. The homologous antigens used in the NI test were reassortant viruses that possessed the A/equine-1 HA and A/Bangkok/79 NA or the A/equine/56 HA and A/USSR/77 NA. These recombinant viruses were kindly supplied by Alan Kendal of the Centers for Disease Control, Atlanta, Ga. Specific rabbit anti-human immunoglobulins and purified A/Bangkok/79 or A/California/78 HA were used in the ELISA tests as described previously (5).

Because the response to challenge with wild-type viruses of the H1N1 or H3N2 subtypes (i.e., level of virus shedding and frequency of illness) were similar for the unvaccinated controls, the data for the two control groups were pooled for analysis. Also, because there were no significant intragroup differences between the H1N1 and H3N2 live virus vaccinees or the subgroups of inactivated virus vaccinees with regard to their respective immune responses to vaccination or their responses to challenge with H1N1 or H3N2 wild-type virus, data for each vaccine subgroup were combined. Thus, composite data from 57 live H3N2 and 14 live H1N1 virus vaccinees were analyzed; their prechallenge antibodies are referred to as infection induced. Similarly, data from the 50 inactivated virus vaccinees were analyzed separately; their prechallenge antibodies are referred to as inactivated virus vaccine induced. Data from 42 unvaccinated control volunteers were also analyzed; their prechallenge antibodies are referred to as naturally acquired.

Because both local and systemic HA IgA and IgG antibodies may be important mediators of resistance, we assessed the contribution of HA antibodies in serum and nasal wash measured by ELISA with anti-Fab (i.e., both IgA and IgG HA antibodies), in addition to HAI in serum, NI in serum, and HA IgA antibodies in nasal wash. The geometric mean titers of live virus vaccine infection-induced NI in serum, ELISA HA Fab in serum and nasal wash, and ELISA HA IgA antibodies in serum and nasal wash before challenge were significantly higher in those volunteers who did not shed virus or become ill (Table 1). HAI geometric mean titers in serum were also significantly higher in vaccinees who did not shed virus. Infection-induced HA Fab antibody in nasal secretions probably represents HA IgA antibody because both local Fab and IgA HA antibodies are

TABLE 2. Antibody predictors of resistance to influenza A virus replication or illness

Immunity induced by:	Resistance to:	Indicated antibody significantly associated with resistance in[a]:			
		Serum		Nasal Wash	
		NI	HAI	HA Fab	HA IgA
Naturally acquired infection (controls)	Virus replication	$P < 0.01$	NS[b]	NS	$P < 0.05$
	Illness	$P < 0.05$	NS	NS	$P < 0.025$
Inactivated virus vaccine	Virus replication	$P < 0.03$	$P < 0.0005$	NS	NS
	Illness	$P < 0.003$	NS	$P < 0.005$	NS
Infection with live virus vaccine	Virus replication	$P < 0.003$	NS	$P < 0.025$	$P < 0.025$
	Illness	NS	NS	NS	NS

[a] Determined by stepwise interactive multiple regression analysis; HA Fab and IgA antibody in nasal wash results were analyzed separately because they correlated significantly in the live virus vaccine group.
[b] NS, Not significantly associated with resistance: $P < 0.05$.

associated with resistance to illness and virus replication. In contrast, the level of inactivated virus vaccine-induced HAI in serum and ELISA HA Fab antibodies in nasal wash before challenge correlated with a decrease in virus replication and frequency of influenza illness. The fact that inactivated virus vaccine-derived Fab (but not IgA) local antibody was associated with a decrease in virus replication and illness suggests that the major contributor to resistance in the nasal compartment is IgG. In addition, among the inactivated virus vaccinees, the levels of ELISA HA Fab antibodies in serum and NI antibody in serum were associated with a decrease in virus replication and illness, respectively. The prechallenge antibody levels in unvaccinated controls who did or did not shed virus, who did or did not become ill, or both were not statistically different.

Analysis of geometric mean titers gave an indication of which antibodies were associated with resistance to virus replication and illness. However, to determine the relative contribution of each antibody population to resistance to virus replication (i.e., virus infection) or illness independent of other antibodies, stepwise interactive multiple regression analysis was performed. Virus index or illness score was employed as the dependent variable, and the following prechallenge antibodies were employed as independent variables: HAI in serum, NI in serum, and ELISA HA Fab in nasal wash or ELISA HA IgA antibodies in nasal wash. Because the levels of ELIAS HA Fab antibody in serum and HAI antibody in serum were highly correlated ($r = 0.6666$), the former was not analyzed in this analysis. The analysis revealed that among the unvaccinated controls, the predictors of resistance to virus replication and illness were NI antibody in serum ($t = -2.4327$, $P < 0.01$ and $t = -1.6959$, $P < 0.05$, respectively) and HA IgA in nasal wash ($t = -1.7331$, $P < 0.05$ and $t = -2.0824$, $P < 0.025$, respectively). These antibodies were probably acquired from prior natural infection with influenza A virus. The significant predictors of live vaccine virus infection-induced resistance to virus replication were NI antibody in serum ($t = -3.0669$, $P < 0.003$) and HA IgA antibody in nasal wash ($t = -1.9213$, $P < 0.025$) or HA Fab antibody in nasal wash ($t = 1.9032$, $P < 0.025$) (Table 2). In contrast, the only significant predictors of inactivated virus vaccine-induced resistance to virus replication were HAI antibody in serum ($t = -3.9128$, $P < 0.0005$ and NI antibody in serum ($t = -1.9853$, $P < 0.03$). The lack of correlation between infection-induced HAI antibody in serum induced by naturally acquired infection or infection with live vaccine virus and resistance to infection with the wild-type challenge virus was most likely a consequence of the low HAI titers present in the serum of the challenged volunteers. The HAI titers were low because each of the volunteers was selected to have low HAI antibody before challenge or vaccination and because the live virus vaccine did not stimulate high levels of this antibody. Others have shown that high levels of HAI antibodies in serum induced by wild-type virus under natural conditions exhibit a significant correlation with resistance to influenza A virus infection (23).

There were no significant predictors of live vaccine virus infection-induced resistance to illness. This was probably due to the small number of ill vaccinees and the low illness scores in this group. In contrast, two significant predictors of inactivated virus vaccine-induced resistance to illness were found: NI antibody in serum ($t = -3.1562$, $P < 0.003$), HA Fab antibody in nasal wash ($t = -2.7871$, $P < 0.005$). The elevated level of NI antibody in serum in some of the inactivated virus vaccinees before challenge was probably derived from previous naturally acquired infection with influenza A wild-type virus.

After identifying the antibodies that correlated with resistance to virus replication or illness, we sought to determine a level of these antibodies that might be predictive of resistance. To do this, we calculated a series of predictive values of these antibody titers by constructing a series of two-by-two tables in which various levels of antibody were compared against occurrence or absence of virus shedding or presence or absence of illness (14). We found a spectrum of antibody levels that was associated with resistance to virus replication or illness by the Fisher exact test. The following antibody titers (reciprocal $\log_2$) correlated most significantly with resistance to illness or virus replication: infection-induced NI titer in serum > 2.0, HA IgA titer in nasal wash > 6.0, HA Fab in nasal wash > 6.3; inactivated vaccine-derived HAI titer in serum > 5.0 and NI titer in serum > 2.0 (Table 2). These cutoff values for HAI and NI antibodies in serum agree with those reported previously (9, 17, 19). No single level of inactivated virus-induced Fab HA antibody in nasal wash was significantly associated with resistance to illness, but the stepwise interactive multiple regression analysis did indicate the importance of this antibody to resistance to illness.

It is clear from our findings that immunity induced by live and inactivated virus vaccines is associated with the induction of different levels of antibody in serum or nasal wash that is associated with resistance. HA antibody in serum measured by HAI and ELISA HA Fab and HA Fab (presumably IgG) antibody in nasal wash appear to be primarily

responsible for resistance to virus replication, illness, or both in vaccinees who received inactivated virus vaccine. NI antibody in serum which was present in some of the live and inactivated virus vaccinees was also associated with resistance. In addition, local HA IgA antibody induced by live virus vaccine was an important mediator of resistance. Our findings suggest that vaccine-induced antibody in serum or nasal secretions can modify infection or illness with wild-type virus if antibody is present in sufficient quantity to exert a biological effect, e.g., virus neutralization (HA-specific antibody) or prevention of virus spread (NA-specific antibody).

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