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Hemagglutinin-Specific Enzyme-Linked Immunosorbent Assay for Antibodies to Influenza A and B Viruses

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An enzyme-linked immunosorbent assay (ELISA) was developed to detect antibodies present in human serum or nasal washes directed against influenza A or B hemagglutinin glycoproteins. The assay was modified to measure the immunoglobulin isotype specificity of the anti-hemagglutinin response in serum and nasal secretions. In the postinfection sera anti-hemagglutinin of the immunoglobulin G isotype was predominant, whereas in nasal secretions the antibody was predominantly immunoglobulin A. The antibody response detected by the ELISA manifested hemagglutinin subgroup specificity. In addition, there was a good correlation between the ELISA antibody titer and the hemagglutination-inhibition or neutralizing antibody titer. The ELISA was more sensitive than the hemagglutination-inhibition assay, and the range of antibody titers measurable by ELISA in human serum was from less than 1:20 for children who had never experienced influenza infection to 1:400,000 for adults convalescing from a secondary infection. With more sensitive tests to detect antibody to the influenza hemagglutinin it should be possible to determine the relative contribution of local and systemic immunity to resistance to influenza virus infection.

Conventional methods of detecting antibody to influenza virus are not sufficiently sensitive to quantitate low levels of viral antibody. For example, vaccinees who received attenuated strains of influenza A virus were resistant to wild-type virus challenge in the absence of detectable serum antibody to the challenge virus (13, 20, 27). Levels of antibody in the nasal secretions regularly tend to be quite low, yet such secretions are of principal interest in studying the immunology of resistance to influenza virus. Thus, there is a need to develop more sensitive techniques capable of detecting low levels of antibody in serum and nasal secretions. Recently, enzyme-linked immunosorbent assay (ELISA) methodology has been used to measure the immune response to influenza A virus (1, 3, 8, 10, 17). However, in these assays, whole virus has been used as an antigen which detects antibodies to both the subtype-specific surface antigens and the type-specific internal antigens (8). Immunity to the hemagglutinin (HA) and neuraminidase surface glycoproteins correlates with resistance, whereas immunity to the internal type-specific antigens does not (15, 23). Therefore, it is important to develop a sensitive ELISA that will detect antibody to the epidemiologically important surface antigens of the influenza A virus (5). In this paper, an HA-specific ELISA is described which can be used to measure antibody in serum and nasal wash specimens.

MATERIALS AND METHODS

Clinical specimens. Live wild-type and attenuated influenza A viruses were given to volunteers as part of a vaccine development program (2). Pre- and postinfection sera and nasal washes were obtained from volunteers or patients who were infected with one of the following agents: (i) A/Hong Kong/123/77 (H1N1) wild-type virus, $10^{4.0}$ 50% tissue culture infectious dose ($TCID_{50}$) (adults) (16); (ii) A/Alaska/6/77 (H3N2) wild-type virus, $10^{4.2}$ $TCID_{50}$ (adults) (14); (iii) influenza B wild-type virus, antigenically similar to B/Hong Kong/8/73 virus (sera from children who had natural infections with influenza B virus were kindly supplied by H.-Y. Kim, Childrens National Medical Center, Washington, D.C); or (iv) A/Udorn/307/72 (H3N2) wild-type, $10^{4.0}$ $TCID_{50}$, or vaccine virus, $10^{6.0}$ to $10^{7.0}$ $TCID_{50}$ (adults) (20). Nasal wash specimens were collected and concentrated as previously described (20).

HAI and neutralization assays. The antigens used in the standard microtiter HA-inhibiton (HAI) assays were (i) A/USSR/92/72 split-product vaccine (Parke, Davis & Co., Hunt Valley, Md.); (ii) recombinant whole virus possessing the A/Texas/1/77 HA and the A/Equine 1/Prague/56 neuraminidase to measure H3-77 antibody; (iii) recombinant whole virus possessing the A/England/42/72 HA and the A/Equine 1/Prague/56 neuraminidase to measure H3-72 antibody; and (iv) whole virus B/Hong Kong/8/73.

The tube neutralization titer test to detect antibodies in nasal wash speciemns was described previously (20).

Purification of HA. Influenza virus grown in the allantoic cavity of embryonated eggs was banded twice in sucrose (12), and the HA was extracted and purified as described previously (19). The purified HA was 95 to 98% free of other influenza antigens. Hyperimmune antiserum raised in sheep to the purified HA contains antibody only to the HA antigen (19). The viruses used to produce the purified HA contained the HA antigen of (i) A/USSR/92/72 (H1N1), (ii) A/Texas/1/77 (H3N2), (iii) B/Hong Kong/8/73, and (iv) A/Udorn/307/72 (H3N2).

Preparation of human immunoglobulins and rabbit anti-human immunoglobulin sera. Serum fractions enriched for immunoglobulin M (IgM) and IgA were prepared from the respective multiple myeloma sera by preparative starch block electrophoresis. Purified IgM was then obtained from fractions devoid of α_2-macroglobulin by ascending gel filtration with Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Purified IgA was obtained by Sephadex G-200 gel filtration of IgA-rich block fractions and collection of the fractions eluting after IgM and before IgG. Purified IgG was obtained by ion-exchange chromatography of pooled normal serum employing diethylaminoethyl cellulose (Whatman Biochemicals Ltd., Maidstone, England) equilibrated with 0.01 M phosphate buffer, pH 8.0. Human $F(ab')_2$ fragments were prepared as previously described (18). Purified myeloma IgE (PS), prepared as previously described (26), was the generous gift of Thomas Waldmann of the National Cancer Institute, National Institutes of Health, Bethedsa, Md. Protein concentrations were determined by absorbance at 280 nm. For use as immunoabsorbents in the purification of antisera, purified immunoglobulins were coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia). For the preparation of antisera, rabbits were initially injected with 500 μg of purified immunoglobulin or $F(ab')_2$ fragments in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) followed by injections of 100 μg of the same proteins emulsified in Freund incomplete adjuvant (Difco) every 2 weeks for at least 6 weeks. Hyperimmune sera were rendered isotype specific by repetitive absorptions with purified immunoglobulins coupled to Sepharose 4B, and the absorptions were monitored by ELISA. Separate myeloma IgA and IgM proteins were employed as immunogens, immunoabsorbents, and as test antigens for antiserum specificity.

ELISA. The ELISA was modified from that of Voller et al. (24) and Yolken et al. (28). The optimal concentration of reagents was determined by checkerboard titrations (25). The type of plastic plate used for the assays was critical. Flat-bottomed "micro-ELISA" plates (Dynatech Laboratories, Inc., Alexandria, Va.) obtained from Griener, Germany, lot no. 81079, were used. Even though all of the plates used came from one lot, it was found that approximately 5 to 10% of them bound test reagents nonspecifically and therefore had high "background" readings. Such defective plates were readily identified, and the corresponding results were discarded.

The ELISA was used to characterize the specificity of rabbit anti-human globulin. In such assays a purified human immunoglobulin (IgG, IgA, IgM, or IgE) was diluted in carbonate buffer (pH 9.8) to a concentration of 100 ng/ml. One hundred microliters of this antigen solution was added to the wells of a microtiter plate which was incubated at 4°C for at least 14 h. Control wells received only carbonate buffer. The outermost row of wells of each plate was not used. A solution containing phosphate-buffered saline with 0.5 ml of polysorbate (PBS-Tween) per liter and 1% fetal calf serum was used to dilute all serum or nasal wash specimens. After three washes with PBS-Tween, 100 μl of diluted rabbit anti-human immunoglobulin was added to antigen-containing and control wells and was serially diluted as described previously (17). The plates were then incubated at room temperature overnight. After three washes, 100 μl of a predetermined dilution of goat anti-rabbit serum conjugated with alkaline phosphatase (Northeast Biomedicals, South Windham, Maine) was added, and the plates were incubated overnight at room temperature. The plates were then washed three times, and 100 μl of *p*-nitrophenol phosphate substrate (1 mg/ml; Sigma 104; Sigma Chemical Co., St. Louis, Mo.) dissolved in 10% diethanolamine buffer was added. After 2 h at 37°C, a multichannel spectrophotometer (Flow Laboratories, Inc., McLean, Va.) was used to measure the yellow chromogen produced by enzymatic cleavage of the substrate. The ELISA titers were calculated by the conventional positive-over-negative (P/N) method in which the endpoint was the highest dilution that gave a P/N ratio equal to or >2. In such a calculation, the optical density of an antigen-containing well (positive) is divided by the optical density of the respective control well (negative).

Similarly, a one-step ELISA was designed to detect antibodies in serum against the purified HA. Purified HA, stored frozen at −70°C, was diluted in carbonate buffer to a concentration of 100 ng/ml. One hundred microliters was added to wells, and the plate was incubated overnight at 4°C. Control wells received only carbonate buffer. For each assay a new sample of frozen HA was used. The test was then performed as described above with the subsequent addition of the following sequence of reagents: human serum; goat anti-human IgG conjugated with alkaline phosphatase (Miles Laboratories, Inc., Elkhart, Ind.); and, finally, substrate. After the addition of each reagent, the plate was incubated overnight at room temperature, except when substrate was added; in this instance the plate was incubated at 37°C for 2 h. The titer of anti-HA antibody was determined by the P/N method described above. The dilutions of reagents were chosen, in part, to give optical density readings in control wells of <0.10.

A two-step ELISA was developed to measure the immunoglobulin isotype (class) specificity of the anti-HA response. In this assay the sequence of reagents from the solid phase up consisted of (i) purified HA, (ii) human serum or nasal wash, (iii) a rabbit anti-human immunoglobulin serum, (iv) goat anti-rabbit serum conjugated with alkaline phosphatase, and (v)

substrate. The incubation times and temperatures were as indicated above, except that the rabbit anti-human immunoglobulin serum was incubated for 5 h. The titers were calculated by the P/N method.

RESULTS

Specificity and sensitivity of the HA-specific ELISA. The serum antibody response of individuals infected with the A/Alaska/6/77 (H3N2), A/Hong Kong/123/77 (H1N1), or a wild-type influenza B virus was determined by using the conventional HAI test and the one-step ELISA, using the purified influenza H3, H1, or B HA in the ELISA (Table 1). Individuals developed detectable antibody only to the HA present in the infecting virus; e.g., individuals infected with the H3N2 influenza A virus had a response to the H3 hemagglutinin but not the the H1 or B hemagglutinin. Thus, the ELISA is like the HAI assay in that it shows subtype specificity, i.e., specificity for the HA.

The ELISA was then compared with the HAI assay to determine which test exhibited the greater sensitivity. The data presented in Table 1 indicate that the mean $\log_2$ rise with the ELISA was greater than that of the HAI assay for the H3, H1, and influenza B antigens. This suggests a greater degree of sensitivity of the ELISA. A comparison of the difference in $\log_2$ rise of each of the 18 pairs of sera tested in the HAI and the ELISA against homologous antigens indicated that the ELISA was, on the average, 2.3 $\log_2$ more sensitive than the HAI assay ($P < 0.001$, Student's t test).

To investigate whether the HAI assay and ELISA were measuring antibody to the same antigen, 24 sera were titrated in both assays, and their respective titers were correlated (Fig. 1). In most cases there was good agreement between the corresponding HAI and ELISA titers. These results suggest that the tests were measuring responses to the same antigens. The slope of each line was >1, the value expected if the two assays have identical sensitivity. Since there were no significant differences among the slopes of the three lines, the ELISA was inferred to be more sensitive for each of the three different HA.

The reproducibility of the determination of ELISA antibody titers was evaluated by testing one serum eight times (four determinations per plate) on 6 different days. The mean ± standard deviation for the 48 determinations was 10.7 ± 0.76 ($\log_2$). The mean ± standard deviations of each of the 6 days were 11.1 ± 0.71; 10.1 ± 0.37; 10.4 ± 0.85; 10.4 ± 0.65; 11.1 ± 0.87; and 10.8 ± 0.54. There was no more than a twofold difference in titer in the same plate on any of the 12 plates tested. An analysis of variance indicated that the greatest source of variation was between plates with small within-plate variation and day-to-day variation.

Modifying the ELISA to determine immunoglobulin isotype specificity of anti-HA response. The one-step ELISA was modified to permit detection of the immunoglobulin isotype of antibody to the influenza HA in human serum. First, the specificity of the rabbit anti-human immunoglobulin antisera used in the two-step ELISA was determined (Table 2). The anti-IgA, and anti-IgM, and anti-IgG antisera were highly specific, except for only a slight cross-reaction of the anti-IgG serum with IgM protein at a dilution of 1:1,600. Next, the dilution of each rabbit serum used in the two-step ELISA was determined by checkerboard titration of several postinfection sera obtained from volunteers infected with the A/Udorn/72 influenza virus on plates containing purified A/Udorn/72 HA. The dilutions of each rabbit anti-human immunoglobulin serum found useful in the two-step ELISA were anti-IgG—1:50,000; anti-IgM—1:5,000; anti-IgA—1:10,000; and anti-FAB—1:

TABLE 1. *Comparison of the HA-specific ELISA and HAI Assay*[a]

Wild-type virus	No. infected	One-step ELISA			HAI		
		Mean $\log_2$ titer[b]		No. with rise[c]	Mean $\log_2$ titer		No. with rise
		Preinfection[d]	Postinfection[d]		Preinfection	Postinfection	
A/Alaska/77 (H3N2)	6	5.2 ± 1.1	9.7 ± 0.8 (4.5)	5	2.5 ± 0.5	4.8 ± 0.7 (2.3)	5
A/Hong Kong/77 (H1N1)	6	6.0 ± 0.4	10.7 ± 0.7 (4.7)	6	2.0 ± 0.0	5.0 ± 0.2 (3.0)	6
B/Hong Kong/72	6	2.8 ± 0.2	9.7 ± 0.6 (6.9)	6	1.0 ± 0.0	4.5 ± 0.2 (3.5)	6

[a] Each serum pair was tested in the ELISA and HAI assay against each of the three antigens. No rises were detected in any of the assays employing a heterologous antigen. Sera were tested by using homologous HA.

[b] All titers are reciprocal and are presented as a mean titer ± standard error. Numbers within parentheses represent the difference between pre- and postinfection titers.

[c] Fourfold or greater rise.

[d] Pre- and postinfection sera were collected 1 month apart.

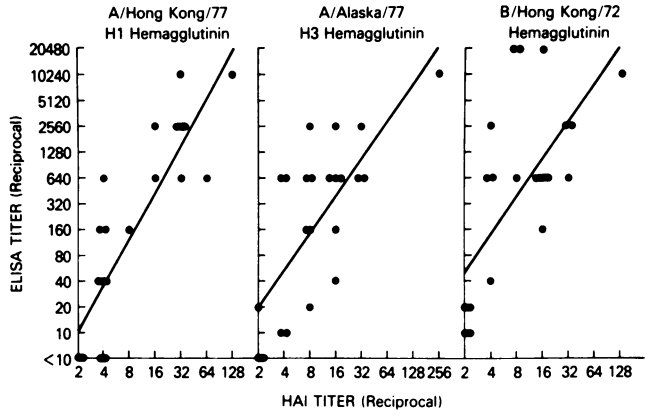

FIG. 1. *Correlation of the ELISA and HAI antibody titers in 24 sera. The slopes (± standard error) of the lines for the H1, H3, and B/Hong Kong/72 HAs are 1.82 ± 0.24, 1.47 ± 0.27, and 1.52 ± 0.31, respectively. The slopes and intercepts of the lines were determined by regression analysis.*

TABLE 2. *Specificity of rabbit anti-human immunoglobulin sera as determined by ELISA*

Rabbit serum	ELISA antibody titer (reciprocal) against indicated human immunoglobulin[a]			
	IgG	IgA	IgM	IgE
Anti-IgG	640,000	<100	1,600	<100
Anti-IgA	<100	64,000	<100	<100
Anti-IgM	<100	<100	102,400	<100
Anti-FAB	640,000	2,560,000	640,000	25,000
Normal	<100	<100	<100	<100

[a] A total of 0.1 ml of a solution of immunoglobulin containing 100 ng/ml was applied to each well.

20,000. At the dilution of rabbit anti-human IgG used, little, if any, cross-reaction with IgM would be expected.

The results of the HAI, one-step ELISA, and two-step ELISA serum antibody response of volunteers infected with the A/Udorn/72 virus are summarized in Table 3. The IgG one- and two-step ELISA were both more sensitive than the HAI response by a magnitude similar to that shown in Table 1. Rises to the H3 HA were detected in these eight serum pairs in the IgG, IgA, and IgM immunoglobulin classes. The levels of antibody present and the magnitude of the rise were greatest using rabbit anti-IgG or anti-FAB sera. Sera from children without previous experience with influenza virus had one- and two-step ELISA anti-HA titers of <1:20.

Since influenza A virus usually localizes in the respiratory tract, it is important to be able to quantitate the influenza A virus antibody response in respiratory secretions. Eleven pairs of pre- and post-inoculation nasal washes were selected for analysis from volunteers who were infected with A/Udorn/72 virus and who showed a spectrum of neutralizing antibody responses (Table 4) (20). For determination of antibody in nasal washes, dilutions of 1:5,000 of rabbit anti-human IgA antibody and 1:20,000 of anti-IgG were used since, at this concentration, the optical density in the control wells (which included all of the sequence of reagents except HA) was <0.10. The post-inoculation levels of ELISA anti-HA antibody were highest in the IgA isotype, and more rises were documented using this anti-IgA serum. Significant quantities of IgG H3 antibody were present with only low quantities of IgM H3 antibody. The $\log_2$ fold rise of the anti-IgA ELISA was 1.5 $\log_2$ greater than that detected by the neutralization test, and the slope (Fig. 2) was >1. These findings suggested a greater sensitivity of the IgA ELISA. Unfortunately, there was only enough of each nasal wash specimen available for testing undiluted in the neutralization assay, but not enough for the ELISA. Consequently, the 1.5 $\log_2$ difference in sensitivity is likely to be an underestimate of the comparative sensitivities of the neutralization assay and the IgA ELISA. Again, there was good correlation between neutralizing antibody titers and IgA ELISA titers (Fig. 2), which suggests that both tests were detecting antibody to the same antigen.

DISCUSSION

Resistance to infection with influenza A virus is associated with the presence of antibodies to the HA and neuraminidase glycoproteins (23). Conventional methods of detecting antibodies to the HA glycoprotein, such as the HAI and neutralization tests, are relatively insensitive and cannot be effectively modified to measure the isotype specificity of the immune response. The present study demonstrates that an ELISA us-

TABLE 3. *Serum HAI and ELISA antibody response to influenza A/Udorn/72 (H3N2) virus infection*

Serum (8 pairs)	HAI[a]			One-step ELISA[b]			Two-step ELISA using indicated rabbit anti-human immunoglobulin serum[c]									
							Anti-IgG			Anti-IgA			Anti-IgM		Anti-FAB		
	Mean $\log_2$ titer[d]	No. with rise[e]	$\log_2$ fold rise[f]	Mean $\log_2$ titer	No. with rise	$\log_2$ fold rise	Mean $\log_2$ titer	No. with rise	$\log_2$ fold rise	Mean $\log_2$ titer	No. with rise	$\log_2$ fold rise	Mean $\log_2$ titer	No. with rise	Mean $\log_2$ titer	No. with rise	$\log_2$ fold rise
Preinfection	2.2 ± 0.3			9.0 ± 1.4			9.2 ± 1.3			6.9 ± 0.9			7.2 ± 0.2		9.2 ± 1.2		
		8	2.4		8	4.1		7	4.9		6	2.8		6	2.7		
Postinfection	4.6 ± 0.3			13.1 ± 1.1			14.1 ± 0.9			9.6 ± 0.5			9.9 ± 0.8		13.4 ± 0.5	7	4.2

[a] HAI antibody tested using recombinant virus containing A/England/42/72 HA and the A/Equine 1/Prague/56 neuraminidase.
[b] One-step ELISA: A/Udorn/72 HA plus human serum plus goat anti-human IgG conjugate plus substrate.
[c] Two-step ELISA: A/Udorn/72 HA plus human serum plus rabbit anti-human immunoglobin plus goat anti-rabbit IgG conjugate plus substrate.
[d] All titers are reciprocal and are presented as a mean titer ± standard error.
[e] Fourfold or greater rise.
[f] Result of mean postinfection titer minus mean preinfection titer. Pre- and post-infection sera were collected 1 month apart.

ing purified HA as antigen does not have these shortcomings.

The antibody detected in the ELISA is directed against the HA antigen, as indicated by the following evidence. First, the antigen is purified to at least 95% homogenicity and induces only an HA antibody response in sheep (19). Second, there is good correlation between the ELISA antibody titer and either the HAI or the neutralization antibody titer; the latter two tests measure antibody directed against the HA antigen (6, 11). Third, the antibody response is specific for the influenza A virus subtype which is determined by surface antigens and not the internal type-specific antigens (5). Last, mouse monoclonal antibody to the HA glycoprotein binds to plates coated with the purified HA, but monoclonal antibody to the neuraminidase glycoprotein does not do so (data not shown). The demonstration that the ELISA titer with purified HA is specific for this antigen is of interest because the antibody responses using whole virus as antigen are not influenza A subtype specific, indicating that internal antigens such as membrane protein and nucleoprotein are detected when whole virus is used as antigen.

Children who have never been infected with influenza A virus have serum titers of less than 1:20 in both the one- and the two-step assays. The low background in the ELISA permits the measurement of small quantities of antibody that were not previously detectable by HAI assay. We have found that the range of the ELISA anti-HA antibody titers in primary influenza A virus infection of children is from less than 1:20 to 1:20,960; in secondary infections in adults ELISA titers up to 1:400,000 have been observed. Comparable levels of antibody are readily measured by an HA-specific radioimmunoassay (22).

The ELISA was modified to measure the immunoglobulin isotype specificity of the antibody participating in anti-HA response in both serum and nasal washes. As might be expected, the ELISA serum anti-HA response was greatest in the IgG isotype, whereas that of the nasal wash was largely in the IgA isotype.

An interesting and unexplained observation in the present study was the finding of anti-HA antibody in the preinfection serum of each of the six volunteers who received A/Hong Kong/77 (H1N1) virus. These volunteers were born after the H1 virus had stopped circulating in 1957 and had not previously been infected with the A/USSR/77 (H1N1), as indicated by their complete susceptibility to infection (16). Thus, their previous exposure would appear to have been limited to H2 and H3 viruses. The level of

TABLE 4. *Nasal wash neutralizing and ELISA antibody response to influenza A/Udorn/72 (H3N2) virus infection*

Nasal wash specimens (11 pairs)	Neutralizing antibody[a]			Two-step ELISA using indicated rabbit anti-human immunoglobulin serum[b]											
				Anti-IgA			Anti-IgG			Anti-IgM			Anti-FAB		
	Mean log_2 titer[c]	No. with rise[d]	Log_2 fold rise[e]	Mean log_2 titer	No. with rise	Log_2 fold rise	Mean log_2 titer	No. with rise	Log_2 fold rise	Mean log_2 titer	No. with rise	Log_2 fold rise	Mean log_2 titer	No. with rise	Log_2 fold rise
Preinfection	0.0 ± 0.0			1.3 ± 0.3			1.0 ± 0.0			1.0 ± 0.0			1.6 ± 0.3		
		8	4.0		11	5.5		8	3.5		1	0.6		10	4.0
Postinfection	4.0 ± 0.5			6.8 ± 0.5			4.5 ± 0.8			1.6 ± 0.4			5.6 ± 0.7		

[a] Neutralizing antibody tested using influenza A recombinant virus possessing the A/England/42/72 HA and the A/Equine 1/Prague/56 neuraminidase.

[b] ELISA antibody tested by using purified influenza A/Udorn/72 HA.

[c] All titers are reciprocal and are presented as a mean titer ± standard error.

[d] Titers corrected to a concentration of 20 mg of IgA/100 ml of nasal wash in pre- and postinfection specimens before calculating rises (20). Fourfold or greater rise in pre- and postinfection nasal washes collected 1 month apart.

[e] Result of mean postinfection titer minus mean preinfection titer.

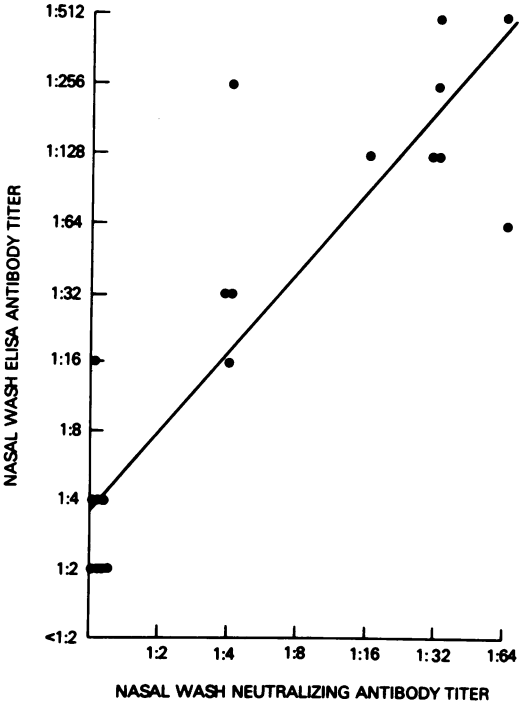

FIG. 2. *Correlation of the ELISA anti-A/Udorn/72 HA antibody titer in the IgA isotype with the neutralization antibody titer. The slope of the line is 1.16 ± 0.13. The slope and intercepts of the lines were determined by regression analysis.*

ELISA antibody was low, with titers ranging between 1:40 and 1:320 (children had titers of less than 1:20 in the same assay). These observations suggest that repeated exposure to H2 and H3 antigens induces low levels of antibody cross-reactive to the H1 glycoprotein that are detectable by the sensitive ELISA test. The presence of such antibody suggests that there are cross-reacting determinants on the HA molecules from different subtypes and that such determinants are not detectable by HAI tests. Clearly, there are large areas of the HA molecule that are shared by the members of different HA subtypes, as indicated by hybridization and sequencing analyses (7, 9, 12). Such determinants might contribute to the cross-reactivity of certain subsets of cytotoxic T cells (4). However, such antibody is not highly protective against the influenza A virus, since each of the volunteers was infected and shed H1N1 virus in high titer; and five of the six volunteers developed clinical influenza (16). It is possible, however, that the anti-HA antibody measured was induced by non-influenza A virus antigens that are cross-reactive with determinants on the H1 HA. With the more sensitive ELISA and radioimmunoassay now available to measure anti-HA antibody, it should be possible to determine the relative contribution of local and systemic immunity in resistance to influenza A or B virus infection.

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