Enzyme-Linked Immunosorbent Assay Compared with Neutralization Tests for Evaluation of Live Mumps Vaccines

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Mumps-specific antibody levels before and after vaccination with live mumps vaccines were determined by enzyme-linked immunosorbent assay (ELISA) and neutralization tests. A correlation was found between neutralization titers and optical density in ELISA. However, postvaccination sera from some vaccinees who failed to seroconvert by neutralization contained significant levels of mumps-specific antibody detectable by ELISA. In some of these serum specimens, the antibody directed to the F polypeptide of mumps virus was predominant. Most sera positive in ELISA neutralized mumps virus upon the addition of fresh guinea pig serum to the virus-serum mixture.

Neutralizing (NT) antibody is currently considered as the most reliable index of the immunity level to mumps virus infection (3, 5). The immunogenicity of live attenuated mumps vaccines has been evaluated by the ability to induce an NT antibody response. Determination of NT antibody is, however, labor intensive and time consuming. Enzyme-linked immunosorbent assay (ELISA), because of its simplicity and rapidity, is gaining increasingly wider acceptance as a serodiagnostic method (9, 13, 18, 20, 21). It has also been used as a sensitive test for vaccination-induced antibody response (9, 19). With the purpose of determining whether ELISA could be used as a substitute for the NT test in the evaluation of vaccines, we tested serum samples obtained before and after vaccination with live mumps vaccines. A small proportion of vaccinees failed to show seroconversion when postvaccination sera were tested by NT test but did seroconvert by ELISA. We therefore studied the nature and the possible protective role of the antibody detected by ELISA but not by the NT test.

MATERIALS AND METHODS

Antigens. Mumps virus Enders strain propagated in the allantoic cavity of embryonated chicken eggs was used for ELISA antigen and radiolabeling. For both purposes, virus was purified from infected allantoic fluid by pelleting and two cycles of banding, first through 20% sucrose onto a 50% (wt/wt) sucrose cushion, then through a 20 to 50% linear sucrose gradient at 25,000 rpm for 4 and 18 h, respectively, in a Beckman SW27 rotor. For the NT test, the same strain propagated in Vero cells was used. Parainfluenza virus types 1, 2, and 3 grown in LLC-MK₂ cells, kindly provided by M. Matsumoto, Department of Virology and Rickettsiology, National Institute of Health, Tokyo, Japan, were used as antigens in hemagglutination inhibition (HI) tests.

Serum specimens. Serum samples were obtained from 77 children before and about 6 weeks after vaccination with live attenuated mumps virus. About 70% of these children were less than 4 years old. A total of 67 children received the Urabe Am-9 strain (22), and the remainder received either the Torii or the Hoshino strain. All of these strains had been developed and licensed in Japan. Despite the absence of an apparent history of mumps infection, all vaccinees 4 or more years old were subsequently found to be seropositive before vaccination.

ELISA. Each well of polystyrene flat-bottomed microtiter plates (M-129A; Dynatech, Plochingen, West Germany) was coated overnight at 4°C with 100 µl of purified mumps virus, diluted overnight in carbonate-bicarbonate buffer (pH 9.6) to a concentration of 0.5 µg of protein per ml and then washed. Washing in this and all subsequent steps was done by four times in 150 µl of phosphate-buffered saline containing 0.05% Tween 20. A total of 100 µl of test serum diluted 1:200 was added in duplicate to antigen-coated wells and left for 2 h at room temperature. The diluent for both test sera and the following enzyme-conjugated secondary antibody was phosphate-buffered saline-0.05% Tween 20 containing 0.1% bovine serum albumin. After washing, 100 µl of peroxidase-conjugated goat anti-human immunoglobulin G (heavy chain specific; Cappel Laboratories, Cochranville, Pa.) diluted 1:4,000 was added. After incubation for 1 h at room temperature and washing, 100-µl volumes of freshly prepared substrate (0.4 mg of *o*-phenylenediamine per ml, 0.012% hydrogen peroxide in citrate-phosphate buffer, pH 5.0) were added. The reaction was allowed to proceed for 30 min at room temperature in the dark before the addition of 25 µl of 2 M sulfuric acid. Optical density (OD) was measured by photometer (Titertek Multiskan; Flow Laboratories, Inc., Rockville, Md.) at 492 nm. A battery of positive and negative reference sera were always included. The standard deviation within the same test or between tests was less than 10% of the mean OD value for the positive reference sera. This variability is comparable to that reported earlier (18, 20).

HI test. HI antibody titers of sera against parainfluenza viruses were determined with 4 hemagglutinating units of antigen and guinea pig erythrocytes. Before the test, nonspecific inhibitors were removed from sera by treatment with a receptor-destroying enzyme of *Vibrio cholerae* (Takeda Chemical Industries, Osaka, Japan). HI titers were expressed as the reciprocals of the highest initial dilutions of sera which completely inhibited hemagglutination.

Electrophoretic analyses of immunoprecipitates. Radioisotopic labeling of purified mumps virus with [³H]succinimidyl propionate (Radiochemical Centre, Amersham, England) was performed by a method described previously (6). To 20 µl of radiolabeled virus (about 400,000 cpm) lysed with 100 µl of RIPA buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01 M Tris hydrochloride [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 1 mM methionine, and 5 × 10⁷ U of Trasylol Aprotinin per 500 ml) (8) was added 20 µl of test serum and the mixture

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FIG. 1. Correlation between neutralization titers and ELISA OD values. OD values of pre- and postvaccination sera were plotted against NT titers. Paired serum samples were obtained from 30 initially seronegative vaccinees who seroconverted in NT tests and from 19 vaccinees who were seropositive by NT tests before vaccination. The horizontal line denotes the cutoff level as defined in the text.

was kept at 4°C overnight. The subsequent procedures of immunoprecipitation were described by Lamb et al. (8). The eluted antigen-antibody complexes were electrophoresed in 10% polyacrylamide gels by the method described by Laemmli (7). The gels were dried and fluorographed (1).

NT tests. NT tests were done in Vero cells. In conventional NT tests, fourfold serial dilutions of sera were mixed with the virus which was diluted to give 20 to 30 PFU per well. The mixture was kept at 35°C for 1 h and inoculated into two wells, each on 24-well (16-mm) Linbro plastic plates. The serum dilution causing 50% plaque reduction was taken as the endpoint. In the complement-mediated NT test, fresh guinea pig serum was added to the virus-serum mixture at a final concentration of 1:400 after the initial incubation period. The final mixture was incubated for an additional 1 h before inoculation.

RESULTS

Correlation between ELISA and NT test. There was a good correlation between ELISA OD values and NT antibody levels in 49 paired serum samples from vaccinees responding to vaccination by an NT antibody rise (Fig. 1). About 60% of the vaccinees were initially seronegative, and the remainder were already seropositive before vaccination but showed an increase in NT antibody titer after vaccination. All prevaccination sera with NT antibody titers of less than 1:4 fell below the cutoff level in ELISA, except one specimen. The

cutoff level was defined as three standard deviations above the mean OD value of negative control serum samples from 50 1-to-3-year-old children without apparent history of mumps infection and with serum NT antibody levels of less than 1:4. The cutoff level was in the range between an OD value of 0.39 and 0.42 in the series of experiments during this study (Fig. 1). With all three strains of mumps vaccine employed, a certain proportion of seronegative vaccinees failed to seroconvert when an NT antibody titer of 1:4 was taken as the threshold. In about 80% of these non-seroconverters, however, a significant increase in OD values was demonstrated in ELISA (Fig. 2). Because mumps and parainfluenza virus antibodies are reported to cross-react occasionally in the complement fixation test and in ELISA (13), it was conceivable that the antibody response to mumps in ELISA was induced by infection with parainfluenza virus. To rule out this possibility, we tested serum samples which were positive in ELISA but not in the NT test for HI activity against parainfluenza viruses. Table 1 shows the results with representative specimens. The antibody rise to mumps virus in ELISA was not correlated with the increase in or the presence of HI antibody to parainfluenza virus. Therefore, the discrepancy between antibody responses in NT tests and in ELISA was not likely due to parainfluenza virus antibody cross-reacting with mumps virus.

Viral polypeptides precipitated by ELISA-positive, NT-negative sera. We wished to determine the viral polypeptide to which the antibody detected by ELISA, but not by the NT test, was directed by polyacrylamide gel electrophoresis of viral polypeptides precipitated by various sera. Mumps virus labeled in vitro with ^3H was disrupted and mixed with 20 μl of undiluted test serum. The resulting antigen-antibody complex was adsorbed to protein A-Sepharose, eluted, and subjected to electrophoresis. When the electrophoretic pattern of radiolabeled mumps virus was compared with those previously reported (4, 10, 14, 17), it was apparent that the method we adopted did not result in uniform labeling of viral

FIG. 2. Antibody response in ELISA not associated with neutralizing antibody response. Pre- and postvaccination OD values of serum samples from 19 initially seronegative vaccinees who did not seroconvert in NT tests.

polypeptides. The polypeptides located near the envelope, HN, F, and M, were more heavily labeled than those located internally. The content or accessibility of residues susceptible to chemical modification also appeared to contribute to the different degree of labeling of polypeptides (Fig. 3, lane 10).

The immunoprecipitates produced by representative serum samples with different reactivity patterns are shown in Fig. 3, lanes 1 to 9. Viral polypeptides precipitated by postvaccination sera positive in ELISA (lanes 1 to 6) included HN, NP, F, and M polypeptides. The major difference between sera with (lane 1 to 3) and without (lanes 4 to 6) NT activity was in the amount of HN polypeptide precipitated. Whereas the former serum samples produced distinct bands of HN polypeptide, barely detectable HN bands were shown by the latter serum samples. On the other hand, similar amounts of F polypeptide was precipitated by both groups of sera. The antibody reacting with NP and M polypeptides was not present regularly. In some of the NT-negative postvaccination sera (e.g., Fig. 3, lane 6 or Table 2, vaccinee 7), the antibody directed to F polypeptides appeared to be responsible for the positive reaction in ELISA. Although the preferential labeling of F over HN polypeptides in this experiment made it difficult to precisely quantify the relative abundance of antibody to the two polypeptides, the predominance of antibody to F polypeptide in this particular serum was confirmed in the experiment in which [³⁵S]methionine-labeled mumps virus-infected Vero cell extract was used as antigen for immunoprecipitation (data not shown).

Complement-mediated neutralization. The above findings suggest that the antibody directed to F polypeptide bound to the virus but was not as efficient in NT as that which bound to HN polypeptide. It was possible, however, that the

FIG. 3. Immunoprecipitation of mumps virus polypeptides by sera with different antibody activity against mumps virus. Mumps virus polypeptides were radiolabeled *in vitro* with ³H, lysed, and precipitated by various sera. The immunoprecipitates were electrophoresed in a polyacrylamide gel, and the gel was fluorographed. Lane M, molecular size markers. Lanes 1 to 3, NT-positive, ELISA-positive postvaccination sera; lanes 4 to 6, NT-negative, ELISA-positive postvaccination sera; lanes 7 to 9, NT-negative, ELISA-negative prevaccination sera; lane 10, ³H-labeled mumps virus.

TABLE 1. Antibody levels to mumps and parainfluenza viruses in sera from 7 people vaccinated with mumps vaccine as determined by NT tests, ELISA, and HI

Vaccinee no.	Mumps		Parainfluenza		
	NT (log ₂)	ELISA (OD ₄₉₂)	HI (log ₂)		
			1	2	3
1					
Pre	3.6	1.55	0 ^a	3	5
Post	7.0	>2	0	3	5
2					
Pre	0	0.23	0	0	5
Post	3.6	1.52	0	0	5
3					
Pre	0	0.19	0	0	6
Post	3.2	1.42	0	0	6
4					
Pre	0	0.26	2	0	7
Post	0	1.05	2	0	6
5					
Pre	0	0.21	0	3	6
Post	0	1.07	0	4	5
6					
Post	0	1.08	0	0	0
7					
Pre	0	0.19	0	0	0

^a 0, <2.

activation of the complement pathway leads to the eventual neutralization of a virus-antibody complex. Table 2 shows the results of such an experiment with representative vaccinees. Vaccinees 4 to 9, who responded to vaccination with various strains by antibody rises detectable by ELISA but not by conventional NT tests, were found to have seroconverted in NT tests when fresh guinea pig serum was added to the preincubated virus-serum mixtures. The guinea pig serum alone at the dilution employed did not affect the infectivity of mumps virus. With NT-positive sera, NT titers were further augmented by the addition of guinea pig serum. There were five vaccinees whose postvaccination sera were negative in ELISA. Three of the samples available for testing were also negative in complement-mediated NT tests. Thus, the presence of antibody detected by ELISA was found to be correlated with NT activity demonstrable in the presence of fresh guinea pig serum.

DISCUSSION

Previous studies showed that serum NT antibody levels of 1:2 or higher against a challenge of 30 to 100 virus 50% tissue culture infective doses, or of 1:4 or higher in 50% plaque reduction tests are associated with protection against natural mumps infections during epidemics (3, 5). We adopted an NT titer of 1:4 in 50% plaque reduction as the threshold in the evaluation of mumps vaccine. Vaccination with live attenuated mumps virus, including those licensed in Japan, induces relatively low levels of NT antibody compared with natural infection. The resulting NT antibody titer does not go much beyond the threshold in many vaccinees and does not reach it in some. Many of those who failed to respond to vaccination by NT antibody rises were, however, actually infected with the vaccine, because antibody specific to mumps virus demonstrable by ELISA was induced. The results of immunoprecipitation showed that NT activity was associated with

TABLE 2. Antibody levels to mumps virus in sera from 12 people vaccinated with various mumps vaccines as determined by ELISA and conventional and complement-mediated NT tests

Vaccinee no.	Vaccine	ELISA (OD ₄₉₂)	NT (log ₂)	Complement-mediated NT ^a (log ₂)
1 (pre)		0.193	0 ^b	0
2 (pre) ^c		0.218	0	0
3 (pre)		0.280	0	0
4				
Pre		0.206	0	0
Post ^d	Urabe-Am9	1.073	0	6.6
5				
Pre		0.261	0	0
Post	Urabe-Am9	1.051	0	5.0
6				
Pre		0.202	0	0
Post	Urabe-Am9	1.077	0	4.4
7				
Pre	Urabe-Am9	0.319	0	0
Post ^e		1.471	0	4.6
8				
Pre		0.307	0	0
Post	Torii	1.115	0	4.0
9				
Pre		0.257	0	0
Post	Hoshino	1.206	0	6.6
10 (post)	Urabe-Am9	1.518	3.6	8.8
11 (post)	Urabe-Am9	1.416	3.2	7.6
12 (post) ^f	Urabe-Am9	>2	7.0	10.6

^a Fresh guinea pig serum was added to a final concentration of 1:400.

^b 0, <2.

^c The immunoprecipitation pattern is shown in Fig. 3, lane 9.

^d The immunoprecipitation pattern is shown in Fig. 3, lane 5.

^e The immunoprecipitation pattern is shown in Fig. 3, lane 6.

^f The immunoprecipitation pattern is shown in Fig. 3, lane 3.

the presence of antibody directed to HN polypeptide. ELISA-positive, NT-negative postvaccination sera contained less antibody to HN polypeptide than did NT-positive sera but did contain comparable amounts of antibody to F polypeptide. The addition of fresh guinea pig serum rendered these sera capable of neutralizing mumps virus. According to Örvell (15), the absorption of mumps convalescent human serum specimens with Tween 80-ether-treated mumps virus depleted HI antibody, leaving non-HI, hemolysis-inhibiting antibody. Non-HI, hemolysis-inhibiting antibody was less efficient in neutralization than was HI antibody, but the neutralizing activity was markedly enhanced by anti-human immunoglobulin G. In a subsequent study, the same author immunized rabbits with purified glycoproteins of mumps virus and confirmed that the HI and the non-HI, hemolysis-inhibiting antibodies represented antibodies directed to HN and F proteins, respectively (16). The latter antibodies did not neutralize infectivity. Importance of antibody to F polypeptide has been emphasized for the defense against paramyxovirus infection (2). The argument was based on the evidence obtained mainly with SV5, in which antibody to F

polypeptide *per se* had neutralizing activity (11, 12). In contrast, antibody to the F polypeptide has not been shown to be very effective in direct neutralization of mumps virus (15, 16). It may contribute to the defense against mumps, however, by other mechanisms. It may neutralize infectivity through activation of complement, as this study has shown. It may play a role in elimination of infected cells expressing viral antigens on their surface through complement-mediated cytotoxicity or antibody-dependent cell-mediated cytotoxicity, or it may inhibit cell fusion, thereby restricting the spread of infection (2). Should these mechanisms proven operative in vivo in protection against infection or development of overt illness, ELISA would provide not only a more convenient but also a more suitable method than NT tests for the evaluation of vaccine and assessment of immunity status.

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