Comparison of a Neutralization Enzyme Immunoassay and an Enzyme-Linked Immunosorbent Assay for Evaluation of Immune Status of Children Vaccinated for Mumps

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A 50% neutralization enzyme immunoassay (N50-EIA) was compared with an indirect enzyme-linked immunosorbent assay (ELISA) for determining mumps virus antibodies in three consecutive serum samples from 138 children vaccinated with a live mumps vaccine at the age (in years) of 1.5. By the N50-EIA, most (132 of 138) preserum samples did not show neutralizing activity. Eight to 12 weeks after vaccination, 17 of the children were still negative, but only 7 remained so after 2.5 years, resulting in a seroconversion rate of 125 of 132 (95%). Over the same period, the neutralization geometric mean titer rose from 3.6 to 9.9. By an indirect ELISA, 128 of 138 preserum samples were found negative. The early and late postvaccination sera of 8 children were ELISA negative, resulting in a seroconversion rate of 120 of 128 (94%). Only two children remained seronegative by both methods. Seven of the late postvaccination serum samples yielded noncorresponding results, reflecting 95% correlation between both methods. Due to cross-reactivity with parainfluenza viruses, the ELISA proved to be less specific, but on the other hand, it showed a greater sensitivity than the N50-EIA.

Although protection against virus infection is determined by cell-mediated and humoral immune responses, the serum level of neutralizing antibodies is still considered to be a reliable index of immunity (1, 4). Therefore, the immunogenicity and, thereby, the implicit efficacy of live attenuated mumps vaccines have often been evaluated by the vaccines' abilities to induce a neutralizing antibody response (10).

However, determination of neutralizing antibodies by plaque reduction and neutralization assays in general is laborious and time consuming (2, 9, 13). Moreover, visual counting of viral plaques or estimating cytopathological effects can be difficult. Therefore, these methods are not suited for screening large numbers of serum samples. Recently, we described an enzyme immunoassay of mumps virus in cell culture with peroxidase-labelled virus-specific monoclonal antibodies, which could be applied for relatively rapid (2 to 3 days), objective titration of mumps virus-neutralizing antibodies (12).

In this study, the 50% neutralization enzyme immunoassay (N50-EIA) was compared with an in-house-developed indirect enzyme-linked immunosorbent assay (ELISA) for the evaluation of live mumps vaccines. For this purpose, three consecutive serum samples from 138 young children were tested, one before and two after vaccination. The second postvaccination serum sample was obtained 2.5 years later in order to investigate the consistency of the immunity level as measured by these two methods.

MATERIALS AND METHODS

Virus. Mumps virus used for the N50-EIA was isolated from a patient as described previously (11). With this virus, Vero cell monolayers in roller bottles were inoculated at a multiplicity of infection of 0.0004. Vero cells were maintained in Dulbecco's minimal essential medium supplemented with 0.2% tryptose, 5% heat-inactivated calf serum, and antibiotics. The medium was buffered with 0.01 M N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid and 0.024 M sodium bicarbonate. The medium was replaced by fresh medium at day 3 at 37°C. At day 5, the monolayers were rinsed (with medium) and subsequently incubated for 8 h at 37°C. Thereafter, the supernatant fluids were pooled and stored at −70°C in small portions. After being thawed, the mumps virus suspensions contained about 2 × 10⁶ PFU per ml. When this procedure is followed, the proportion of infectious virus particles in the virus suspensions is supposed to be high.

Mumps virus antigen to be used in the ELISA was prepared by propagating the Enders strain in Vero cells, which were inoculated at a multiplicity of infection of 0.003. The cell monolayers were incubated for 7 days at 34°C. After the medium was replaced at day 3, the virus was harvested daily. The virus was isolated from the pooled supernatant fluids by low-speed centrifugation to remove crude cell debris followed by ultracentrifugation for 2 h at 25,000 rpm in a Beckman rotor 30. The pellet was resuspended in phosphate-buffered saline (PBS) (pH 7.2), sonicated, and stored in small portions at −70°C for use in the ELISA. The mumps virus preparation contained 4 × 10⁶ PFU/ml and a protein concentration of 0.8 mg/ml. Plaque titration and other general virological methods have been described previously (12, 13).

MAb. The production and characterization of mumps-specific monoclonal antibody (MAb) UM 10B (immunglobulin G2a) have been described previously in another paper (12). Horseradish peroxidase (HRP) was conjugated to unpurified (ascitic fluid) MAb UM 10B by using the periodate method (8). The freshly prepared conjugates, diluted 1:10 in
PBS, were stored in 1-ml portions at 4°C after a thymol crystal (Sigma, St. Louis, Mo.) was added. Immediately before being used, the conjugate was diluted in PBS plus 0.05% Tween 20. The end dilution of conjugate varied between 1:10,000 and 1:30,000.

**Vaccine.** Mumps vaccine, lot numbers 2755 B, 0664 H, and 775 E, was obtained from Merck Sharp & Dohme. Each dose of the vaccine contained about 5,000 cell culture-infective doses of the Jeryl Lynn strain of mumps virus. The virus is produced in primary cell cultures derived from chicken embryos. One hundred fifty-three children, 18 months of age, living in two suburban townships close to Amsterdam were enrolled in the field trial and immunized with live attenuated mumps virus vaccine according to the manufacturer’s instructions.

**Sera.** Sera were obtained just before the vaccination of children, 8 to 12 weeks later, and 2.5 years thereafter. Immunizations and blood sampling were done by physicians and nurses from the local departments of municipal health. All sera were stored at −20°C until used. The consecutive sera were tested at the RIVM by the ELISA with a single (1:100) dilution of serum. Enough serum was available from 138 children to perform independently a full titration of neutralizing antibody by the N\textsubscript{50}-EIA at the University Hospital of Utrecht. Afterwards, the results obtained with both the ELISA and the N\textsubscript{50}-EIA were compared.

**N\textsubscript{50}-EIA of mumps virus-neutralizing antibodies.** A modified version of the N\textsubscript{50}-EIA earlier described was performed (12). Infectious doses of about 5,000 PFU of mumps virus (0.022 ml) were added to 0.022 ml of 1/1, 1/3, 1/10, 1/30, 1/100, 1/500, and 1/1,000 dilutions of human serum in 96-well plates (Costar, Cambridge, Mass.). The virus-serum mixtures were incubated for 2 h at 37°C, and, subsequently, 0.02 ml of each mixture was added into the wells of other 96-well plates. Then, 0.1 ml of a suspension of Vero cells (1.5 × 10\textsuperscript{5} cells per well) was added to each well and the plates were incubated for either 2 or 3 days at 37°C in a humidified CO\textsubscript{2} incubator. Subsequently, the cells were fixed by the addition of 0.1 ml of 0.05% glutaraldehyde (E. Merck AG, Darmstadt, Germany) for 10 min at room temperature. The plates were then washed with tap water, rinsed with PBS, and finally shaken dry. Subsequently, HRP-labelled mumps virus-specific MAb UM 104 minus HRP plus 0.5% Tween 20 was added to each of the plates, and the mixture was incubated for 1 h at 37°C. Thereafter, the plates were washed three times with tap water and shaken dry. The substrate for HRP (tetramethylbenzidine urea peroxidase) was added in 0.05-ml quantities and incubated for 30 min at room temperature. The reaction was stopped with 0.1 ml of 0.18 M H\textsubscript{2}SO\textsubscript{4}. A\textsubscript{450} values were read in a Titertek Multiskan (Flow Laboratories, Irvine, Scotland). The inhibition of virus multiplication by neutralizing antibody is calculated as a percentage of the control: % inhibition = 100 − [(A\textsubscript{450} serum dilution − A\textsubscript{450} cell control)/(A\textsubscript{450} virus control − A\textsubscript{450} cell control)] × 100. The titer of immune serum can be arbitrarily defined as the reciprocal dilution causing 50% inhibition.

**Indirect ELISA.** After being thawed, the ELISA antigen was diluted 1:300 in 0.1 M Na\textsubscript{2}CO\textsubscript{3} (pH 9.6), added in 0.1-ml portions to the wells of 96-well plates (Costar), and incubated overnight at 22°C. In between all incubation steps, the plates were washed three times with PBS plus 0.05% Tween 20 in a Titertek microplate washer 120 (Flow). Subsequently, serum dilutions (0.1 ml) were added twice to the wells and incubated for 2 h at 37°C. All sera were diluted 1:100 in 2 mM Tris buffer plus 1% Tween 20. Next, 0.1 ml of a conjugate (prepared at the RIVM) of HRP and sheep anti-human immunoglobulin G, diluted 1:6,000 in 2 mM Tris buffer plus 1% Tween 20 plus 2% normal sheep serum, was added to the wells and incubated for 2 h at 37°C. Finally, 3.3×10\textsuperscript{5} tetramethylbenzidine (Sigma) as a substrate for HRP (0.1 ml) was added, and the reaction was stopped after 10 min by the addition of 0.1 ml of 2 M H\textsubscript{2}SO\textsubscript{4}. A\textsubscript{450} values were measured with a Titertek Multiskan. The ELISA of parainfluenza viruses was performed in the manner described above. A possible cross-reaction due to serum components contaminating the ELISA antigens was excluded by the preincubation of individual sera with fetal calf serum. The parainfluenza viruses were obtained from the Laboratory of Virology (RIVM).

**RESULTS**

The distribution of the absorbance values obtained with the ELISA and that of the reciprocal titers obtained with the N\textsubscript{50}-EIA are illustrated in Fig. 1. The results are summarized in Table 1. Mumps-neutralizing antibodies were not detected in 132 of 138 (96%) prevaccination serum samples. In sharp contrast, five preserum samples from six children that were found to be positive by N\textsubscript{50}-EIA contained high levels of neutralizing antibodies, and, accordingly, high absorbance values were measured by the ELISA. Serum from the sixth child was negative by the ELISA. After these five serum samples were excluded, the arithmetic mean ELISA absorbance value of 133 prevaccination serum samples was 0.106 (standard deviation, 0.059). Normally, this would result in a cutoff value of 0.283 (mean + three standard deviations). However, because the population of absorbance values of presera possessed rather a log normal distribution (Fig. 1), it was not correct to calculate a representative arithmetic mean, and therefore the geometric mean was used. When the cumulative normal distribution was applied for the calculation of the 95% level, the cutoff absorbance value proved to be 0.230. When this cutoff value was used, 128 of 138 (93%) prevaccination serum samples were considered to be negative by the ELISA. When the results of both methods were compared (Table 2), 127 prevaccination serum samples (92%) were found to be negative and 5 were found to be highly positive by both tests. The results of six serum samples by the two tests did not agree (6 of 138 = 4.3%).

In the early postvaccination sera, 17 children had not developed detectable levels of neutralizing antibodies (Table 1). The ELISA results were better in that regard; only eight serum samples remained below the cutoff value, and five of these were also neutralization negative (Table 2). The results of 15 serum samples did not correspond in both tests (15 of 138 = 10.9%). Of these 15 serum samples, the three that were ELISA negative were just below the cutoff value, while the 12 neutralization-negative samples had various ELISA absorbance values above the cutoff value.

The results of the late postvaccination sera showed better agreement between the two methods: only seven serum samples were noncorresponding (7 of 138 = 5.1%). Neutralizing activity in seven serum samples could not be detected (Table 2), and four of these were negative by both methods. The remaining three serum samples were ELISA positive in both the early and late postvaccination stages. From the eight ELISA-negative late postvaccination serum samples, four did contain relatively low levels of mumps-neutralizing antibody. However, three of these four were mumps antibody positive in the ELISA and in the N\textsubscript{50}-EIA of the corresponding early postvaccination sera (results not explicitly shown). So, initial ELISA-positive sera may decline to an ELISA-negative status, at least when a single dilution...
FIG. 1. Distribution over arbitrarily chosen intervals of neutralization titers and absorbance values from pre- (solid bars), early post- (stippled bars), and late postvaccination (hatched bars) sera as obtained by the N₅₀-EIA (A) and the ELISA (B). The frequency in each interval of the population of 138 children is denoted. In order to keep scales similar in the figure, the frequency of prevaccination sera in panel A is only 100, while it is in reality 132.

(1/100) of serum is used. In contrast, regression from neutralization positive to neutralization negative did not occur.

The neutralization titers of the late postvaccination sera were conspicuously higher than those of the early sera, as indicated by the shift in distribution to the higher neutralization titers (Fig. 1). This might be due to the slow development of neutralizing antibodies after vaccination. The shift in distribution to higher absorbance values of early and late postvaccination sera is also present in the ELISA but is not as obvious as in the N₅₀-EIA. This is also reflected by the geometric mean titer (GMT) values of both methods (Table 1). While the correlation of absorbance values and corresponding neutralization titers of individual serum samples is often rather difficult, the means of certain intervals like those in Fig. 1 correlate reasonably.

In Fig. 2, exemplary N₅₀-EIA and ELISA results are given for sera from six children. The child given the code BA 023 (see also Table 3) is one of the two children who remained seronegative by either test. In contrast, another child (BA 094) had a strongly mumps-neutralizing preserum, and,
unsurprisingly, no rise in neutralization titer occurred upon immunization. Children BA 049 and BU 079 (Table 2) showed initially no seroconversion but developed neutralizing antibodies later concomitantly with a further rise in absorbance value. A significant rise in neutralizing antibody titer is exemplified by the results obtained with two other children (BU 015 and BU 026).

The sera from 16 children with neutralization-negative early postvaccination sera were tested again by the N$_{50}$-EIA. The results of the two neutralization tests and the ELISA are presented in Table 3. As shown in this table, the neutralization-negative presera remained negative in the second test, but 2 of the 16 initially neutralization-negative early postvaccination serum samples became positive, although the titers, 1 and 2, were low. The early seroconversion as determined by the ELISA of sera from children BA 049, BA 041, BA 012, BA 040, BA 053, BU 079, and BA 073 is indicative of a greater sensitivity for the ELISA than for the N$_{50}$-EIA. The last children developed neutralizing antibodies later than most children.

A possible cross-reaction of parainfluenza virus antibodies was determined with the 10 mumps-positive prevaccination serum samples in the ELISA. Therefore, antigen of parainfluenza virus types 1, 2, 3, 4a, and 4b and that of simian virus 5 and Sendai virus was used in an indirect ELISA in which cross-reaction with contaminating calf serum components was excluded. From the five double-positive preserum samples (positive by both the ELISA and the N$_{50}$-EIA), four were found negative for any type and one serum sample showed triple reactivity with Sendai and parainfluenza types 2 and 3, indicating a natural mumps infection for these children before vaccination. From the other five ELISA-positive but N$_{50}$-EIA-negative preserum samples, three reacted with most parainfluenza types and one serum sample showed absorbance with parainfluenza type 3. Only one

### TABLE 1. Results of N$_{50}$-EIA and ELISA of pre-, early post-, and late postvaccination sera from 138 children and their GMT

<table>
<thead>
<tr>
<th>Type of sera</th>
<th>No. (%) of serum samples</th>
<th>GMT$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive by: N$_{50}$-EIA</td>
<td>Negative by: ELISA</td>
</tr>
<tr>
<td></td>
<td>N$_{50}$-EIA</td>
<td>ELISA</td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>6 (4)</td>
<td>10 (7)</td>
</tr>
<tr>
<td>Post-vaccination</td>
<td>Early</td>
<td>121 (88)</td>
</tr>
<tr>
<td></td>
<td>Late</td>
<td>131 (95)</td>
</tr>
</tbody>
</table>

$^a$ The cutoff neutralization titer in the N$_{50}$-EIA is 1.

$^b$ The cutoff absorbance value in the ELISA is 0.230.

$^c$ GMT is determined from the total number of children and includes the five highly double-positive preserum samples. The negative value of the N$_{50}$-EIA (<1) was arbitrarily made 0.1 for calculation purposes.

### TABLE 2. Correlation of N$_{50}$-EIA and ELISA of pre-, early post-, and late postvaccination sera from 138 children

<table>
<thead>
<tr>
<th>Type of serum and result of ELISA$^b$</th>
<th>No. (%) of serum samples with the following result by N$_{50}$-EIA$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Pre-vaccination</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Early post-vaccination</td>
<td>118 (85)</td>
</tr>
<tr>
<td>Positive</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Negative</td>
<td>4 (3)</td>
</tr>
</tbody>
</table>

$^a$ The cutoff neutralization titer in the N$_{50}$-EIA is 1.

$^b$ The cutoff absorbance value in the ELISA is 0.230.

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FIG. 2. N$_{50}$-EIA and ELISA results obtained with sera from six children. The N$_{50}$-EIA and the ELISA were performed as described in the text. Representative examples of antibody titration by the N$_{50}$-EIA (lines) and antibody detection by the ELISA (single symbols) are indicated. O and ●, presera; △ and ▲, early postvaccination sera; □ and ▼, late postvaccination sera. In the N$_{50}$-EIA, single absorbance values, measured at 450 nm at each dilution of serum, are interconnected by lines (broken lines, presera; solid lines, postvaccination sera). The virus control without serum (0% neutralization) is the mean of six absorbance values. The cell control, which equals 100% neutralization by serum, is also the mean of six absorbance values. Control absorbance values in each N$_{50}$-EIA plate were determined and vary slightly, as indicated in the figure. Neutralization titers could be calculated by graphic interpolation.
serum sample of these five was found negative. A parainfluenza virus infection can be assumed to have occurred in four of these five children before the mumps vaccination.

DISCUSSION

In the present study, we compared the N$_{50}$-EIA with the common indirect ELISA. A full titration (1 of 1 to 1/1,000) with the N$_{50}$-EIA was performed with three consecutive serum samples from 138 children. Independently, antibodies to mumps virus in single dilutions (1/100) of the same sera were determined by the ELISA. Both assays were performed in single experiments to achieve a realistic comparison of the two methods. As expected, the great majority of the preserum samples from 18-month-old children contained no mumps antibodies. Five children had high absorbance values and some of the highest serum neutralization titers measured in all (3 × 138) serum samples. This must be due to a recent natural mumps infection. Remarkably, four of these five preserum samples showed no cross-reaction with parainfluenza viruses. Five neutralization-negative preserum samples were positive by the ELISA. The five corresponding children, however, did seroconvert upon vaccination concomitantly with a rise of absorbance value in the ELISA. Four of these five preserum samples contained parainfluenza antibodies that cross-react with mumps virus in the less specific ELISA, which measures a broader spectrum of antibodies than the N$_{50}$-EIA. The assumption of the presence of cross-reactive antibodies in some human sera is confirmed by these results (3, 5, 7, 11).

The five highly double-positive preserum samples were excluded from the calculation of the ELISA cutoff value. Their exclusion did not have much influence on the GMT value (0.101 versus 0.093) but was important for the standard deviation used for the calculation of the 95% level with the cumulative normal distribution:

\[
\text{正常}{\text{值}} = \frac{10^{\left(\frac{x - \text{GTM}}{\text{SD}}\right)}}{2}
\]

On the contrary, the arithmetic mean and standard deviation changed dramatically after this exclusion (0.132 ± 0.147 versus 0.106 ± 0.059). The influence of the five neutralization-negative, ELISA-positive preserum samples on the 95% level calculation was negligible, but on the arithmetic mean it was still large, as expected. With the 95% level as the cutoff value (0.230), there is no need to exclude these five preserum samples from the population.

The neutralization titers of the first postvaccination sera are generally low, as reflected by a GMT value of 3.6 and by a rather high percentage of negative sera (12%). The ELISA proved to be more sensitive in detecting mumps antibodies at that stage (6% negative sera) than the neutralization test, which is in agreement with previous findings (6, 9, 10). Probably the time of sampling after vaccination (8 to 12 weeks) is relatively short for determining by the N$_{50}$-EIA the actual seroconversion rate. The development of the titer of neutralizing antibodies seems to be rather slow, at least after vaccination. The late postvaccination sera showed a much better rise in neutralization titer (GMT value, 9.9) and a lower percentage of negative sera (5%). The rise in ELISA-detectable mumps antibodies took place earlier after vaccination. We found an almost fivefold rise in the GMT values of pre- and early postvaccination sera and only a small increase thereafter in the late postvaccination sera. It is, however, possible that the late increase is partly due to community-acquired infections.

In this study, all early neutralization-positive children remained positive at the late time point. In the ELISA, however, from the eight late negative serum samples, five exhibited a decline from a positive status in the early postvaccination stage. The five serum samples may contain low levels of mumps-neutralizing antibodies, as determined by the positive N$_{50}$-EIA in three cases, or no levels at all, as indicated by negative N$_{50}$-EIA in two cases. The latter may be due to some aspecific binding of early sera in the ELISA (false positives), and the former may be due to the measurement of a single dilution (1:100). From the seven neutralization-negative serum samples, three were clearly positive in
the ELISA of both postvaccination samples. Despite these
title differences, only seven serum samples showed final
noncorresponding results, which means there is 95% corre-
lation between both methods. Moreover, only two children
remained negative by both tests. It should be noted, how-
ever, that establishing a correlation between the results from
individual serum samples was rather difficult, because dif-
ferent sets of antibodies are measured by the two methods.
The early conversion rate as determined by the N50-EIA was
115 of 132 (87%), and the late conversion rate was 125 of 132
(95%). For the ELISA, the early and late rate was 120 of 128
(94%).

The main conclusion of the present study is that the
N50-EIA and the ELISA are useful for monitoring serocon-
version rates after large-scale mumps vaccinations. The
ELISA proved to be more sensitive than the N50-EIA, but it
might be less specific. Simultaneous performances of both
assays may be useful to select discordant sera for further
serological analysis.

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REFERENCES

epidemic: correlation of insusceptibility to mumps with serum
plaque neutralizing and hemagglutination-inhibiting antibodies.
2. Ennis, F. A., R. D. Douglas, G. L. Stewart, H. E. Hopps, and
H. M. Meyer. 1968. A plaque neutralization test for determin-
of mumps and parainfluenza type-1 virus infections by enzyme
immunoassay, with a comparison of two different approaches
4. Hilleman, M. R., R. E. Weibel, E. B. Buynak, J. Stokes, and
Protective efficacy as measured in a field evaluation. N. Engl. J.
antibodies against mumps, parainfluenza 2 and Newcastle dis-
6. Leinikki, P. O., I. Shekarchi, N. Tzan, D. L. Madden, and J. L.
Sever. 1979. Evaluation of enzyme-linked immunosorbent assay
Determination of IgG- and IgM-class antibodies to mumps virus
by solid-phase enzyme immunoassay. J. Virol. Methods 4:249–
257.
body, a new method of conjugation. J. Histochem. Cytochem.
immunosorbent assay compared with neutralization tests for
ical standardization of a test for susceptibility to mumps. J.
linked immunosorbent assay for mumps and parainfluenza type
Microbiol. 11:319–323.
12. Van Tiel, F. H., C. A. Kraaijeveld, J. Baller, T. Harmsen,
T. A. M. Oosterlaken, and H. Snippe. 1988. Enzyme immunoas-
say of mumps virus in cell culture with peroxidase-labelled virus
specific monoclonal antibodies and its application for determi-
13. Wagenvoort, J. H. T., M. Harmsen, B. J. Khader Boutahar-
Trouw, C. A. Kraaijeveld, and K. C. Winkler. 1980. Epidemi-