

## Enzyme-Linked Immunospot Assay Detection of Mumps-Specific Antibody-Secreting B Cells as an Alternative Method of Laboratory Diagnosis<sup>∇</sup>

Donald R. Latner,<sup>1\*</sup> Marcia McGrew,<sup>1</sup> Nobia Williams,<sup>1</sup> Luis Lowe,<sup>1</sup> Roniel Werman,<sup>2</sup> Eli Warnock,<sup>2</sup> Kathleen Gallagher,<sup>1</sup> Peter Doyle,<sup>3</sup> Sandra Smole,<sup>4</sup> Susan Lett,<sup>4</sup> Noelle Cocoros,<sup>4</sup> Alfred DeMaria,<sup>4</sup> Raimond Konomi,<sup>4</sup> Cedric J. Brown,<sup>1</sup> Paul A. Rota,<sup>1</sup> William J. Bellini,<sup>1</sup> and Carole J. Hickman<sup>1</sup>

*Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333<sup>1</sup>; Office of the Chief Operating Officer, Office of Health and Safety, Centers for Disease Control and Prevention, Atlanta, Georgia 30333<sup>2</sup>; University Health and Counseling Services, Northeastern University, Boston, Massachusetts 02115<sup>3</sup>; and Massachusetts Department of Public Health, Jamaica Plain, Massachusetts 02130<sup>4</sup>*

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Although high measles, mumps, and rubella (MMR) vaccination coverage has been successful in dramatically reducing mumps disease in the United States, mumps (re)infections occasionally occur in individuals who have been either previously vaccinated or naturally infected. Standard diagnostics that detect virus or virus-specific antibody are dependable for confirming primary mumps infection in immunologically naive persons, but these methods perform inconsistently for individuals with prior immune exposure. We hypothesized that detection of activated mumps-specific antibody-secreting B cells (ASCs) by enzyme-linked immunospot (ELISPOT) assay could be used as a more reliable diagnostic. To test this, a time course of virus-specific ASC responses was measured by ELISPOT assay following MMR vaccination of 16 previously vaccinated or naturally exposed adult volunteers. Mumps-specific ASCs were detectable in 68% of these individuals at some point during the first 3 weeks following revaccination. In addition, mumps-specific ASCs were detected in 7/7 previously vaccinated individuals who recently had been infected as part of a confirmed mumps outbreak. These data suggest that ELISPOT detection of mumps-specific ASCs has the potential for use as an alternative method of diagnosis when suspect cases cannot be confirmed by detection of IgM or virus. In addition, it was determined that mumps-specific memory B cells are detected at a much lower frequency than measles- or rubella-specific cells, suggesting that mumps infection may not generate robust B-cell memory.

Prior to 2006, surveillance data suggested that elimination of endemic mumps virus circulation in the United States might be forthcoming as the number of mumps cases had dropped precipitously from over 185,000 in 1968 to less than 300 per year from 2001 to 2005 (13, 46). In fact, high coverage with the measles, mumps, rubella (MMR) vaccine led to the declared elimination of endemic measles (10, 12, 27, 33) and rubella (9) viruses in the United States during the years 2000 and 2004, respectively. However, during 2006 there was a resurgence of mumps in the United States that occurred mostly in the mid-western states. Interestingly, a large proportion of the >6,500 reported cases occurred among 18 to 24 year olds, of whom the majority had previously received two doses of MMR vaccine (2, 8, 30). The outbreak of 2006 was not an anomaly. Another large outbreak began in June 2009 in the northeast and continued into the summer of 2010. As of October, >3,800 cases have been reported since the outbreak began and 75% of cases have occurred among individuals who received two doses of

MMR vaccine (11, 14). Although it has now been well documented that individuals who have been either previously vaccinated or naturally infected with mumps can be (re)infected with wild-type virus (22, 31, 32, 48), the biologic basis for their susceptibility is not completely understood. There is considerable debate as to whether it is due to primary vaccine failure (failure to respond to immunization), secondary vaccine failure (waning immunity), priming of inefficient immune responses (production of predominantly nonneutralizing antibodies), or differences in the ability of vaccine-induced antibody to neutralize viruses of different genotypes (although there is only one recognized serotype) (34, 39).

Clinical diagnosis of mumps infection in individuals who have been either previously vaccinated or naturally infected is challenging because parotitis, the hallmark symptom of mumps, can be attributed to a number of etiologies that range from salivary calculi, dental sepsis, and Sjögren's syndrome to other virus infections, such as parainfluenza virus, Epstein-Barr virus, and cytomegalovirus (3, 19, 21, 28). Furthermore, acute parotitis may only be present in 60 to 70% of mumps cases, but can range from 50 to 95%, depending on the immunity of the population (35, 36). Nonspecific respiratory symptoms are also commonly associated with mumps infection (40 to 50% of cases), and up to 20% of cases are asymptomatic (15, 20). In the absence of epidemiologic links to a confirmed

\* Corresponding author. Mailing address: Measles, Mumps, Rubella, and Herpesvirus Laboratory Branch (MMRHLLB), National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, 1600 Clifton Road, MS-G18, Atlanta, GA 30333. Phone: (404) 639-2771. Fax: (404) 639-4056. E-mail: dlatner@cdc.gov.

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TABLE 1. Summary of diagnostic results following vaccination<sup>a</sup>

Volunteer	No. of previous MMR vaccinations	Result for:								
		Mumps			Measles			Rubella		
		Initial IgG	IgM	ASCs	Initial IgG	IgM	ASCs	Initial IgG	IgM	ASCs
A	2	+	-	-	+	-	-	+	-	+
B	2	+	-	+	+	-	+	+	-	+
C	2	+	-	+	+	-	+	+	-	+
D	2	+	-	+	+	-	+	+	-	+
E	2	+	-	+	+	-	+	+	-	+
F	2	+	-	-	+	-	-	+	-	+
G	2	+	-	-	+	-	-	+	-	+
H	2	+	-	-	+	-	+	+	-	+
I	2	IND	-	+	+	-	+	+	-	+
J	2	+	-	+	+	-	+	+	-	+
K	3	+	-	+	+	-	+	+	-	+
L	2?	+	-	+	+	-	+	+	-	+
M	1-3?	+	(+)	+	+	-	+	+	-	+
N	0	+	-	+	+	-	-	+	-	+
O	0	-	+	+	+	-	-	+	-	-
P	0	+	-	-	+	-	-	+	-	-

<sup>a</sup> A list of volunteers who participated in the study, their vaccination histories, and test results are shown. IgG ELISA results are indicative of serum samples taken immediately prior to administration of vaccine. IgM ELISA and ASC ELISPOT results indicate if a positive response was detected at any time point during the first 3 weeks following vaccination. The positive IgM result shown in parentheses is likely a false positive, as discussed in the text. IND, indeterminate.

outbreak or importation from an area of endemicity, mumps may not necessarily be considered the most likely etiology for parotitis, especially among previously vaccinated individuals.

These clinical challenges are further compounded by difficulties in confirming mumps infection by laboratory testing. The standard laboratory diagnostic methods are direct detection of virus or viral RNA through culture or real-time reverse transcription-PCR (RT-PCR), detection of mumps-specific IgM, or detection of a 4-fold rise in IgG titer by a quantitative or semiquantitative method such as a plaque-reduction neutralization test (PRNT). These methods are reliable for confirmation of mumps in patients encountering the virus for the first time. However, the IgM response and viral shedding that occur in persons who have been previously vaccinated or naturally infected appear blunted in duration and intensity, making detection of both unreliable (5, 38). Similarly, it is unusual to detect a 4-fold rise in IgG titer in persons who have been previously exposed because they typically have a demonstrable IgG titer at the time of clinical presentation (38). Therefore, the lack of a reliable laboratory test to confirm infection in vaccinated individuals may lead to an underestimation of disease incidence. These diagnostic issues are not unique to mumps, but are shared with varicella-zoster (29) and pertussis (45).

To address this problem, we hypothesized that the detection of virus-specific antibody-secreting B cells (ASCs [plasmablasts]) by enzyme-linked immunospot (ELISPOT) assay could be used as an alternative diagnostic method based on reports that these cells are present in the circulation only following recent activation by antigen (4, 18, 40-44, 47). We initially tested the sensitivity and specificity of this approach by measuring the level of MMR-specific memory B cells in normal healthy adult volunteers. Then, we tested the feasibility of using this method as a diagnostic in two different ways (under Institutional Review Board [IRB] approval). First, we measured the time course of the ASC response to mumps, measles,

and rubella after administration of MMR vaccine to adult volunteers who had been either previously vaccinated with MMR or previously infected. Second, we detected mumps-specific ASC responses in 7/7 patients who were part of a confirmed mumps outbreak in the northeastern United States. Together, these data indicate that ELISPOT detection of ASCs may be a reliable alternative method for confirming mumps (re)infection in individuals who have prior immune exposure to the virus through vaccination or natural infection.

## MATERIALS AND METHODS

**Human subjects and study design.** The studies described below were approved by the CDC Human Research Protections Office and Institutional Review Board. In the first study, 16 CDC employee volunteers received a standard dose of MMR-II vaccine (Merck & Co., Inc., Whitehouse Station, NJ) following informed consent. The cohort ranged in age from 25 to 58 years, with an average age of 37 years. Ten of the 16 volunteers had previously received two age-appropriate doses of MMR vaccine (Table 1). One volunteer (K) had previously received MMR vaccine three times. Two volunteers (L and M) recalled that they received 1 to 3 doses of MMR vaccine but had no records. Three volunteers (N, O, and P), ages 39 to 57, either had never been vaccinated or had no record and no recollection of previous MMR vaccination. Each of these latter three volunteers reported they had rubella disease, and two of these three (N and P) reported they had measles. None of the 16 volunteers had any knowledge or record of having mumps. Volunteers N and P were mumps IgG positive at the prevaccination time point, suggesting they had been either previously vaccinated or infected. All of the remaining volunteers were also mumps IgG positive, except for I and O, who were mumps IgG indeterminate and negative, respectively. Volunteer O, however, had a positive T-cell proliferative response to mumps at the prevaccine time point, suggesting prior exposure (data not shown). Volunteer I did not have a T-cell proliferative response to mumps at the prevaccination time point, but was mumps IgG positive by 7 days postinfection. All volunteers were IgG positive for measles and rubella at the prevaccination time point.

Whole-blood and serum specimens were taken just prior to administration of the vaccine and at approximately days 7, 14, and 21 postvaccination. Buccal swab specimens were taken at the prevaccination, day 7, and day 14 time points for analysis by real-time RT-PCR (1, 6, 24).

Five cell preparation tubes (CPTs; 8 ml) (catalog no. 362761; Becton Dickinson, Franklin Lakes, NJ) containing whole blood and one 5-ml serum specimen were collected immediately prior to administration of the vaccine and at approx-

imately days 7, 14, and 21 postvaccination. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood, and MMR-specific antibody-secreting B cells were detected by ELISPOT assay as described below. Total IgG-secreting B cells were also measured, and Vero cell lysate was used as a negative control antigen. Virus-specific serum IgG and IgM levels were measured by enzyme-linked immunosorbent assay (ELISA). In addition, oral fluid specimens were taken with an OraCol swab (Malvern Medical Developments, Ltd., Worcester, United Kingdom) at the prevaccination, day 7, and day 14 time points for virus RNA detection by real-time RT-PCR. Swabs were rinsed with 1 ml Dulbecco's modified Eagle's medium (DMEM) plus 2% fetal calf serum (FCS), and the fluid was frozen at  $-70^{\circ}\text{C}$  until RNA extraction as described below.

In the second study, individuals who had a recent confirmed or suspected case of mumps were recruited to donate 8 to 10 ml of whole blood and an oral fluid specimen following informed consent. These individuals met the clinical case definition for mumps and had a date of symptom onset between 7 and 28 days prior to specimen collection. All but one of these individuals had previously received two age-appropriate doses of MMR vaccine. The remaining individual reported having received two doses, but only had documentation for one dose. The whole-blood specimens were tested by ELISPOT assay for mumps-specific ASCs. RNA extracted from oral fluid samples was tested by real-time RT-PCR for the presence of mumps virus.

**Virus, antigens, cells, and antibody.** A wild-type mumps virus isolate (MuVs-USA06-IA, genotype G) (6) was grown on Vero cells in DMEM (2% fetal calf serum) until the cytopathic effect was complete. Infected cells were freeze-thawed two times while still in the flask. The lysate was collected and then clarified by centrifugation at  $800 \times g$ , and virus was quantified by titration on Vero cells. The titer of virus present in the supernatant fluid was approximately  $1 \times 10^7$  PFU/ml and was used at a 1/10 dilution in phosphate-buffered saline (PBS) to coat ELISPOT plates. An uninfected Vero cell lysate was identically prepared and diluted for use as a negative control antigen. Measles and rubella antigens were purchased from Meridian Life Science, Inc., Memphis, TN (catalog no. 7604 and 6123, respectively) and were also prepared from infected Vero cells. Measles and rubella antigens were diluted to 1/10 and 1/50 for coating ELISPOT plates.

Goat anti-human IgG capture antibody was obtained from Invitrogen Life Technologies Corp., Carlsbad, CA (catalog no. H17000). The antigens were diluted in PBS for coating 96-well ELISPOT plates (catalog no. MAHAN-4550; Millipore, Temecula, CA) at the following concentrations: mumps virus stock, 1:10; measles virus, 1:10; rubella virus, 1:20; Vero cells, 1:10; goat anti-human IgG capture antibody, 1:250.

**PBMC preparation.** CPT tubes of whole blood were spun at  $1,800 \times g$  for 20 min at  $25^{\circ}\text{C}$  with no brake. The buffy coat was collected, pooled, and washed four times in 50 ml calcium- and magnesium-free PBS and then resuspended at  $5 \times 10^6$  cells/ml in RPMI medium plus 10% fetal calf serum.

**Memory B-cell assay and ASC ELISPOT assay.** PBMCs were stimulated and memory B-cells detected by ELISPOT assay essentially as previously described (16). Briefly,  $5 \times 10^5$  PBMCs were cultured in each well of a 24-well plate in 1 ml of RPMI 1640 medium (Gibco) containing 10% heat-inactivated fetal calf serum (Atlas Biologicals) and  $1 \times$  penicillin-streptomycin (Gibco) and supplemented with 2 mM L-glutamine (Gibco). Cultures were maintained at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 6 days. Mitogen-stimulated cultures also contained 1/10,000 fixed *Staphylococcus aureus* Cowan cells (Sigma), 6  $\mu\text{g}/\text{ml}$  phosphothioated CpG oligonucleotide (Operon), 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, and 1/100,000 pokeweed mitogen (PWM) extract (kindly provided by Rafi Ahmed, Emory University). At the end of the culture period, cells were washed three times in complete medium and resuspended in 200  $\mu\text{l}$  complete medium (without mitogen) before application to the antigen-coated ELISPOT plates.

When testing for antibody-secreting B cells (ASCs), PBMCs were not stimulated prior to detection by ELISPOT assay, but instead were tested directly after isolation from whole blood. PBMCs ( $10^6$ ) were applied to each well of the ELISPOT plate and were titrated in the positive and negative IgG capture wells to facilitate counting.

Detection of activated B cells by ELISPOT assay was performed as previously described (16, 17). ELISPOT plates (96 wells) were coated with virus antigen or total IgG capture antibody (at the dilutions indicated above) overnight at  $4^{\circ}\text{C}$  and were stored up to 7 days at  $4^{\circ}\text{C}$ . Immediately before use, plates were washed four times with PBS plus 0.05% Tween 20, washed four times with PBS, and then blocked for 2 h at  $37^{\circ}\text{C}$  in RPMI medium plus 10% fetal calf serum. PBMCs ( $1 \times 10^6$  total) were placed into each well of the washed and blocked ELISPOT plates. For measurement of the total number of IgG-secreting ASCs, cells were titrated (1:3 dilutions). Cells were incubated in the plate overnight at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ . The next day, cells were first washed from the plate four times with PBS and then four times with PBS plus 0.05% Tween 20. Biotinylated IgG detection antibody

(catalog no. H10015; Invitrogen Life Technologies Corp., Carlsbad, CA) was diluted 1:1,000 in PBS plus 1% fetal calf serum and applied to the plate for 1 h at  $25^{\circ}\text{C}$ . Plates were then washed four times with PBS plus 0.05% Tween 20, and streptavidin-horseradish peroxidase (HRP) conjugate (catalog no. A2004; Vector Laboratories, Burlingame, CA) was diluted 1:1,000 in PBS plus 1% fetal calf serum (FCS) and applied to the plates for 1 h at  $25^{\circ}\text{C}$ . Plates were washed four times with PBS plus 0.05% Tween 20 and then four times with PBS.

A fresh 20-mg/ml stock of 3-amino-9-ethylcarbazole (AEC [catalog no. A5754]; Sigma-Aldrich, St. Louis, MO) dissolved in *N,N*-dimethylformamide (catalog no. D-4551; Sigma-Aldrich, St. Louis, MO) was prepared, further diluted to a final concentration of 300  $\mu\text{g}/\text{ml}$  in 0.1 M sodium acetate buffer (pH 5.0), and passed through a 0.45- $\mu\text{m}$  syringe filter immediately before use. Hydrogen peroxide was added to a final concentration of 0.03%, and the solution was added to each well. The colorimetric reaction was allowed to proceed until spots were clearly evident on positive-control, total-IgG-capture wells ( $\sim 5$  min). The reagent and plate backing material were removed, and the reactions were quenched by being submerged in distilled water.

**ELISA.** Mumps, measles, and rubella IgG was measured with commercially available ELISA kits (Wampole Laboratories, Princeton, NJ). Mumps IgM capture ELISA was performed by a method similar to that described for measles (23). Rubella IgM testing was performed with a commercially available capture ELISA kit (Diamedix, Miami, FL).

**RNA extraction and RT-PCR.** RNA was extracted from  $10^6$  purified PBMCs and from oral fluid samples by using the Qiagen viral RNA minikit (Qiagen, Inc., Valencia, CA) as specified by the manufacturer. Real-time RT-PCR protocols for the detection of mumps, measles, and rubella were performed as previously described (1, 6, 24). The genotype of mumps virus was determined based on the sequence of the gene coding for the short hydrophobic (SH) protein (25, 26).

**Statistical analysis.** Receiver operating characteristic (ROC) analysis was performed with Prism software (GraphPad Software Inc., La Jolla, CA) to estimate the sensitivity and specificity of the ELISPOT test and to establish cutoff values for detection by using the results from the third-dose MMR vaccination study. Briefly, the numbers of background spots detected on wells coated with uninfected Vero cell lysate (negative control) were subtracted from the numbers of antigen-specific spots detected at the prevaccination and 1-week-postvaccination time points. The numbers of background-corrected antigen-specific spots that were detected at the prevaccination time point were considered negative test results, and the numbers detected at the 1-week-postvaccination time point were considered positive test results. The non-zero numbers from both the negative and positive test results were compiled and then ranked, and the average difference between each value in the data set was determined and used as a cutoff to estimate sensitivity and specificity. The sensitivity (percentage of individuals scoring higher than the cutoff at 1 week postvaccination) and the specificity (percentage of individuals scoring below the cutoff at the prevaccination time point) were determined for each of these intermediate cutoff values. Based on these results, a cutoff threshold was chosen that gave the optimal sensitivity and specificity for each antigen, as indicated in Fig. 2 and in the text.

Repeated-measures analysis of variance (ANOVA; Proc-Mixed model) was performed with Statistical Analysis Software (SAS) (SAS Institute, Inc., Cary, NC) to determine the significance of differences among three data sets. These include: (i) the averages of antigen-specific memory B cells (Fig. 1), (ii) the average levels of antigen-specific ASCs following third-dose MMR vaccination, and (iii) the average level of mumps-specific ASCs from the naturally occurring cases compared to the average level of mumps-specific cells following third-dose vaccination. Data from the third-dose MMR vaccination study were transformed by using the Box-Cox method to stabilize variance (7).

## RESULTS

**MMR-specific memory B cells.** As an initial test of the antigens and the method, we detected MMR-specific memory B cells from normal healthy adult volunteers (under an IRB-approved protocol) who had been either previously vaccinated or naturally infected. Because memory cells are quiescent and do not secrete antibody unless they are restimulated, PBMCs were cultured in the presence of polyclonal mitogens for 6 days prior to detection. The positive control wells were coated with anti-human immunoglobulin capture antibody instead of virus antigen. Cells that were cultured for 6 days in the absence of mitogen were used as a negative control and were applied to

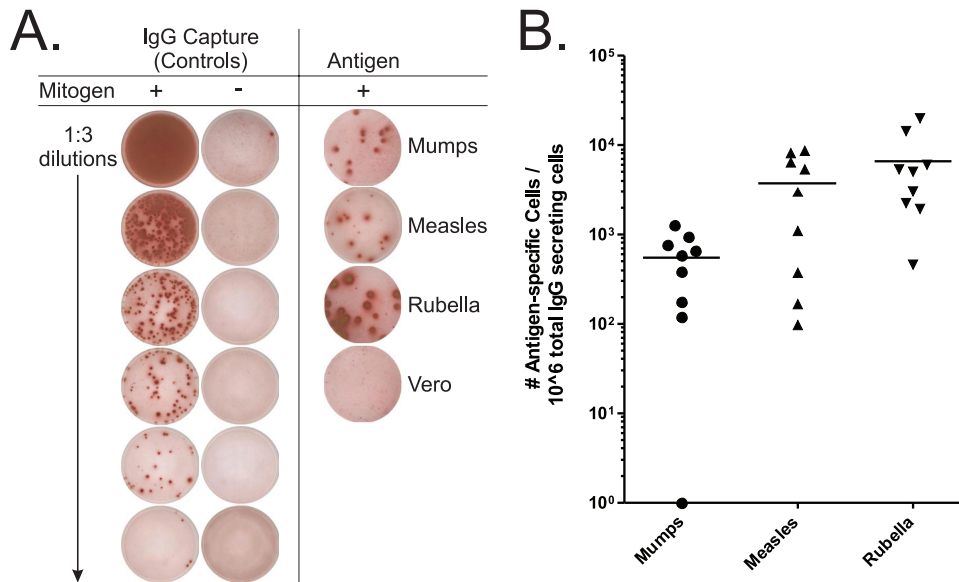


FIG. 1. MMR-specific memory B-cell frequencies. (A) Representative memory B-cell ELISPOTs from one donor that are reactive to each antigen are shown. (B) Average numbers of memory B cells specific for each antigen are shown from nine donors. Background responses to Vero cell lysate (negative control) were subtracted. Comparison of the means by repeated-measures ANOVA and Tukey's posttest indicated that the average number of memory B cells specific for mumps was significantly lower than the average number of rubella-specific memory B cells ( $P < 0.05$ ). Instances in which zero antigen-specific cells were detected were graphed with an artificial value of 1 to serve as a baseline placeholder on the log scale. The horizontal line represents the mean of each group.

wells coated with Ig capture antibody. Additional negative controls were mitogen-stimulated cells that were applied to wells coated with uninfected Vero or Sf9 cell lysates (these wells were used for background correction). An anti-human IgG secondary antibody was used for detection. As many replicate wells as possible were tested in each case and were limited by the number of cells recovered from the blood collection. Examples of ELISPOT results from one donor are shown in Fig. 1A. Cells in the positive control (immunoglobulin capture) wells were titrated to facilitate counting.

The graphs in Fig. 1B represent the average numbers of memory B cells that are specific for mumps, measles, and rubella (whole virus) from nine donors. Four of the individuals were tested three separate times, three individuals were tested twice, and two individuals were tested once. The number of background cells/spots that reacted with uninfected Vero lysate was subtracted from the number of cells that reacted with the virus antigens. When no antigen-specific cells (spots) were observed from a given individual, the number was artificially set to "1" so it would appear as a placeholder on the log-scale dot plot. The horizontal lines represent the mean of each group. The average numbers of memory B cells for all antigen groups were compared by repeated-measures ANOVA, and they were determined to be significantly different with a  $P$  value of 0.0016. Tukey's multiple-comparison posttest showed that the average mumps-specific memory B-cell level was significantly lower than the average rubella level ( $P < 0.05$ ).

**ASC detection following MMR vaccination.** We next tested the feasibility of using ELISPOT detection of virus-specific ASCs as a diagnostic tool by vaccinating CDC employee volunteers with a standard dose of MMR-II vaccine (Merck & Co., Inc., Whitehouse Station, NJ) under an IRB-approved protocol. This allowed us to attempt to measure the time

course of the ASC response to all three live-attenuated viruses in the vaccine and estimate the length of time following infection that the ASCs can be detected in circulation. Although our primary interest was in detecting the mumps-specific B-cell response, we also measured the measles and rubella B-cell responses as internal positive controls. (Please refer to Materials and Methods and Table 1 for a more thorough description of the vaccination status of each volunteer.) Virus-specific IgG and IgM levels were measured at each time point. Individuals who were IgG seropositive prior to vaccination and those who developed an IgM response following vaccination are indicated in Table 1.

Virus-specific B cells were measured at each time point by ELISPOT assay. Background correction was performed with an uninfected Vero cell lysate as the negative control antigen, since each virus preparation was grown on Vero cells. The number of virus-specific spots at each time point is shown as a fraction per  $10^6$  PBMCs (Fig. 2). The fraction of ASCs that responded to each virus ranged widely among volunteers. In general, the individuals who had a detectable ASC response to one virus had a similar relative level of response to the other viruses, with the relative magnitudes of the responses being rubella  $>$  measles  $>$  mumps. The peak of the ASC response appeared to be at 1 week postvaccination, and the average numbers of spots per  $10^6$  PBMCs for each antigen at this time point were as follows: mumps, 1.04; measles, 2.84; and rubella, 9.39.

Although the sample size was small, we performed receiver operating characteristic (ROC) analysis to estimate the sensitivity and specificity of the assay and to determine the threshold of detection for each antigen as described in Materials and Methods. The optimum sensitivity and specificity for each antigen at the 1-week postvaccination time point were as follows:

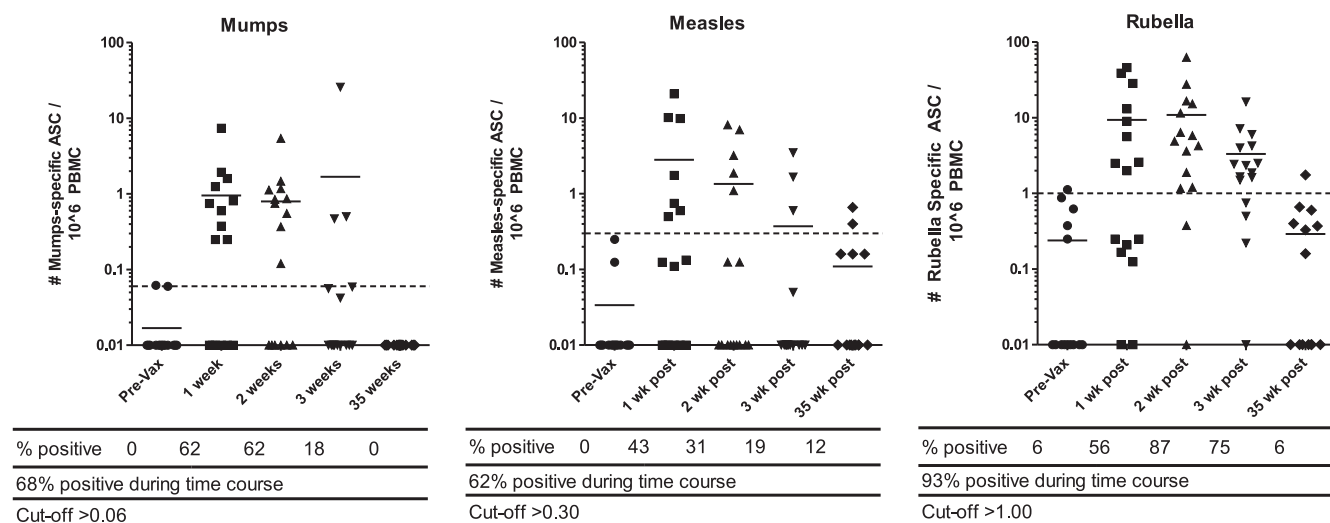


FIG. 2. Time course of ASC response following third-dose MMR vaccination. The numbers of mumps-specific ASCs per  $10^6$  PBMCs are shown for each individual at each time point indicated following vaccination with MMR. Horizontal lines indicate the mean for each group. Instances in which zero antigen-specific cells were detected were graphed with an artificial value of 0.01 to serve as a baseline placeholder on the log scale. Average ASC responses at each time point postvaccination were compared to the prevaccination time point for each antigen by repeated-measures ANOVA and Tukey's posttest. The data were transformed prior to analysis by the Box-Cox method to stabilize variance. Significant differences were observed between the prevaccination time point and the first-week-postvaccination samples for mumps ( $P < 0.0001$ ), rubella ( $P < 0.0001$ ), and measles ( $P = 0.0004$ ). The difference between prevaccination and second-week-postvaccination ASC levels was significant only for mumps ( $P < 0.0001$ ) and rubella ( $P < 0.0001$ ). ROC analysis was performed as described in Materials and Methods, and cutoff values were established for each antigen (dotted lines).

mumps, 62% sensitivity and 93% specificity; measles, 43% sensitivity and 93% specificity; and rubella, 56% sensitivity and 92% specificity. Based on this analysis, cutoff values were established for each antigen, and the percentages of individuals with a positive response are indicated below each graph in Fig. 2. The kinetics of the response varied among individuals, and not every person had a positive response at each measurement. Overall, the percentages of individuals who had a positive response during at least one time point were as follows: mumps, 68%; measles, 62%; and rubella, 93%.

To determine if the postvaccination ELISPOT results have positive predictive value for recent exposure to each virus, the average number of ASCs that reacted with each antigen across all time points was compared by repeated-measures ANOVA of data that were transformed by the Box-Cox method to stabilize variance. Significant differences were observed between the prevaccination time point and the first-week-postvaccination samples for mumps ( $P < 0.0001$ ), rubella ( $P < 0.0001$ ), and measles ( $P = 0.0004$ ). The difference between prevaccination and second-week-postvaccination ASC levels was significant only for mumps ( $P < 0.0001$ ) and rubella ( $P < 0.0001$ ) (Fig. 2).

Real-time RT-PCR was performed to detect mumps, measles, and rubella viruses from oral fluid samples taken during the first three visits (prevaccination through day 14 postvaccination) and PBMC specimens that were taken during the first four visits (up to day 22 postvaccination). None of the viruses were detected by RT-PCR in any sample, but amplification of RNase P mRNA from each sample indicated that the RNA extraction yielded intact RNA (data not shown).

The prevaccination serum sample from volunteer M was positive for mumps IgM; however, the optical density (OD)

values for the initial sample were similar to those of the subsequent samples from this individual, suggesting that these were false-positive results. The serum sample taken from volunteer O at day 21 postvaccination was positive for mumps IgM. None of the remaining samples was positive for measles or rubella IgM.

**Mumps-specific ASC detection from mumps cases.** Although the number of mumps-specific ASCs detected by ELISPOT assay following third-dose MMR vaccination had a statistically significant positive predictive value for exposure to mumps virus, we hypothesized that infection by wild-type mumps virus may induce a more robust ASC B-cell response than vaccination. From mid-March to April 2009, a small cluster of mumps cases occurred in the northeastern United States among individuals who were all two-dose MMR vaccine recipients. One of the individuals had recently traveled to Ireland and was clinically diagnosed with mumps after returning. This case was confirmed by a 4-fold rise between acute and convalescent IgG titer by a semiquantitative immunofluorescence assay. Six additional individuals, some of whom had direct contact with the index case, subsequently presented with parotid swelling and other symptoms consistent with mumps. Two of these individuals were RT-PCR positive for mumps, and one of these two was also IgM positive (Table 2). The remaining four individuals with suspect cases were either IgM negative or indeterminate. Due to sample availability, not every individual was tested by standard diagnostic methods upon their initial visit(s), as indicated in Table 2.

These seven individuals consented to participation in the study following local IRB approval, and a single 10-ml whole-blood specimen and oral fluid sample were obtained from each. The number of days between the onset of symptoms and

TABLE 2. Summary of naturally infected mumps cases<sup>a</sup>

Donor	Result from:					No. of days after onset
	Initial visit(s)			ASC testing		
	RT-PCR	IgM	4× IgG	RT-PCR	ELISPOT	
1	NA	—	+	—	+	28
2	NA	IND	NA	—	+	12
3	+	—	NA	—	+	8
4	NA	—	NA	—	+	8
5	NA	—	NA	—	+	8
6	+	+	NA	—	+	7
7	NA	—	NA	+ (G)	+	7

<sup>a</sup> The results from standard, routine mumps testing by real-time RT-PCR, ELISA (IgM), and immunofluorescence assays (4× rise in IgG between paired sera) are shown for each of the natural mumps infection cases reported in the text. Samples labeled “initial visit” were obtained during the patients’ initial visit(s) to the clinician. Samples labeled “ASC testing” were obtained subsequent to the initial samples, and the day post-symptom onset on which they were obtained is indicated. These samples were used for detecting mumps ASC ELISPOTs shown in Fig. 3. The letter “G” indicates the PCR product from donor 7 was sequenced and found to be mumps genotype G. NA, not available; IND, indeterminate.

the time of collection of these specimens ranged from 7 to 28 days. PBMCs were purified from each whole-blood sample and were tested for mumps ASCs by ELISPOT assay. The numbers of mumps-reactive ASCs from each of these individuals are shown in Fig. 3 and are plotted against the times of blood collection post-symptom onset. Convalescent-phase blood specimens were not available from these individuals for follow-up ELISPOT testing. However, the numbers of mumps-reactive spots from these wild-type infection cases were compared with the results from the each time point of the third-dose vaccination study. Analysis of the data by repeated-measures ANOVA indicated that the average level of mumps-specific ASCs from the outbreak specimens was significantly higher than the mean mumps-specific ASC levels from each time postvaccination ( $P < 0.0001$ ; data not shown).

Oral fluid samples were collected at the same time as the whole-blood specimens that were obtained for ASC testing. These oral samples were tested by real-time RT-PCR, and one was positive for mumps. Sequence analysis indicated that this virus was a representative of genotype G (GenBank accession no. GU056309), the most frequently detected mumps genotype currently circulating in Ireland (37).

## DISCUSSION

Public health agencies have been persistently frustrated by the challenges of using standard laboratory diagnostic methods to reliably confirm mumps (re)infection of individuals who have been previously vaccinated or naturally infected. This is perhaps best illustrated by two reports which describe the use of ELISA to detect mumps-specific IgM and real-time RT-PCR to detect the viral RNA from suspected mumps cases during outbreaks at the University of Kansas (5) and the University of Virginia (38) during 2006. All of the patients with suspect cases from the University of Virginia had previously received two doses of MMR vaccine, and 97% of the patients with cases from the University of Kansas had two doses; the remainder received one dose. In both studies, the rate of IgM

detection among suspect cases of infection was 12 to 13%. Similarly, the rate of detection by RT-PCR was 31 to 35%. Furthermore, the University of Kansas study (5) suggested that virus shedding is minimal after the first 3 days of symptom onset. In many cases, it is not possible or practical to obtain specimens for RT-PCR in this brief time frame. Even if samples are collected during the first 3 days of onset, RT-PCR requires careful shipping, storage, and processing of the specimen to prevent RNA degradation. Although detection of a 4-fold rise in IgG titer between acute- and convalescent-phase serum specimens has also been used to diagnose recent infection, we have found this method to be similarly unreliable as acute-phase specimens from vaccinated individuals typically have intermediate to high levels of IgG (our unpublished observations). Resolution of these diagnostic challenges is of the utmost importance because it confounds basic prevention measures (such as the quarantine of cases) and limits the accuracy of the epidemiologic assessment of outbreaks. A high number of false-negative results will lead to an underestimation of disease incidence. Moreover, asymptomatic spread among case contacts may expand and prolong an outbreak.

Based on several published observations that antibody-secreting B cells (ASCs/plasmablasts) are detectable in circulation only following recent activation by antigen (4, 41, 44, 47), we hypothesized that mumps-specific ELISPOT detection of these cells could be used as a more reliable alternative to standard diagnostic methods. In the absence of a sustained mumps outbreak, we first examined the numbers and kinetics of ASCs produced by volunteers who were recently vaccinated with MMR-II. This dose of vaccine was the third dose of MMR received by 10 of the 16 volunteers. We found that mumps-specific ASCs could be detected in 12 of 16 (68%) individuals from 1 to 3 weeks postvaccination. Volunteer O, who was mumps IgG negative at the prevaccination time point, had 4-fold more mumps ASCs present at 3 weeks postvaccination than the next-highest response from any other person (volun-

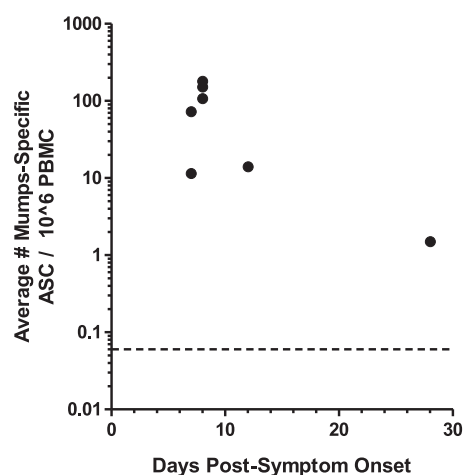


FIG. 3. ASC ELISPOTs from vaccinees with natural disease. The average number of mumps-specific ASCs per  $10^6$  PBMCs is shown plotted against the time post-symptom onset that ELISPOT testing was performed on blood samples collected from each of seven individuals who were infected by wild-type mumps virus. The dotted line represents the cutoff value for mumps ( $>0.06$ ) that was established based on the ROC analysis of the third-dose MMR data (Fig. 2).

teer B) at any time point and was the only person to have a 4-fold rise in IgG titer between the prevaccination and day-21-postvaccination samples (data not shown). Other than this one example, the number of mumps-specific ASCs detectable following vaccination did not appear to correlate with IgG titer, age, or any other known parameter. The timing and duration of the rubella- and measles-specific ASC responses were similar to those for mumps. However, the average number of rubella-specific ASCs was approximately 5-fold higher per volunteer than that observed for both measles and mumps. Statistical analysis indicated that the detection of virus-specific ASCs has predictive value for confirming recent exposure to mumps, measles, and rubella.

It is unclear why certain individuals did not have a detectable ASC response to vaccine, but this could be explained in a number of ways. First, it is possible that the numbers of their ASCs peaked and declined between specimen collection time points. Alternatively, the attenuated viruses in the vaccine may have been neutralized before a measurable ASC response could be elicited or measured. However, it was generally noted that individuals who responded well to one component of the vaccine also responded relatively well to the other two components. Although two separate cohorts of individuals were used to quantify memory B-cell numbers and the ASC response to a third dose of MMR, it is interesting that the hierarchy of memory B-cell levels mirrored that of the ASC response to vaccine. We observed more rubella-specific memory B cells and ASCs than those specific for measles, and there were more measles-specific cells observed than for mumps. Thus, the level of ASC response following infection may likely be directly related to the number of memory B cells that are available to respond in a given individual. While it may be expected that the B-cell response will vary significantly among viruses/antigens, these data could suggest that mumps elicits poor B-cell memory. If this is true, it may be a basis for susceptibility to reinfection, especially if the majority of the memory response is directed to nonneutralizing virus epitopes.

Given the frequency with which we were able to detect mumps-specific ASC responses among individuals who had been recently vaccinated and the relatively broad window of time during which these cells were present, we suspected that mumps-specific ASC detection following wild-type virus infection of previously vaccinated individuals would be possible. Furthermore, we anticipated that this method might be particularly useful when these cases cannot be confirmed by conventional testing (4). This was demonstrated by testing samples that were obtained from seven two-dose vaccinees who were recently infected. Mumps ASCs were detected in all seven cases, but only three of them were positive by standard testing. It is possible that the difference between rate of ASC detection among the naturally infected individuals (100%) and the rate of ASC detection among the individuals who were challenged with the vaccine (68%) could be attributed to the force and/or route of infection. These rates of ASC detection by ELISPOT assay compared very favorably with the RT-PCR and IgM serology results from both studies described here and published results for IgM (12 to 13%) and RT-PCR (31 to 35%), as noted above (5, 38).

In contrast to virus shedding, which rapidly diminishes (within 3 days) after symptom onset (5, 38), mumps-specific

ASCs were present and detectable out to 28 days post-symptom onset in one individual and ASCs were easily detected in individuals who were 7 to 12 days post-symptom onset. It is worth noting, however, that the timing of the peak of the ASC response to mumps infection has not yet been well established and may vary considerably among individuals. In one study (47), the ASC response to influenza vaccine peaked at day 7 postvaccination and was barely detectable by day 14. While it is important to more accurately determine the optimal timing for sample collection, these data suggest that 5 to 10 days after onset may provide the best opportunity for detection. Although these results are encouraging and provide proof of concept, additional testing is needed to more carefully determine the best cutoff value and to further validate this method before it is implemented as a diagnostic tool.

Unlike testing for 4-fold rises in IgG between paired acute- and convalescent-phase serum specimens, which requires a significant time delay, ASC detection by ELISPOT assay requires only one sample and is extraordinarily sensitive. The lower limit of detection will vary among donors in accordance with the total number of cells assayed and the number of cells that respond to the negative control antigen. However, we have routinely observed limits of detection as low as 1 cell per  $10^7$  PBMCs.

Overall, we have found the ELISPOT method for detecting virus-specific ASCs to be more sensitive throughout a longer period of time than RT-PCR or IgM ELISA for detecting recent mumps infection in individuals who have been either previously vaccinated or naturally infected. While this is not intended as a replacement for IgM and RT-PCR testing, it may be useful for diagnosing cases that cannot be otherwise confirmed by standard methods, for initially confirming cases in an outbreak, and for testing asymptomatic case contacts. As previously reviewed (4), this assay can be easily adapted for use with other infectious agents. Similar diagnostic challenges exist for varicella-zoster (29) and pertussis (45), and the assay can be completed in as few as 16 to 24 h.

Although this method is promising, some potential limitations exist. First, ASCs do not survive for an extended length of time after blood collection. This may require processing and testing of the blood in a timely manner after phlebotomy to maintain ASC viability. Second, the method may not be well suited for use on infants or small children because they may be unable to initiate a mature B-cell response and because the minimum volume of blood required (approximately 5 ml) may not be acceptable. However, the very young are less likely to be vaccinated, in which case standard diagnostics would be the better choice. Finally, there exists a possibility of cross-reactivity between B cells specific for mumps and other viruses such as parainfluenza viruses 2 and 4. This might pose a challenge particularly for the diagnosis of sporadic suspect cases, but could potentially be addressed by the use of recombinant antigens.

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