The nucleoprotein of the respiratory syncytial virus (RSV-N) is immunogenic and elicits an IgG response following infection. The RSV-N gene was cloned into a mammalian expression vector, pREN2, and the expressed luciferase-tagged protein (Ruc-N) detected anti-RSV-N-specific IgG antibodies using a high-throughput immunoprecipitation method (the luciferase immunoprecipitation system [LIPS]-N_{RSV} assay). The specificity of the assay was evaluated using monoclonal antibodies (MAbs) and monospecific pre- and postimmunization rabbit antisera. Blood serum samples from chimpanzees and humans with proven/probable RSV infection were also tested. The pre- and postimmunization serum samples from rabbits given human metapneumovirus (HMPV) or measles virus were negative when tested by the LIPS-N_{RSV} assay, while antisera obtained after immunization with either the RSV-A or RSV-B strain gave positive signals in a dose-dependent manner. RSV-N MAb 858-3 gave a positive signal in the LIPS-N_{RSV} assay, while MAb 213-1 against other paramyxovirus nucleoproteins or RSV-F or RSV-G did not. Serum samples from chimpanzees simultaneously immunized with vaccinia-RSV-F and vaccinia-RSV-G recombinant viruses were negative in the LIPS-N_{RSV} assay; however, anti-RSV-N IgG responses were detected following subsequent RSV challenge. Seven of the 12 infants who were seronegative at 9 months of age had detectable anti-RSV-N antibodies when they were retested at 15 to 18 months of age. The LIPS-N_{RSV} assay detects specific anti-RSV-N IgG responses that may be used as a biomarker of RSV infection.

**MATERIALS AND METHODS**

**Cells and viruses.** COS-1 cells were grown in Dulbecco’s modified Eagle’s minimal (DMEM) (Gibco, Grand Island, NY) medium, while Vero and HEP-2 cells were grown in Eagle’s medium containing Earl’s salts (EMEM) (Cellgro, Manassas, VA), as previously described (7, 8). RSV strain A2 was obtained and prepared as described previously (9), and RSV-B1 was similarly amplified once in Vero cells. Cold-passaged RSV-cp52 was amplified twice in Vero cells at 32°C and purified for animal immunizations as described previously (9). The monoclonal antibody-resistant mutant 1142 (MARM-1142) was selected, plaque purified, amplified, and the sequence of the fusion glycoprotein was deduced as previously described (10).

**Sera and antibodies.** The rabbits were immunized with purified UV-inactivated RSV-A2 administered subcutaneously using 10^5 50% tissue culture infective dose (TCID_{50}) equivalents, first in Freund’s complete adjuvant at 6 sites and then using Freund’s incomplete adjuvant every 3 weeks, for a total of 3 injections. Blood serum samples were collected before the first immunization and 4 weeks after the last boost. The same

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Address correspondence to Judy A. Beeler, judy.beeler@fda.hhs.gov.

* Present address: Thembi Mdluli, Purdue University, Weldon School of Biomedical Engineering, West Lafayette, Indiana, USA.

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protocol was used to obtain rabbit anti-RSV-cp52, anti-RSV-B1, and anti-measles virus sera. The rabbits were similarly immunized with affinity-purified RSV-F (10 μg/dose) or RSV-G (10 μg/dose) administered by subcutaneous injection using affinity-purified RSV-F and RSV-G proteins obtained from the WHO Reagent Bank for RSV and parainfluenza virus 3 (PIV3). The animal immunizations were performed under protocols approved by the Center for Biological Evaluation and Research (CBER) Institutional Animal Care and Use Committee.

Anti-N-specific IgG murine monoclonal antibodies were tested, including anti-RSV-N 858-3 (Chemicon International, Temecula, CA), anti-HMPV-N (Novus Biological, Littleton, CO) (11), anti-measles-N (12), anti-influenza-nucleoprotein (NP) (Millipore, USA), and anti-mumps-N (13). Monoclonal anti-RSV-F (1200 and 1243) and anti-RSV-G (1197) IgG antibodies were obtained and characterized as previously described (7).

Anonymous serum samples were previously obtained from subjects 6 to 18 months of age, following parental informed consent, and were stored frozen; the FDA Research in Human Subjects Committee gave approval for the testing. Human immunoglobulin, RSV IgG Lot 1 (RSV Lot 1), a pooled human reference immunoglobulin, was obtained from BEI Resources and used at a starting concentration of 1% IgG, except in tests used to determine the limit of detection, in which case RSV Lot 1 was tested in triplicate at concentrations ranging from 10% to 0.000001% IgG in parallel with IgG-depleted human immunoglobulin (IgGΔ) (Sunny Lab, United Kingdom). The serum and immunoglobulin samples were also tested using a plaque reduction neutralization (PRN) assay versus RSV-A2, as previously described (14, 15). The endpoint titers were calculated using the Spearman-Kärber method (16).

Chimpanzee serum samples were previously generated under approved protocols at the NIH. Briefly, chimpanzees (2 per group) were given RSV-MARM-1142 or recombinant vaccinia viruses expressing the RSV-F or RSV-G proteins (17). Chimpanzees no. 329 and no. 338 (group 1) were infected by combined intratracheal (i.t.) and intranasal (i.n.) inoculation on day 0 with MARM-1142. Chimpanzees no. 1,475 and no. 1,479 (group 2) were simultaneously inoculated subcutaneously on day 0 with vaccinia-RSV-F and vaccinia-RSV-G recombinant viruses. All chimpanzees were infected with RSV-A2 administered i.n. and i.t. on day 28 using 10⁶ TCID₅₀ per site, as reported previously. The serum samples were obtained preimmunization (day 0), prechallenge (day 28), and postchallenge on day 42 (group 1) or day 56 (group 2).

**FIG 1 Western blot assay confirms the expression of Ruc and Ruc-N antigen in COS-1 lysates.** COS-1 cell monolayers were transfected with pREN2 or pREN2+RSV-N and cell lysates harvested. FLAG-tagged proteins were precipitated using anti-FLAG-coated Sepharose beads, and precipitated proteins were separated by subcutaneous injection using affinity-purified RSV-F and RSV-G proteins obtained from the WHO Reagent Bank for RSV and parainfluenza virus 3 (PIV3). The animal immunizations were performed under protocols approved by the Center for Biological Evaluation and Research (CBER) Institutional Animal Care and Use Committee.

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**Generation of Renilla luciferase–tagged RSV-A2 nucleoprotein construct.** A mammalian expression vector, pREN2, was used to generate Renilla luciferase (Ruc)–tagged RSV-N protein (Ruc-N) (6). The RSV-N gene was inserted into the pREN2 polycloning site downstream of the sequences for the FLAG epitope and Renilla luciferase, such that the expressed fusion protein was tagged at its amino terminus (8). HEp-2 cell monolayers were infected with RSV-A2 and mRNA extracted from the cell lysates using an RNeasy minikit (Qiagen, Valencia, CA). RSV strain A2 (GenBank accession no. M11486.1) was used to design primers for cDNA synthesis and PCR amplification of the N gene insert. An EcoRI restriction site at the 5’ end and an XhoI site at the 3’ end facilitated cloning. The primers were 5’-GGGCTCAGAAGAGAAAACGCTCATCATCATCATTAC (reverse) and 5’-AAGGAAATTCGAGATGGCTCTTAGCAAAAGTCAAG (forward). Real-time PCR (RT-PCR) (ProntoScript AMV first strand cDNA synthesis kit; New England BioLabs, Ipswich, MA) was used to synthesize and amplify RSV-N cDNA, followed by gel electrophoresis and cloning into pCRJ-TOPO prior to amplification in Escherichia coli (Invitrogen, Carlsbad, CA). The resulting TOPO-insert vector was prepared using the Qiagen maxi kit and digested using EcoRI and XhoI restriction enzymes. The insert was extracted and purified using QIAquick (Qiagen), ligated into EcoRI/XhoI-cut pREN2 vector, and amplified in E. coli. The resulting pPREN2+RSV-N was prepared using a Maxiprep kit (Qiagen). The sequence and integrity of the DNA construct were confirmed using automated DNA sequencing.

**Renilla luciferase–nucleoprotein expression and characterization.** COS-1 cell monolayers were transfected with pREN2 or with pREN2+RSV-N, as previously described (8). Renilla light units (RLU) per 20 μl of harvested lysates were determined using a luminometer (SpectraMax L; Molecular Devices, Sunnyvale, CA). To confirm the expression of Ruc-N fusion protein, COS-1 cell lysates transfected with plasmid pREN2 or pPREN2+RSV-N were harvested and the tagged proteins precipitated using anti-FLAG-coated Sepharose beads (Clontech, Mountain View, CA); the proteins were separated by SDS-PAGE using 4 to 12% polyacrylamide gel and then transferred to nitrocellulose using iBlot (Invitrogen).
The blots were probed with rabbit anti-FLAG IgG (Sigma-Aldrich, St. Louis, MO) and with rabbit anti-RSV-A2 antisera, and specific bands were detected using peroxidase-conjugated goat anti-rabbit IgG antibody (KPL, Gaithersburg, MD) and LumiGLO substrate (KPL).

**LIPS assay to detect RSV-N-specific IgG antibodies.** COS-1 cell lysates containing Ruc-tagged RSV-N protein (Ruc-N) or Ruc only (without insert) were obtained and the LIPS assay performed as previously described (8). Briefly, a master plate was prepared by diluting serum samples 1:10 in assay buffer A (50 mM Tris [pH 7.5], 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) in a 96-deep-well polypropylene plate. Next, the following were added in triplicate to each well of a round-bottom 96-shallow-well polypropylene plate: 40 μl of buffer A, 10 μl of diluted serum from the master plate, and 50 μl of Ruc-RSV-N antigen from crude COS-1 lysate diluted in buffer A, such that 1 x 10⁵ RLU were added per well, followed by 1 h incubation at room temperature on a rotary shaker. The antigen-antibody mixtures (100 μl/well) were then transferred to 96-well high-throughput sequencing (HTS)-filter plates containing 5 μl of a 30% suspension of UltraLink protein A/G beads and further incubated for 1 h at room temperature on a rotary shaker. The filter plates were washed with buffer A (8X) and phosphate-buffered saline (PBS) (2X) using a vacuum manifold and dried. The RLU/well content was determined following the addition of coelenterazine (100 μl/well; Promega, Madison, WI) in a SpectraMax L luminometer. The RLU values from the wells containing beads, antigen, and buffer A but no serum served as buffer blanks and were averaged and subtracted from the values from all other wells. The data from the triplicate wells were then averaged to generate an RLU value for each sample. A positive response was defined as a value of >5 standard deviations above the mean value obtained with control serum tested against Ruc-N antigen, as recommended (8). The antisera were tested in at least two independent assays and in parallel against Ruc antigen without insert to confirm that the reactivity was specific for the RSV-N antigen.

**Production and purification of recombinant RSV-N protein in E. coli.** Maxiprep plasmid for pCRII-TOPO-RSV-N was digested using EcoRI and XhoI restriction enzymes to extract the RSV-N cDNA insert, which was purified by agarose gel electrophoresis and cloned into pET 28a (+) vector (Novagen, Madison, WI) containing the 6X His tag downstream of the T7 promoter. This vector was transformed into E. coli strain...
BL21 cells (DE3) (Novagen) and the expression of recombinant RSV-N protein induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Novagen). The cells were centrifuged at 4,500 rpm for 30 min at 4°C, the pellets resuspended in lysis buffer, and RSV-N protein purified using nickel-nitrilotriacetic acid (NTA) agarose (Qiagen). RSV-N protein production was confirmed by Western blotting using monoclonal anti-RSV-N IgG antibody.

**Statistical analysis.** A two-tailed t test was performed to compare the mean RLU values obtained for the assays using pre- and postinfection/immunization sera obtained from animal infections/immunizations.

**RESULTS**

**Characterization of Ruc-N protein in COS-1 lysates.** Rabbit anti-FLAG antibody detected the FLAG epitope present at the 5′ end of expressed Ruc antigen at ~40 kDa in the COS-1 lysate transfected with pREN2 plasmid, as expected (Fig. 1), as well as a specific band at ~80 kDa in the COS-1 lysates transfected with pREN2+RSV-N (Fig. 1). Rabbit anti-RSV polyclonal serum similarly revealed a specific band at ~80 kDa, consistent with the estimated molecular weight of Ruc-tagged RSV-N.

**Assay specificity.** Serum samples from rabbits immunized with purified UV-inactivated RSV-A2 were positive for anti-RSV-N IgG antibodies, while serum samples obtained prior to immunization and from rabbits immunized with affinity-purified RSV-F or RSV-G were negative in the LIPS-N<sub>R</sub> assay (P < 0.05, Fig. 2A).

RSV-N-specific IgG antibody responses were tested using serial dilutions of rabbit antisera obtained from animals before and after immunization with RSV-A2, HMPV, or measles virus (Fig. 2B). The signals obtained using all preimmune, anti-HMPV, and anti-measles antisera were at background levels at all dilutions tested (range, 300 to 900 RLU). In contrast, rabbit anti-RSV-A2 antisera gave positive signals in a dose-dependent manner at all dilutions tested up to and including 10<sup>−3</sup> (4,557 RLU); these values were significantly above the cutoff set using all preimmune rabbit antisera (mean + 5 standard deviations [SD] = 1,397 RLU); rabbit anti-RSV-A2 did not react when tested against Ruc antigen alone.

Similarly, a specific IgG response was detected when RSV-N-specific MAb 858-3 was tested using the LIPS-N<sub>R</sub> assay (~120,000 RLU), while MAbs specific for RSV-F, RSV-G, or that were directed against other myxovirus and paramyxovirus N proteins were negative in this assay (all readings < 2,000 RLU) (Fig. 2C).

To further demonstrate the specificity of the assay, rabbit anti-RSV-A2 antisera was first incubated with serial dilutions of purified recombinant RSV-N prior to testing in the LIPS-N<sub>R</sub> assay. As shown in Fig. 2D, the RLU signal obtained following incubation of the serum with the highest concentration of recombinant RSV-N tested was decreased by ~95% compared with the value obtained in the absence of soluble recombinant RSV-N protein (mean ± SD RLU = 203,243 ± 53,630 RLU), while no inhibition was seen following incubation with bovine serum albumin; the RLU signals for rabbit anti-RSV-A2 were inversely related to the concentration of soluble recombinant RSV-N protein used for the preincubation.

**LIPS-N<sub>R</sub> assay detects RSV-N-specific IgG antibodies elicited by either subtype A or B RSV strains.** Since the pREN2+RSV-N construct was obtained using the RSV-N gene derived from a subtype A strain, we next compared the ability of Ruc-N antigen to bind IgG antibodies using serum samples from rabbits immunized with either subtype A (RSV-A2) or subtype B (RSV-cp52, RSV-B1) RSV strains. Rabbit pre- and postimmunization serum samples were evaluated using the LIPS-N<sub>R</sub> assay. Specific anti-N IgG antibody responses were detected in antisera from rabbits immunized with UV-inactivated RSV-A2 (at dilutions as high as 10<sup>−3</sup>), or with RSV-cp52 or RSV-B1 (at dilutions as high as 10<sup>−4</sup>). The bold dashed line represents the cutoff for a positive result and is equal to the mean + 5 SD above the value obtained for the preimmun serum sera diluted 1:10.

**FIG 3** LIPS-N<sub>R</sub> assay detects IgG antibodies elicited following exposure to either subtype A (RSV-A2) or subtype B (RSV-cp52, RSV-B1) RSV strains. Rabbit pre- and postinmunization serum samples were evaluated using the LIPS-N<sub>R</sub> assay. Specific anti-N IgG antibody responses were detected in antisera from rabbits immunized with UV-inactivated RSV-A2 (at dilutions as high as 10<sup>−3</sup>), or with RSV-cp52 or RSV-B1 (at dilutions as high as 10<sup>−4</sup>). The bold dashed line represents the cutoff for a positive result and is equal to the mean + 5 SD above the value obtained for the preimmun serum sera diluted 1:10.

**LIPS-N<sub>R</sub> detects antibodies in human serum elicited by natural infection.** Human serum (100 samples) obtained from subjects 6 to 18 months of age were tested using the LIPS-N<sub>R</sub> assay to determine if the test could discriminate between positive and negative samples from infants and toddlers and to see if we could detect seroconversion among RSV-naive subjects using paired samples. In this assay, the cutoff for a positive result was 8,000 RLU (mean + 5 SD) for the test on IgG<sub>Δ</sub>. Of the samples tested, 36/100 had signals below the assay cutoff, while the remaining samples were positive by the LIPS-N<sub>R</sub> assay (range, 10,000 to 335,214 RLU; Fig. 4A). Among the seronegative samples, 12 from infants 9 months of age had a paired sample obtained 6 or 9 months thereafter. Seven of the 12 paired samples (58%) had detectable RSV-N IgG antibodies in samples collected at 15 or 18 months of age (Fig. 4B), while 5 of 12 paired samples remained negative at the later time point. Eleven of the 12 paired samples had sufficient quantities remaining for PRN testing. When the samples collected at 9 months of age were tested, 9/11 subjects had PRN titers of <1:40, and two infants had 50% neutralization dose (ND<sub>50</sub>) titers of 1:55 and 1:57. Likewise, in the samples collected at 15 to 18 months of age, the PRN ND<sub>50</sub> titers were negative (<1:40) or low (1:65) for the 5 subjects with no detectable anti-RSV-N IgG by the LIPS-N<sub>R</sub> assay, while the 6 subjects with a positive LIPS-N<sub>R</sub> assay result had ND<sub>50</sub> titers of >1:248 (range, 1:258 to 1:1,376) (Fig. 4C).
RSV Lot 1 was also tested over a wide range of IgG concentrations using both the LIPS-NRSV assay and a traditional PRN test in order to compare the sensitivities in detecting RSV-specific responses. As shown in Fig. 5, an anti-RSV-N-specific IgG response was detected at concentrations as low as 0.001% IgG, while no reactivity was seen at any dilution when tested against Ruc antigen alone. In contrast, PRN antibodies were detected at concentrations as low as 0.01% IgG. IgGΔ did not inhibit RSV in the PRN test (data not shown).

LIPS-NRSV detects anti-N antibodies in immunized animals following experimental challenge with live virus. Serum samples from 4 chimpanzees were tested for anti-RSV-N-specific antibody...
responses using the LIPS-N$_{RSV}$ assay, and the results are shown in Fig. 6. All preimmunization sera were negative for RSV-neutralizing antibodies, as reported previously (17), and signals detected using the LIPS-N$_{RSV}$ assay were $\leq 500$ RLU. Accordingly, the assay cutoff for a positive result was set at $1,115$ RLU (mean $\pm$ SD) using data obtained from the tests on the preimmune sera from all 4 chimpanzees. In contrast, serum samples obtained on study day 28 from chimpanzees infected i.n./i.t. with RSV-MARM-1142 had detectable anti-RSV-N IgG antibody responses (26,000 and 28,000 RLU), whereas day 28 serum samples from chimpanzees concomitantly immunized with vaccinia-RSV-F and vaccinia-RSV-G recombinant viruses had no detectable anti-RSV-N antibodies, as expected. Following challenge with live RSV-A2, all 4 chimpanzees had high levels of anti-RSV-N-specific IgG antibodies (with values of 41,000, 52,000, 17,000, and 40,000 RLUs in chimpanzees no. 329, 338, 1,475, and 1,479, respectively), and the anti-RSV-N IgG antibody responses in chimps previously inoculated with RSV-MARM-1142 were boosted 1.5- to 1.8-fold after rechallenge with RSV-A2 virus.

DISCUSSION
We developed a simple, rapid, and specific LIPS assay to detect IgG antibody responses against RSV-N that may be used as markers of infection or exposure. Many studies have demonstrated that RSV-F protein elicits both neutralizing antibody responses and cytotoxic T lymphocytes that are protective against subsequent infection (4, 5, 18). In addition, a passively administered monoclonal antibody that binds to a single epitope on the RSV-F glycoprotein can protect high-risk infants from severe disease (19). RSV-G protein also elicits a neutralizing antibody response that is protective, and for these reasons, many recent RSV candidate vaccines contain or express RSV-F or a combination of RSV-F and RSV-G protein antigens (4, 20). Since these candidate vaccines lack RSV-N, monitoring the anti-RSV-N IgG response among subjects 6 months to 2 years of age can help to identify an RSV-naive population prior to vaccine administration and potentially confirm natural infections and exposures that occur during the RSV season following immunization.

Currently, commercially available RSV ELISA kits using whole virus or lysates from infected cells contain the entire repertoire of RSV antigens; these assays are not designed to detect a protein-specific response. An ELISA that detects anti-N responses using vector-expressed antigen has been developed; however, this assay is not commercially available (21). A serological assay capable of detecting a virus-specific response to a nonvaccine antigen might serve as a complement to other virus detection methods for determining RSV attack rates during clinical trials, since subjects enrolled in these studies sometimes fail to be evaluated during the early period of an illness and intermittent shedding of RSV can also complicate the ability to identify infection even if sensitive methods are used to detect virus, antigen, or genome.

As a proof of concept, tests on preimmune, postimmune, and postchallenge antisera were performed using a PRN assay and using the LIPS-N$_{RSV}$ assay. Anti-N-specific IgG antibodies were detected at dilutions up to and including 0.001% IgG in the LIPS-N$_{RSV}$ assay, while plaque counts were reduced by 50% or more at dilutions up to and including 0.01% IgG. IgG (unspiked) inhibited plaque counts by $\sim 20$ to 40% when tested in the PRN assay.
postchallenge chimpanzee antisera demonstrate that the LIPS-N<sub>RSV</sub> assay can distinguish serum samples from animals with documented RSV exposure from samples obtained from RSV-naive animals; they also show that anti-RSV-N IgG antibodies may develop following experimental infection in spite of high levels of serum-neutralizing antibodies elicited after immunization with RSV-F and RSV-G antigens.

The LIPS-N<sub>RSV</sub> assay specifically detects anti-RSV-N antibodies induced in response to either RSV infection or immunization with whole-virus antigens. Monospecific rabbit polyclonal antibodies raised against RSV were positive in the LIPS-N<sub>RSV</sub> assay while preimmune sera and antibodies elicited in response to HMPV and measles virus were not. Likewise, an anti-RSV-N monoclonal antibody was positive in this assay, while monoclonal antibodies specific for RSV-F, RSV-G, or the N proteins of other myxoviruses and paramyxoviruses did not react. Additionally, signals were significantly reduced by preincubating anti-RSV antiserum with soluble purified RSV-N protein prior to testing, providing further confirmation of the specificity of the assay. The LIPS-N<sub>RSV</sub> assay was also able to detect antibodies elicited by either subtype A or B RSV.

Although we were not able to test serum samples from infants before and after their first documented RSV infection, paired serum samples from infants and toddlers demonstrated that the LIPS-N<sub>RSV</sub> assay can identify RSV-naive infants and detect sero-responses due to subsequent probable RSV exposures. There was a good correlation between the results obtained using the LIPS-N<sub>RSV</sub> assay with those obtained using a traditional PRN test, with 6 of 9 samples positive by both the PRN and LIPS-N<sub>RSV</sub> assays, for a positive predictive value of 67%; there were no false positives when the PRN results were compared to those obtained using the LIPS-N<sub>RSV</sub> test.

Due to transcriptional attenuation, there is an abundance of nucleoprotein produced in RSV-infected cells, and RSV-N is highly conserved among different RSV strains; therefore, RSV-N protein seems to be a good candidate antigen for use in developing a serological assay to monitor RSV exposures (1, 3, 5, 21, 22). Nucleoprotein antigen has been used to develop serological assays for a variety of paramyxoviruses and pneumoviruses; anti-N responses are routinely used to assess exposure to measles virus in epidemiological studies (12, 23–27). A side-by-side comparison of responses are routinely used to assess exposure to measles virus in epidemiological studies (12, 23–27). A side-by-side comparison of responses are routinely used to assess exposure to measles virus in epidemiological studies (12, 23–27). A side-by-side comparison of responses are routinely used to assess exposure to measles virus in epidemiological studies (12, 23–27). A side-by-side comparison of responses are routinely used to assess exposure to measles virus in epidemiological studies (12, 23–27).

In conclusion, the LIPS-N<sub>RSV</sub> assay represents progress in using a serological assay to detect prior RSV exposure. This test is a rapid, sensitive, and specific high-throughput assay that may help to monitor infections among RSV-naive infants following the receipt of mono- or bivalent RSV-F or RSV-F+G vaccines.

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