Evidence of Avian Leukosis Virus Subgroup E and Endogenous Avian Virus in Measles and Mumps Vaccines Derived from Chicken Cells: Investigation of Transmission to Vaccine Recipients

SHIRLEY X. TSANG, WILLIAM M. SWITZER, VEDAPURI SHANMUGAM, JEFFREY A. JOHNSON, CYNTHIA GOLDSMITH, ANTHONY WRIGHT, ALY FADLY, DONALD THEA, HAROLD JAFFE, THOMAS M. FOLKS, AND WALID HENEINE

HIV and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research, and Infectious Disease Pathology Activity, National Center for Infectious Diseases; Centers for Disease Control and Prevention, Atlanta, Georgia 30333; Avian Disease and Oncology Laboratory, U.S. Department of Agriculture, East Lansing, Michigan 48823; and Harvard Institute for International Development, Cambridge, Massachusetts 02138

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Reverse transcriptase (RT) activity has been detected recently in all chicken cell-derived measles and mumps vaccines. A study of a vaccine manufactured in Europe indicated that the RT is associated with particles containing endogenous avian retrovirus (EAV-0) RNA and originates from the chicken embryonic fibroblasts (CEF) used as a substrate for propagation of the vaccine. We investigated the origin of RT in measles and mumps vaccines from a U.S. manufacturer and confirm the presence of RT and EAV RNA. Additionally, we provide new evidence for the presence of avian leukosis virus (ALV) in both CEF supernatants and vaccines. ALV pol sequences were first identified in particle-associated RNA by amplification with degenerate retroviral pol primers. ALV RNA sequences from both the gag and env regions were also detected. Analysis of hypervariable region 2 of env revealed a subgroup E sequence, an endogenous-type ALV. Both CEF- and vaccine-derived RT activity could be blocked by antibodies to ALV RT. Release of ALV-like virus particles from uninoculated CEF was also documented by electron microscopy. Nonetheless, infectivity studies on susceptible 15B1 chicken cells gave no evidence of infectious ALV, which is consistent with the phenotypes of the ev loci identified in the CEF. PCR analysis of ALV and EAV proviral sequences in peripheral blood mononuclear cells from 33 children after measles and mumps vaccination yielded negative results. Our data indicate that the sources of RT activity in all RT-positive measles and mumps vaccines may not be similar and depend on the particular endogenous retroviral loci present in the chicken cell substrate used. The present data do not support transmission of either ALV or EAV to recipients of the U.S.-made vaccine and provide reassurance for current immunization policies.

Human vaccines made from live attenuated viruses have been used effectively worldwide to reduce and prevent morbidity and mortality from many viral infections. Most strains of the attenuated viruses were developed by adapting virus strains to certain culture conditions on primary animal-derived cells. The attenuated measles and mumps vaccines licensed in the United States are produced by a single manufacturer with primary chicken embryonic fibroblasts (CEF) (43). Measles and mumps vaccines are usually combined with attenuated rubella virus which is produced with human diploid cells, and the trivalent vaccine (MMR) is administered during childhood (14). MMR has been highly efficacious in preventing disease since the early 1970s (13, 30, 43). The general manufacturing regulations for these chicken-derived vaccines require that all avian embryo cell cultures used for propagation of vaccine strains originate from a closed, specific-pathogen-free, healthy flock which has been screened for absence of known chicken bacterial pathogens and viruses, including both avian retrovirus groups: the reticuloendotheliosis viruses (REV) and the avian leukosis-sarcoma viruses (ALV) (43). At least six subgroups of ALV (A to E and J) have been identified in chickens based on differences in the envelope sequences. Only subgroup E viruses are expressed from endogenous sequences that are part of the chicken germ line; all other subgroup viruses are exogenous. The endogenous sequences are usually referred to as endogenous viral (ev) loci (32, 35).

Reverse transcriptase (RT), an enzyme present in retroviruses, was recently detected in live attenuated vaccines from several manufacturers (8). The RT-positive vaccines were all derived from chicken cells and included measles, mumps, and yellow fever vaccines (8, 34, 42). RT activity was not detected in the rubella vaccine produced in human cells (42). RT was identified in the vaccines by using a newly developed RT assay that uses PCR amplification as a detection system. This improved method has been found to be up to 1 million-fold more sensitive than conventional non-PCR-based RT assays (23, 33). The level of RT activity present in these vaccines was low and could not be detected by conventional RT assays (42). Both the evidence showing association of RT with particles and the observed sensitivity to specific RT inhibitors, such as zidovudine triphosphate, suggest a retroviral origin rather than non-specific polymerase activity (8, 34, 42).

Weissmahr et al. recently examined the origin of RT in a CEF-derived measles vaccine from a European manufacturer.
By using a novel PCR-based method designed to detect unknown particle-associated RNA sequences with conserved primer targets, the rRNA primer binding site from the long terminal repeat (LTR), Weissmahr et al. identified RNA sequences related to the endogenous avian virus (EAV-0). They also demonstrated that enzymatically active RT and EAV-0 RNA were physically present together, which was interpreted in indirect evidence for the presence of EAV virions. Additional proof for the existence of EAV-0 retrovirus particles, including visualization of virions by electron microscopy and demonstration of EAV RT activity that was distinct from other avian retroviral RT, was not shown (42).

EAV proviral sequences are present in the genome of all chickens, including line 0 chickens that have been bred to eliminate any endogenous viral (ev) loci related to ALV (20). EAV sequences are transcriptionally active during embryogenesis but were not known to be associated with RT-positive particles (11). The findings of Weissmahr et al. of RT-associated particles containing EAV RNA may explain the origin of RT activity in ev- chicken embryos reported 20 years ago (2-4). The implications of EAV-0 RNA for vaccine recipients remain unknown.

Since all licensed vaccine producers follow similar manufacturing regulations regarding screening of source chickens for exogenous ALV and REV infections, it is likely that any RT activity found to be associated with these vaccines will be of endogenous origin. However, it is not known whether RT activity in vaccines from different manufacturers also originates from particles with EAV-0 RNA. Expression of endogenous retrovirus particles by chick cells and the characteristics of such particles both are highly variable and depend largely on the specific genetic profiles of the endogenous retroviral loci (e.g., EAV and ALV) of the chicken substrate used. For instance, at least 30 ALV loci have been identified in different chicken strains, and each chicken can carry multiple ev loci. ALV loci confer a range of different phenotypes: expression of infectious EAV, expression of viral proteins, or no detectable expression of any virus-related protein or RNA (32, 35). Therefore, RT in MMR vaccines propagated in chick cell substrates with different endogenous retroviral loci may be associated with endogenous particles that have distinct biologic properties and thus pose different risks to vaccine recipients.

Therefore, it is important to study the origin of RT in all licensed measles and mumps vaccines produced by various manufacturers. We focus in this study on the chicken-derived MMR vaccine used in the United States. Identification of the avian retroviruses responsible for the RT in this vaccine and assessment of their risks of transmission to vaccine recipients are necessary for a full understanding of the biological significance of this RT activity. This information may be important for policy decisions regarding the use of RT-positive vaccines. We found evidence of both endogenous ALV and EAV in the U.S.-made MMR vaccine but were unable to propagate ALV in vitro or to demonstrate transmission of these avian retrovirus sequences to vaccine recipients.

**MATERIALS AND METHODS**

**Vaccines, CEF, and CEF culture supernatant.** CEF which had not been inoculated with any vaccine virus strains (referred to hereafter as uninoculated or control CEF) were provided by a U.S. MMR vaccine manufacturer. CEF were pooled from chicken embryos derived from a closed, specific-pathogen-free flock of White Leghorn chickens maintained by the manufacturer. Culture supernatants of the cells were provided by the same manufacturer. Both CEF and CEF culture supernatants were shipped frozen and aliquoted after arrival. Aliquots were stored at −30°C until use. Lyophilized U.S.-made MMR vaccines were purchased and were stored at 4°C prior to use. The MMR vaccines were reconstituted in diluent according to the instructions of the vaccine manufacturer.

**Detection of RT activity.** Detection of RT activity in cell-free culture supernatants or vaccines was performed by the PCR-based Amp-RT assay, as described previously (23). Testing was performed on 10 μl or less. Qualitative determination of Amp-RT products was done by Southern blot hybridization, as previously described (25, 45).

**Purification of particle-associated RNA.** A volume of 250 ml of culture supernatant of uninoculated CEF was used for each CEF RNA extraction. Supernatants were first clarified by centrifugation at 1,000 rpm for 10 min. Viral particles were then pelleted by ultracentrifugation at 100,000 × g (Beckman Ti 60 rotor) for 1 h at 4°C. For MMR vaccines, 50 doses were each dissolved in 0.5 ml of diluent and virus particles were ultracentrifuged as was done with the CEF supernatants. Ultracentrifuged pellets from either preparation were resuspended in 1 ml of phosphate-buffered saline, and free RNA and DNA were digested with 250 ng of RNase (Boehringer Mannheim, Indianapolis, Ind.) per μl and 5 U of DNase I (Boehringer Mannheim) for 1 h at 37°C in the presence of 10 mM MgCl2. Particles were pelleted by another round of ultracentrifugation, pellets were lysed with 1 ml of RNA Stat60 buffer (TelTest B Inc., Friendswood, Tex.), and RNA was isolated and purified according to the instructions for use of RNA Stat60 (TelTest B Inc.). Nuclease acids were washed with 70% ethanol, air dried, and dissolved in 50 μl of distilled water—double-distilled water. Isolated RNA was treated with RNase-free DNase I for 1 h at 37°C, and DNase I was inactivated by heating at 95°C for 10 min. To check for the absence of cellular mRNA, we performed an RT-PCR analysis with chicken β-actin primers for each RNA preparation. Only isolated RNA with undetectable chicken β-actin sequences was used for the study.

**RT-PCR analysis.** An RNA template-specific RT-PCR was used for initial detection of the pol sequence in the particle-associated RNA (37). Primers mopF and mopR were used; they were derived from highly conserved retroviral pol sequences corresponding to KVLPOG and YMDDL2, respectively. This assay format is known to selectively amplify RNA target sequences in the presence of target DNA contamination. RNA was reverse transcribed at 37°C for 2 h by a mopF primer containing a 530 tag (mopR30), and RT was inactivated by heating at 95°C for 10 min. The 130- and forward primer mopF at a high annealing temperature of 65°C.

RNA isolated from 5 to 10 ml of CEF supernatant or 1 dose of MMR was applied to a 20-μl RT reaction mixture containing 50 mM Tris-HCl (pH 8.3), 10 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol; 400 μM deoxynucleoside triphosphate, 1 U of RNase inhibitor, and 10 U of murine leukemia virus RT (Promega, Madison, Wis.) with 80 ng of reverse primer (R) at 37°C for 2 h, followed by 95°C for 10 min. The cDNA product from the RT step was added to a PCR mixture (total volume of 100 μl) containing 2.5 U of Taq DNA polymerase (Perkin-Elmer, Foster City, Calif.) and 1.0× PCR buffer (Perkin-Elmer) with 100 ng of forward primer (F). The cycling conditions were 95°C for 5 min; 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C; and a final step of 7 min at 72°C. For each RT-PCR, a control amplification in which no added RNA was included to detect target RNA contamination. Additionally, negative controls containing water were also included.

Primers used in RT-PCR for ALV pol were as follows: mopF, 5′-TGGAAGCTTTCA/GACA/GCGTGGA3′; mopR, 5′-ACCAAAATTAT/GATCAAC3′; primer2, 5′-GGAGGCAGAACGATCCGATCGAC3′; primer3, 5′-TTTGGTGAAACTACCTTG3′. The specific probe for HIV-1 pol was 5′-AATTCCCCAGAGTTGAGAGAAG3′. Primers used in RT-PCR for ALV gag and env sequences were abag5SF (5′-ACGCTGGTCAGACGATCTGAG3′) and abag5SR (5′-CCCTTAAACCAACAAA3′); gagf, 5′-GGTCTGATATGCTA3′; and carf, 5′-CCCTTAAACCAACAAA3′. The internal oligonucleotide probe used for hybridization of the ALV env product was 5′-GGTGGAGATGCTA3′. The specific probe for the chicken β-actin primer targets were CAF (5′-GAGGAATCCGTGCCTGTCCTTT3′) and carF (5′-GGAGGAGTATGCTAAG3′). The specific internal probe for the chicken β-actin RT-PCR product was 5′-GGAGGAGTATGCTAAG3′. Primers used in RT-PCR for EAV 3′ pol were EAVF1 (5′-TCGTCTGATATGCTAAG3′) and EAVR1 (5′-GATGGAATCCGCTGTCCTTT3′) and carF (5′-GGAGGAGTATGCTAAG3′). The specific internal probe for the chicken β-actin RT-PCR product was 5′-GGAGGAGTATGCTAAG3′. The specific probe for the chicken β-actin primer targets were CAF (5′-GAGGAATCCGTGCCTGTCCTTT3′) and carF (5′-GGAGGAGTATGCTAAG3′). The specific internal probe for the chicken β-actin RT-PCR product was 5′-GGAGGAGTATGCTAAG3′.

**Cloning and sequencing.** RT-PCR products of ALV pol and env were cloned into pT5Blue vector (Novagen, Madison, Wis.). Plasmid DNA from recombinant clones was extracted with the Midi prep kit (Qiagen, Chatsworth, Calif.) and sequenced by either Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio) or an automated sequencer (Perkin-Elmer Applied Biosystems Division). RT-PCR products of ALV gag and both EAV pol and EAV element were directly sequenced as an automated sequencer (Perkin-Elmer Applied Biosystems Division). ALV gag, EAV pol, and EAV element sequences were used for nucleic acid homology searches of the GenBank and EMBL sequence databases.
fixed in buffered 2.5% glutaraldehyde, postfixed with 1% buffered osmium, and en bloc stained with 4% uranyl acetate. The pellets were dehydrated through a graded series of alcohol and propylene oxide and embedded in Epon substitute and Epon 812 (29). Sections were stained with uranyl acetate and lead citrate.

Neutralization of RT activity by anti-RT antisera. The ability of antisera to block RT activity was measured by quantitative Amp-RT assays, which use an enzyme-linked immunosorbent assay (ELISA)-based, nonradioactive oligonucleotide-primer hybridization system to quantitate Amp-RT products (22). The RT neutralization reactions were set up with serial 10-fold dilutions of 1 μl of antisera (i.e., postimmune serum). Control reaction mixtures were made with similar dilutions of the appropriate preimmune serum. Reactions included 9 μl of the sample and 1 μl of serum. The level of RT tested in all samples was adjusted to be in the linear range of the quantitative Amp-RT assay (22). The anti-avian myeloblastosis virus (AMV) RT antisera and its preimmune serum were purchased from Quality Biotech Incorporated-Resource Laboratory (Carneden, N.J.).

In vitro infectivity assays. Samples of CEF culture supernatant were tested for infectious exogenous and endogenous ALV, as described by Faddy and Witter (21). Briefly, either 1 ml of CEF supernatants concentrated from 250 ml as described above, or 1 ml of uncentrifuged supernatant was inoculated on 15B1 cells in 35-mm plates. Six inoculated 15B1 cultures, including three from the unconcentrated and three from the concentrated CEF supernatants, were monitored for production of ALV p27 protein. At 7 to 9 days postinoculation, cell lysates were tested for the presence of ALV p27 antigen by ELISA, as previously described (21). Control cultures were inoculated with 4,000 infectious units of RAV-0, a prototypic infectious endogenous ALV isolate.

Proviral DNA PCR. Peripheral blood mononuclear cells (PBMC) or other cell lysates were prepared at a concentration of 10^6 cells per ml of lysis buffer (50 mM KCl, 10 mM Tris-Cl [pH 8.3], 1 mg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween 20), supplemented with 60 μg of proteinase K per ml at 56°C for 1 h, followed by 94°C for 10 min. Aliquots of lysates from 150,000 PBMC from MMR recipients or U.S. blood donors were PCR amplified for ALV env and EAV env-like sequences. The sensitivities of both PCR assays were measured by spiking known plasmid copies that contain either ALV or EAV target sequence into background DNA lysate from 150,000 PBMC. Both assays were found to have a detection threshold of one copy (see Fig. 7). Standard amplification conditions of 35 cycles were used, as described above. The PCR products were detected by Southern blot hybridization of 20% end-labeled internal probes. The primers used for PCR detection of integrated proviral ALV sequence were ALV Pol clone 11698 (5'-TATATGATTTCTTATGGTGA-3') and ALV Env clone 4821 (5'-TTTCCACACATACCGGATCATTA-3'); the specific probe was ALVENP1 (5'-AACAGGAAATACGACACAGGGC-3'). The primers used for EAV were EAVF10 (5'-ACAGAAGATCAGATGACGGCCG-3') and EAVER10 (5'-GCGCTGATATGCCTGATACCT-3'); the specific probe was EAVP1 (5'-CCGTGTGTCACCCACAAACATT-3'). Genomic DNA from CEF was also isolated, using the Easy-DNA kit (Invitrogen, San Diego, Calif.), and proviral ALV DNA was amplified according to the Expand long-template PCR system (Boehringer Mannheim). pBRS-a-2 is a Ruppin-Schmidt A strain of ALV plasmid DNA used as a positive control. The primer pairs used for proviral DNA pol-env and env 3' LTR PCR were ALVPolf (5'-ATGACTCCTTGTCCTCACTTT-3') and RSEH1R (5'-AAC GGCGCCTGTTGGAACAC-3') and ENVPolf2 (5'-ACAGGTGGTGCAGCAGGATTT-3') and REVLRSp (5'-TGTTGTAAGTGGATAATGCGG-3') for env 3' LTR. The specific probe used for env-E were 5'-TCTG GAGATGTCAGACAGCAAGTACC-3'.

Typeability of CEF by locus-specific PCR analysis. CEF DNA was analyzed for the presence of env loci by using PCR-based methods described by Benkel (6). These validated methods are locus specific and use PCR primers that target env LTRs and the unique flanking sequences of integration sites. The amplifications produce diagnostic fragments of specific lengths which are indicative of the presence or absence of the locus in chicken cells. CEF can be typed as homozygous negative or positive for each locus. Heterozygosity is also indicated by the presence of both negative and positive diagnostic fragments. We tested for the presence of six env loci known to exist in White Leghorn chickens. The loci tested (and their corresponding phenotypes) are ev-1 (Gag ρ [very low level particle producer]), ev-2 (infectious particles), ev-3 (Gag ρ Env-), ev-6 (Gag-Env-), ev-7 (RT- [noninfectious particles]), ev-9 (Gag Env-), ev-12 (infectious particles).

Vaccine recipients and other test populations. Thirty-three children with documented dates of MMR vaccination were included in the study. Pre-MMR samples were obtained during the 6 months before the first MMR vaccination. Post-MMR samples were obtained 6 to 12 months after the first vaccination with MMR. Ninety-nine anonymous U.S. blood donors were also included and used as controls.

Nucleotide sequence accession numbers. ALV sequences determined in this study have been deposited in GenBank under accession no. AF605789, AF087830, and AF087831.

RESULTS

RT activity in CEF supernatants and CEF-derived vaccines. We determined the presence of RT activity by using Amp-RT, which can detect RT activity from approximately 1 to 10 human immunodeficiency virus type 1 (HIV-1) virions (22). RT activity was detected in several lots of monovalent measles and mumps vaccines, as well as in trivalent MMR vaccines from a U.S. vaccine manufacturer. Testing of endpoint 10-fold dilutions from a reconstituted 0.5-ml MMR vaccine showed that the detectable RT activity titer is equivalent to 0.01 μl of vaccine (Fig. 1A, lanes 5 to 8). RT was also detected in supernatants of CEF that had not yet been inoculated with any vaccine virus (Fig. 1A, lane 4). These results suggest that CEF is the source of RT in the vaccines. Amp-RT activity was also found on sucrose-banded CEF supernatants, with peak activity in a fraction with a density of 1.15 g/ml, suggesting that this activity is associated with retrovirus particles (data not shown).

Detection and identification of ALV pol RNA in CEF supernatants. To characterize the nucleotide sequence of any putative retrovirus expressed in CEF culture, we analyzed the particle-associated RNA that was extracted from un inoculated CEF supernatants. The method used for isolating this RNA selects for pelletable particle-associated RNA and eliminates free DNA or RNA contaminants of cellular origin by pretreatment with RNases and DNases prior to extraction of particle-associated RNA. We have confirmed the absence of contamination of these RNA preparations with either cellular DNA or RNA. The negative PCR result (Fig. 1B) for chicken β-actin mRNA, a highly expressed transcript in chicken cells, reflects the lack of contamination with cellular mRNA. Also, the non-detectable RT-PCR product in the control reactions with no RT demonstrates the absence of contamination with any DNA in the CEF RNA (lane 5 of Fig. 1C).

To identify the presence of unknown retroviral sequences in the particle-associated RNA, we used an RT-PCR strategy that employs primers derived from two sequences (KVLPOG and YMDDL) that are conserved in all known retroviral polymerase (pol) genes (36). These primers permit the identification of a wide spectrum of pol-related sequences to amplify virus-specific internal sequence (36, 44). A similar strategy with primers from the YMDD motif of RT and another highly conserved motif in the protease gene has also been successfully used to amplify pro-pol sequences from uncharacterized retroviruses (31, 38). A positive PCR signal of the expected size was observed in RT-PCRs containing CEF RNA as well as in the HIV-1 positive control (Fig. 1C, lane 1 and 2, respectively). No PCR products were seen in the control reactions with no added RT (Fig. 1C, lanes 5 and 6). This result demonstrates the absence of DNA that can be amplified by these generic pol primers, thus confirming that the RT-PCR product in the CEF reaction originated from RNA only. Hybridization with an internal HIV-1-specific probe was positive only with the RT-PCR product of the HIV-1 control (Fig. 1D, lane 2), not with that from CEF RNA (Fig. 1D, lane 1). This finding confirms the identity of the HIV-1 PCR product. We have cloned the RT-PCR pol fragment from CEF RNA and sequenced three representative clones. All internal 90-bp sequences from the three clones were identical and were 97 to 100% homologous to pol sequences of various ALV isolates (7, 12).

Identification of endogenous ALV. To confirm the presence of ALV RNA in CEF supernatants and to identify the subgroup of ALV, additional particle-associated gag and env ALV sequences were amplified from CEF RNA by ALV-specific RT-PCR analysis. Since ALV subgroups have distinct envelope glycoproteins which confer different tropisms to the virus for both avian and nonavian cells, defining the env subgroup of the ALV in CEF RNA is important for predicting cellular tropism (10). Both env and gag sequences were detected (Fig. 1D).
2A lane 1), and both amplifications originated from RNA, since PCRs that were not reverse transcribed had no detectable products (Fig. 2A, lane 4). The env sequence selected for amplification included hypervariable region 2 (hr2) of the envelope (gp85) gene of ALV, which is a subgroup-specific region and thus can allow the differentiation among all five known chicken ALV subgroups (A to E) (10, 16). The gag and env RT-PCR products were cloned and sequenced. The gag sequence showed the highest homology (96 to 98%) to ALV gag sequences (12, 40). The hr2 sequence was highly related to endogenous subgroup E env sequences (99% homology at the nucleotide level) (19) but less related to hr2 sequences of exogenous subgroups A to D (74 to 84% homology) (9, 10, 19).

Two proviral regions encompassing a 3-kb pol-env fragment and a ~1.6-kb env 3’ LTR sequence were also analyzed by PCR amplification of CEF DNA. Both products were amplified, and both hybridized with an env-E-specific probe (data not shown). These data confirm the presence of large endogenous ALV sequences in these CEF.

Visualization of C-type ALV-like particles in CEF. Uninoculated CEF culture fluids were examined by thin-section electron microscopy (EM). We found low numbers of C-type retrovirus particles in both pellets from CEF supernatants (Fig. 3A and B) and cells (Fig. 3C). The particles had a diameter of 90 nm and consisted of an outer envelope, a distinct inner membrane, and a central electron-dense nucleoid. These morphological features are consistent with the ultrastructural characteristics of ALV virions (5). Observation of a few particles is consistent with both the relatively low sensitivity of EM analysis and the low level of RT in the CEF. These results reflect a low rate of virus expression by CEF cells.

Inability to propagate ALV from CEF supernatants. To determine if the endogenous ALV RNA sequences in RT-positive control CEF supernatants are associated with replication-competent ALV virions, the CEF supernatants were applied to cultures of chicken 15B1 cells, which are known to be highly susceptible to infection with any exogenous or endogenous ALV (18). All six cultures had undetectable ALV p27 at day 9 postinfection. In contrast, the control culture inoculated with

FIG. 1. (A) Detection of RT activity in uninoculated CEF supernatants and measurement of RT titer in MMR by the Amp-RT assay. Lane 1, HIV-1 as positive control; lane 2, culture medium as negative control; lane 3, water as assay reagent control; lane 4, culture supernatant of uninoculated CEF; lanes 5 to 8, end-point 10-fold dilutions of 1 μl of reconstituted MMR. (B) RT-PCR analysis of chicken β-actin mRNA sequences. Lane 1, particle-associated CEF RNA isolated from uninoculated culture supernatant; lane 2, cellular RNA from CEF cells; lane 3, assay reagent control. (C) RT-PCR amplification by generic retroviral pol primers by an RNA template-specific method. Lane 1, particle-associated RNA from CEF supernatants; lane 2, HIV-1 RNA as positive control; lane 3, RNA from supernatant of uninfected cell line A3.01 as negative control; lane 4, water as assay reagent control; lanes 5 to 8, duplicate RT-PCR controls of lanes 1 to 4, respectively, done with no RT in the reaction mixture; M, molecular weight marker. (D) Southern-blot hybridization of RT-PCR products shown in panel C with a specific HIV-1 pol probe.

FIG. 2. Analysis of ALV and EAV RNA sequences from uninoculated CEF supernatants. (A) RT-PCR products of ALV gag and env RNA sequences. CEF, RNA from CEF supernatant; RSV-B, Rous sarcoma virus B as positive control. (B) RT-PCR products of EAV pol and EAV element RNA. M, molecular weight marker; H2O, reagent control.
RAV-0 was positive for ALV p27 protein (data not shown). These findings do not support the presence of infectious ALV particles in the RT-positive CEF fluids.

**ev loci in CEF DNA.** The results of locus-specific PCR analysis of CEF DNA identified the presence of ev-1, ev-3, and ev-6 ALV-E loci. No evidence of ev-2, ev-7, ev-9, ev-12, or ev-21 was found. Representative results are shown in Fig. 4. The CEF DNA was found to be heterozygous for ev-1, as indicated by the presence of both positive (295-bp) and negative (505-bp) PCR fragments (Fig. 4). Similarly, ev-3 is also heterozygous, as a positive band (190 bp) and a very faint negative band (270 bp) were both seen.

**Detection of EAV-0 RNA in CEF supernatants.** Because of recent reports of particle-associated EAV-0 RNA sequences in measles vaccines produced in Europe (42), we analyzed the presence of EAV-0 RNA in our CEF RNA preparations. We first amplified the EAV-0 5’ R-U5 RNA sequence by RT-PCR with EAV-specific primers, as previously reported, and confirmed the presence of EAV-0 RNA in our CEF RNA (data not shown) (42). Based on these data, we have reexamined the presence of EAV sequences in the RT-PCR product obtained from CEF RNA by the highly conserved generic retroviral pol primers (Fig. 1C). Southern blot hybridization of this PCR product with an EAV-specific internal oligonucleotide probe (the sequence was kindly provided by Merck Corp.) was positive, indicating that EAV sequences are present and may have been amplified along with the ALV pol sequences (data not shown). The failure to identify EAV in this PCR product may be due to the limited analysis of three clones from this pol product. Two other EAV sequences were successfully amplified from CEF RNA by RT-PCR with EAV-specific primers (Fig. 2B). The first sequence is a 315-bp fragment from the EAV element (transmembrane envelope TM-like) region, and the second is a 472-bp fragment from the pol region. Sequence analysis of the EAV element fragment showed high relatedness to EAV-0 (94.3% [GenBank accession no. X59844]) but little homology to the env sequences of subgroup J of ALV (60.4%) and RAV-0 (52.8%), a known endogenous ALV strain. A 96.6% homology to EAV was also seen in the EAV pol sequence, as described below.

**Demonstration of ALV-like RT activity in CEF culture and measles vaccine.** We measured the ability of anti-ALV RT antibodies to inhibit RT activity in both CEF culture fluids and monovalent measles vaccine. Antiserum raised against RT of AMV, a strain of exogenous ALV, completely blocked the RT activity in both CEF and the vaccine, while preimmune serum had little or no blocking activity (Fig. 5A and B). Similar reductions in RT activity were seen with AMV RT, while no blocking effect was observed with HIV-1 RT, an enzyme that is distantly related to ALV (Fig. 5C and D). The observed inhibition of RT activity suggests the presence of ALV-like RT activity in both the CEF and the measles vaccine. However, these data do not necessarily confirm that the neutralizable RT activity is due to ALV RT only. EAV and ALV RTs are about 65% homologous (in 450 bp of 3’ pol [see below]) and may therefore be antigenically cross-reactive. To determine whether the anti-AMV RT antiserum used may have some neutralizing activity against the EAV RT, we tested RT-positive supernatants from cultured ev-negative line 0 chicken cells. The antiserum was able to block the RT activity in this supernatant in a fashion similar to that of the AMV RT, CEF, and measles vaccine (data not shown). The observed cross-neutralization between ALV and EAV RT suggests that the anti-AMV RT antiserum is not sufficiently specific to differentiate between both enzymes. ALV- or EAV-specific monoclonal antibodies may be necessary for such testing.

**Detection of ALV and EAV RNA sequences in MMR vaccine.** Measles and mumps vaccines are prepared by diluting CEF-derived vaccine harvests that may possibly result in eliminating either ALV or EAV. To determine whether MMR vaccines contain ALV, EAV, or both sequences, we examined particle-associated RNA from a single-dose MMR vaccine by RT-PCR. We detected the presence of three ALV RNA sequences from gag, pol, and env as well as two EAV sequences from the env-like EAV element and pol regions. Representative results are shown in Fig. 6. The EAV 3’ pol sequence amplified from the MMR was sequenced, and a 96.6% homology to previously reported EAV sequences was observed, thus confirming high relatedness to EAV. These findings indicate that dilution of vaccine harvest preparations does not eliminate either ALV or EAV.
Evaluation of transmissibility of ALV and EAV sequences to MMR recipients. To investigate whether any possible persistent infection with ALV and EAV retroviral sequences occurs after exposure to MMR vaccination, we screened for the presence of ALV and EAV proviral sequences in PBMC obtained from 33 children documented to have received MMR. Pre- and post-MMR vaccination samples, as well as randomly selected PBMC samples from 99 U.S. blood donors, were all tested by PCR for ALV env and EAV-0 env-like EAV element sequences. Despite the use of highly sensitive PCR assays, none of the 165 samples tested were positive for either ALV or EAV sequences (Fig. 7). These data do not support transmission of ALV or EAV sequences to MMR vaccinees.

DISCUSSION

This study was designed to identify the source of particle-associated RT activity in the U.S.-made MMR vaccine and to evaluate the implications of the findings for vaccine recipients. The data presented here confirm the presence of RT activity in the U.S.-made MMR vaccine and show that the CEF substrate used is the source of the RT activity. However, the molecular
Our findings of particle-associated RNA sequences of EAV-0 support those of Weissmahr et al., who examined CEF fluids from a European vaccine manufacturer (42). The negative results of PCR screening of MMR vaccine recipients does not support transmission of either ALV or EAV to these children. The lack of evidence of infectious ALV suggests also the absence of ev loci in CEF DNA that are associated with expression of endogenous infectious ALV (e.g., ev-2, ev-10, ev-12, and ev-21) (35). These data are consistent with ev-1, ev-3, and ev-6, which were identified in the CEF DNA. ev-3 and ev-6 have large deletions in the gag-pol and 5′ LTR gag regions, respectively, and therefore cannot independently generate mature particles (35). ev-1 possesses a full-length genome but produces low levels of noninfectious particles (17). Additional analysis of CEF DNA to identify the presence of other ev loci may be necessary.

Recently, an ALV subgroup J strain was identified in chickens and was found to possess a recombinant genome consisting predominantly of an exogenous-type ALV backbone and an EAV-like env gene (1, 39). The evidence in our study for the presence of both EAV and ALV RNA raise questions on whether the detected EAV and ALV RNAs are copackaged in the same particles or are parts of a recombinant genome similar to that seen in subgroup J ALV. While our present data cannot address the question of ALV-EAV copackaging, several observations argue against the presence of a subgroup J-like virus in the CEF tested. First, we have not found evidence of ALV replication in 15B1 cells, which are known to be susceptible to infection with subgroup J virus (21). Second, the particle-associated ALV env sequences identified in the CEF supernatants were closest to the endogenous ALV sequences identified in the CEF fluids and MMR vaccines. The differences in the source of RT among the various RT-positive vaccines should not be unexpected, since different vaccine manufacturers use different chick cell substrates with distinct endogenous retroviral loci. Therefore, our data highlight the importance of studying independently the origin and risks of each of the currently used RT-positive vaccines.

We found no evidence of infectious ALV that can replicate in indicator chicken 15B1 cells, despite the use of concentrated CEF inocula. These data suggest the presence of noninfectious endogenous ALV particles in the CEF fluids. Nonchicken cells, such as turkey embryo fibroblasts and Japanese quail primary embryonic cells, have been used previously as susceptible cells for ALV subgroup E replication (27, 34). Further evaluation of infectious ALV from CEF fluids and MMR vaccine on these nonchicken cells may be warranted to confirm the present results seen on the chicken 15B1 cells.
ected by routine screening for ALV infection and by the ability of this virus to quickly cause disease in chickens. Our inability to detect either ALV or EAV sequences in PBMC samples from any vaccine recipient provides more direct evidence for the absence of infection with either ALV or EAV. These negative results were observed despite the use of highly sensitive PCR assays that employ specific primers and probes derived from vaccine-associated EAV and ALV sequences. Confirmation of these results by serologic screening, however, may be necessary. The relatively small number of MMR recipients studied here and the single tissue (PBMC) analyzed from these subjects limit the strength of our conclusions regarding transmissibility. Therefore, additional laboratory surveillance of EAV and ALV infection in recipients of this as well as other chicken-derived vaccines may be prudent. Nevertheless, the observed lack of evidence of either ALV or EAV infection in the vaccine recipients is consistent with the noninfectious nature of ALV seen in this study and with other in vitro data reported recently on the inability of other RT-positive uninoculated CEF fluids to infect a variety of human and other mammalian cells, including PBMC (27, 34).

Little or no information is available from previous human studies on the prevalence of antibodies to subgroup E ALV. A few studies have looked at risks of ALV infections in humans by serologic testing for antibodies to ALV and have reported mostly negative or false-positive results (15, 25, 26). In many previous studies, the use of exogenous subgroup-specific neutralization assays limited the utility of the data in addressing risks of human infection with subgroup E ALV. A more recent study reported positive Western blot results to ALV gag proteins in poultry workers and some persons with no occupational exposure to ALV (24). However, additional evidence of active ALV infection in these persons, including virus isolation and detection of ALV sequences, was lacking. No serologic testing for antibodies to EAV in humans has been previously reported.

Documentation of ALV and EAV in MMR raises questions about the adequacy of using the present CEF as substrates for vaccine propagation and whether changing to a cell substrate that has no detectable RT activity is necessary. CEF originating from line 0 chickens may provide a more suitable avian cell substrate that does not express any ALV. However, we found that culture fluids of CEF cells from line 0 chickens were also RT positive, most likely due to EAV expression. Therefore, obtaining an RT-negative substrate may require a change from chicken cells to RT-negative cells from a different species. Since the cell substrate is also critical to the attenuation of the vaccine viruses, changing the cell type may have an unpredictable effect on both the safety and the efficacy of the vaccine. Thus, any suggestion to change cell substrates must be approached cautiously by balancing potential unknown and theoretical risks associated with ALV-EAV against the risk of possibly compromising established vaccine safety or efficacy.

There is currently no evidence that exposure to ALV through vaccination is associated with adverse events. Our inability to demonstrate infectious virus or to find any ALV-EAV sequences in recipients of the U.S.-made MMR vaccine supports the safety of this vaccine product and suggests that these endogenous viruses are not xenotropic. The endogenous subgroup E ALV virus is not known to be oncogenic in domestic fowl, and EAV-0 has not been associated with any known diseases in chickens (28). While exposure to exogenous-type ALV in preparations of yellow fever vaccine has been previously demonstrated with vaccine recipients, no increase in tumors has been found among the vaccinees (41). The proven efficacy of MMR and the absence of evidence of any harm due to MMR vaccination support current immunization policies.

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REFERENCES

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