

Diagnostic Potential of Baculovirus-Expressed Rubella Virus Envelope Proteins

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The envelope glycoproteins E1 and E2 of rubella virus were abundantly expressed in *Spodoptera frugiperda* Sf9 insect cells by using a baculovirus expression vector. The recombinant protein products were purified by immunoaffinity chromatography and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblotting, and enzyme immunoassay (EIA). The purified recombinant antigen consisted of the envelope polypeptides, corresponding to the viral E1 and E2 proteins, and a polyprotein precursor (molecular mass, 90 to 95 kDa). The antigen was reactive with human convalescent-phase sera in immunoblot analysis, and the reactivity correlated well ($r = 0.861$) with that of a whole-virus antigen when tested by EIA by using a total of 106 rubella virus immunoglobulin G-positive and -negative serum specimens. When the sera from patients with recent rubella virus infection were tested with the recombinant glycoproteins by EIA, the correlation was not as close ($r = 0.690$). However, all of the 26 serum specimens were reactive with the recombinant antigen. The results demonstrate that these bioengineered antigens have a potential for use in routine diagnostic assays of rubella virus immunity and recent infection.

Rubella virus (RV) is an enveloped, positive-stranded RNA virus that belongs to the family *Togaviridae* (22). Rubella is normally a mild, self-limited disease, but it may cause fetal damage if it is acquired during the first trimester of pregnancy. Thus, serological tests to determine the immune status in women of childbearing age and tests to diagnose recent RV infections in pregnant women are of great importance.

RV consists of three structural proteins: a nonglycosylated capsid protein, C (molecular mass, 33 kDa), and two envelope glycoproteins, E1 (molecular mass, 58 kDa) and E2 (molecular mass, 42 to 47 kDa) (19, 28, 30). The hemagglutination activity (9, 24, 30) and a major epitope with viral neutralization activity (9, 24, 31) have been localized to the E1 protein. E1 has been shown to contain at least six antigenic epitopes (5, 31), and it has been demonstrated that the antigenicity of the E1 protein is independent of its glycosylation (8). The E2 glycoprotein has not been as well characterized, and the difficulties in generating anti-E2 monoclonal antibodies suggest that immunogenicity of the E2 protein is poor or that the epitopes may be hidden under the E1 protein in the envelope spike complexes (8). However, it has also been demonstrated that the E2 protein contains a neutralization epitope (5). The natural antibody response to E1 is predominant in most patients with RV infections, but in patients with the congenital rubella syndrome, the antibodies to E2 were found to be more abundant (11).

Baby hamster kidney cells (BHK-21) (29) or African green monkey kidney (Vero) cells (1) are used in the conventional methods of growing RV. However, by those methods, the yield of virus remains low, despite the improvements in the cell culture systems for large-scale production (26). Alterna-

tive approaches for the production of RV antigens in vitro have been presented. The bioengineered structural proteins of RV have been produced by genetic expression in *Escherichia coli* (25) or by using simian virus 40 (3, 18) or pCMV5 (7) expression vectors in COS cells. The possibility of using the synthetic peptides as antigens in serological tests has also been studied (14).

Baculovirus expression vectors, which use the polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), have been used for the expression of foreign prokaryotic or eukaryotic genes. The recombinant genes are expressed as fusion or nonfusion proteins during viral infection, and most recombinant protein products appear to undergo normal posttranslational modifications (15, 16).

We have previously shown (20) that the E1 and E2 proteins expressed in *Spodoptera frugiperda* Sf9 insect cells, using a baculovirus expression vector, reacted with human rubella antibodies. We therefore expressed the corresponding E1 and E2 proteins of RV, using a baculovirus expression vector construct, to study whether these recombinant protein products can be used in the diagnosis of RV infection.

MATERIALS AND METHODS

Sera. A total of 106 serum specimens were examined for RV immunoglobulin G (IgG) antibodies by using recombinant RV enzyme immunoassay (EIA) and RV EIA test kits. Sera were obtained from randomly selected healthy blood donors (The Finnish Red Cross Blood Transfusion Service); over 90% of the serum specimens were statistically known to have RV IgG antibodies. RV IgG antibody-negative sera were obtained from Boston Biomedica Inc. (Mansfield, Mass.). In addition, 26 serum specimens from patients diagnosed to have recent RV infection (Department of

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Virology, University of Helsinki, Helsinki, Finland) were tested.

Cells and virus. *S. frugiperda* Sf9 cells were propagated as monolayer cultures at 27°C in TNMFH medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Flow Laboratories, Irvine, Calif.), as described previously (20). A recombinant baculovirus (Ac701-RVE) containing the E2-E1-coding region of RV, including the sequence encoding the E2 signal peptide, was used. The recombinant proteins were expressed in infected cells under the transcriptional regulation of the polyhedrin gene promoter (20).

The Therien strain of RV grown in B-Vero cell cultures was purified as described previously (19) and was used as the reference antigen in immunoblotting experiments.

Purification of the recombinant proteins. At 48 to 72 h postinfection, cells were pelleted by low-speed centrifugation ($400 \times g$, 2 min), washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.9% NaCl [pH 7.4]), and resuspended in TNE buffer (10 mM Tris hydrochloride, 0.15 M NaCl, 1 mM EDTA [pH 7.8]) containing 1% Nonidet P-40 and 0.2 mM phenylmethylsulfonyl fluoride. After sonication, the sample was clarified by centrifugation ($5,000 \times g$, 5 min) and applied to an immunoaffinity column containing rabbit anti-RV IgG antibodies (10 mg, protein A purified) linked to Sepharose 4B (Pharmacia, Uppsala, Sweden). The recombinant proteins were incubated overnight at 4°C with the affinity matrix, and the unbound proteins were washed with TNE buffer. The recombinant proteins were eluted with 50 mM lithium diiodosalicylate (pH 8.0; elution rate, 36 ml/h). The fractions, 1 ml each, were collected, diluted (1:10) with PBS, and tested by EIA as described below. The immunoreactive fractions were pooled and concentrated (Centricon microconcentrators; Amicon, Danvers, Mass.).

Sodium dodecyl sulfate (SDS)-PAGE and immunoblotting. Wild-type baculovirus (AcNPV strain E2)-infected *S. frugiperda* Sf9 cells, recombinant virus (Ac701-RVE)-infected Sf9 cells, purified recombinant proteins, and purified RV were separated under reducing or nonreducing conditions by polyacrylamide gel electrophoresis (PAGE; 10% [wt/vol]), as described by Laemmli (13), transferred to a nitrocellulose sheet (27), and tested for their immunoreactivities as described previously (21). The human and rabbit sera were used at dilutions of 1:50.

Whole-virus EIA. RV IgG and IgM antibodies were measured with RV EIA test kits (Rubella IgG EIA and Rubella IgM EIA; Labsystems, Helsinki, Finland).

Recombinant RV EIA. Microtitration wells (Labsystems) were coated with purified recombinant RV antigen (100 μ l per well) at 4°C overnight. Test sera were diluted 1:50 with PBS containing 1% bovine serum albumin and Tween 20 and incubated for 1 h at 37°C in recombinant RV antigen-coated wells. Unbound proteins were removed by rinsing the wells three times with a PBS-Tween 20 washing solution. RV-specific IgG antibodies were detected by incubation for 1 h at 37°C with alkaline phosphatase-conjugated anti-human IgG antibodies (Labsystems). The quantity of bound enzyme conjugate was measured by incubation with the substrate *p*-nitrophenylphosphate. The enzyme reaction was stopped after 30 min with 1 M NaOH, and the amount of bound RV-specific IgG antibodies was determined by measuring the A_{405} (Multiscan MC; Labsystems). A reagent blank and negative and positive controls were included in each test run. The results were expressed as EIUs (for the definition of EIUs, see the legend to Fig. 4).

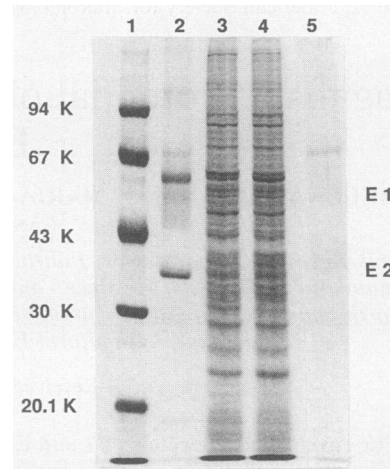


FIG. 1. SDS-PAGE analysis of the recombinant RV antigen produced in baculovirus-infected *S. frugiperda* Sf9 cells. Lane 1, molecular weight markers (in thousands; K); lane 2, purified RV; lane 3, AcNPV (wild-type virus, strain E2)-infected Sf9 cells; lane 4, Ac701-RVE (recombinant virus)-infected Sf9 cells; lane 5, immunoaffinity-purified recombinant RV antigen. The proteins corresponding to the viral E1 and E2 proteins are indicated.

RESULTS

Purification of recombinant RV glycoproteins. The recombinant RV proteins were purified by immunoaffinity chromatography by using rabbit anti-RV antibodies. The antigenic activities of the proteins were found to remain intact when the elution was done with 0.05 M lithium diiodosalicylate, as has also been demonstrated for purification of whole-virus antigen (2) and for purification of bioengineered E1 protein (25). The recovery of the purification could not be detected by measuring the A_{280} because of the presence of lithium diiodosalicylate. The fractions were tested by EIA by using RV-negative and -positive sera, and the fractions showing good responses with positive samples and low reactivities with negative samples were pooled and concentrated. This antigen was then analyzed by SDS-PAGE (Fig. 1).

The purified recombinant proteins E1 and E2 (Fig. 1, lane 5) migrated somewhat faster than those derived from purified RV did (Fig. 1, lane 2). This is most likely due to differences in glycosylation between the two systems, as has been suggested previously (20). Cellular extracts of *S. frugiperda* Sf9 cells infected with wild-type (AcNPV) as well as recombinant (Ac701-RVE) baculoviruses before purification are shown in Fig. 1, lanes 3 and 4, respectively. The recombinant proteins could hardly be identified from cellular extracts before purification.

Identification of the purified recombinant proteins. The identities of the recombinant polypeptides were further established by immunoblot analysis by using rabbit anti-RV antibodies (Fig. 2). From extracts of whole *S. frugiperda* Sf9 cells infected with the recombinant baculovirus, the E1 protein as well as a few minor additional proteins could be seen (Fig. 2, lane 3). In lane 4 of Fig. 2, which represents the purified recombinant antigen, the polyprotein precursor and the E1 and E2 proteins were clearly identified. In addition, a protein migrating somewhat slower than E1, which was also present in wild-type baculovirus-infected cells (Fig. 2, lane 2) and recombinant baculovirus-infected cells (Fig. 2, lane 3), was seen.

Figure 3 shows the reactivities of human RV IgG-positive

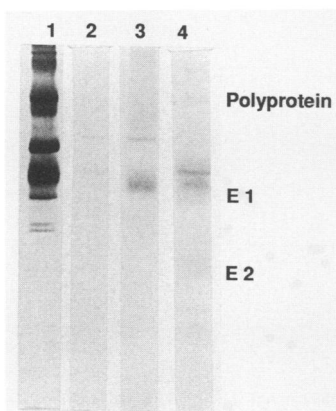


FIG. 2. Immunoblot analysis of nonreduced recombinant RV antigen by using rabbit anti-RV antibodies. Lane 1, purified RV; lane 2, AcNPV (wild-type virus, strain E2)-infected *S. frugiperda* Sf9 cells; lane 3, recombinant RV-infected Sf9 cells; lane 4, purified recombinant RV antigen. The proteins corresponding to the viral E1 and E2 proteins and the polyprotein precursor are shown.

sera with purified recombinant antigen in immunoblot analysis. These sera were reactive with the E1 protein, while the IgG-negative serum did not show any reactivity.

Recombinant proteins in EIA. The antigenic properties of recombinant E1 and E2 proteins were finally evaluated by EIA. Figure 4 shows the correlation of the reactivities of RV IgG antibody-positive and -negative sera between recombinant RV and whole-virus antigen by using a total of 106 serum samples. The reactivities of these two different antigens correlated with a coefficient of $r = 0.861$.

IgG antibodies of some serum specimens from cases of recent RV infection reacted strongly with the capsid protein (C) (Fig. 5), which was not expressed in our recombinant system. Because of these differences in IgG reactivities, the panel of 26 serum specimens from patients with recent RV infection was tested. Figure 6 shows the correlation of the reactivities of the IgG antibodies between recombinant RV and whole-virus antigen by using these sera, which were tested earlier and shown to contain RV IgM antibodies. All of the corresponding sera were positive with the recombinant antigen, although some variability in reactions ($r =$

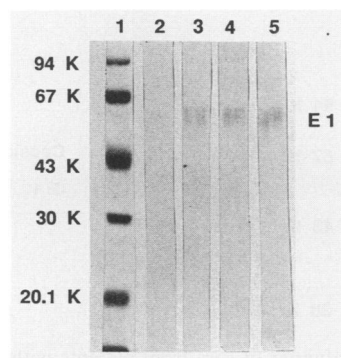


FIG. 3. Immunoblot analysis of nonreduced purified recombinant RV antigen with human serum samples. Lane 1, molecular weight markers (in thousands; K); lane 2, reactivity of an RV-negative serum sample; lanes 3 to 5, reactivities of different RV IgG antibody-positive sera. The protein corresponding to the viral E1 protein is shown.

0.690) could be seen, when they were compared with the whole-virus antigen.

DISCUSSION

In the present study, we abundantly expressed the E1 and E2 envelope glycoproteins of RV in recombinant baculovirus (Ac701-RVE)-infected *S. frugiperda* Sf9 cells and examined the immunological characteristics of this antigen when it was purified by immunoblotting and EIA.

The recombinant antigen (Fig. 1, lane 5) contained, after staining with Coomassie blue, a diffuse band that migrated somewhat faster than the authentic E1 protein (58 kDa) of RV did. Also, a band corresponding to the E2 protein of RV was shown to migrate faster than authentic E2 protein (42 to 47 kDa). This is in agreement with the earlier findings of Oker-Blom et al. (20) and is believed to be caused by differences in glycosylation between Sf9 and vertebrate cells, as has also been demonstrated in a recent study in which the influenza virus hemagglutinin was expressed (12). In addition, the antigen contained a protein which could be seen as a band above the E1 protein. This protein is most likely the same cellular protein which exists in whole-virus preparations and may be captured by the rabbit anti-RV antibodies with recombinant antigen in immunoaffinity chromatography.

Recombinant polypeptides were identified by immunoblotting by using rabbit anti-RV antibodies (Fig. 2). In extracts from *S. frugiperda* Sf9 cells infected with the recombinant baculovirus (Fig. 2, lane 3), the E1 protein and a few additional cellular proteins could be seen; they were also reactive in wild-type baculovirus-infected cells (Fig. 2, lane 2).

Lane 4 of Fig. 2 represents the purified recombinant antigen. In addition to the E1 and E2 polypeptides, there exists an immunologically reactive protein (90 to 95 kDa) that is presumed to be a polyprotein precursor. It has been reported that the cDNA encoding the spike proteins of RV is expressed in recombinant baculovirus-infected Sf9 cells as a polyprotein, which is processed to form two smaller proteins that are equivalent to the E1 and E2 proteins of RV (20). The cellular protein, which was reactive with rabbit anti-RV antibodies, was also detectable by immunoblot analyses.

The antigenic characteristics of the recombinant proteins were further analyzed by immunoblotting with human RV antibody-positive sera (Fig. 3). These sera showed reactivities with the E1 protein, although the reactions were weak. Under these conditions, which presumably depend on the quantity of the proteins, there were no visible reactions with the polyprotein or the E2 protein.

To study the suitability of the recombinant antigen as a diagnostic reagent in RV antibody assays, a total of 106 serum samples, including RV antibody-positive and -negative serum samples, were tested by EIA (Fig. 4). The correlation of the reactivities between whole-virus antigen and the recombinant antigen was good ($r = 0.861$) in this material that showed similar antigenic activities.

We demonstrated by immunoblotting that, in some cases of recent RV infection, the IgG antibodies reacted strongly with the capsid protein and that the reactivities with other structural proteins remained low (Fig. 5). We tested sera from patients with recent RV infection and with RV IgM antibodies with recombinant antigen to determine whether this difference in reactivities with structural proteins was visible in EIA. Also, it has been shown that, early after RV infection, the avidity of IgG antibodies is low and increases

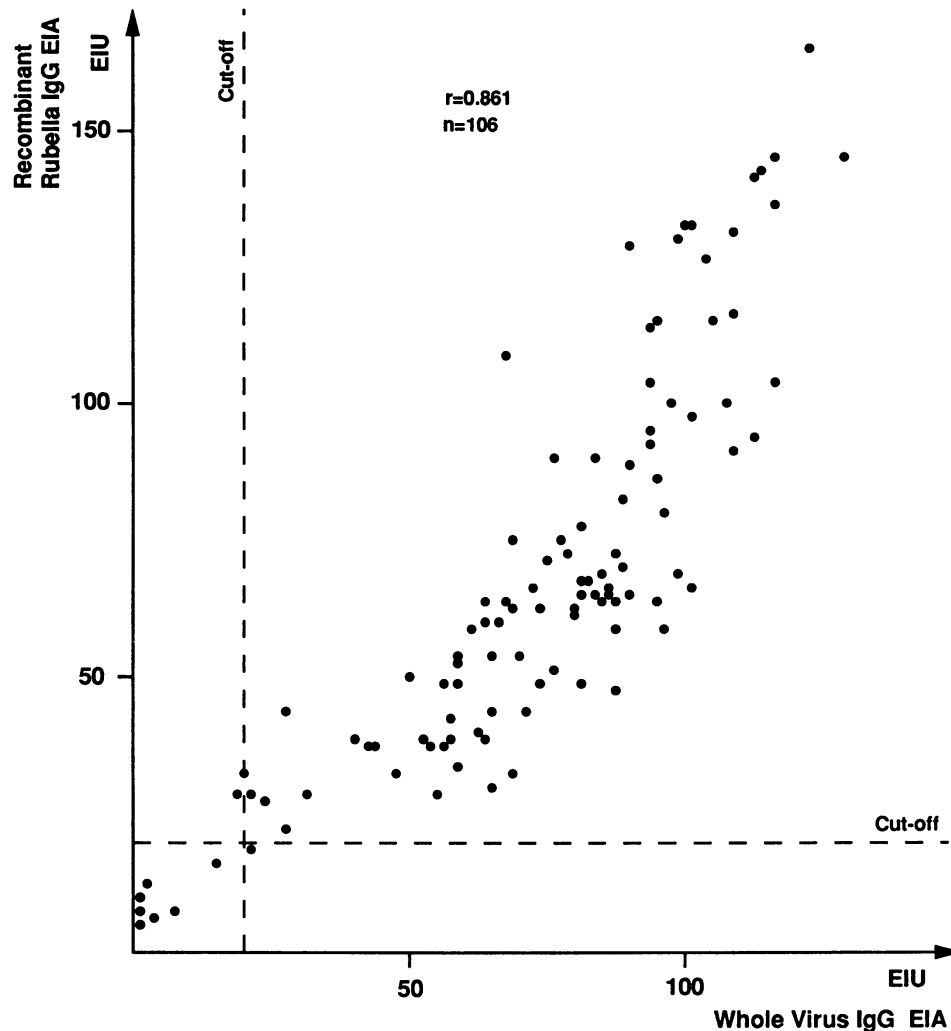


FIG. 4. Correlation of the reactivities of the RV IgG antibody-positive and -negative sera between recombinant RV and whole-virus antigen by EIA. $EIU = [(A_{\text{sample}} - A_{\text{rb}})/(A_{\text{pc}} - A_{\text{rb}})] \times 100$, where A_{sample} is the mean absorbance of the patient sample, A_{rb} is the mean absorbance of the reagent blank, and A_{pc} is the mean absorbance of the positive control.

with time (4, 6, 17). However, all of the sera used in this study were reactive, although the correlation of reactivity between recombinant and whole-virus antigen ($r = 0.690$) was not as close as that with convalescent-phase sera.

Our preliminary results of using the recombinant antigen in the IgM assays indicated that the reactivities of IgM antibodies were weak with the recombinant proteins in indirect EIA (data not shown). We have not tested the reactivities of the recombinant antigen in an antibody capture EIA, in which the antigen can be labeled, as has been done previously with whole-virus antigen (23), or can be used with labeled anti-RV antibodies. In this type of assay, the recombinant proteins may be more reactive with IgM antibodies.

In conclusion, we expressed RV envelope proteins in recombinant baculovirus-infected lepidopteran insect cells and purified the proteins by immunoaffinity chromatography. Our results demonstrate that their antigenicities in RV IgG EIA correlate well with those of whole-virus antigen. Thus, the recombinant proteins have a potential for use in the routine detection of human RV IgG antibodies.

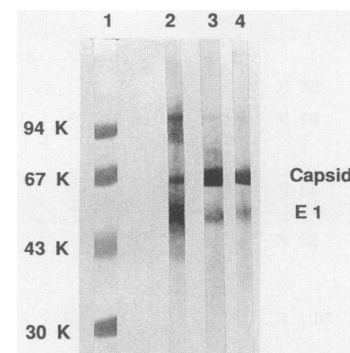


FIG. 5. Reactivities of sera from patients with recent RV infections as determined by immunoblot analyses by using nonreduced whole-virus antigen. Lane 1, molecular weight markers (in thousands; K); lane 2, reactivities of RV IgG antibodies in serum from a patient with remote RV infection; lane 3 and 4, reactivities of RV IgG antibodies in sera from patients with recent RV infections. The capsid and E1 proteins are indicated.

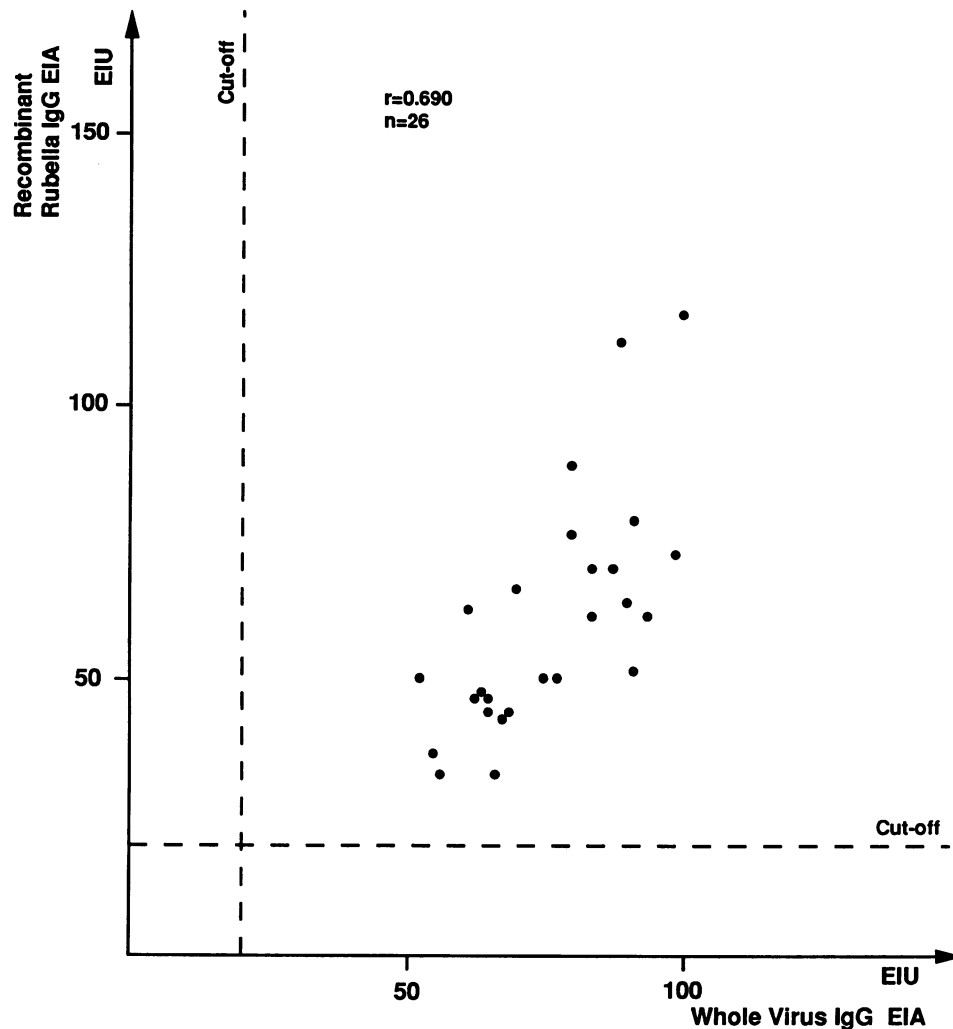


FIG. 6. Correlation of the reactivities of RV IgG antibodies between recombinant and whole-virus antigen in EIA when tested with sera from patients with a recent RV infection. See the legend to Fig. 4 for the definition of EIU.

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