Antibody Response to Epstein–Barr Virus Rta Protein in Patients with Nasopharyngeal Carcinoma

A New Serologic Parameter for Diagnosis

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BACKGROUND. Nasopharyngeal carcinoma (NPC) is associated closely with Epstein–Barr virus (EBV). The authors previously reported that an EBV immediate-early gene, *BRLF1*, was expressed frequently in NPC tumors, and a significant elevation in immunoglobulin G (IgG) antibodies directed against *BRLF1* gene product Rta was detected in NPC sera by a radioactive immunoprecipitation assay. To simplify and to make the detection more quantitative, an enzyme-linked immnunosorbent assay (ELISA) was developed in this study.

METHODS. Antigen domains of Rta were identified further using an immunoprecipitation assay. Two glutathione-S-transferase (GST) recombinant Rta fragments (R150-GST and R185-GST) were prepared subsequently and were used as antigens in the ELISA. Serum samples derived from 51 patients with NPC patients, 115 non-NPC ENT patients, and 47 healthy volunteers were examined for the presence of antibodies directed against Rta.

RESULTS. Among the patients with NPC, 74.5% showed a positive IgG response to R150-GST, and 62.7% showed a positive IgG response to R185-GST, with 80.4% positive for either fragment. In contrast, the reactions were positive in only 8.5% of healthy volunteers and 13.0% of control patients. When using a mixture of the two recombinant Rta proteins as coating antigens, the IgG positive responses were 82.3%, 10.6%, and 14.8%, respectively, in patients with NPC, healthy volunteers, and control patients. It is noteworthy that 51.0% of the NPC sera showed a positive immunoglobulin A (IgA) response, with none of the control patients showing obvious reactivity. Both the IgG response and the IgA response to Rta protein in patients with NPC were correlated with the IgA response to EBV early antigens and virus capsid antigens, the classic serologic markers used to diagnose NPC.

CONCLUSIONS. The ELISA method described for the detection of IgG antibodies directed against recombinant Rta proteins is simple and reliable and may be useful as a serologic parameter for the screening and diagnosis of patients with NPC. *Cancer* 2001;92:1872–80. © *2001 American Cancer Society.*

KEYWORDS: Epstein–Barr virus, *BRLF1* transcription activator, nasopharyngeal carcinoma, enzyme linked immunosorbent assay, serologic test.

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Epstein–Barr virus (EBV) is a ubiquitous γ-herpes virus that infects more than 90% of the human population and establishes a lifelong viral persistence in the host. The EBV genome is a 172,000 base-pair (bp), double-stranded DNA that encodes more than 100 genes, including at least 10 latent genes and more than 80 lytic genes. A spectrum of malignancies of lymphoid and epithelial cell origin are associated with EBV, such as Burkitt lymphoma, T-cell lymphoma, Hodgkin disease, gastric carcinoma, and nasopharyngeal carcinoma (NPC). NPC is the most consistently EBV-associated tumor, with a

100% detection rate of EBV genes in patients with the undifferentiated type of NPC.[1] The etiologic link between NPC and EBV was established originally on the basis of serologic evidence. The serum titers of immunoglobulin A (IgA) antibodies directed against the EBV early antigens (EA) and viral capsid antigens (VCA) were much greater in patients with NPC compared with matched controls.[2] These serologic tests have proven to be of diagnostic and prognostic value.[3,4] The presence of the EBV genome in almost every NPC malignant epithelial cell was confirmed regardless of geographic origin.[5,6] The monoclonality of the viral DNA indicated that the malignancy had arisen from a clonal expansion of a single EBV-infected progenitor cell.[7] It has been generally accepted that the EBV infection in patients with NPC is predominantly latent, with consistently detectable mRNAs of latent genes (*EBNA-1*, *LMP1*, *LMP2*, and *EBER*) in tumor cells.[1,8] Most previous studies have concentrated on the possible oncogenic role of EBV latent antigens in NPC. However, increasing evidence suggests that EBV lytic infection occurs in NPC and probably plays an important role in the development of this tumor. The evidence of EBV reactivation in NPC includes the isolation of infectious EBV from tumor cells,[9] the presence of the linear form of the EBV genome,[7] and the detection of cell free EBV DNA in plasma from most patients with NPC, indicating a productive virus release into the blood.[10] Recently, we demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) that the EBV lytic gene *BRLF1*, one of the immediate-early genes crucial for the initiation of EBV lytic cycle, was expressed frequently in NPC tumor biopsies. Furthermore, immunoglobulin G (IgG) antibodies directed against the *BRLF1* transcription activator (Rta) in patients with NPC also were detected, suggesting *BRLF1* expression at the protein level.[11]

NPC is the most common form of nasopharyngeal malignancy, with more than 80,000 new cases each year.[12] Humoral immune response to both EBV latent and lytic antigens has been observed frequently in patients with NPC. Although the protective value of these EBV specific IgA and IgG antibodies usually is insignificant, their unusual serologic characteristics have been applied to assist in the diagnosis and prognosis of patients with NPC. The detection of IgA antibodies directed against EA and VCA traditionally is conducted by an indirect immunofluorescence assay (IFA) that is inconvenient and technically demanding. More recently, serologic tests using various EBV recombinant antigens have been developed. These antigens include the EBV-encoded alkaline DNase, DNA polymerase, thymidine kinase,[13] ribonucleotide reductase,[14] membrane antigen,[15] transcription activa-

tor Zta,[16] and latent antigen EBNA1.[17] However, the sensitivity and specificity of these tests evaluated at different laboratories were inconsistent.[13,18,19] To date, the conventional IFA used for detecting IgA directed against EA and VCA still seems to be the most reliable serologic marker for NPC.[19] Patients with early-stage NPC respond well to radiotherapy, with a 5-year survival rate of 70–80%, whereas the survival rate in patients with advanced disease drops to 20–40%.[3] Therefore, the development of a more sensitive and convenient assay for screening high-risk populations would be of great benefit.

Using an immunoprecipitation assay, we previously reported that the IgG antibodies against Rta were increased significantly in patients with NPC compared with healthy controls.[11] In the current study, we explored further the diagnostic value of the Rta specific antibodies for NPC by establishing an enzyme-linked immunosorbent assay (ELISA). Two recombinant proteins that had antibody-binding regions in Rta were produced and were used subsequently as antigens in the ELISA test. NPC and control sera were examined. The results demonstrate that the IgG antibody titers directed against Rta proteins are significantly higher in NPC. This ELISA test is sensitive and specific, and it may be useful as a diagnostic and screening parameter.

MATERIALS AND METHODS
NPC and Control Sera
Serum samples were taken from untreated patients attending the ENT clinics at Singapore National University Hospital and Singapore General Hospital, and informed consent was obtained from each patient. Fifty-one patients were confirmed histologically with undifferentiated NPC. The patient control group included 115 non-NPC ENT patients with complaints, such as epistaxis, tinnitus, vertigo, otitis media, sinusitis, and vasomoto rhinitis, but who were histologically negative for NPC. In addition, 47 healthy volunteers were included. All sera were stored at −20 °C until use.

Plasmid Construction
Plasmid pKT-R150C (Fig. 1) containing the 450-bp C-terminal fragment of *BRLF1* was subcloned from pKT-R222C[11] by polymerase chain reaction (PCR). The primers used were 5′-ATCCCATGGAATTCCCGT-GGGCCAACCGG-3′ (sense), and 5′-TGCTCTAGACTA-AAATAAGCTGGTGTCAAAAATAG-3′ (antisense). Nco I and Xba I restriction enzyme sites were introduced into the primers, and the amplified fragment was cloned subsequently into Nco I-Xba I digested pKT vector. Two additional internal truncates of *BRLF1*, R150I and R185I, were subcloned into pGEX-5X-3 ex-
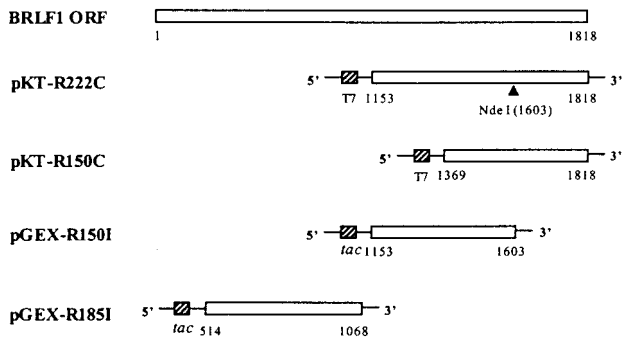
FIGURE 1. Plasmid construction. *BRLF1* ORF indicates the 1818-base-pair (bp), full-length open reading frame of the immediate-early gene *BRLF1*. Plasmid pKT-R150C was subcloned from pKT-R222C for in vitro transcription and translation. pGEX-R150I and pGEX-R185I each contained an internal truncate of *BRLF1* and were expressed subsequently in bacteria to generate two recombinant glutathione-S-transferase (GST) fusion Rta proteins, R150-GST and R185-GST.

pression vector (Amersham Pharmacia Biotech Inc., Piscataway, NJ) under the control of the *tac* promoter (Fig. 1), which could be induced using isopropyl β-D-thiogalactoside (IPTG). pGEX-R150I contained a 450-bp internal *BRLF1* fragment from nucleotides 1153–1603, which was obtained by cutting with Pvu II and Nde I from pKT-R222C plasmid.[11] After blunting the ends with the Klenow fragment of DNA polymerase, the inserts were ligated into the Sma I-linearized pGEX-5X-3 (Fig. 1). The pGEX-R185I was generated by subcloning the 555 bp of *BRLF1* internal truncate from pKT-R435C plasmid[11] into pGEX-5X-3 (Fig. 1). The Pvu II-Apa I-digested inserts were blunted with the Klenow fragment and subsequently cloned into the Sma I-linearized pGEX-5X-3. Both inserts were cloned in frame with the upstream start codon for fusion protein glutathione-S-transferase (GST) and were confirmed by sequencing.

In Vitro Transcription, Translation, and Immunoprecipitation

The plasmid pKT-R150C was transcribed and translated in vitro in the presence of [^{35}S]-methionine using the transcription and translation (TnT) system (Promega, Madison, WI) to generate a 150-amino-acid, C-terminal Rta protein, R150C, according to the manufacturer's instructions and a procedure described previously.[11] Protein R150I was obtained by TnT reaction using the Nde I linearized plasmid pKT-R222 (Fig. 1). Immunoprecipitation assay was performed as described previously.[11]

Expression and Purification of the Recombinant R150-GST and R185-GST Fusion Proteins

E. coli BL21 (DE3) cells (Amersham Pharmacia Biotech Inc.) were transformed separately with pGEX-R150I, pGEX-R185I, and pGEX-5X-3. The recombinant clones were grown overnight at 37 °C in 20 mL of Luria-Bertani (LB) broth with 100 μg/mL of ampicillin. One liter of fresh LB medium was inoculated with the overnight culture at 1:100 dilution and incubated at 30 °C with vigorous shaking until the level of the optical density at 600 nm (OD)$_{600}$ reached 0.6–0.8. IPTG (Bio-Rad Laboratories, Hercules, CA) was added to a final concentration of 0.1 mM, and the culture was grown for 3 hours. All subsequent steps were performed at 4 °C except where indicated. Cells were harvested by centrifugation at ×4000 *g* for 10 minutes, and resuspended in 100 mL of lysis buffer (PBS containing 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride), and disrupted by sonication. The supernatant was collected by centrifugation at ×12,000 *g* for 15 minutes, and 1 mL of the 50% slurry of glutathione sepharose 4B (Amersham Pharmacia Biotech Inc.) equilibrated with PBS was added. After incubation for 30 minutes at room temperature with rotation, the matrix was loaded into a disposable column (Bio-Rad Laboratories) and washed three times with 10 mL of lysis buffer. The recombinant GST fusion proteins and free GST proteins were eluted with 1–3 mL of elution buffer (50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione; Aldrich Chemical Company, Milwaukee, WI) by incubation for 10 minutes at room temperature. The protein concentration was determined using the microtiter plate protocols provided by the Bio-Rad protein assay kit (Bio-Rad Laboratories). Aliquots of the elution were stored at −20 °C until use.

### Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

To analyze protein expression, the various samples collected were denatured by boiling in a solution containing 2% sodium dodecyl sulfate (SDS); 1% β-mercaptoethanol; 50 mM Tris-HCl, pH 6.8; 10% glycerol; and 0.3% bromophenol blue for 5 minutes followed by separation on duplicate SDS-polyacrylamide gel electrophoresis (PAGE) gels (10% polyacrylamide). After electrophoresis, one of the gels was stained with Coomassie brilliant blue, and the other gel was transferred to nitrocellulose membranes (Stratagene, Cedal Creek, TX) using a semidry electrophoretic transfer cell (Bio-Rad Laboratories). The blotted membrane was incubated in blocking buffer (5% nonfat milk, 20 mM Tris, 150 mM sodium cloride, and 0.1% Tween-20, pH 7.6) overnight at 4 °C with gentle shaking and then reacted

with 1:10,000 diluted goat anti-GST polyclonal antibody (Amersham Pharmacia Biotech Inc.) for 1 hour at room temperature with shaking. Horseradish peroxidase (HRP)-conjugated rabbit antigoat immunoglobulin (Dako A/S, Glostrup, Denmark) at 1:10,000 dilution was added and incubated for 1 hour. Signals were detected by using an ECL Plus Western blotting detection system (Amersham, Buckinghamshire, United Kingdom) and exposing to an X-ray film.

To confirm the antigenicity of the recombinant Rta fragments, four IgG-Rta positive serum samples and four negative serum samples that were identified in our previous study[11] were employed to perform Western blot analysis. A mixture of R150-GST, R185-GST, and free GST was loaded into a single preparation well and separated by SDS-PAGE. After electric transfer and blocking, the blotted membrane was cut into strips, and each strip was reacted separately with a 1:1000 diluted serum sample. Anti-GST antibody (1:10,000 dilution) was included as a control. HRP-conjugated rabbit F(ab′)$_2$ antihuman IgG (Dako A/S) at 1:1000 dilution or rabbit antigoat Ig at 1:10,000 dilution was used as the secondary antibody. The remaining detection procedures were the same as those described above.

ELISA

The purified R150-GST and R185-GST were used separately or in combination to coat 96-well polystyrene microtiter plates (Immuno II; Dynatech, South Wundhanm, ME) at a volume of 100 μL per well by incubation overnight (16–18 hours) at room temperature. The proteins were prediluted in carbonate buffer, pH 9.6, at a final concentration of 1 μg/mL, and the optimal concentration was determined by titration against standard IgG-Rta positive sera.[11] The plates were washed five times with PBS supplemented with 0.05% Tween-20 (PBST), and nonspecific binding sites were blocked with 100 μL per well of Tris-based diluents containing 1% bovine serum albumin (Sigma, St. Louis, MO), 3% normal goat serum, and 1% skim milk for 1 hour at room temperature. The plates were washed another five times before 100 μL of diluted serum (1:50) in Tris-based diluents were added. The plate was incubated for 1 hour at 37 °C followed by six washes in PBST. HRP-conjugated goat F(ab′)$_2$ antihuman IgG (1:500 dilution) or rabbit antihuman IgA (1:500 dilution; Dako A/S) was added at 100 μL per well and incubated for 30 minutes at 37 °C. The plate was then washed six times in PBST, and a colorimetric reaction was developed with 100 μL per /well of enzyme substrate o-phenylenediamine (Sigma) at 0.5 mg/mL dissolved in 0.1 M citrate/phosphate buffer, pH 5.5, containing H$_2$O$_2$. After a 15-minute incubation
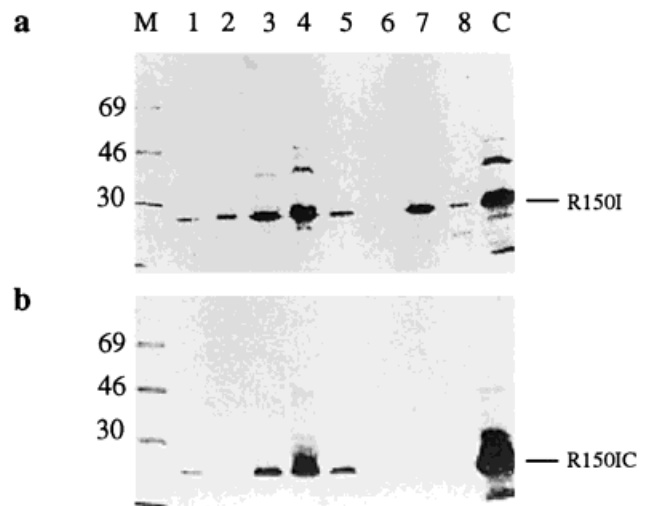
FIGURE 2. Defining antigen regions in Rta C-terminal part using immunoprecipitation assay. (a) The 150-amino-acid internal truncate of Rta (R150I) was generated by TnT reaction containing [^{35}S]methionine using the Nde-I linearized pKT-R222C plasmid (lane C; see Fig. 1). Among the eight immunoglobulin G (IgG)-Rta positive sera, seven were positive, and one was negative for precipitation of R150I, the same that was observed previously using R222C as the antigen.[11] (b) pKT-150C plasmid was translated in vitro to generate a 150-amino-acid C-terminal truncate of Rta (R150C) (lane C). IgG antibody directed against this region was detected using the same panel of sera that was used in a. Only four sera (lanes 1, 3, 4, and 5) were capable of precipitating this protein (lanes 1–8). Lane M: molecular mass markers (in kiloDaltons).

in the dark at 37 °C, the reaction was stopped by adding 50 μL per well of 4 N H$_2$SO$_4$. The OD was measured at 490 nm with a 620 nm reference filter. IgG-Rta positive and negative control sera were included in each test. All tests were carried out in duplicate. The cut-off point of IgG reactivity was at an OD value of 0.20 for both R150-GST and R185-GST, 0.33 for the combination of both proteins, and 0.12 for IgA.

IFA

IgA antibodies directed against EBV EA and VCA were detected using IFA as described previously.[20]

Statistical Analysis

Differences in ELISA OD mean values between the groups were compared using a Student unpaired, two-tailed t test. A comparison of the positive rates of antibodies directed at recombinant Rta proteins between patients with NPC and the two control groups was performed with the chi-square test. Linear correlations between the ELISA OD values of antibodies directed at recombinant Rta proteins and the IgA titers
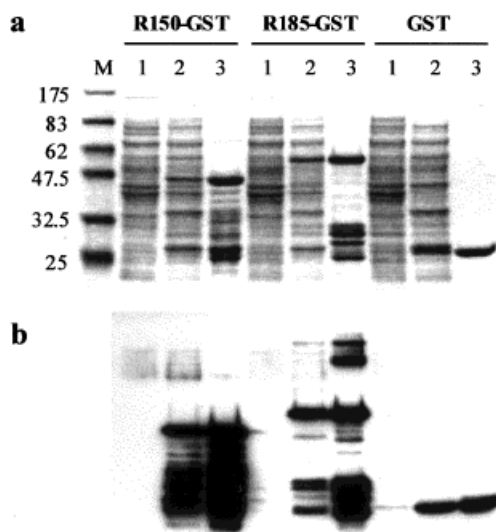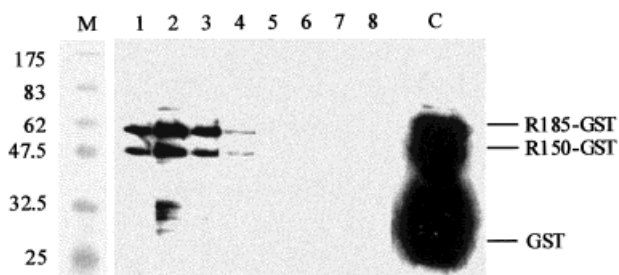
FIGURE 3. Analysis of the expression and purification of the glutathione-S-transferase (GST) recombinant Rta proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. (a) A 10% SDS-PAGE gel stained with Coomassie brilliant blue. (b) Western blot recognized by an anti-GST antibody. Plasmids pGEX-R150I, pGEX-R185I, and pGEX-5X-3 were transformed separately into BL21 (DE3) cells. Expression of the GST recombinant proteins R150-GST, R185-GST, and free GST was induced by 0.1 mM isopropyl β-D-thiogalactoside in Luria-Bertani (LB) medium. Supernatant of cell lysates before and after induction were compared as shown in lanes 1 and 2 for each protein. The recombinant proteins were purified by affinity chromatography using the glutathione sepharose 4B matrix (lane 3). Lane M: molecular mass markers (in kiloDaltons).


FIGURE 4. Western blot analysis to confirm the antigenicity of the glutathione-S-transferase (GST) recombinant Rta proteins (R150-GST and R185-GST). A mixture of R150-GST, R185-GST, and free GST were separated on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The anti-GST antibody reacted to all the three GST-containing proteins (lane C). Four immunoglobulin G (IgG)-Rta positive sera recognized both R150-GST and R185-GST without cross reactivity to the free GST protein (lanes 1–4), and four IgG-Rta negative sera failed to react to these proteins (lanes 5–8). Lane M: molecular mass markers (in kiloDaltons).

directed at EA or VCA were performed with a Spearman rank test.

RESULTS

Defining Antigen Regions at the Rta C-Terminal

We previously described that the antigen regions reactive to the IgG antibodies in NPC sera were located at the C-terminal two-thirds of Rta. To define further the antigen regions within the 222-amino-acid C-terminal part, we divided this region into two overlapping fragments with each containing 150 amino acids (Figs. 1, 2). The internal truncate R150I was the TnT product of a linearized pKT-R222C plasmid, which had been cut with Nde I at the two-thirds position of the original insert prior to the TnT reaction (Figs. 1, 2a, lane C). The C-terminal R150C was generated by subcloning the corresponding open reading frame of *BRLF1* into pKT vector under the control of T7 polymerase promoter followed by in vitro TnT synthesis in the presence of [35S] methionine (Fig. 2b, lane C). These two radiolabeled, overlapping proteins were used as antigens in an immunoprecipitation assay using eight serum samples that were examined previously.[11] The results showed that the pattern of serum reaction to the internal R150I proteins was the same as the original R222C fragment: seven positive reactions and one negative reaction (Fig. 2a, lanes 1–8).[11] In contrast, the sera in lanes 2, 7, and 8 in Figure 2a that were positive for precipitating R222C[11] failed to precipitate the R150C fragment (Fig. 2b). It is obvious that, within the R222C region, the main antigen epitope binding the reactive antibodies in NPC sera is located at the R150I fragment; thus, this fragment was used in later experiments for developing the ELISA.

Expression and Purification of the Recombinant Proteins R150-GST and R185-GST

The *BRLF1* open reading frames encoding R185I and R150I were cloned separately into pGEX-5X-3 expression vector. The expression of GST fusion proteins was conducted in *E. coli.* Figure 3 shows that both 45-kiloDalton (kDa) R150-GST and 49.2-kDa R185-GST, which resulted from the fusion of the 27-kDa GST to the 18-kDa R150I or the 22.2-kDa R185I, respectively, were detected in the supernatant of the cell lysates, whereas only the 27-kDa GST protein was produced from the vector-transformed cells. Recombinant proteins were purified by affinity chromatography using the glutathione sepharose 4B matrix. The yield after purification was about 4–6 mg per liter culture for both GST fusion proteins. The antigenicity of the recombinant proteins was confirmed by Western blot analysis using IgG-Rta positive and negative sera. Figure 4 shows that the mixture of R150-GST, R185-GST, and free GST proteins were separated by SDS-PAGE and detected by the anti-GST antibody as a control
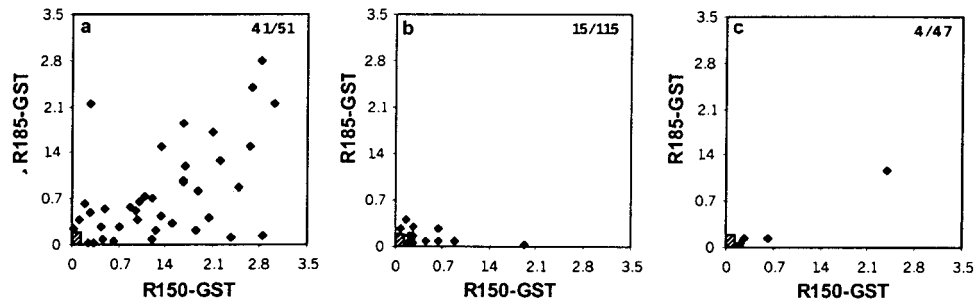
FIGURE 5. Immunoglobulin G (IgG) antibody response to the two glutathione-S-transferase (GST) recombinant Rta protein fragments (R150-GST and R185-GST) determined by enzyme-linked immnunosorbent assay. The shaded area indicates the location of negative samples in which the optical density (OD) values were < 0.20 for both IgG-R150 and IgG-R185. (a) Of 51 serum samples from patients with nasopharyngeal carcinoma (NPC), 38 were positive for IgG-R150, and 32 were positive for IgG-R185, and 41 sera reacted to either one of the two Rta proteins (80.4%). The mean OD values were 1.07 ± 0.92 for IgG-R150 and 0.61 ± 0.70 for IgG-R185 ($P > 0.05$). (b) Fifteen of 115 patient control sera were positive for either IgG-R150 or IgG-R185 (13.0%), with 13 sera reacting to R150-GST, and 4 reacting to R185-GST. (c) Only 4 of 47 serum samples from healthy volunteers were positive for IgG-R150, and 1 also reacted to R185-GST. The positive rate was significantly higher in NPC sera than in the control sera ($P < 0.01$).

TABLE 1
Enzyme-Linked Immunosorbent Assay Detection of Antibodies Directed Against Recombinant R150 and/or R185 Glutathione-S-Transferase Fusion Proteins in Patients with Nasopharyngeal Carcinoma and Controls

| Antibody | NPC ($n = 51$) | | Healthy controls ($n = 47$) | | Patient controls ($n = 115$) | |
	Positive	Sensitivity (%)	Positive	Specificity (%)	Positive	Specificity (%)
IgG-R_{150}/R_{185}	41	80.4	4	91.5	15	87.0
IgG-$R_{150+185}$	42	82.3	5	89.4	17	85.2
IgA-$R_{150+185}$	26	51.0	0	100.0	0	100.0
IgA-EA	43	84.3	—	—	2	98.3
IgA-VCA	49	96.1	—	—	36	68.7

NPC: nasopharyngeal carcinoma; IgG-R_{150}/R_{185}: immunoglobulin G (IgG) antibodies responding to one of the two glutathione-S-transferase (GST) recombinant Rta protein fragments (R_{150} and R_{185}); IgG-$R_{150+185}$: IgG antibodies responding to both GST recombinant Rta protein fragments; IgA: immunoglobulin A; EA: early antigens; VCA: virus capsid antigens.

(Fig. 4, lane C). All four IgG-Rta positive sera could recognize both recombinant proteins without interaction with the free GST protein (Fig. 4, lanes 1–4), whereas all four IgG-Rta negative sera failed to produce any positive signals in the Western blot analysis (Fig. 4, lanes 5–8). The results indicate that the recombinant Rta-GST fusion proteins could be used as antigens to develop an ELISA to detect the specific antibodies against Rta because of their representative antigenicity and the absence of cross reactivity to GST in human sera.

Detection of Antibodies Directed Against Rta by ELISA

To detect the antibodies directed against Rta, the recombinant proteins used to coat the 96-well plates were 100 ng per well, and the serum dilution was 1:50 according to a checkerboard titration (data not shown). First, the distinct antibody response to each of the two Rta recombinant fragments was deter-

mined. R150-GST or R185-GST was coated separately onto a 96-well microtiter plate, and the corresponding IgG antibodies (IgG-R150 or IgG-R185) in serum samples were detected by ELISA. Sera from 51 patients with NPC, 115 control patients, and 47 healthy volunteers were tested: The results are shown in Figure 5 and Table 1. ELISA OD readings > than 0.20 were defined as positive, and the shaded areas in Figure 5 indicate the sera that were negative for both proteins. Of the patients with NPC, 38 patients were positive for IgG-R150 (74.5%), and 32 patients were positive for IgG-R185 (62.7%). The mean OD values (± standard deviation) were 1.07 ± 0.92 for IgG-R150 and 0.61 ± 0.70 for IgG-R185 (Fig. 5a, Table 1). Forty-one sera (80.4%) reacted positively to either one of the two recombinant proteins, and, among them, 29 sera (70.7%) were positive for both proteins. In contrast, only 15 of 115 sera from control patients (13.0%) reacted positively to either of the two Rta fragments (Fig.
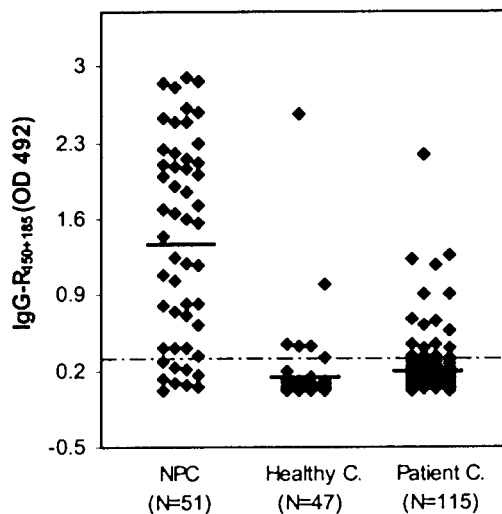
FIGURE 6. Detection of immunoglobulin G (IgG) antibodies (IgG-R$_{150+185}$) responding to the two glutathione-S-transferase (GST) recombinant Rta protein fragments (R150-GST and R185-GST) in patients with nasopharyngeal carcinoma (NPC) and in controls, including healthy volunteers (Healthy C.) and control patients (Patient C.), by enzyme-linked immnunosorbent assay (ELISA). ELISA was conducted using a mixture of R150-GST and R185-GST as coating antigens. The dashed line indicates the cut-off value for optical density (0.33). Forty-two of 51 NPC sera samples (82.3%) were positive for IgG-R$_{150+185}$, which was significantly higher than the positive rate in healthy volunteers (10.6%) and control patients (14.8%; $P < 0.01$). The mean OD values for each group are indicated by bars: 1.41 ± 0.92 for patients with NPC, 0.15 ± 0.40 for healthy volunteers, and 0.22 ± 0.30 for control patients. The differences in the mean OD value between NPC patients and controls were statistically significant ($P < 0.01$).

5b, Table 1), with 13 samples positive for IgG-R150 (11.3%) and 4 samples positive for IgG-R185 (3.4%). Similarly, among the sera from 47 healthy volunteers, only 4 samples reacted positively to R150-GST (8.5%), with one of them also reacting to R185-GST (Fig. 5c, Table 1). The positive rate was significantly higher in NPC sera than in control sera ($P < 0.01$). These results indicate that there is indeed a much higher IgG response in patients with NPC toward Rta compared with control patients and volunteers, and a combination of R150-GST and R185-GST proteins for coating ELISA plates may simplify the test while maintaining its sensitivity.

Next, we determined the serum reactivity to a mixture of R150-GST and R185-GST proteins by using both proteins as antigens to coat the ELISA plates. IgG antibodies directed at the mixed recombinant Rta proteins (defined as IgG-R$_{150+185}$) were detected in the same panel of serum samples used previously: The results obtained are illustrated in Figure 6 and Table 1. The cut-off point of the ELISA OD reading was determined at 0.33 in this test. Forty-two of 51 NPC sera

(82.3%) reacted positively to the combined Rta protein fragments, with a mean ± standard deviation OD value of 1.41 ± 0.92 (Table 1). In contrast, only 5 of 47 healthy volunteers (10.6%) and 17 of 115 control patients (14.8%) were positive for IgG-R$_{150+185}$ ($P < 0.01$), with mean OD values of 0.15 and 0.22, respectively, for these two groups ($P < 0.01$; Table 1). The presence of IgA antibodies directed against the combination of R150-GST and R185-GST proteins (defined as IgA-R$_{150+185}$) was examined in the serum samples. Twenty-six of 51 NPC sera (51.0%) were positive (cut-off point, 0.12) for IgA-R$_{150+185}$, but neither of the two control groups revealed a positive IgA anti-Rta response ($P < 0.01$; Table 1).

The IgA antibodies directed against EBV lytic gene products, EA and VCA, are used currently as serologic diagnostic parameters for NPC. The IgA antibody titers reacting to EA and VCA in the 51 NPC sera and 115 control sera were determined by IFA, and the results are summarized in Table 1. IgA-EA was positive in 84.3% of NPC patients, comparable to IgG-R$_{150+185}$ ($P > 0.05$), but in only 1.7% of the control sera, significantly lower than the positive rate of IgG-R$_{150+185}$ in the same group ($P < 0.01$). IgA-VCA was positive in 96.1% of NPC patients, significantly higher than IgG-R$_{150+185}$ ($P < 0.01$), whereas its positive rate in the control group was 31.3%, which was significantly higher than that of IgG-R$_{150+185}$. A correlation was observed between the ELISA OD values for IgG-R$_{150+185}$ and IgA-EA (correlation coefficient [r] = 0.575; $P < 0.01$) and IgA-VCA (r = 0.326; $P < 0.05$) titers (Fig. 7). IgA-R$_{150+185}$ also was correlated positively with both IgA-EA (r = 0.678; $P < 0.01$) and IgA-VCA (r = 0.414; $P < 0.01$; Fig. 7).

DISCUSSION

We demonstrated previously that the IgG antibody response to the EBV immediate-early gene product Rta was increased significantly in patients with NPC compared with healthy volunteers; thus, this serologic characteristic has the potential to be developed into a diagnostic parameter for patients with NPC.[13] In vitro translated, radioactive Rta was used for the immunoprecipitation detection. The yield of this preparation was low and expensive, and the immunoprecipitation assay was inconvenient and difficult to quantify.[13] Therefore, our objective was to develop an ELISA method for the detection of the anti-Rta antibodies.

Mapping the antigen regions of Rta within the 222 amino acids at the C-terminal demonstrated two antibody-binding regions: R150I and R150C. All sera that recognized R222C also could react to the internal truncate R150I, but only a proportion of these sera could react to the C-terminal truncate, R150C. Based on these
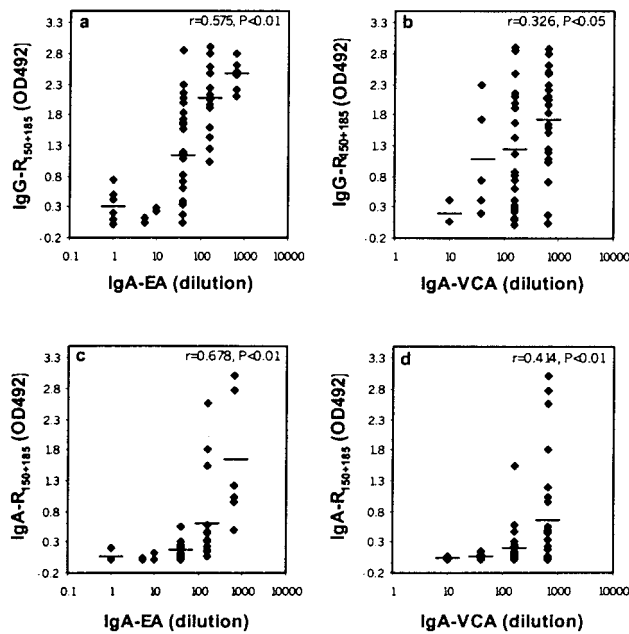
FIGURE 7. Correlations between the optical density (OD) values of immunoglobulin G (IgG) antibodies (IgG-R$^{150+185}$) and immunoglobulin A (IgA) antibodies (IgA-R$_{150+185}$) responding to the two glutathione-S-transferase (GST) recombinant Rta protein fragments (R150-GST and R185-GST) and the titers of IgA-early antigens (IgA-EA) or IgA-virus capsid antigens (IgA-VCA) in patients with nasopharyngeal carcinoma. (a) IgG-R$_{150+185}$ versus IgA-EA. (b) IgG-R$_{150+185}$ versus IgA-VCA. (c) IgA-R$_{150+185}$ versus IgA-EA. (d) IgA-R$_{150+185}$ versus IgA-VCA. A positive correlation was observed for all four combinations.

results, R150I was used as a substitute for R222C. Furthermore, another fragment of Rta, R185I, that could bind to the antibodies, as described previously,[13] also was included. The *E. coli*-produced, recombinant fusion proteins retained the Rta antigenicity, and, similar to previous reports,[21,22] the GST fragment in the fusion protein did not reveal any reactivity to the sera in our results and, thus, was not removed from the recombinant protein fragments.

Purified R150-GST and R185-GST were used subsequently to coat ELISA plates for the detection of specific anti-Rta antibodies. To identify further the antibodies for each of these two antigens, IgG antibodies directed against R150-GST and R185-GST were determined separately by ELISA. The results indicated that the antibody responses to the two proteins were mostly overlapping but with obvious exceptions. Therefore, these two Rta protein fragments were combined to increase the sensitivity of the ELISA method. In this test, IgG-R$_{150+185}$ was positive in 82.3% of NPC serum samples but in only 10.6% of healthy volunteer serum samples and 14.8% in control patient serum samples. Furthermore, an IgA responses were de-

tected in 51.0% of NPC serum samples, but in none of the sera from healthy volunteers or control patients.

IgA-EA and IgA-VCA traditionally have been the serologic parameters used most often for the diagnosis of patients with NPC due to their relatively high sensitivity and specificity, despite the inconvenience of IFA. Various efforts have been made to replace this IFA method with simplified ELISA methods. However, most tests have failed to maintain a comparable specificity and sensitivity.[18,19] The current study used an antigen Rta protein for ELISA that was shown to be expressed much more frequently in patients with NPC.[13] Because Rta is expressed in EBV lytic replication, similar to EA and VCA, it was conceivable that the antibody response would have a similar tendency. Indeed, this was true. The experimental data revealed a good correlation between these two methods. However, although the positive rate of IgG-R$_{150+185}$ in NPC was comparable to that of IgA-EA, it was less specific. Thus, IgG-R$_{150+185}$ does not seem to be a parameter that may replace IgA-EA completely for the diagnosis of NPC. However, it is important to note that, whereas most NPC sera responded equally well to EA and Rta, about 12% of the NPC sera showed reactivity to only one (Fig. 7), indicating a complementary feature of these two serologic parameters. Therefore, considering the convenience and reliability of the present ELISA method, it is of benefit to use IgG-R$_{150+185}$ in combination with IgA-EA in the serologic analysis of NPC. Furthermore, in laboratories that need to examine a large number of sera for NPC screening or diagnosis, the present ELISA method may be used preferentially to reduce the workload. Nevertheless, positive results should be interpreted with caution. Finally, the significance of IgG-R$_{150+185}$ for the monitoring of disease activity in patients with NPC should be studied in the future.

The preliminary results from this study indicate that multiple antigen regions located in the EBV Rta protein and the humoral immune responses against each region are distinct and complementary. The ELISA test to detect serum IgG-R$_{150+185}$ levels may be useful as a serologic parameter to assist in the diagnosis of NPC alone or in combination with IgA-EA.

REFERENCES

1. Rickinson AB, Kieff E. Epstein–Barr virus. In: Fields BN, Knipe DM, Howley PM, editors. Fields virology, 3rd ed, vol 2. Philadelphia: Lippincott-Raven, 1996:2397–446.
2. Henle G, Henle W. Epstein–Barr virus specific IgA serum antibody as an outstanding feature of nasopharyngeal carcinoma. *Int J Cancer* 1976;17:1–7.
3. Vokes EE, Liebowitz DN, Weichselbaum RR. Nasopharyngeal carcinoma. *Lancet* 1997;350:1087–91.

4. De-Vathaire F, Sancho-Garnier H, De-The H, Pieddeloup C, Schwaab G, Ho JHC, et al. Prognostic value of EBV markers in the clinical management of nasopharyngeal carcinoma (NPC): a multi-center follow-up study. *Int J Cancer* 1988;42: 176–81.

5. Wolf H, Zur-Hausen H, Becker V. EB viral genomes in epithelial nasopharyngeal carcinoma cells. *Nature* 1973;244: 245–47.

6. Huang DP, Ho JHC, Henle W, Henle G. Demonstration of Epstein–Barr virus associated nuclear antigen in nasopharyngeal carcinoma cells from fresh biopsies. *Int J Cancer* 1974;14:580–8.

7. Rabb-Traub N, Flynn K. The structure of the termini of the Epstein–Barr virus as a marker of clonal cellular proliferation. *Cell* 1986;47:883–89.

8. Brooks L, Yao QY, Rickinson AB, Young LS. Epstein–Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* 1992;66:2689–97.

9. Trumper PA, Epstein MA, Giovanella BC, Finerty S. Isolation of infectious EB virus from the epithelial tumor cells of nasopharyngeal carcinoma. *Int J Cancer* 1977;20:655–62.

10. Lo YMD, Chan LYS, Lo KW, Leung SF, Zhang J, Chan ATC, et al. Quantitative analysis of cell-free Epstein–Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res* 1999;59:1188–91.

11. Feng P, Ren EC, Liu D, Chan SH, Hu H. Expression of Epstein–Barr virus lytic gene BRLF1 in nasopharyngeal carcinoma: potential use in diagnosis. *J Gen Virol* 2000;81:2417–23.

12. Parkin DM, Stiemsward J, Muir CS. Estimates for the worldwide frequency of twelve major cancers. *Bull WHO* 1984;62: 163–82.

13. Littler E, Baylis SA, Zeng Y, Conway MJ, Mackett M, Arrand JR. Diagnosis of nasopharyngeal carcinoma by means of recombinant Epstein–Barr virus proteins. *Lancet* 1991;337: 685–9.

14. Gan YY, Fones-Tan A, Chan SH, Tsao SY, Li B, Tan WH. Molecular cloning and expression of Epstein–Barr virus antigens in the lambda gt11 expression vector: antibodies towards proteins from the BORF2 and BKRF4 reading frames in nasopharyngeal carcinoma patients. *Intervirology* 1994; 37:233–5.

15. Uen WC, Luka J, Pearson GR. Development of an enzyme-linked immunosorbent assay (ELISA) for detection IgA antibodies to the Epstein–Barr virus. *Int J Cancer* 1988;41:479–82.

16. Yip TTC, Ngan RKC, Lau WH, Poon YF, Joab I, Cochet C, et al. A possible prognostic role of immunoglobulin-G antibody against recombinant Epstein–Barr virus BZLF-1 transactivator protein ZEBRA in patients with nasopharyngeal carcinoma. *Cancer* 1994;74:2414–24.

17. Foong YT, Cheng HM, Sam CK, Dillner J, Hinderer W, Prasad U. Serum and salivary IgA antibodies against a defined epitope of the Epstein–Barr virus nuclear antigen (EBNA) are elevated in nasopharyngeal carcinoma. *Int J Cancer* 1990;45:1061–4.

18. Liu MY, Chang YL, Ma J, Yang HL, Hsu MM, Chen CH, et al. Evaluation of multiple antibodies to Epstein–Barr virus as markers for detecting patients with nasopharyngeal carcinoma. J *Med Virol* 1997;52:262–9.

19. Chan SH, Soo MY, Gan YY, Fones-Tan A, Sim PS, Chew CT. Epstein–Barr virus (EBV) antibodies in the diagnosis of NPC—comparison between IFA and two commercial ELISA kits. *Singapore Med J* 1998;39:263–5.

20. Feng P, Chan SH, Ooi EE, Soo MYR, Loh KS, Wang D, et al. Elevated blood levels of soluble tumor necrosis factor receptors in nasopharyngeal carcinoma: correlation with humoral immune response to lytic replication of Epstein–Barr virus. *Int J Oncol* 1999;15:167–72.

21. Thomas LM, Huntington PJ, Mead LJ, Wingate DL, Rogenson BA, Lew AM. A soluble recombinant fusion protein of the transmembrane envelope protein of equine infectious-anemia virus for ELISA. *Vet Microbiol* 1992;31:127–37.

22. McCauliffe DP, Yin H, Wang LX, Lucas L. Autoimmune sera react with multiple epitopes on recombinant 52 and 60 kDa Ro (SSA) proteins. *J Rheumatol* 1994;21:1073–80.