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Epstein-Barr Virus Nuclear Antigen 1 Linear Epitopes That Are Reactive with Immunoglobulin A (IgA) or IgG in Sera from Nasopharyngeal Carcinoma Patients or from Healthy Donors

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The entire amino acid sequence of the unique region of the EBNA 1 protein was synthesized as a set of 41 20-residue peptides with an overlap of 10 amino acids. The peptides were tested in the enzyme-linked immunosorbent assay for reactivity with immunoglobulin A (IgA) and IgG in sera from 50 patients with nasopharyngeal carcinoma (NPC) as compared with 36 serum samples from healthy Epstein-Barr virus (EBV)-seropositive donors and 5 serum samples from EBV-negative donors. The most immunoreactive peptide for both IgA and IgG binding was localized to the glycine-alanine repeat domain of the antigen. In the unique regions, 16 immunoreactive peptides were found. Of these, four were reactive with IgG but not IgA and three peptides were reactive with IgA but not IgG in NPC sera. In addition, several IgA and IgG epitopes on the carboxy-terminal region were specifically reactive with NPC sera, but unreactive with sera from healthy EBV-positive donors. The results suggest that EBV serology specific for individual epitopes may provide additional useful information not available by conventional serology with whole antigens or the EBNA complex.

Epstein-Barr virus (EBV) transformation is regularly accompanied by the expression of the EBV-determined nuclear antigen (EBNA), which can be detected by anticomplement immunofluorescence in all EBV-carrying immortalized cultured cells (27) and in the two EBV-associated human tumors Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (18). EBNA is an entity composed of at least six antigens, designated EBNA 1 through 6 (5, 28). In NPC biopsies, only EBNA 1 is consistently expressed by the malignant cells (8).

EBNA 1 is encoded by the *Bam*HI K fragment of the EBV genome (15) and is a DNA-binding phosphoprotein (11) that functions in the maintenance of the episomal state of the EBV genome (32). Anticomplement immunofluoresence-detectable antibodies to EBNA are a characteristic of the healthy EBV carrier state. Elevated EBNA titers are seen in Burkitt's lymphoma and NPC, whereas the EBNA titers are low or nil in immunodeficient patients with EBV-carrying lymphoproliferative disorders (14). Approximately 30% of EBNA 1 consists of a glycine-alanine repeat region which is flanked by unique regions (1). Studies with synthetic peptides have shown the glycine-alanine repeat to be an important antigenic site (6, 7, 29). Using an EBNA 1 fusion polypeptide lacking the glycine-alanine repeat region, Milman et al. (24) demonstrated that the unique carboxy-terminal region is also antigenic.

Elevated serum immunoglobulin A (IgA) antibodies against the EBV viral capsid and diffuse early antigen are useful diagnostic markers for NPC (13). In addition, IgA reactivity to several EBV-encoded proteins is selectively raised in NPC, namely, DNase (2), DNA polymerase (21), thymidine kinase (20), and ribonucleotide reductase (10).

Recently, we reported that 91% of NPC sera contain specific IgA to EBNA 1 glycine-alanine peptide compared with 13% of normal human sera and 11% of sera from patients with other malignancies (9). In 86% of NPC sera, we also detected IgA binding to a recombinant carboxy-terminal EBNA 1 protein lacking the glycine-alanine repeat. The present study was designed to define other immunoreactive epitopes in the EBNA 1 protein by systematic peptide synthesis. The positions of both the IgA- and IgG-reactive epitopes for sera from NPC patients and EBV-positive healthy individuals are reported.

MATERIALS AND METHODS

Sera. Ninety-one serum samples were tested in this study: 36 were from EBV-positive healthy individuals, and 50 were from patients with histologically proven NPC, collected during follow-up clinics at University Hospital, Kuala Lumpur. All normal sera and sera from cancer patients were predominantly from Malaysian Chinese, aged 30 years and older. The five EBV-negative sera were collected at the National Bacteriology Laboratory, Stockholm, Sweden.

Peptide synthesis. Forty-two 20-amino-acid peptides with a 10-amino-acid overlap, representing the deduced amino acid sequence of the unique regions of EBNA 1 were synthesized by using t-Boc amino acids (Bachem, Bubendorf, Switzerland) and *p*-methylbenzhydrylamine resin (Fluka, Buchs, Switzerland), in accordance with the multiple solid-phase peptide synthesis method of Houghten (16). Removal of the protecting groups from the formyl-tryptophan and methionine sulfoxide residues was achieved by cleavage with 25% hydrogen fluoride (31). The peptides were then cleaved from the resin with liquid hydrogen fluoride, using a multivessel apparatus (17). To note the position in the protein, the putative initiation codon was designated amino acid 1.

Synthetic peptide 1 corresponds to amino acids 2 to 21 in the *Bam*HI K fragment; peptide 2, to amino acids 12 to 31; peptide 3, to amino acids 22 to 31; up to peptide 9, with amino acids 72 to 91 at the glycine-alanine repeat junction.

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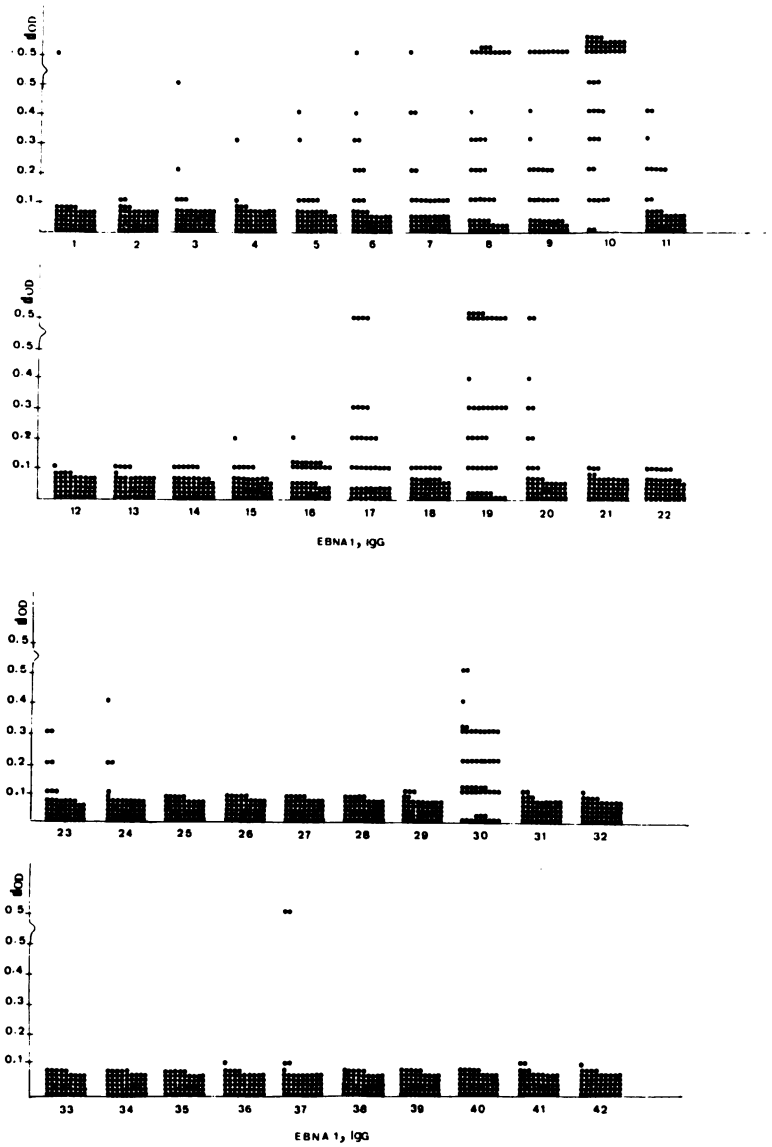

FIG. 1. Serum IgG reactivity to EBNA 1 protein in 50 NPC serum samples. Peptide number is along the abscissa. Peptide 1 represents the amino terminus of the viral protein, and each consecutive peptide is 10 amino acids closer to the carboxy terminus. Peptide 9 is in the junction of the unique amino-terminal region and the glycine-alanine repeat. Peptide 10 (p107) is the main epitope of the glycine-alanine repeat (7). Peptide 11 is in the junction of the glycine-alanine repeat and the unique carboxy-terminal region, and each consecutive peptide is then 10 amino acids closer to the carboxy terminus, which is peptide 42. Each dot represents the mean optical density of the duplicates of one serum sample after subtraction of the absorbance of the same serum sample reacted with uncoated wells (dOD).

Peptide 10 (p107), derived from the IR3 of the *Bam*HI K fragment, consists exclusively of alanines and glycines coupled to a terminal cysteine residue (6). Peptide 11, at the carboxy side of the glycine-alanine junction, corresponds to amino acids 332 to 351, peptide 12 corresponds to amino acids 342 to 361, and so on up to the carboxy-terminal peptide (peptide 42), with amino acids 642 to 661.

Enzyme-linked immunosorbent assay (ELISA). The synthetic peptides, diluted to 20 μg/ml in 10 mM carbonate buffer, pH 9.6, were added (50 μl/well) to microtiter wells (Immulon II; Dynatech Industries, McLean, Va.) and kept overnight at 4°C. After two washes with phosphate-buffered saline (PBS)–0.05% Tween 20 (PBS-T), the plates were blocked with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) in PBS for 60 min at room temperature. The plates were washed twice in PBS-T, and human sera diluted 1:50 in PBS-T–1% bovine serum albumin buffer were added and allowed to react for 120 min at room temperature. After three washes with PBS-T, a horse radish peroxidase-labeled monoclonal antibody against IgA (Dako, Copenhagen, Denmark) diluted 1:5,000 in PBS-T–bovine serum albumin buffer was incubated in the plates for 60 min at room temperature. Development of the colorimetric reaction was as described previously (9). For IgG detection, an anti-human IgG alkaline phosphatase conjugate (Dako) diluted 1:1,000 in PBS-T–bovine serum albumin buffer was used. The enzyme reaction was initiated by the addition of 1 mg of phosphatase substrate (Sigma) per ml in 0.1 M diethanol-

TABLE 1. Detection of significantly elevated IgA and IgG antibodies against EBNA 1 synthetic peptides among 50 NPC serum samples, 36 EBV-positive NHS samples and 45 serum samples from individuals with cancers other than NPC (OC)[a]

Peptide no.	Peptide sequence	% IgA positive[b]			χ^2 (df = 1)[c]		% IgG positive[b]		χ^2 (df = 1)[c]
		NPC	NHS	OC	NPC vs OC	NPC vs NHS	NPC	NHS	NPC vs NHS
6	GRPGAPGGSGSGPRHRDGVR	4.0	2.8	ND[d]		NS	14.0	0.0	*
7	SGPRHRDGVRRPQKRPSCIG	20.0	0.0	ND		**	10.0	8.3	NS
8	RPQKRPSCIGCKGTHGGTGA	34.0	5.5	0.0	***	**	42.0	13.9	***
9	CKGTHGGTGAGAGAGGAGAG	70.0	2.8	2.2	***	***	34.0	11.1	**
10	AGAGGGAGGAGAGGGAGGAGC	82.0	2.8	10.5[e]	***	***	86.0	69.4	NS
11	GAGGGAGAGGAGAGGGGRGR	52.0	2.8	0.0	***	***	16.0	2.8	*
13	GGSGGRGRGGSGGRGRGGSG	8.0	0.0	0.0	NS	NS	0.0	0.0	NS
16	RGGSRERARGRGRGRGEKRP	24.0	8.3	ND		NS	2.0	8.0	NS
17	RGRGRGEKRPRSPSSQSSSS	2.0	0.0	ND		NS	28.0	11.1	NS
19	GSPPRRPPPGRRPFFHPVGE	22.0	0.0	0.0	**	**	56.0	27.8	*
20	RRPFFHPVGEADYFEYHQEG	0.0	0.0	ND		NS	14.0	0.0	*
24	PGEGPSTGPRGQGDGGRRKK	10.0	0.0	0.0	NS	NS	6.0	5.5	NS
30	TWVAGVFVYGGSKTSLYNLR	50.0	22.2	0.0	***	**	46.0	44.4	NS
37	AEVLKDAIKDLVMTKPAPTC	2.0	0.0	ND		NS	4.0	0.0	NS

[a] OC: gastrointestinal, 22; head/neck, 9; breast, 13; cervix, 1.
[b] Percentage of sera giving delta absorbance of >0.2.
[c] NS, nonsignificant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.
[d] ND, not done.
[e] Data from a previous study (9).

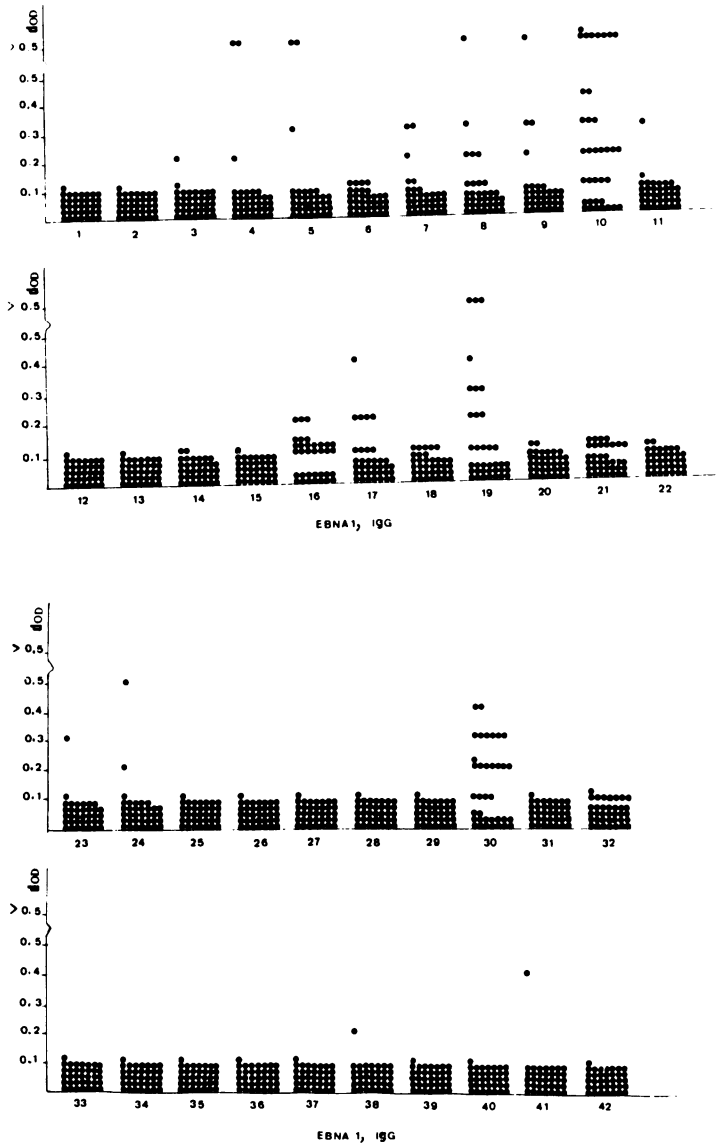

FIG. 2. Serum IgG reactivity to EBNA 1 protein in 36 EBV-positive NHS serum samples. Peptide designation was as in the legend to Fig. 1. Five EBV-negative serum samples were negative for all peptides. dOD, see legend to Fig. 1.

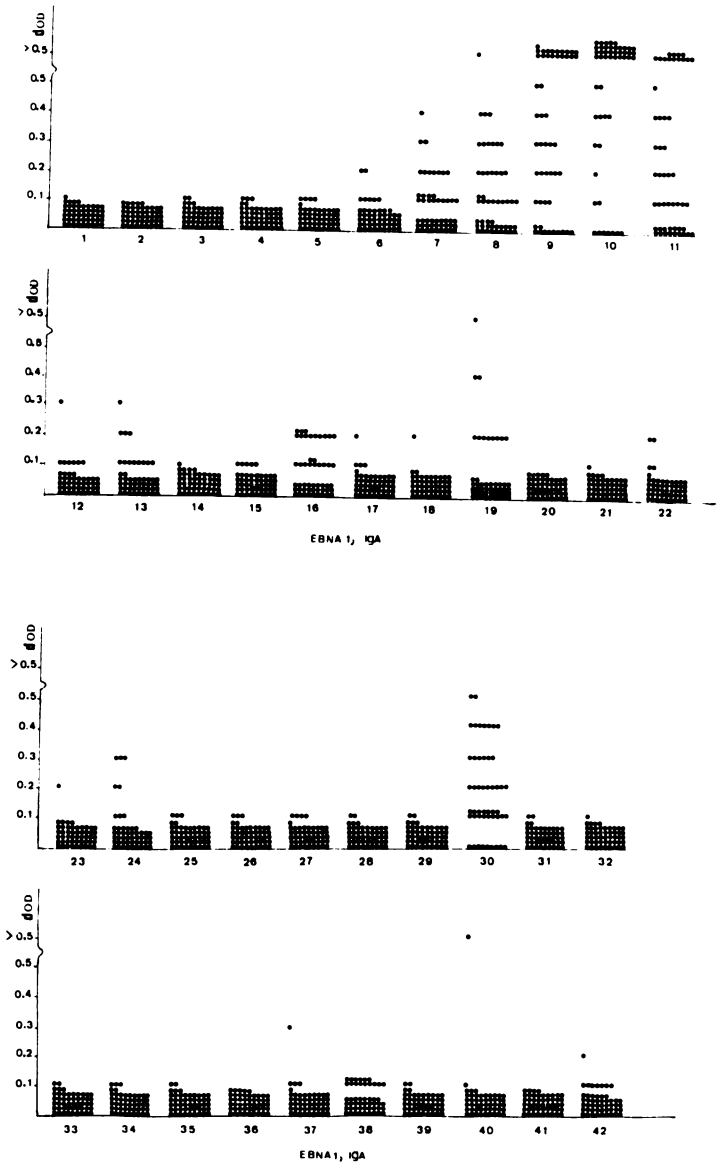

FIG. 3. Serum IgA reactivity to EBNA 1 protein in 50 NPC serum samples. See legend to Fig. 1 for details, including definition of dOD.

TABLE 2. EBNA 1 linear epitopes preferentially recognized by IgA or IgG antibodies

Peptide no.	NPC		χ^2 (df = 1)[a]	
	% IgA positve[b]	% IgG positive[b]	IgA	IgG
6	4.0	14.0	—	—
7	20.0	10.0	—	—
8	34.0	42.0	—	—
9	70.0	34.0	***	
10	82.0	86.0	—	—
11	52.0	16.0	***	
13	8.0	0.0	*	
16	24.0	2.0	**	
17	2.0	28.0		***
19	22.0	56.0		***
20	0.0	14.0		*
24	10.0	6.0	—	—
30	50.0	46.0	—	—
37	2.0	4.0	—	—

[a] —, nonsignificant; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.
[b] Percentage of sera giving delta absorbance of >0.2.

amine buffer (pH 9.6)–1 mM $MgCl_2$, and the plates were read at 405 nm after the colorimetric reaction was stopped with 3 N NaOH.

Specific IgA or IgG antibody to EBNA 1 peptides was defined as a change in absorbance (difference between peptide-coated wells and wells coated only with buffer) of >0.2. All sera were tested in duplicate.

RESULTS

Several EBNA 1 peptides were reactive with IgG antibodies in NPC sera (Fig. 1). The most pronounced IgG binding (86%; Table 1) was to the glycine-alanine peptide 10 (p107 in reference 6). Peptides 9 and 11, which make up transition sequences at the amino and carboxy ends of the repeat region, respectively, were also reactive. In addition, there were several IgG epitopes downstream towards the carboxy terminus, namely, peptides 17, 19, 20, 23, 24, 30, and 37. On the amino-terminal side of the repeat region, IgG was also detected against peptides 3, 4, 5, 6, 7, and 8 in NPC sera. Except for peptides 6, 20, and 37, sera from healthy,

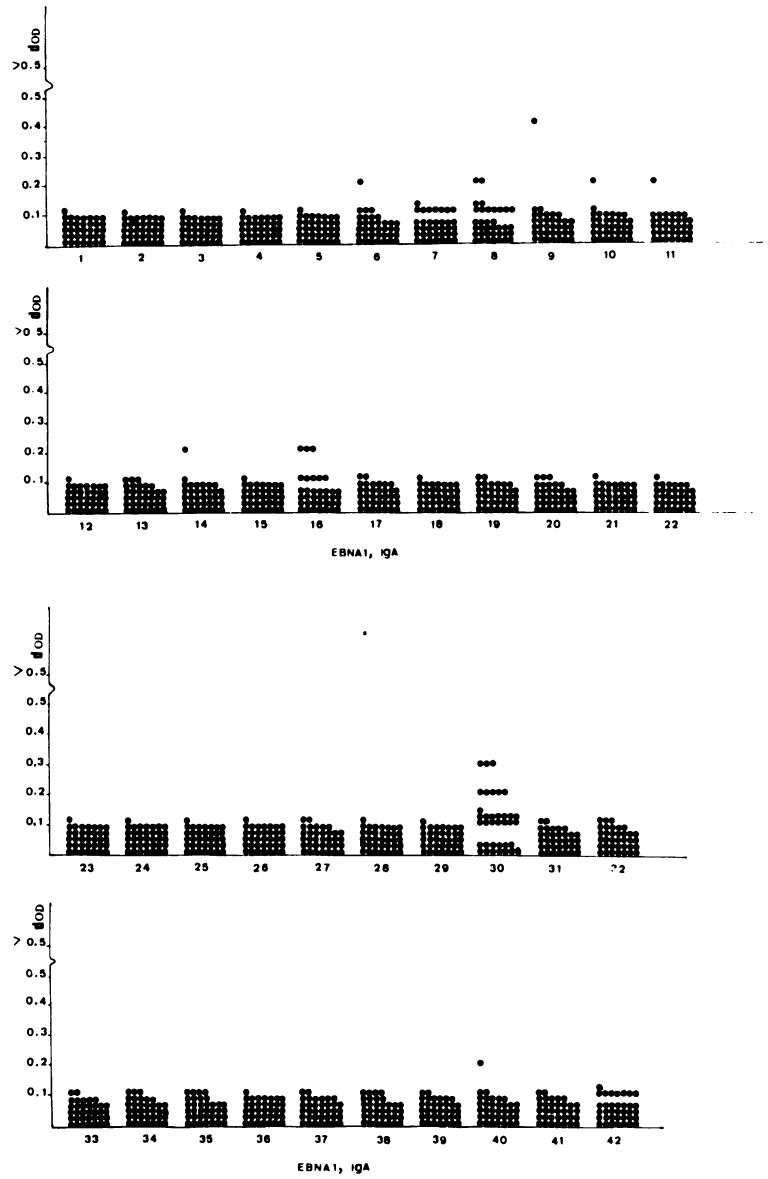

FIG. 4. Serum IgA reactivity to EBNA 1 protein in 36 EBV-positive NHS serum samples. Five EBV-negative serum samples were negative for all peptides. See legend to Fig. 1 for details, including definition of dOD.

EBV-positive donors also contained IgG-reactive antibodies to similar EBNA 1 epitopes (Fig. 2; Table 1), with particularly strong binding to peptides 10, 19, and 30. None of the EBV-negative sera were IgG reactive against any of the EBNA 1 peptides. This suggests that the majority of these epitopes are specific for EBNA and not cross-reactive with unrelated antigens.

The majority of IgG-reactive epitopes were also recognized by specific IgA antibodies in NPC sera (Fig. 1 and 3). However, several peptides (peptides 3 and 5 on the amino side and peptides 17 and 20 on the carboxy side) were reactive with IgG but not with IgA. On the other hand, peptides 13, 16, and 22 were mainly bound by IgA antibodies in NPC sera but not by IgG. The preferential reactivity of the immunogenic EBNA 1 peptides for IgA or IgG in NPC sera is presented in Table 2.

The most immunogenic IgA epitope was, again, the glycine-alanine peptide 10/p107 (Fig. 3). In a previous study, we found that 91% of NPC patients and 13% of normal healthy subjects had IgA antibodies against peptide p107 (9). In the present study, we used a simplified ELISA method with slightly lower sensitivity. Our present finding that 82% of NPC sera and 3% of normal healthy sera (NHS) contain IgA to this peptide is therefore in good agreement with our previous study.

In contrast to the IgG reactivity in EBV-positive NHS (Fig. 2, 70% against peptide 10; Table 1), the IgA reactivity to the repeat region sequences (peptides 9, 10, and 11) was minimal (Fig. 4, <3%; Table 1). None of the EBV-negative NHS showed IgA binding to any of the EBNA 1 peptides. Peptides 13, 19, and 24 appeared to be IgA-reactive epitopes restricted for NPC, since the EBV-positive normal sera were

unreactive. These peptides were also not bound when tested against 45 serum samples from a variety of cancer patients other than those with NPC (Table 1).

DISCUSSION

Our present epitope mapping of EBNA 1 shows that the protein contains multiple linear epitopes. Rumpold et al. (29) argued that only the glycine-alanine region and not the carboxy-terminal region was immunoreactive. In contrast, Milman et al. (24) reported strong immunoreactivity with a fusion protein representing the unique carboxy-terminal region. Our study demonstrates that the glycine-alanine repeat is the most immunoreactive linear epitope, for both IgG and IgA serum antibody binding, but that sequences in both the amino-terminal and the carboxy-terminal unique regions contained distinct IgG- and/or IgA-reactive epitopes.

EBV-positive sera, both NPC and NHS, possessed IgG reactivity to several peptide sequences along the EBNA 1 protein. Such IgG-reactive epitopes of EBNA 1 in NHS, e.g., peptides 10, 19, and 30, could be useful as target antigens in ELISA for serological investigation of EBV-immune status, as has been shown previously in the case of peptide 10/p107 (6, 7). ELISA for p107 is already established as a convenient and rapid immunological test that complements the traditional immunofluorescence assay for IgG to the viral capsid antigen as an indicator of previous EBV infection (19). However, the p107 ELISA reactivity is not absolutely correlated to the EBNA antibody titers measured by anticomplement immunofluorescence (19), and it would therefore be interesting to see whether a combination of our immunoreactive EBNA 1 peptides in ELISA could improve this correlation.

In NPC sera, the IgA and the IgG immunoreactivities are not necessarily against the same epitope. For example, peptides 3, 5, and 20 were IgG restricted and peptides 13 and 22 were limited to IgA binding alone. This may be related to aspects of viral antigen processing and presentation (23) and the different immunobiological response between local IgA immunity in the nasopharynx and B-cell reactivity. In previous studies on another virus, we also found that the IgA-, IgG-, and IgM-reactive epitopes are not necessarily identical (4).

Serological diagnosis of virus diseases by using specific, defined linear epitopes has been shown to adequately reflect the antibody response to the native viral protein in several systems (3, 25, 26, 30). Unfortunately, NPC sera that were IgA unreactive for peptide 10/p107 (18%; Table 1) also remained unreactive against other epitopes of EBNA 1 protein. This means that it was not possible to exploit the use of a combination of peptides, including IgA disease-specific sequences, to increase the sensitivity of the peptide 10/p107 IgA ELISA (9) for NPC diagnosis. However, peptide 10/p107 is not completely devoid of IgA reactivity in NHS, possibly reflecting the small amount of cross-reaction with normal cellular proteins described previously (12, 22), and it is therefore noteworthy that several other peptides (notably peptide 9) showed equal or greater specificity for NPC in IgA ELISAs. Our finding that certain epitopes of EBNA 1 are associated with viral disease rather than merely viral immunity suggests that disease-related serology, also for EBV-related diseases other than NPC, will be more useful if individual epitopes are used rather than whole proteins.

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