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Disease-related Differences in Antibody Patterns Against EBV-encoded Nuclear Antigens EBNA 1, EBNA 2 and EBNA 6

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Antibodies to Epstein–Barr virus (EBV) nuclear antigen family (EBNA) and three of its individual members, EBNA 1, EBNA 2 (A and B) and EBNA 6, were measured by anticomplement immunofluorescence (ACIF) in sera of 75 healthy controls, 13 patients with chronic EBV infection, 38 with non-Hodgkin lymphoma (NHL), 23 with Hodgkin's disease (HD), 105 with nasopharyngeal carcinoma (NPC) and 7 patients with infectious mononucleosis (IM). Their anti-EBV lytic antigens were also measured. We observed that: (1) anti-EBNA 2A and E6 rose in parallel 4–6 weeks after IM, followed by anti-EBNA 1 at 3–6 months, (2) all seropositive individuals had anti-EBNA 1; 74% also had anti-EBNA 2A and E6, (3) anti-EBNA 1 accounted for most of the anti-EBNA reactivity in non-IM sera. Striking disease-associated differences were noted on the humoral responses to the lytic and transformation-associated antigens. Compared to the controls, anti-EBNA 1, -EBNA 2A and -EBNA 6 were simultaneously four to 10 times higher in chronic reactivations, whereas only anti-EBNA 1 was elevated (10 times) in NPC. Individual EBNA titres were normal in NHL or HD patients.
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INTRODUCTION

EPSTEIN–BARR VIRUS (EBV)-encoded antigens can be categorised into growth transformation-associated antigens and lytic antigens. At least six different proteins of the nuclear antigen complex, EBNA 1–6 (abbreviated E1, E2, etc.), and two of the latent membrane antigens (LMP 1 and 2) (reviewed in [1]) are expressed in growth transformed (immortalised) B cells [2]. E1 binds to the origin of latent viral DNA replication. This binding is essential for the maintenance of the viral genomes in the free episomal form [3]. E2 plays an essential role in B cell activation [4]. Two antigenically distinct allelic forms of E2, designated EBNA 2A and 2B, have been identified [5]. E2A carrying viral isolates are more ubiquitous than E2B carriers. The latter are less prevalent and often occur in dual infections with E2A carriers [5, 6]. The functions of EBNA 3–6 are not known. In some EBV-infected cell lines, a small minority of the cells switch on the lytic cycle, manifested by the appearance of the early antigen complex (EA-D and EA-R) and the late viral capsid antigen (VCA).

The cellular expression of growth transformation-associated antigens differs, depending on the cell phenotype. EBV-transformed B cells of normal origin (LCL) express all eight growth transformation antigens. Phenotypically representative Burkitt's lymphoma (BL) cells express only E1 [7]. Nasopharyngeal

carcinoma (NPC) cells express only E1 and the LMP complexes [8]. Rescue of virus by cocultivation of BL or NPC with cord blood cells leads to the full expression of all eight proteins in the derived LCL [9, 10]. We have previously argued [11] that these differences in viral expression reflect the differential regulation of the virus in the corresponding normal host cells during latent persistence.

Viral expression during latency will have to be studied directly, by immunohistochemical and molecular techniques at the cell level. As a complement to such studies, measurement of antibody formation to various virally encoded proteins provides additional information that is not available otherwise. The earlier serological work, particularly by the Henles, has shown, for instance, that healthy seropositive persons form antibodies regularly against proteins of the VCA, membrane antigen (MA) and EBNA complexes, but not against the EA complex. Anti-EA antibodies could be taken as indicators for active processes, like acute infection or the progressive growth of EBV-carrying tumours. In a later serological study [12], interesting discordances were found between anti-E1 and anti-E2A antibodies. Anti-E2A were found to precede anti-E1 by weeks to months after primary infection. During convalescence, anti-E2A often fell or disappeared whereas anti-E1 increased and persisted at stationary levels during a life time in healthy individuals. In patients with immunosuppressive or chronic conditions, the anti-E1 to anti-E2 ratios were generally below 1.0 compared to >1.0 in the healthy population [12].

The main objectives of the present study were to (1) examine the interrelationships amongst the antibodies against E1, E2A, E2B and E6, (2) analyse their relationship to what was previously known as anti-EBNA and (3) detect possible disease associated anti-EBNA profiles. We have examined these antibodies in patients with infectious mononucleosis (IM), chronic EBV reactivations, non-Hodgkin lymphoma (NHL), Hodgkin's disease (HD), NPC and in healthy seropositives. In parallel, we have also measured their antibodies to EA-D, EA-R and VCA for references.

MATERIALS AND METHODS

Sera

Acute phase sera from 7 IM patients (VCA-IgM positive and/or EBNA conversion) were collected at appoximately weeks 2, 4, 6, 13 and 20 after onset. Sera from 13 patients with chronic lymphadenopathy of unknown aetiology were selected as representative of EBV reactivation, as judged by anti-EA titres above 1:20 and anti-EBNA higher than 1:80 to exclude patients with active primary infections (whose elevated anti-EA may be accompanied by low or absent IgM-VCA and anti-EBNA titre). Sera from 23 HD and 38 NHL patients were from Radiumhemmet, Karolinska Institute. Sera from 105 patients diagnosed with NPC and 75 healthy controls were from the University of Malaya, Kuala Lumpur.

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EBV-specific serology

Anti-VCA, EA-D and EA-R (abbreviated as D and R) were detected by indirect immunofluorescence according to Henle *et al.* [13]. Antibodies to EBNA were measured by anticomplement immunofluorescence (ACIF) using Raji cells (EBNA-Raji) as described by Reedman and Klein [14], with guinea-pig complement (Diamedix Corp., Miami, Florida, U.S.A.) and fluorescein-isothiocyate-conjugated goat anti-guinea pig C3b (Organon Teknica Cappel) substituted for the human complement/anti-human complement reagents. Guinea-pig and human complement yielded identical results in parallel assays. The wide availability of the guinea-pig reagents obviated the need to screen for EBV seronegative donors. Raji cells (positive for EBNA 1, 2A, 3, 4) were used to detect anti-EBNA(Raji). BJAB, an EBV negative B cell line, was used as negative antigen control to identify the sera with anti-nuclear antibodies. Such sera were excluded from analyses involving the various anti-EBNA. An ACIF titre of 1:5 or greater is considered significant.

Transfectants

Monospecifically transfected DG-75 cells were used to assay antibodies against EBNA 1, 2A and 6. The cells were stably transfected with recombinant selection vectors that carry the BamHI K fragment (the pBk1 plasmid), a subfragment of the BamHI WYH region (nucleotides 44664 to 50628; the pE A6 plasmid) [15], or a subfragment of the EcoRI B fragment (nucleotides 97027 to 102756; the pB E4 plasmid) [16] of B95-8 EBV DNA. Rat-1 cells, an established line of fibroblasts, transfected with the BamHI Y fragment of Ag-876 EBV were the source for the EBNA 2B (E2B) assays. Antigen expressions in these transfectants were stably and continuously maintained in RPMI 1640 with 10% bovine calf serum for at least 3 years. Cell smears were prepared from exponentially growing cultures by spreading cell suspensions, at density of 1×10^7/ml, onto glass cover slips, air drying rapidly, and fixing for 1 min in an equal mixture of acetone and methanol. All sera with anti-nuclear antibodies reacted equally to BJAB, DG75 and Rat-1 cells in preliminary assays. Hence, only BJAB was used as negative antigen control in the present study. The specificities of the antigen smears were checked with a panel of monospecific sera characterised previously by immunoblotting [17].

All of the sera were tested for IgG antibodies to VCA, D, R (VCA-G, D-G and R-G), IgA antibodies to VCA and D (VCA-A, D-A), antibodies to EBNA(Raji), EBNA 1, EBNA 2A, EBNA 2B and EBNA 6 (EBNA, E1, E2A, E2B and E6, respectively).

RESULTS

Table 1 summarises the prevalence and geometric mean antibody titres (GMT) to the various EBV antigens. Significant disease-associated differences were observed.

Antibodies to lytic antigens

Of the non-IM sera in the survey, all 254 were from EBV seropositive individuals. Almost without exception, IgG antibodies to VCA, D and R antigens were more prevalent and higher in titre among patients with chronic reactivations, HD, NHL and NPC, compared to the healthy controls. As shown in Table 1 and Fig. 1, the patients with reactivations had VCA titres five times above the controls; their D and R antibodies were 15 and 41 times higher, respectively, compared to the controls. In comparison, NHL and HD patients showed four to 10-fold elevated titres to VCA, D and R. The most striking

Table 1. Distribution of EBV antibodies

	Controls			Reactivated			HD			NHL			NPC		
Antigens	No.(+)	(%)	GMT	No.(+)	(%)	GMT	No.(+)	(%)	GMT	No.(+)	(%)	GMT	No.(+)	(%)	GMT
Lytic		*n* = 75			*n* = 13			*n* = 23			*n* = 38			*n* = 105	
VCA-A	2	(2)	20		Not done		3	(13)	127	8	(21)	123	83	(79)	128
VCA G	75	(100)	303	13	(100)	1422	23	(100)	1359	38	(100)	1714	105	(100)	4255
D-A	0	(0)			Not done		1	(4)	80	5	(3)	105	51	(49)	80
D-G	3	(4)	5	11	(85)	78	9	(39)	20	15	(39)	58	79	(75)	509
R-G	1	(1)	5	8	(62)	207	5	(22)	52	14	(37)	38	5	(5)	139
Control															
BJAB	1	(1)	40	0	(0)		3	(13)	32	0	(0)		39	(37)	104
Nuclear		*n* = 74			*n* = 13			*n* = 20			*n* = 38			*n* = 66	
EBNA-Raji	73	(99)	245	13	(100)	751	20	(100)	197	38	(100)	123	66	(100)	2454
E1	73	(99)	199	13	(100)	675	19	(96)	135	33	(87)	132	66	(100)	1882
E2A	58	(78)	21	13	(100)	116	14	(61)	31	24	(63)	40	48	(73)	27
E6	61	(82)	19	13	(100)	175	17	(86)	35	24	(63)	39	49	(74)	20
E2B	44	(60)	16	6	(46)	28	9	(39)	15	15	(39)	23	33	(50)	19

differences were observed among the NPC patients whose titres to VCA, D and R titres were 14, 101 and 28 times above those of the controls, respectively. While antibodies to VCA were elevated in all patient categories, the presence of anti-D and R showed some noteworthy qualitative differences among the various groups. For example, anti-D and R were both present in the majority of the patients with reactivation; they were also present in equal numbers of HD and NHL patients; while anti-D, but not anti-R, was the most common EA antibody in NPC patients, in accord with earlier results [18].

Antibodies to transformation antigens

Of the 254 non-IM sera, 43 NPC and 1 control sera could not be evaluated for EBNA or the individual EBNA components due to the presence of confounding antinuclear antibodies as detected with the BJAB (EBV-negative) cell line. Of the remaining 211 sera, 210 (99.5%) had detectable EBNA and E1 titres, 157 (74%) with anti-E2A, 107 (50%) with anti-E2B, 164 (78%) with anti-E6.

Correlations between EBNA and E1 activities

Prior to the identification of the six individual antigens within the EBNA family, 'anti-EBNA' referred to the nuclear staining obtainable by the ACIF reaction against Raji cells. Previous studies have not established if and to what extent anti-EBNA is predominantly influenced by one particular member of the family. We have, therefore, plotted the total EBNA(Raji) titres against anti-E1, and also anti-E2A of sera from 74 controls and 60 NPC patients without anti-nuclear antibodies. Figure 2a shows the close correlation between the anti-EBNA and anti-E1 titres. In contrast, no such correlation existed between anti-EBNA and anti-E2A (Fig. 2b), -E2B or -E6 (not shown). Identical anti-EBNA/E1 relationships were found in all patient groups (data not shown).

Correlation between anti-E2 and anti-E6

From Table 1, it was noted that the distribution of E2A and E6 antibodies was similar both in prevalence and titre for all seropositive subject groups, suggesting a possible correlation between the two. Figure 3 is a comparative plot of anti-E2A vs. anti-E6 of 75 control and 60 NPC patients. The data suggested a moderate correlation between the two, in that 75% of the sera had anti-E2A and anti-E6 titres within 4-fold of each other. Eighteen sera (13%) had either anti-2A or E6. Similar correlation was seen in other patient groups.

Dominance of anti-E2A in acute IM sera

In contrast to the seropositive individuals with EBV infections in their distant past, anti-E2A, not E1, antibodies dominate during the first 5 months after onset of primary infection. Figure 4 shows the sequence of seroconversion to E2A, E6 and E1 in the 7 IM patients with measurable EBNA titres. Although this sampling of IM patients was small, the data suggest that anti-E2A antibodies were the earliest to appear, followed very closely by anti-E6. At 2 weeks after onset, 4 out of 7 patients had measurable anti-E2A compared to two out of seven for anti-E6. At 14 weeks, all 7 patients seroconverted to EBNA 2A, 5 to EBNA 6 and 3 to EBNA 1. 3 of the 7 IM patients had barely detectable anti-E 2B at 1:5 and were not included in our analysis.

Differential anti-EBNA profiles in EBV reactivations and in malignancies

Antibodies to the growth transformation-associated antigens showed disease-associated differences (Fig. 5). Three patterns emerged from the analyses of antibodies to the individual EBNA components. The first, observed only among patients with

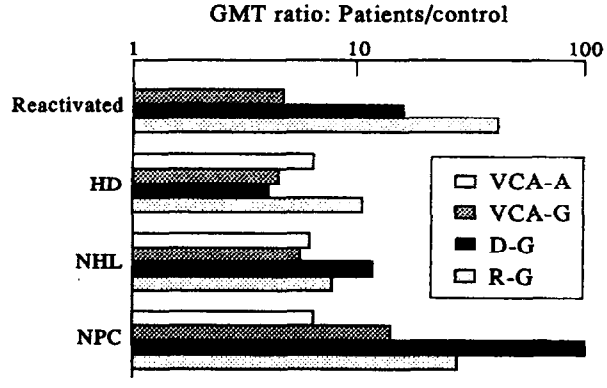

Fig. 1. Ratio of the geometric mean titres to EBV-associated lytic antigens in patients with reactivations, HD, NHL and NPC compared to controls.

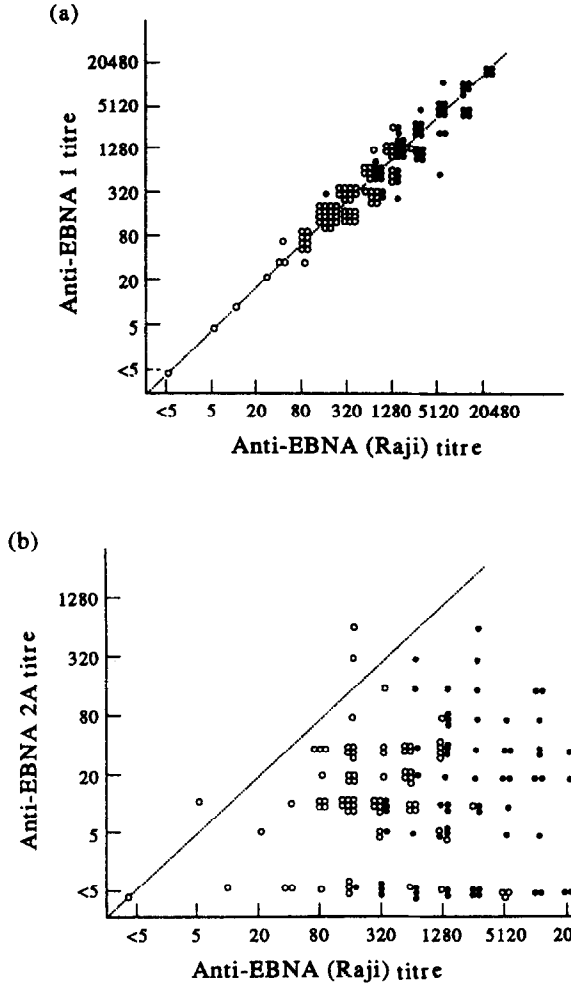

Fig. 2. Comparative plots of anti-EBNA (Raji) titres vs. anti-E1 (2A) and anti-E2A (2B) in controls (○) and NPC patients (●). The dotted line represents an antibody ratio of 1.0.

reactivations, showed significant increases in antibodies to E1, E2A, and E6 (3.4, 5.5 and 9.1-fold, respectively). The second was observed for the NPC sera, where anti-E1 increased 9.5-fold, while anti-E2A and E6 remained normal. The third pattern was apparent for both the HD and NHL patients, where anti-

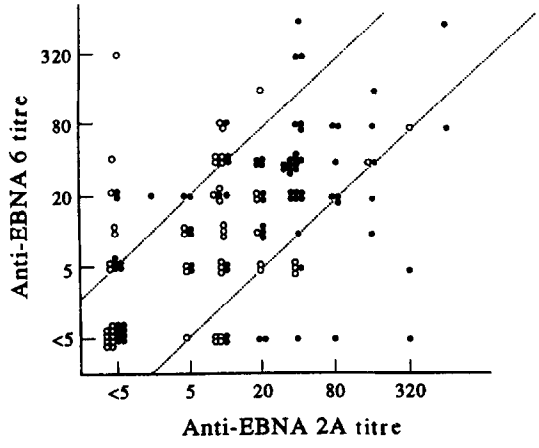

Fig. 3. Comparative plot of anti-E2A titres vs. anti-E6 in controls (○) and NPC patients (●). The area bounded by the dotted lines contains titre ratios of 4 or less.

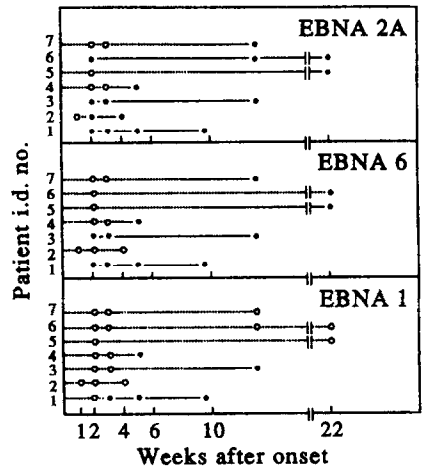

Fig. 4. Seroconversion of IM patients to EBNA-2A, 6 and 1. Seroconversion is indicated as a change from the dotted to solid line. (○----○ negative); (●——● positive).

E1, E2A and E6 were not significantly different from those of the controls. Anti-E1 titres for the NHL patients, however, were consistently on the lower end of the spectrum, with geometric mean titres about half those observed for the controls.

Prevalence of anti-E2A vs. E2B

In the healthy control group, 55% of the adults have detectable anti-E2A and anti-E2B, 23% anti-2A only, 4% anti-2B only, and 18% have no anti-E2A or E2B. The prevalence of these two markers was not significantly different in the patient population from that of the controls. In patients with both anti-E2 reactivities, no correlations between the titres were observed.

DISCUSSION

Differential EBV antigen expression in different disease conditions may reflect the role of EBV in the progression of these diseases. Determination of the corresponding antibodies in patients with various conditions, in turn, might provide information on the *in vivo* viral events. With this possibility in mind, we measured antibodies to a comprehensive panel of EBV lytic and latent antigens. The well characterised antibodies to the lytic antigens served as reference in the patient populations under investigation, against which the antibody profiles to the EBNA components could be evaluated. The subject groups were

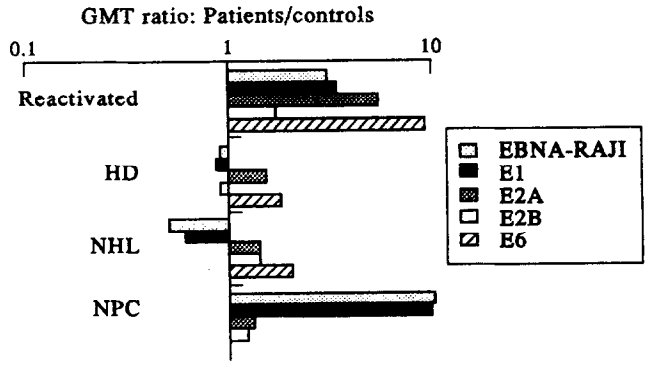

Fig. 5. Ratio of the geometric mean titres to EBV-associated growth transformation antigens in patients with reactivations, HD, NHL and NPC compared to controls.

selected to include patients with a wide spectrum of EBV-associated conditions, ranging from primary infection, to uneventful recovery (healthy seropositive controls), to benign reactivation, to tumours with occasional associations with EBV (NHL and HD) and to a tumour known to be closely associated with EBV (NPC).

Our results showed that all the patient groups, to varying extent, had increased EBV lytic infections, as indicated by the increased prevalence and titres to VCA, D and R antigens. Increases in these antibodies were moderate for the patients with reactivations, with HD and NHL, but were dramatic for NPC patients (Table 1, Fig. 1) compared to the normal controls.

In contrast to the somewhat uniform responses seen with the lytic antigens, the spectra of antibodies to the various EBNA components showed wide variations among the four patient groups (Fig. 5). The most striking differences were observed in patients with NPC. Whereas their anti-EBNA(Raji) levels were 10-fold above normal, the anti-E2A and -E6 were not elevated. Their anti-EBNA(Raji) titres could be entirely accounted for by the increased anti-E1. This finding is in line with the observation that only EBNA-1, but not EBNA 2–6, is expressed in NPC tumours [8, 19].

By comparison, donors with chronic reactivations showed elevated titres to E1, E2A and E6. This may reflect the proliferation of lymphoblastoid cells that express the total spectrum of EBNA antigens. In HD and NHL patients, anti-E1 titres were slightly lower than normal, with no differences seen in anti-E2A or -E6 titres. The lack of anti-E2A and -E6 elevation is in line with the absence of these proteins from EBV carrying Hodgkin's lymphomas that have been found to express EBV-encoded small RNA (EBER) transcripts and LMP [20, 21]. There are no reports on E1 expression in HD. Further elucidation in this point would require additional cytologic, serologic as well as epidemiological investigations.

In addition to the potential implications in the disease-associated antigen expression, our data highlighted, in some detail, the apparent 'dissociation' of the humoral responses to the latency-associated antigens and the lytic antigens in the infected host. While all the patient categories (chronic reactivation, HD, NHL and NPC) showed increased antibody titres to the lytic antigens, antibodies to the latent antigens differed in a disease-associated manner.

In the process of determining the disease-associated relationships of the various antibodies to the EBNA complex, we made several observations concerning the sequences of their appearance during primary infection. Henles and coworkers previously showed that anti-EBNA 2A appeared within 3 months of IM whereas anti-E1 appeared only after the third month. Anti-E2A were detectable 2 years later at low, residual titres in 71% of the patients. Antibodies to E1, on the other hand, were stationary throughout the 48 months of observation. Their study did not include sera taken sufficiently early after the onset of the disease to detect the time when anti-E2 antibodies appeared. In our study, seroconversion to EBNA 2A occurred within the first weeks in 4 out of 7 patients. By week 14, all 7 patients had seroconverted. In comparison, only 2 patients seroconverted to EBNA 6 during the first 2 weeks and 5 out of 7 by week 14, when only 3 out of 7 had measurable anti-E1.

Several other generalisations could also be made from our data:

(1) Antibodies against E2A and E6 varied in parallel. This is consistent with the coordinate regulation of the two antigens, spliced from the same common transcript. Their presence and level coincide to within two serum dilutions in 75% of the sera (Fig. 3). (2) Anti-E1 accounted for essentially all of the anti-EBNA in convalescent or later phase sera as measured by the conventional ACIF assay using Raji cells. (3) Dual infections of type A and B viruses are quite common. Half of the 254 individuals sampled in our study (regardless of their geographic origin) had demonstrable antibodies to both viruses, compared to 23% with only type A and 4% with only type B infections. These serological data are in accord with recent virological studies [6, 22]. In our ACIF procedure, cross reactions between EBNA 2A and 2B appear to be minor. Cross reactions could be excluded in 27% of the sera tested that had antibodies to only one type. In the remaining 55% of the sera that have antibodies to both virus types, there was no correlation between the titres. The antibody titre to one type was independent of the titre obtained for the second type, a pattern not consistent with the presence of cross reactions.

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A Cross-linking Assay Allows the Detection of Receptors for the Somatostatin Analogue, Lanreotide in Human Breast Tumours

Grégoire Prévost, Philippe Provost, Valérie Sallé, Monique Lanson and François Thomas

Hypothalamic somatostatin and its synthetic analogues inhibit the cell growth of several tumour models. The somatostatin analogue lanreotide (somatuline or BIM23014C) inhibits both the *in vivo* and *in vitro* cell growth of various mammary tumours. In order to evaluate the presence of receptors for lanreotide in breast tissue, samples from 41 female and 2 male patients were analysed by a cross-linking assay. All the samples examined possessed at least one subtype of lanreotide binding polypeptide, however, different polypeptide patterns were observed. The two major complexes had molecular weights of 57 kD and 42 kD. The previously demonstrated antiproliferative activity of lanreotide and the high percentage of positive tumours supports the use of lanreotide in clinical trials. However, the role of each receptor subtype in the control of breast cell proliferation requires further characterisation.
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INTRODUCTION

HYPOTHALAMIC SOMATOSTATIN [1] and its analogues [2] inhibit both the *in vivo* and *in vitro* cell growth of various human cell lines including small cell lung cancer, pancreatic carcinoma, prostatic carcinoma and breast carcinoma [3–7]. The antiproliferative effect of these molecules is the result of their direct activity on tumour growth and their inhibition of hormone secretion (growth hormone, prolactin, and insulin-like growth factor) [8]. Multiple subtypes of the somatostatin receptor have been observed by chemical cross-linking in various cells such as pituitary adenomas [9] or breast cancer cell lines [3]. The molecular heterogeneity of the somatostatin binding polypeptides has been elucidated by a quantitative autoradiographic study of the rat brain and the adenohypophysis demonstrating the presence of different subtypes of receptors in these tissues [10]. Recently, more than two different genes coding for somatostatin binding polypeptides have been cloned demonstrating the existence of various somatostatin receptors [11]. The somatostatin analogue, Lanreotide (somatuline or BIM23014C) [12] is an efficient *in vivo* and *in vitro* antiproliferative agent in several mammary models. Between 15 and 90% of breast tumours express somatostatin receptors [3, 8, 13, 14]. This variation may be due to differences in labelled ligands and in the methodology used for their detection (cryostat-section autoradiography, chemical cross-linking, *in vivo* scintigraphy, filtration-retention). The heterogeneity of somatostatin receptor distribution in breast biopsy specimens may also account for this difference [15]. In order to evaluate the potential direct activity of the somatostatin analogue lanreotide in breast tumours, lanreotide receptors have been analysed by the cross-linking assay in 43 surgical samples (patients: 41 females and 2 males).

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MATERIALS AND METHODS

Materials

The somatostatin analogue lanreotide (BIM-23014C or somatuline) was provided by Ipsen-Biotech (Paris, France). The labelled peptide [^{125}I] lanreotide (592–740 GBq/mmol) was obtained from F. Dray and C. Tiberghien (Hospital Cochin, Paris, France). Ethylene glycol-bis (succinimidyl succinate) (EGS) was obtained from the Sigma Chemical Co. (France).

Tumour tissue samples

Specimens were obtained from 43 patients who had surgery for breast cancer in the Department of Gynecology (Hospital