Acta virol. **34** : 132 — 138, 1990

ANTIBODY AGAINST SYNTHETIC PEPTIDE DERIVED FROM EPSTEIN-BARR VIRUS-DETERMINED NUCLEAR ANTIGEN 1 (EBNA-1) IN CHILD NON-HODGKIN'S LYMPHOMA

P. SOVA, K. ROUBALOVÁ, M. WEINREB[1], E. HAMŠÍKOVÁ, R. KODET[2], V. KRCHŇÁK[3], J. ROUBAL

Institute of Sera and Vaccines, 101 03 Prague 10, [1]Department of Pediatric Oncology, Faculty Hospital Motol, [2]Department of Pathology, Medical School of Pediatrics, Charles University, Prague, and [3]Pharmaceuticals Prague, Czechoslovakia

Received March 13, 1989

Summary. — Antibody reactivity against a synthetic peptide derived from Epstein-Barr virus nuclear antigen 1 (EBNA-1) was determined in 56 cases of child non-Hodgkin's lymphoma and 31 controls. The patients were divided into subgroups based on tumour location and histology and the antibody responses in the various groups were compared. A significant increase in both IgG and IgM antipeptide titres was detected in patients with tumours localized in the abdomen. High IgG titres were also noted in Burkitt-type, lymphoblastic, and centroblastic lymphomas. On the other hand, low or nil IgG titres were found in unclassified malignant lymphomas, in four cases of centroblastic-centrocytic lymphoma and in lymphomas located in the mediastinum. Surprisingly, the occurrence of antipeptide IgM antibody was highest in those tumours, where IgG titres were low, i.e. in subjects with mediastinal tumours and in unclassified malignant lymphomas. However, with the exception of tumours localized in the abdomen and unclassified tumours, the IgM titres in positive individuals were low and comparable with titres found in a part of healthy controls.

Key words: *Epstein-Barr virus (EBV)*; *EBV-determined nuclear antigen*; *EBNA-1*; *synthetic peptide*; *child non Hodgkin's lymphoma*

Introduction

Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis (IM) (Henle *et al.*, 1968), is known to be associated with Burkitt's lymphoma (Nonoyama *et al.*, 1973; Reedman *et al.*, 1974) and carcinomas originating in the Waldayer ring (Klein *et al.*, 1974; Wilmes and Wolf, 1981; Břicháček *et al.*, 1984). Immunocompromised individuals represent a high-risk group in which EBV infection can evolve into opportunistic lymphoproliferative diseases (Filipowich *et al.*, 1980; Hanto *et al.*, 1981; Purtilo *et al.*, 1981) that range from fatal acute IM to chronic disorders (Purtilo *et al.*, 1977; Britton

et al., 1978). Indeed, polyclonal EBV-positive lymphomas can be frequently seen in patients with primary or acquired immune deficiency. They have been detected in patients suffering from the XLP-syndrome (Purtilo, 1981), in organ-transplant recipiens (Hanto *et al.*, 1981), and patients with AIDS (Purtilo, 1987). Poorly controlled opportunistic lymphoproliferation can sometimes lead to the evolvement of true monoclonal malignancy.

Recently, we reported on serological findings indicating that EBV infection is frequently activated in juvenile patients suffering from non-Hodgkin's lymphoma (NHL). Moreover, some of the patient subgroups constituted according to tumour histology and location, exhibited a more pronounced EBV-related serology than others. Preliminary results on anti-EBNA-1 responses suggested that various subgroups may also differ in the level of anti-EBNA-1 antibody (Roubalová *et al.*, 1988). EBNA-1 is a latent protein that is expressed in all EBV-immortalized lymphoblastoid cells (Reedman and Klein, 1973). Almost all people who have experienced EBV infection possess antibodies against EBNA-1. Their absence in EBV-seropositive individuals might reflect a certain immune dysfunction (Purtilo *et al.*, 1977; Henle and Henle, 1981; Purtilo, 1981; Hanto *et al.*, 1981). However, increased anti-EBNA-1 titres might also have significance for evaluating virus interaction with the host. In the present paper we analysed in some detail the IgG and IgM antibody responses to a synthetic peptide derived from EBNA-1 in various subgroups of child NHL.

Materials and Methods

Patients. A group of 56 children with NHL, whose sera were collected before beginning therapeutic treatment, was assembled at the Department of Child Oncology, Faculty of Pediatrics, Charles University, Prague. The diagnoses were based on clinical findings and histological examination. Histological evaluation was performed at the Department of Pathology, School of Pediatrics, Charles University, Prague, using Kiel classification (Lennert, 1981). The age of the patients ranged from 2 to 15 years; 61 per cent of the group were boys. A control group of 31 subjects, sex and age-matched with one half of the patients, was assembled from children hospitalized with orthopedic problems. Table 1 shows the distribution of patients ac-

Table 1. Patients subgrouped according to tumour location and histology

Tumour location (n)	Number of patients with indicated type of lymphoma histology					
	Burkitt type	lympho-blastic	centro-blastic	centroblastic-centrocytic	unclas-sified	unknown
Abdomen (14)	4	5	2	0	2	1
Mediastinum (12)	0	2	0	1	4	5
Neck (20)	4	6	2	3	1	4
Generalized (10)	2	3	0	0	0	0
Total group	10	16	4	4	7	15

cording to tumour location and to the type of lymphoma histology; these criteria were used for dividing the patients into several subgroups. As shown in the last column of Table 1, histological examination could not be performed in about 1/4 of patients.

The peptide. The synthetic peptide used represented a glycine-and-alanine-rich antigenic determinant of EBNA-1 that is coded for by the IR-3 repeat in the EBV-genome *Bam*HI-K fragment (Dillner *et al.*, 1984; Rhodes *et al.*, 1985). It was synthesized by the solid-phase technique (Merrifield, 1963) and purified by high-pressure liquid chromatography. Before use it was conjugated to bovine serum albumin (BSA, Serva) by means of glutaraldehyde. The conjugate was dialysed against 50 mmol/l Tris-HCl (pH 8.0), distributed into aliquots and stored at — 20 °C. Control antigen was prepared by glutaraldehyde treatment of BSA only. Specificity of the peptide was confirmed on a panel of EBV-positive and EBV-negative human sera.

Sera. Patient sera were heat-inactivated and the content of anti EBNA-1 antibody was determined by ELISA using the synthetic peptide as antigen. For IgG determination sera were routinely diluted 1 : 20 and 1 : 100; for IgM determination, they were screened at dilution 1 : 50. All sera used for IgM determinations were absorbed with control antigen, i.e. glutaraldehyde-treated BSA. The absorption was done as follows: 175 μg of control antigen in concentrated solution was added to 10 μl of serum. Then dilution buffer (see ELISA test below) was added to 500 μl final volume. The mixture was incubated 1 hr at room temperature and overnight at 4 °C. Unless absorbed, about one quarter of sera reacted with the conjugate non-specifically. No non-specific reactions were noted for the IgG class.

Antibody titres were calculated from ELISA-determined absorbance. They were normalized to standard (one for IgG and one for IgM determinations), whose titres were considered to equal 1000. The formula used for calculation was:

$$\text{Titre} = \frac{\text{Absorbance obtained with tested serum}}{\text{Absorbance obtained with standard serum}} \times 1000$$

ELISA. Mercelisa plates (Dynatech) were coated with conjugated peptide by adding 2 μg of conjugate in 0.1 ml of bicarbonate buffer, pH 9.6 per well. The plates were incubated 1 hr at room temperature and overnight at 4 °C. Free binding sites on the polystyrene were saturated with 1% BSA prepared in the same buffer. After washing the plates, 100 μl volumes of human sera diluted as indicated and absorbed with control antigen if necessary were added and the plates were incubated for 1 hr. To minimize non-specific binding, the phosphate-based dilution buffer (pH 7.2) contained 0.5 mol/l NaCl, 1% BSA and 1% Triton X-100. After several washings, 100 μl of peroxidase-conjugated swine antihuman IgG or IgM (Sevac Prague) diluted 1 : 2500 times was added. Following another 1 hr the washed plates were filled with substrate mixture containing orthophenylene diamine. The colour reaction was stopped with 2 mol/l H_2SO_4 and measured at 492 nm. The detailed scheme of the procedure employed and the composition of the buffers have been described elsewhere (Vestergaard *et al.*, 1974; Roubalová *et al.*, 1988).

Results

We measured the IgG and IgM antibody response to the EBNA-1-derived synthetic peptide in 56 children suffering from non-Hodgkin's lymphoma (NHL). Their sera were collected before the administration of therapy.

Among the healthy controls, 87.1 per cent of individuals were anti-EBNA-1-positive in the IgG class (Table 2). On the other hand, sera of only 67.9% of the patients reacted by IgG binding to the peptide. This might have reflected an immunodeficient condition among the patients. As shown in Table 2, the mean IgG response against the peptide was stronger among the patients than in control children but the difference was low. However, significant differences in antibody levels emerged when the patients were subgrouped according to tumour location and histology. The titres were considerably increased in children with tumours located in the abdomen

Table 2. Antipeptide IgG antibody in children with non-Hodgkin's lymphoma [1]

Patient group	Number of patients tested	Positives per cent	GTM[2]	
NHL (total)	56	67.9	97	(N.S.)[6]
Control children	31	87.1	72	—
Tumours in abdomen	14	92.8	387	($p < 0.01$)
neck	20	70.0	107	(N.S.)
mediastinum	12	33.3	16	($p < 0.01$)
generalized	10	70.0	100	(N.S.)
Lymphoma[3,4] histology:				
Burkitt type	13	84.6	240	($p < 0.02$)
lymphoblastic	14	92.8	281	($p < 0.02$)
non-classified[5]	7	28.6	16	($p < 0.01$)
centroblastic	4	100.0	787	($p < 0.02$)
centroblastic-centrocytic	4	50.0	15	($p < 0.05$)

[1] All sera were collected before treatment of patients.
[2] For calculating geometric mean titres (see Materials and Methods), negative sera were considered to have a titre equal to 1.
[3] It was not possible to examine histologically all patients tested; hence the total number of patients examined is lower than 56.
[4] Minor histological subgroups that contained less than 4 individuals were not included in the study.
[5] This subgroup comprises patients who do not fit into the other subgroups including the minor subgroups.
[6] Statistical significancy of the differences between respective and control GMTs, evaluated by a t-test, is given in the parenthesis.
N.S. — non-significant.

and children with Burkitt-type, lymphoblastic, and centroblastic lymphomas. On the other hand, IgG titres were low or missing in patients with mediastinal lymphomas, malignant unclassified lymphomas, and centroblastic-centrocytic lymphomas.

Table 3 shows IgM responses to the EBNA-1-derived synthetic peptide by various patient subgroups. As the incidence of IgM antibody was lower than that of IgG, the geometric mean titres (GMTs) presented in Table 3 were calculated from the positive sera only. Among the NHL patients, 15, i.e. 26.8% were IgM-positive against the peptide. Surprisingly, 19.3% of control children also reacted with the peptide in the IgM class. Moreover, the mean titres in IgM-positive individuals of both groups were about the same: 383 and 471, respectively. The highest IgM titres were found in about 30 per cent of patients with abdominal tumour location. All four IgM-positive patients of this subgroup were among the six individuals possessing the highest titres (above 500).

Table 3. Antipeptide IgM antibody in child non-Hodgkin's lymphoma[1]

Patient group	Number of patients tested	Positive per cent	GMT of positive sera only[2]
NHL (total)	56	26.8	383
Control children	31	19.3	471
Tumours in abdomen	14	28.6	814
neck	20	20.0	298
mediastinum	12	41.7	331
generalized	10	20.0	N.C.[3]
Lymphoma histology:			
Burkitt type	13	15.4	N.C.
lymphoblastic	14	7.1	N.C.
non-classified	7	57.1	510
centroblastic	4	N.C.[4]	N.C.
centroblastic-centrocytic	4	N.C.[4]	N.C.

[1] All sera were collected before treatment of patients and were absorbed with control antigen.
[2] GMT was calculated from positive sera only.
[3] N.C. — Not calculated because of low number of positive cases and/or patients tested.
[4] Only one positive patient was found in either group.

Somewhat raised IgM titres were also recorded in patients with malignant unclassified lymphomas. This subgroup and that with tumours in the mediastinum had the highest rates of IgM-positive persons: 57.1 and 41.7%, respectively, despite of their titres not being so high as in IgM-positive patients with abdominal tumours. Surprisingly, these two subgroups had low or zero titres of IgG antibodies against the peptide. No conclusions could be drawn on a possible relation between IgM titres and tumour histology because of the low number of positive individuals and/or cases.

Discussion

In child NHL, IgG titres against EBNA-1-derived synthetic peptide were only slightly raised above the levels found in normal controls; this was in line with our preliminary results (Roubalová *et al.*, 1988). The NHL patients exhibited somewhat increased antipeptide IgM incidence over controls, but the IgM titres were comparable in both groups.

Distribution of the patients into subgroups based on tumour location and tumour histology revealed significant differences in antipeptide-antibody levels: both IgG and IgM antipeptide titres were increased in patients with tumours in the abdomen. High IgG titres were also associated with Burkitt's type, lymphoblastic, and centroblastic lymphomas. Recently, we described (Roubalová *et al.*, 1988) that the responses against certain EBV antigens

other than EBNA-1 were also the highest in subjects with Burkitt's type and abdomen-located lymphomas.

On the other hand, low IgG titres were detected in children with unclassified malignant lymphomas, in all the cases with centroblastic-centrocytic lymphomas, and in lymphomas located in the mediastinum. Surprisingly, the patients with mediastinal lymphomas and unclassified NHLs displayed antipeptide IgM antibody more frequently than others. The nature of the differences between IgG and IgM response is unknown. One can only speculate that the patients with low IgG antibody levels might have had a defect in switching from IgM to IgG synthesis or might have developed IgM autoantibodies that cross-reacted with the gly-ala peptide. Both possibilities could have also occurred in combination.

It is not understood why antipeptide IgM antibodies were relatively frequent in the control group of normal children. Since the sera were absorbed with control antigen (see Materials and Methods), they seem to have been gly-ala-peptide specific. It is noteworthy that IgM antibodies reacting with the EBNA-1 peptide are frequently found in the acute phase of IM (Smith *et al.*, 1986; Geltosky *et al.*, 1987). These antibodies cross-react also with several cell proteins (Rhodes *et al.*, 1987) and may persist in a certain proportion of individuals for a rather long time. Our results indicate that about 20 per cent of healthy children may have low titres of similar autoantibodies. Moreover, their frequency might well be increased in some patient groups.

As described here and in our previous report (Roubalová *et al.*, 1988), pronounced EBV-related serological findings were detected in certain subgroups of children NHL. This may indicate a closer relationship between these subgroups and EBV. Despite our having found viral DNA and EBNA-1 directly in lymphoma tissue in one instance (unpublished observation), it is probable that in the majority of cases the serological findings might rather have been a consequence of EBV activation due to the immune suppression caused by the lymphoma itself. However, the study of EBV behaviour in child NHL could help with elucidation of the *in vivo* condition that leads to virus activation and the possible superimposition of EBV activation on the underlying lymphoma.

References

Britton, S., Andersen-Anvret, M., Gergely, G., Henle, W., Jondal, M., Klein, G., Sandsett, B., and Svedmyr, E. (1978): Epstein-Barr virus immunity and tissue distribution in fatal case of infectious mononucleosis. *Med. Inteligence* **298**, 89—92.

Břicháček, B., Hirsch, I., Šíbl, O., Vilikusová, E., and Vonka, V. (1983): Association of some supraglotic laryngeal carcinomas with EB virus. *Int. J. Cancer* **32**, 193—197.

Dillner, J., Sternas, L., Kallin, B., Alexander, H., Ehlin-Henriksson, B., Jorvall, H., Klein, G., and Lerner, R. (1984): Antibodies against a synthetic peptide identify the Epstein-Barr virus nuclear antigen. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4652—4656.

Filipowich, A. H., Spector, B. D., and Kersey, J. (1980): Immunodeficiency in humans as a risk factor in the development of malignancy. *Prevent. Med.* **9**, 252—259.

Geltosky, J. E., Smith, R S , Whalley, A., and Rhodes, G. (1987): Use of a synthetic peptide-based ELISA for the diagnosis of infectious mononucleosis and other diseases. *J. clin. Lab. Analysis* **1**, 153—162

Hanto, D. W., Frizzera, G., Purtilo, D. J., Sakamoto, K., Sullivan, J. L., Saemundsen, A. K., Klein, G., Simmons, R. L., and Najarian, J. S. (1981): Clinical spectrum of lymphoproliferative disorders in renal transplant recipients and evidence for the role of Epstein-Barr virus. *Cancer Res.* **41**, 4253—4261.

Henle, W., Henle, G., (1981): Epstein-Barr virus-specific serology in immunologicaly compromised individuals. *Cancer Res.* **41**, 4222—4225.

Henle, W., Henle, G., and Diehl, V. (1968): Relation of Burkitt's tumor-associated herpesvirus to infectious mononucleosis. *Proc. natn. Acad. Sci. U.S.A.* **59**, 94—101.

Klein, G., Giovanella, B. C., Lindahl, T., Fialkow, P. J., Singh, S., and Stehlin, J. S. (1974): Direct evidence for presence EBV-DNA and nuclear antigen in malignant epithelial cells from patients with poorly differentiated carcinoma of nasopharynx. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4737—4741.

Lennert, K. (1981): *Histocompatibility of non-Hodgkin's lymphomas (based on Kiel classification).* Springer — Verlag, New York.

Merrifield, R. B. (1963): Solid phase peptide synthesis. I. The synthesis of tetrapeptide. *J. Am. chem. Soc.* **85**, 2149—2154.

Nonoyama, M., Huang, D. H., Pagano, J. S., and Singh, S. (1973): DNA of Epstein-Barr virus detected in tissue of Burkitt's lymphoma and nasopharyngeal carcinoma. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3265—3268.

Purtilo, D. T. (1981): Immune deficiency predisposing to Epstein-Barr virus-induced lymphoproliferative syndrome as a model. *Advanc. Cancer Res.* **34**, 279—312.

Purtilo, D. T., DeFlorio, D., Hutt, L. M., Phawan, J., Yang, J. P., Otto, R., and Edwards, W. (1977): Variable phenotypic expression of an X-linked recessive lymphoproliferative syndrome. *New Engl. J. Med.* **297**, 1077—1081.

Purtilo, D. T. (1987): Opportunistic cancers in patients with immunodeficiency syndromes. *Arch. Pathol. Lab. Med.* **111**, 1123—1129.

Reedman, B. M., and Klein, G. (1973): Cellular localization of an Epstein-Barr virus (EBV) — associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. *Int. J. Cancer* **11**, 499—520.

Reedman, B. M., Klein, G., Pope, J. H., Walters, M. K., Hilgers, J., Singh, S., and Johansson, B. (1974): EBV-associated complement-fixing and nuclear antigens in Burkitt lymphoma biopsies. *Int. J. Cancer* **13**, 755—763.

Rhodes, G., Carson, A. D., Valbracht, J., Houghten, R. and Vaughan, J. H. (1985): Human immune responses to synthetic peptide from the Epstein-Barr virus nuclear antigen. *J. Immunol.* **134**, 211—216.

Rhodes, G., Rumpold, H., Kurki, P., Patrick, K. M., Carson, D. A., and Vaughan, J. H. (1987): Autoantibodies in infectious mononucleosis have specificity for the glycine-alanine repeating region of the Epstein-Barr virus nuclear antigen. *J. exp. Med.* **165**, 1026—1040.

Roubalová, K., Weinreb, M., Roubal, J., Kodet, R., Koutecký, J., and Vonka, V. (1988): EpsteinBarr virus (EBV) antibodies in children with non-Hodgkin's lymphomas. *Acta virol.* **32**, 339—348.

Smith, R. S., Rhodes, G., Vaughan, J. H., Horwitz, C. A., Geltosky, J. E., and Whalley, A. S. (1986): A synthetic peptide for detecting antibodies to Epstein-Barr virus nuclear antigen in sera from patients with infectious mononucleosis. *J. infect. Dis.* **154**, 885—889.

Vestergaard, B. F., Graubale, P. C., and Spanggard, H. (1977): Titration of herpes simplex virus antibodies in human sera by the enzyme-linked immunosorbent assay (ELISA). *Acta Pathol. microbiol. Scand.* (B) **85**, 466—468.

Wilmes, E., and Wolf, H. (1981): Tonsillen Karcinome und Epstein-Barr Virus. *Laryng. Rhinol. Othol.* **62**, 7—10.