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Humoral immune response in Epstein–Barr virus infections. II. IgG subclass distribution in African patients with Burkitt's lymphoma and nasopharyngeal carcinoma

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SUMMARY

Native Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) patients from Kenya were examined with regard to the serum concentrations and distribution of the four IgG subclasses, total IgG, and antibody activities to Epstein–Barr (EB) virus associated antigens. The results were compared with corresponding data of an African control group. As revealed by indirect immunofluorescence techniques, the patients displayed a pattern of IgG and IgA antibodies to EB virus associated antigens which is characteristic for these diseases. No significant differences could be detected between the total IgG levels of the diagnostic groups. The mean total IgG concentrations of our Kenyan patients were two to three times as high as those found in four different groups of Europeans, which is consistent with results of previous studies on Gambian, Nigerian, and Congolese Bantu populations. Quantitative determination of the four IgG subclasses by radial immunodiffusion revealed a unique pattern in the BL group which was characterized by a decreased proportion of IgG2 and significantly lower absolute IgG2 values as compared with the controls. The IgG subclass distribution pattern in the African NPC sera was essentially identical with that of European NPC and African control sera. The pathogenetic implications of these findings are discussed.

Keywords Epstein–Barr virus Burkitt's lymphoma nasopharyngeal carcinoma IgG sublcasses Kenya

INTRODUCTION

Like native Burkitt's lymphoma (BL), undifferentiated nasopharyngeal carcinoma (NPC) was linked to Epstein–Barr (EB) virus by seroepidemiological studies (Old *et al.*, 1966; Henle *et al.*, 1969, 1970; De Schryver *et al.*, 1969; Gunvén *et al.*, 1970) and by the demonstration of EB virus DNA in tumour cells (zur Hausen *et al.*, 1970; Kaschka-Dierich *et al.*, 1976). While geographical and meteorological co-factors (altitude, temperature, humidity), favouring transmission of malaria, and probably genetic predisposition were shown to play an important role in the aetiology and pathogenesis of BL (Burkitt, 1962; Booth *et al.*, 1967), the development of NPC appeared to be essentially determined by genetic and cultural influences (Simons *et al.*, 1975a, 1975b; for reviews see de-Thé, 1979 and Epstein & Achong, 1979).

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It was shown in the mouse system that the growth dynamics of allogeneic tumours is influenced by the humoral immune response of the host. Takasugi & Hildemann (1969) characterized a tumour specific antibody belonging to the IgG2 subclass which enhanced the growth of allogeneic tumours. As similar mechanisms might be operative in neoplastic diseases of humans, we hold it relevant to investigate the serum IgG subclass distribution and subclass characteristics of specific antibodies in these conditions.

A first step was made when we reported on the IgG subclass distributions in infectious mononucleosis (IM) and European NPC (Kaschka, Hilgers & Skvaril, 1982). The present investigation was designed to supply information on total IgG and IgG subclass concentrations as well as IgG and IgA antibody activities to EB virus associated antigens in serum specimens from Kenyan BL and NPC patients.

MATERIALS AND METHODS

Patients. The study includes 42 African subjects divided into three groups (Table 1). The first group consists of 18 NPC patients; the second group comprises 10 cases of EB virus associated BL, and the third group is formed by 14 individuals suffering from EB virus unrelated diseases of the head and neck regions (controls). Clinical examinations and blood sampling were carried out at the Department of Head and Neck Surgery, Kenyatta National Hospital, Nairobi, Kenya.

EB virus associated NPC was diagnosed on the basis of clinical, histological and serological criteria. All tumours were classified as anaplastic or undifferentiated carcinomas. In the corresponding serum specimens distinctly elevated IgG and IgA antibody activities were recorded to VCA and EA. Furthermore, high anti-EB virus nuclear antigen (EBNA) titres could be demonstrated (Table 2).

The diagnosis of BL was established on clinical, histological, and cytological grounds. The tumours were characterized as poorly differentiated lymphocytic lymphomas containing variable numbers of non-malignant histiocytes and thereby showing the typical 'starry sky' appearance.

Immunofluorescence techniques. Antibody titres to the viral capsid antigen (VCA) and to the D (i.e. diffuse) and R (i.e. restricted) components of EA were determined separately for the IgG and IgA immunoglobulin classes using established indirect immunofluorescence techniques (Henle & Henle, 1966; Henle *et al.*, 1969; Henle, Henle & Klein, 1971). Fluorescein isothiocyanate (FITC) labelled goat anti-human IgG (heavy and light chain) and anti-human IgA (α chain) reagents were obtained from Hyland Laboratories (Los Angeles, California, USA).

All the sera were titrated for antibodies to EBNA by a slightly modified anti-complement immunofluorescence (ACIF) procedure (Reedman & Klein, 1973). Each specimen was tested on smears of two cell lines: (1) the African BL derived, EB virus genome positive non-producer lymphoblastoid cell line Raji (Pulvertaft, 1965; Epstein *et al.*, 1966), which expresses EBNA, and (2) for control the American BL derived, EB virus genome negative lymphoblastoid cell line Ramos (Klein *et al.*, 1975) which does not express EBNA. One batch of cells was used throughout the whole study, respectively.

Prior to preparation of smears the cells were washed twice in balanced salt solution (BSS), then resuspended in hypotonic solution (HS: 0·016% (wt/vol.) $MgCl_2$, 0·015% $CaCl_2$, 0·276% glycerol, adjusted to pH 7·0) to which four drops of fetal calf serum had been added per ml. One drop of concentrated cell suspension was spread on each slide, air dried at room temperature for 15 min, and fixed in newly prepared methanol/acetone (1:2) at $-20°C$ for at least 5 min. Human serum which did not contain any detectable antibody activity to EB virus associated antigens, as judged by the indirect immunofluorescence techniques described, and which gave no reaction with Raji cells in the ACIF test served as a source of complement. The smears were treated with serial dilution of patient sera at 37°C for 15 min, washed, and incubated with a 1:10 dilution of complement at 37°C for 15 min. After a further wash, the smears were stained with a 1:20 dilution of FITC conjugated anti-human β_{1C}/β_{1A} globulin (Hyland Laboratories, Los Angeles, California, USA) at room temperature for 30 min, rinsed, counterstained with Evans blue (100 mg/l) at room temperature for 5 min, washed again, and mounted in BSS/glycerol (1:1).

Appropriate anti-EB virus negative and positive control sera were tested simultaneously. All

Table 1. Clinical data of East African patients with BL, NPC and EB virus unrelated diseases of the head and neck regions (controls)

Group	Patient No.	Initials	Age (years)	Sex	Diagnosis
Controls	1	D. N.	15	f.	dacryadenitis
	2	S. K.	45	m.	sinusitis
	3	O. J.	9	f.	adenoid
	4	F. M.	35	m.	tumour of the glomus jugulae
	5	T. O.	78	m.	parotid tumour
	6	J. G.	32	m.	carcinoma of the larynx
	7	K. M.	51	m.	carcinoma of the larynx
	8	M. M.	47	m.	carcinoma of the larynx
	9	E. M.	68	m.	carcinoma of the larynx
	10	M. K.	42	m.	carcinoma of the tongue
	11	K. M.	66	m.	carcinoma of the palate
	12	O. J.	41	f.	carcinoma of the lip
	13	G. S.	30	m.	Kaposi's sarcoma
	14	A. V.	50	f.	sarcoma of the neck
BL	15	M. M.	12	m.	BL
	16	T. M.	7	m.	BL
	17	M. J.	9	f.	BL
	18	J. O.	8	m.	BL
	19	M. O.	13	m.	BL
	20	S. H.	7	m.	BL
	21	M. N.	18	m.	BL
	22	E. O.	7	m.	BL
	23	M. G.	not known	m.	BL
	24	P. B.	3	f.	BL
NPC	25	D. M.	30	m.	NPC
	26	J. I.	46	m.	NPC
	27	P. M.	22	m.	NPC
	28	J. A.	39	m.	NPC
	29	E. K.	61	m.	NPC
	30	P. M.	71	m.	NPC
	31	S. M.	21	m.	NPC
	32	M. K.	20	m.	NPC
	33	P. G.	57	m.	NPC
	34	T. K.	67	m.	NPC
	35	P. S.	55	m.	NPC
	36	A. N.	63	m.	NPC
	37	J. N.	47	m.	NPC
	38	W. N.	21	m.	NPC
	39	M. G.	50	f.	NPC
	40	I. B.	30	m.	NPC
	41	J. N.	27	m.	NPC
	42	D. M.	70	m.	NPC

Table 2. Geometric mean titres* and ranges† of antibodies directed against VCA, EBNA and the D and R components of EA in East African patients with BL, NPC and EB virus unrelated diseases of the head and neck regions (controls)

Group	Anti-EA(D) IgG	Anti-EA(D) IgA	Anti-EA(R) IgG	Anti-EA(R) IgA	Anti-VCA IgG	Anti-VCA IgA	Anti-EBNA
Controls $n=14$	< 10	< 10	< 10	< 10	170 (60; 500)	< 10	55 (5; 450)
BL $n=10$	10 (0·5; 500)	< 10	20 (0·5; 1200)	< 10	800 (50; 12500)	10 (1·5; 50)	110 (25; 500)
NPC $n=18$	50 (0·5; 3500)	20 (1; 400)	30 (1; 800)	< 10	1000 (80; 12500)	60 (2; 2200)	80 (6; 900)

* Titres are given as reciprocal values.

† Ranges have been calculated after $e^{\bar{y} \pm 2S_y}$ where $y_L = \ln(x_L)$.

serum specimens were coded before being tested and the clinical data were withheld until the titres had been determined to avoid any bias in titration.

IgG subclass determinations. IgG subclass concentrations were measured by radial immunodiffusion in agar plates which contained the respective subclass specific antisera, as described previously (Skvaril, Roth-Wicky & Barandun, 1980; Kaschka *et al.*, 1982).

The total IgG concentrations of our serum specimens were determined as the sum of the four IgG subclass concentrations which gave somewhat lower results than direct measurements of total IgG by radial immunodiffusion (Morell & Skvaril, 1971; Kaschka *et al.*, 1979) or by nephelometry (Kaschka *et al.*, 1982).

Statistical analysis. The results of the immunofluorescence tests were expressed as geometric mean titres and ranges. The concentrations of the four IgG subclasses and of total IgG were given as mean values and standard deviations. The Kruskal–Wallis test served for the evaluation of differences between groups for any parameter. If the global test showed significant differences ($P < 0{\cdot}05$) pairwise comparisons according to Holm (1979) were performed for the $\alpha = 0{\cdot}05$ level of significance.

RESULTS

Antibody titres to VCA, EA, and EBNA

Table 2 shows the results obtained by the various immunofluorescence techniques. The anti-EA response in EBV associated BL is characterized by a complete lack of specific IgA antibodies and markedly elevated IgG anti-EA (R) titres. In one BL patient (T.M., No. 16) an IgG anti-EA (D) titre of 1 : 1280 has been observed which is an unusually high value in comparison with typical BL cases described by Henle & Henle (1979).

In NPC we find an intermediate IgG antibody titre directed against EA (D) and somewhat lower IgA anti-EA (D) and IgG anti-EA (R) activities. The geometric mean IgG anti-VCA titre reaches the six-fold value of the controls and is markedly higher than the corresponding titre of the BL patients. As far as IgA anti-VCA is concerned, the absolute values of the antibody titres are considerably lower than the respective IgG titres, but the difference between the NPC and control groups is even more distinct. In contrast, the geometric mean titre of anti-VCA IgA antibodies in the BL group is essentially identical with the control value.

Both, the African BL and NPC patients, exhibit distinctly elevated geometric mean titres for anti-EBNA, as compared with the African controls. These antibody patterns to EB virus associated antigens are in accordance with those reported by Henle & Henle (1976, 1979).

Total IgG concentrations

No significant differences can be recorded between the total IgG concentrations of the Kenyan BL, NPC and control sera (Table 3). The respective empirical distribution functions are almost congruent (Fig. 1).

The greatest standard deviation is observed in the control group (Table 3), and it appears noteworthy that patients G.S. (No. 13) and A.V. (No. 14), who exhibit the highest serum IgG concentrations in this group, both suffer from sarcomas (Table 1).

As compared with European NPC patients studied by Kaschka *et al.* (1982), the mean total IgG levels of our African BL an NPC groups are twice as high. Analogous results were obtained independently by different investigators examining a variety of physiological and pathological conditions in Europeans and Africans (Cohen & McGregor, 1963; Rowe *et al.*, 1968; Turner & Voller, 1966, Michaux, 1966).

IgG subclass distributions

Tables 3 & 4 show the IgG subclass concentrations and their distributions. The mean value for IgG2 is significantly lower in the BL group than in the NPC patients and in the controls (Table 3). For IgG1, IgG3 and IgG4 the differences between groups do not reach statistical significance.

Table 3. Total serum IgG and IgG subclass concentrations in East African patients with BL, NPC, and EB virus unrelated diseases of the head and neck regions (controls)

Group		Total IgG (g/l)	IgG1 (g/l)	IgG2 (g/l)	IgG3 (g/l)	IgG4 (g/l)
Controls	Mean	33·36	23·14	6·37	2·87	0·98
$n=13$	s.d.	16·32	13·83	3·34	0·92	0·64
	range*	(21; 64)	(13; 46)	(3·1; 7·4)	(1·7; 3·7)	(0·3; 1·7)
BL	Mean	30·16	24·20	2·38†	2·65	0·93
$n=10$	s.d.	11·46	9·36	1·19	1·28	1·04
	range*	(16; 42)	(12; 33)	(1·3; 2·7)	(1·1; 3·5)	(0·2; 2·5)
NPC	Mean	28·70	19·50	5·71	2·81	0·68
$n=18$	s.d.	11·82	10·05	3·77	1·81	0·32
	range*	(17; 48)	(11; 40)	(2·8; 9·3)	(1·2; 5·7)	(0·3; 1·1)

* Symmetrical distribution free 80% range computed from the cumulative distribution functions.

† Significantly different from the control group ($P<0·005$).

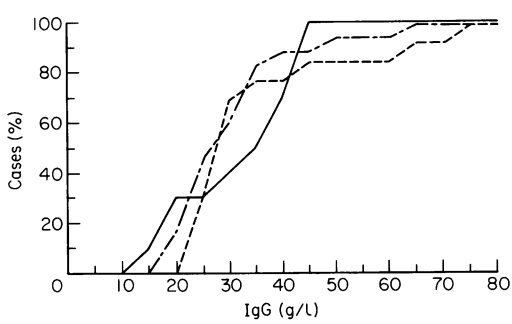

Fig. 1. Empirical distribution functions for total IgG in East African patients with BL, NPC and EB virus unrelated diseases of the head and neck regions (controls) (—— = BL; ·-·-·-· = NPC; - - - - = controls)

Table 4. Serum IgG subclass distributions* in Europeans and Africans with different EB virus associated diseases and in various control groups

Group	IgG1 (%)	IgG2 (%)	IgG3 (%)	IgG4 (%)
WHO reference serum pool 67/97†	60·0	29·4	6·5	4·1
108 healthy male European adults†	60·9	29·6	5·3	4·2
Anti-EB virus negative Europeans‡	59·5	29·1	7·2	4·3
Anti-VCA positive Europeans‡	64·7	23·6	6·8	4·9
African controls	69·4	19·1	8·6	2·9
Europeans with IM‡	73·0	17·8	5·2	4·0
Europeans with NPC‡	70·6	20·5	6·1	2·8
Africans with NPC	67·9	19·9	9·8	2·4
Africans with BL	80·2	7·9	8·8	3·1

* For reasons of comparability, the relative proportions (percentages) refer to total IgG values calculated as the sum of the four IgG subclass concentrations, respectively.

† Calculated from the data reported by Morell & Skvaril (1971).

‡ Calculated from the data reported by Kaschka *et al.* (1982).

The relative proportions of the four IgG subclasses in Kenyan individuals suffering from BL, NPC, and EB virus unrelated diseases of the head and neck regions are summarized in Table 4. This information is confronted with analogous data from European patients with EB virus associated diseases and different control groups (Kaschka *et al.*, 1982). While marked differences have been detected between the European and African NPC patients with regard to the absolute values of their total serum IgG and IgG subclass concentrations (see above), the relative distributions of the four IgG subclasses are essentially similar in both groups. The BL patients have the highest percentage of IgG1 and lowest percentage of IgG2.

The empirical distribution functions for IgG2 illustrate the similarity between the NPC and control goups and the differences between these and the BL patients (Fig. 2).

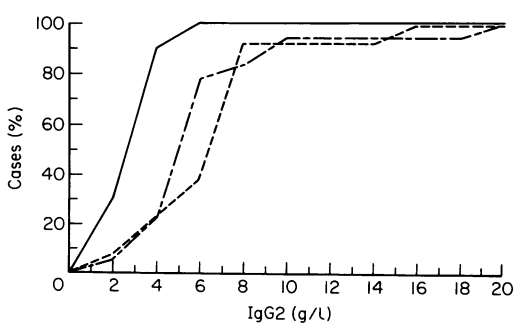

Fig. 2. Empirical distribution functions for the IgG2 subclass concentrations in East African patients with BL, NPC, and EB virus unrelated diseases of the head and neck regions (controls). (——= BL; ·-·-·-·= NPC; - - - - = controls)

DISCUSSION

It has been established that Nigerian, Gambian, and Congolese Bantu populations exhibit higher total serum IgG levels than Europeans (Cohen & McGregor, 1963; Turner & Voller, 1966, Michaux, 1966, Rowe *et al.*, 1968). Our observations are accordant with these findings and extend them to a population in Kenya.

The elevated serum IgG levels in Africans have been shown to result from an increased IgG synthesis (Cohen & McGregor, 1963) which could be due to genetic and environmental factors (Turner & Voller, 1966; Rowe *et al.*, 1968, Henle & Henle, 1979).

The most striking features in the IgG subclass distribution pattern of our African BL patients are a significantly lower mean IgG2 concentration and a markedly decreased IgG2 proportion of total IgG as compared with the African NPC and control groups (Tables 3 & 4, Fig. 2). Analogous differences are to be found between the African BL patients and various groups of healthy Europeans (Table 4).

Whereas European NPC patients displayed significantly elevated serum concentrations of total IgG and of the IgG 1 subclass as compared with two different healthy control groups (Kaschka *et al.*, 1982), no such differences could be detected between our Kenyan NPC and control populations (Tables 3 & 4, Fig. 1). Although considerably higher total IgG and IgG subclass levels were recorded in the African than in the European NPC sera (Table 3; Kaschka *et al.*, 1982), the relative distributions of the four IgG subclasses are similar in both groups (Table 4).

As displayed in Table 2, EB virus specific antibodies of the IgA class reach considerable titres in the NPC group. They are mainly directed against VCA and against the D component of EA. These results are in accordance with earlier data reported by Henle & Henle (1976) who, additionally, observed a correlation between IgA antibody titres to EB virus associated antigens and the tumour burden in NPC. Comparing the EB virus specific antibody titres of African and European NPC patients (Table 2; Kaschka *et al.*, 1982), we find them to be in the same order of magnitude.

The question arises whether the IgG subclass distribution pattern detected in our African BL sera is caused by immunoglobulin secretion of the tumour cells or by the humoral immune response to tumour antigens or, possibly, by tumour unrelated factors. Examining large numbers of BL biopsies, Fialkow *et al.* (1973) and Gunvén *et al.* (1980) were able to show that the majority of cells carried surface immunoglobulins. These immunoglobulins were either true constituents of the cell membrane so that the respective idiotypes could serve as clonal markers or, in a few cases, they resulted from outside coating of the cells with IgG (Gunvén *et al.*, 1980; Klein *et al.*, 1968). *In vitro* studies on BL derived cell lines showed that a minor fraction of the lines did not produce immunoglobulins and that the others usually did not secrete them, but rather contained a high amount of immunoglobulins at the cell surface. The stage of differentiation of BL lines with regard to immunoglobulin production was reminiscent of that of resting B cells (for review see Nilsson, 1979).

The peak incidence of BL was observed in the age group of 6–7 years (Burkitt, 1963). Our BL patients range in age from 3 to 18 years with a mean value of 9·0 years. Since, in contrast, the African control persons have a mean age of 41·4 years with a minimum of 9 and a maximum of 78 years (Table 1), the question of comparability of the two diagnostic groups requires discussion. Rowe *et al.* (1968) found that Gambian children attained adult total serum IgG concentrations by about the fifth year of age. In a European population studied by Morell, Skvaril & Barandun (1975) adult IgG levels were reached already within the second year of life. Considering that the mean age of our BL group is distinctly higher, we can pre-suppose adult IgG concentrations. Thus, a comparison of this group with the African controls concerning their total IgG levels appears to be justifiable. As of yet, no information is available on the age related development of the four IgG subclass concentrations in Africans. Investigating this question in European and American children, respectively, Schur, Rosen & Norman (1979) and Oxelius (1979) were able to demonstrate that IgG1, IgG3 and IgG4 attained adult levels around the seventh year of life whereas IgG2 did not reach adult values before puberty.

Based on these findings, we conclude that the IgG subclass distribution pattern detected in our

BL patients is probably age specific rather than being due to a humoral immune response to tumour antigens, immune complex formation, or tumour specific regulatory mechanisms (e.g. generation of enhancing or inhibitory antibodies).

The subclass properties of specific IgG antibodies directed against EB virus associated antigens are currently investigated.

Whereas the role of immunogenetic processes in the aetiology and pathogenesis of NPC is still unclear (Klein, 1979, de-Thé, 1979), the consistent appearance of specific chromosomal translocations in BL has suggested that they might play a role in malignant transformation (for review see Klein, 1981). Regularly, immunoglobulin genes are located upon the chromosomes involved. The recent detection of the human c-myc onc gene in regions of BL chromosomal translocation and, at the same time, in the vicinity of immunoglobulin heavy chain loci (Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982; for review see Klein, 1983) might be a first step in elucidating the aetiology of BL.

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