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Humoral immune response in Epstein-Barr virus infections. I. Elevated serum concentration of the IgG1 subclass in infectious mononucleosis and nasopharyngeal carcinoma

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SUMMARY

Using radial immunodiffusion we measured IgG subclass concentrations and studied their distribution in serum samples from patients with infectious mononucleosis (IM) and nasopharyngeal carcinoma (NPC), two Epstein-Barr virus (EBV)-associated diseases, in comparison with two control groups [completely anti-EBV negative persons and subjects carrying antibodies to the viral capsid antigen (VCA)]. Antibody titres to VCA and to the early antigen (EA) were determined by indirect immunofluorescence and revealed characteristic patterns for the respective diagnostic groups. Nephelometric assays served for quantitating total protein, albumin, total IgG, IgA and IgM in all the sera. In the IM and NPC groups the concentration of IgG1 was significantly elevated by more than 50% whereas the other three subclasses remained unchanged as compared with the controls. Correspondingly, we found a significant increase of total IgG in IM and NPC. In IM, the only disease where VCA-specific IgM antibodies have been reported to occur, IgM levels were markedly elevated. Our data suggest that the IgG1 subclass plays an important role in the humoral immune response to EBV-determined antigens and that it is possibly involved in the control of virus infection.

INTRODUCTION

Epstein-Barr virus, a B lymphotropic herpes virus acting as a polyclonal B cell activator (Jondal & Klein, 1973; Pattengale, Smith & Gerber, 1974; Rosén *et al.*, 1977), has been identified as the causative agent of IM (Henle, Henle & Diehl, 1968). It is regularly associated with two human tumours: (1) Burkitt's lymphoma (BL), a monoclonal B cell malignancy preferentially occurring in East African children (Fialkow *et al.*, 1973; Gunvén *et al.*, 1980) and (2) NPC, an epithelial tumour containing varying amounts of T lymphocytes which is remarkably frequent in southern China, where it exhibits an obviously genetically determined relationship with certain HLA antigens (zur Hausen *et al.*, 1970; Klein, 1979). These diseases are characterized by different antibody patterns to EBV-determined antigens (Henle & Henle, 1979).

Recently, subclass restrictions of the IgG response to various antigens have become evident in a number of human diseases (Riesen, Skvaril & Braun, 1976; Mortimer & Widdowson, 1979;

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Kaschka *et al.*, 1979; Siber *et al.*, 1980; Beck, 1981). Stimulated by these reports, we studied the IgG subclass distribution in the sera of patients with IM and NPC as compared with two control groups and correlated these data with the total protein and immunoglobulin concentrations as well as with the antibody titres to EBV-related antigens.

MATERIALS AND METHODS

Patients.

One hundred and twenty-one persons were investigated including 26 cases of acute infectious mononucleosis (IM), 40 cases of nasopharyngeal carcinoma (NPC) (from West Germany), 31 anti-viral capsid antigen (VCA) positive controls and 24 completely anti-Epstein-Barr virus (EBV) negative controls.

IM was diagnosed on clinical, haematological and serological grounds. The clinical syndrome included fever, lymph node enlargement, pharyngitis and liver dysfunction. Peripheral lymphocytosis and numerous atypical lymphocytes were usually detectable. In all IM patients the diagnosis was confirmed by an at least four-fold rise in antibody titres to VCA and EA.

The diagnosis of EBV-associated NPC was established clinically, histologically and serologically. All these cases were classified as anaplastic or poorly differentiated carcinomas. In the corresponding sera a marked elevation of antibody titres to VCA and EA could be demonstrated (Table 1). Two out of the 40 NPC patients were recently diagnosed and still untreated cases, whereas 36 subjects had been treated by irradiation and two by surgery plus irradiation. All treated patients were in a state of remission.

The anti-VCA positive control group was constituted from healthy persons having got over IM at least 1 year ago.

A second control group (anti-EBV negative group) included healthy individuals without a history of IM and without detectable serum antibodies to VCA and EA, as judged by the indirect immunofluorescence techniques described below.

Immunofluorescence techniques. Antibody titres to VCA and EA were determined by modifications of the indirect immunofluorescence techniques described by Henle & Henle (1966). For VCA tests we used smears of the producer cell line P3HR-I. EA was induced in NC 37 cells by treatment with the co-carcinogen and tumour promoter 12-*0*-tetradecanoylphorbol-13-acetate (TPA, Sigma chemicals, Munich, West Germany) in a concentration of 20 ng/ml culture fluid for 60 hr according to zur Hausen *et al.* (1978). One batch of cells was used throughout the whole study, respectively. Anti-EBV negative and positive control sera were tested simultaneously with the patient sera. NPC sera obtained independent from this study served as positive controls. All the

Table 1. Geometric mean titres* and ranges† of antibodies to EA and VCA in IM and NPC as compared with two control groups

	VCA	EA
anti-EBV negative	<8	<8
anti-VCA positive	70 (15; 380)	<8
IM	270 (50; 1450)	40 (10; 210)
NPC	1660 (330; 8400)	150 (10; 2400)

*Titres are given as reciprocal values.

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

patient sera were coded before being tested for VCA and EA antibodies and the clinical data were withheld until the titres were known to avoid any bias in titration. Fluorescein isothiocyanate (FITC)-labelled anti-human IgG was obtained from Behringwerke (Marburg, West Germany) and diluted 40-fold in phosphate-buffered saline (PBS) before use.

Nephelometric assays. Serum protein and immunoglobulin concentrations were measured by means of nephelometric assays using a Beckman nephelometer (70° forward scattering, wavelength 400–550 nm; Beckman instruments, Fullerton, California, USA). For total protein determination serum samples were diluted 200-fold and precipitated in the presence of 2·2 M trichloroacetic acid (Merck, Darmstadt, West Germany) under continuous stirring. During this process, the intensity of scattered light was measured as described by Reiber (1980). Standard human serum (Behringwerke, Marburg) served for calibration.

The quantitation of albumin, total IgG, IgA and IgM was performed by means of kinetic nephelometric assays according to Sternberg (1977) measuring the intensity of scattered light during immunoprecipitin reactions with the respective antisera. For all these reactions we used the specific antisera and standard solutions included in the Beckman immunochemistry reagent kit (Beckman instruments).

IgG subclass determinations. IgG subclass concentrations were determined by radial immunodiffusion in agar plates which contained the respective subclass-specific antisera, as described previously (Skvaril, Roth-Wicky & Barandun, 1980). World Health Organization (WHO) reference serum pool 67/97, the IgG subclass concentrations of which are known, served for calibration (Morell & Skvaril, 1971). Subclass-specific antisera were generated in sheep by immunization with purified myeloma proteins of the respective subclasses followed by absorption with IgG proteins of the remaining three subclasses in order to eliminate antibodies directed against antigenic determinants common to all IgG subclasses. The effectiveness of the absorption was confirmed and the antisera were tested by double immunodiffusion against purified monoclonal proteins of all four subclasses and both (κ and λ) light chain types and also by immunoelectrophoresis using the same antigens. Myeloma proteins of the four IgG subclasses were prepared by chromatography on diethylaminoethyl (DEAE)-Sephacel or by preparative electrophoresis followed by Sephadex G-200 gel filtration. Both gels were obtained from Pharmacia chemicals (Uppsala, Sweden).

Statistical analysis. The results of the immunofluorescence tests (anti-VCA and anti-EA titres) were expressed as geometric mean titres and ranges. The concentrations of total serum protein, albumin, total IgG, IgA, IgM and IgG subclasses were given as mean values and standard deviations. The Kruskal–Wallis test was applied for determining 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 and EA

In all patients of the four diagnostic groups the humoral immunity to VCA and EA was investigated. Table 1 shows that the respective antibody titres reach maximum values in the NPC group and somewhat lower levels in the group with acute IM. The persons having got over IM a considerable time ago (anti-VCA positive group) show markedly lower anti-VCA titres than the IM and NPC groups and, in contrast to the latter ones, have no detectable antibodies to EA. These results are in good accordance with earlier reports (Henle & Henle, 1979). It seems noteworthy that there are only small overlaps between the titre ranges of the different diagnostic groups.

Serum protein and immunoglobulin profiles

The serum protein and immunoglobulin profiles show distinctly elevated IgG concentrations in the IM and NPC groups as compared with the controls (Table 2). In the NPC group we observe the highest IgA levels, but as the individual serum IgA concentrations are distributed over a wide range we get a large standard deviation, and the difference from the controls does not reach statistical significance. The highest serum IgM concentrations are to be found in IM patients. The difference in

Table 2. Serum protein profiles and immunoglobulin concentrations in IM, NPC, and two control groups

		anti-EBV negative	anti-VCA positive	IM	NPC
Total Protein (g/l)	n	15	21	17	35
	$\bar{x}$	82·8	78·2	83·5	89·2†
	s.d.	11·2	15·9	9·5	11·4
	range‡	(65·4, 104·7)	(47·0, 97·7)	(65·6, 100·1)	(72·0, 107·6)
Albumin (g/l)	n	15	21	17	35
	$\bar{x}$	48·7	43·1	45·6	49·9
	s.d.	8·0	11·9	7·7	6·7
	range‡	(32·6, 59·4)	(19·7, 59·8)	(35·6, 59·0)	(39·8, 57·8)
IgG (g/l)	n	14	21	14	34
	$\bar{x}$	13·2	10·7	14·5†	15·6*†
	s.d.	7·0	4·1	3·0	4·5
	range‡	(7·9, 29·4)	(3·3, 15·5)	(11·1, 19·5)	(8·8, 23·4)
IgA (g/l)	n	15	21	17	35
	$\bar{x}$	2·8	2·4	2·4	3·9
	s.d.	1·4	1·5	1·2	3·1
	range‡	(1·4, 5·3)	(0·7, 5·2)	(1·0, 4·8)	(1·4, 13·3)
IgM (g/l)	n	15	21	17	35
	$\bar{x}$	1·8	1·5	2·2†	2·1
	s.d.	0·9	0·7	0·8	1·0
	range‡	(0·8, 3·6)	(0·6, 2·6)	(1·1, 3·4)	(1·1, 4·3)

*Significantly different from the anti-EBV negative group.

†Significantly different from the anti-VCA positive group (Kruskal-Wallis test and Holm's pairwise comparisons; $\alpha = 0{\cdot}05$).

‡Symmetrical distribution-free 95% range computed from the cumulative distribution functions.

IgM between this group and the anti-VCA positive control group is statistically significant (Table 2). In the anti-VCA positive control group we observe the lowest and in the NPC patients the highest mean values for total protein, albumin and IgG. The differences in total protein and IgG levels between these two groups are also statistically significant (Table 2). Figs 1 and 2 show the empirical distribution functions for IgG and IgM in the four diagnostic groups.

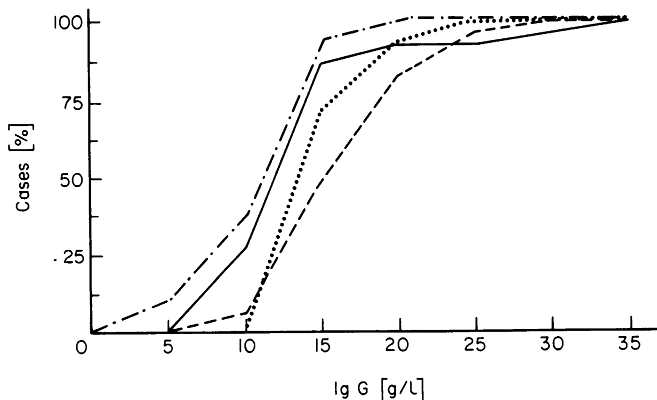

Fig. 1. Empirical distribution functions for total IgG in the four diagnostic groups. (—— = anti-EBV negative; —·— = anti-VCA positive; ···· = IM; – – – = NPC).

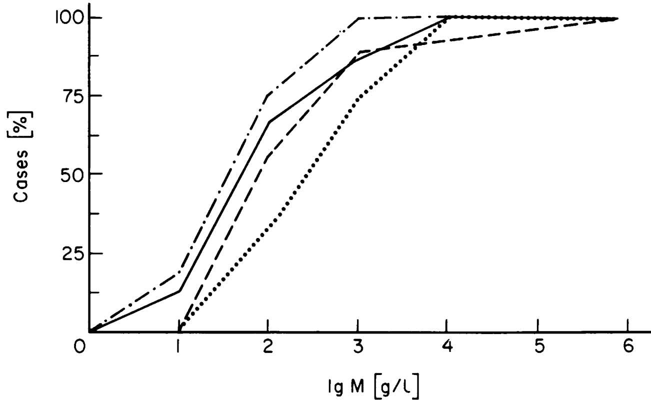

Fig. 2. Empirical distribution functions for IgM in the four diagnostic groups. (For explanation of lines see Fig. 1).

IgG subclass distribution

All the sera were examined with regard to the concentrations of the four IgG subclasses. Our results are summarized in Table 3 and Fig. 3. The statistical analysis of these data revealed a significant elevation of the IgG1 concentration in the IM and NPC groups by more than 50% as compared with both control groups (Table 3). As far as IgG2, IgG3, and IgG4 are concerned, no significant differences could be demonstrated between IM, NPC and the control groups. In IM 70% of total

Table 3. IgG subclass concentrations in IM, NPC, and two control groups

		anti-EBV negative	anti-VCA positive	IM	NPC
IgG1	*n*	23	27	23	39
(g/l)	x̄	6·13	6·45	9·86*†	9·90*†
	s.d.	2·89	2·34	3·30	5·57
	range‡	(2·43, 13·29)	(1·86, 9·87)	(5·32, 16·98)	(4.71, 19·83)
IgG2	*n*	23	27	23	39
(g/l)	x̄	3·00	2·35	2·41	2·87
	s.d.	1·49	1·40	1·28	1·39
	range‡	(1·21, 5·71)	(0·21, 4·54)	(0·74, 5·48)	(0·94, 5·68)
IgG3	*n*	23	27	23	39
(g/l)	x̄	0·74	0·68	0·70	0·86
	s.d.	0·71	0·48	0·50	0·44
	range‡	(0·16, 2·67)	(0·15, 1·66)	(0·16, 2·03)	(0·31, 1·77)
IgG4	*n*	23	27	23	39
(g/l)	x̄	0·44	0·49	0·54	0·39
	s.d.	0·39	0·57	0·63	0·45
	range‡	(0·03, 1·38)	(0·07, 1·88)	(0·10, 1·98)	(0·09, 1·62)

*Significantly different from the anti-EBV negative group.

†Significantly different from the anti-VCA positive group (Kruskal-Wallis test and Holm's pairwise comparisons; $\alpha = 0{\cdot}05$).

‡Symmetrical distribution-free 95% range computed from the cumulative distribution functions.

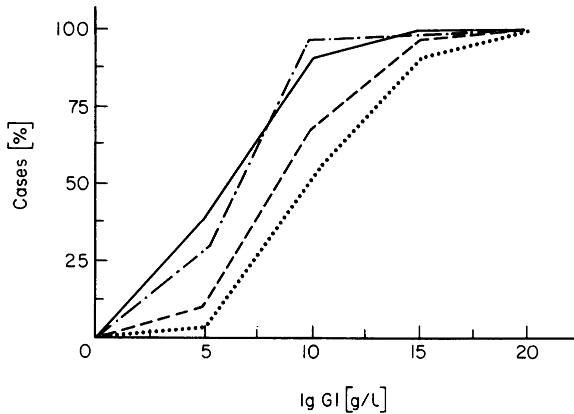

Fig. 3. Empirical distribution functions for IgG1 in the four diagnostic groups. (For explanation of lines see Fig. 1).

IgG belong to the IgG1 subclass, whereas the relative proportion of IgG1 in NPC is 68%, in the anti-EBV negative group 46%, and in the anti-VCA positive group 58%. For comparison, the percentage of IgG1 in 108 healthy male adults amounted to 61% (Morell & Skvaril, 1971).

DISCUSSION

The principal subject of our present communication is the distribution of the four IgG subclasses in IM, NPC and two control groups. We found significantly elevated levels of the IgG1 subclass in both IM and NPC patients, whereas the concentrations of the other three subclasses showed no major differences from the controls (Table 3, Fig. 3). Therefore we have to assume that this elevation of IgG1 is the cause of a corresponding elevation of total IgG in the IM and NPC groups (Table 2, Fig. 1). Taking the elevated antibody titres to VCA and EA (Table 1) into consideration, these observations might indicate that the IgG1 subclass plays a predominant role in the specific antibody response to EBV-determined antigens.

In our study the highest mean IgA concentration was observed in the NPC group (Table 2). However, because of a large standard deviation the difference from the controls did not reach statistical significance. Wara *et al.* (1975) were able to demonstrate elevated serum IgA levels in NPC patients, and Henle & Henle (1976) found that specific IgA antibody titres to VCA and to the D (i.e. diffuse) component of the non-structural EA complex were higher in this group than in controls. Moreover, IgA antibody titres turned out to correlate with the tumour burden in NPC.

The number of untreated NPC cases included in this study was very small (two) and did not allow a statistical comparison of data with the treated cases.

The serum IgM concentration was significantly elevated in the IM group as compared with the anti-VCA positive control group (Table 2, Fig. 2). This finding probably corresponds to the fact that IM is the manifestation of a primary infection with EBV. The occurrence of VCA-specific antibodies belonging to the IgM class has been described exclusively in IM (Henle & Henle, 1979).

Calculating the sum of the four IgG subclass concentrations in each diagnostic group, we should expect a result equal to the total IgG value. In fact this is not the case. Because of a considerably higher specificity of the IgG subclass antisera used in the radial immunodiffusion assay (Skvaril *et al.*, 1980) as compared with the anti-human IgG reagent used in the nephelometric assay (Reiber, 1980) total IgG values are somewhat higher than expected. A detailed discussion of this matter has been published elsewhere (Kaschka *et al.*, 1979).

At present, little is known about the distribution of virus antibody activity among human IgG subclasses. In multiple sclerosis, possibly a 'slow virus' disease, and in subacute sclerosing panencephalitis IgG1 from cerebrospinal fluid and brain extracts seems to contain measles antibody

activity (Vandvik, Natvig & Wiger, 1976; Kaschka *et al.*, 1979). Beck (1981), examining laboratory personnel with especially high antibody titres to rubella, polio (types I, II, and III) and herpes simplex type I viruses, found the main portion of serum antibody activity to these viruses to reside in the IgG3 subclass. Antibodies to rubella, like polio an RNA virus, consisted almost totally of IgG3. Therefore, it is hard to understand why this author concludes that IgG3 plays a special role in the antibody response to DNA viruses. In sera from African BL patients a considerable part of the antibody activity to the EBV-associated nuclear antigen (EBNA) appears to be associated with the IgG3 subclass, as has been shown by absorption studies with Staphylococcus protein A (Luka, J., personal communication). Thus, a number of experimental data support the notion that the four IgG subclasses might have specific physiological roles in the immune system, but it would be premature to attribute certain functions exclusively to one subclass.

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