

## Polyspecific Natural Antibodies and Autoantibodies Secreted by Human Lymphocytes Immortalized With Epstein-Barr Virus

By Jean M. Seigneurin, Brigitte Guilbert, Marie J. Bourgeat, and Stratis Avrameas

Recent studies have shown that autoreactive B cells and autoantibodies are present in pathological as well as in normal situations. In the present study, we immortalized human B cell lines from normal individuals and from patients with malignant or benign dysglobulinemia with Epstein-Barr virus and examined, after cloning, the autoantibody reactivities of the immunoglobulins secreted by these cells. Forty-two supernatants were analyzed by enzyme-immunoassay on a panel of 13 self and non-self antigens: trinitrobenzenesulfonic acid (TNP), DNA, L-glutamine, L-alanine, L-tyrosine (GAT), actin, myosin, tubulin, albumin, renin, spectrin, transferrin, thyroglobulin, myoglobin, peroxidase, and by immunofluorescence in tissue sections. Fourteen (33%) of the immunoglobulin-secreting cell lines were found to have an autoantibody function; seven secreted IgM, six IgA, and one IgG. The

light chains were of the kappa type in 11 cases. The vast majority of these clones reacted with more than five antigens of the panel and all of them reacted with TNP. No correlation was found between a given isotype and an antibody specificity. More than half of these antibodies also reacted with cellular antigens present in tissue sections. None of the four cell lines secreting monoclonal antiviral antibodies reacted with any of the antigens of the panel. The results indicate that immunoglobulins secreted by human monoclonal lymphoid cell lines can have polyspecific autoantibody functions, similar to those found in normal human polyclonal antibodies, in human monoclonal paraproteins and in natural monoclonal antibodies synthesized by murine or rat clones obtained from physiologically normal animals.

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**S**TUDIES THAT we have performed during the last few years have revealed the presence of natural antibodies in humans, rats, and mice.<sup>1-7</sup> The main characteristic of the majority of these natural antibodies and of human monoclonal paraproteins from patients with lymphoproliferative diseases was their polyspecificity, that is, they were able to react with more than two self and/or non-self antigens.

Previous studies have shown that lymphoblastoid cell lines (LCLs) established after immortalization of human B lymphocytes by Epstein-Barr virus (EBV)<sup>8,9</sup> have been used to produce human monoclonal antibodies to haptens, bacterial or viral antigens and other determinants.<sup>10-13</sup> Occasionally, autoantibodies with rheumatoid factor activity or directed against cellular antigens have been detected in LCLs from subjects with evidence of autosensitization.<sup>14</sup> It has been also demonstrated that the autoantibodies reacting with antigens in multiple organs were synthesized by EBV-activated B cells.<sup>15,16</sup> Similarly, Winger et al<sup>17</sup> observed specific antibodies to a variety of autoantigens secreted by EBV-transformed B cell lines in individuals without prior deliberate immunization. Robinson and Stevens<sup>18</sup> recently reported that several clones derived from patients who lacked clinical and serological evidence of autoimmunity produced antibodies reacting with cellular antigens. Thus, studies by us and other groups strongly suggest that autoantibodies and autoreactive B cell clones constitute a normal and physiological status.

In this work using well defined and characterized antigens we studied the specificity of immunoglobulins secreted by several LCLs established from patients with malignant or benign dysglobulinemia or from healthy individuals. We found that the vast majority of the antibodies secreted by LCLs were able to react with more than two self and non-self antigens and corresponded therefore to the natural antibodies previously described.<sup>1-7</sup> Furthermore, as was found for the human monoclonal paraproteins,<sup>3,19</sup> either IgM, IgA, or IgG could possess such a polyreactivity.

### MATERIALS AND METHODS

**Antigens.** Bovine serum albumin (BSA), whale skeletal muscle myoglobin, porcine thyroglobulin, calf thymus native double-

stranded DNA, and trinitrobenzenesulphonic acid (TNP) were purchased from Sigma Chemical Co, St Louis. L-glutamine, L-alanine, L-tyrosine, (60:30:10) co-polymer (GAT) was purchased from Miles Yeda, Ltd, Rehovot, Israel. Human albumin was purchased from Schwartz Mann, Cambridge, MA. Human transferrin (purified) was purchased from Behringwerke A.G., Marburg, FRG. Horseradish peroxidase (grade I, RZ = 3) was obtained from Boehringer, Mannheim, FRG. Calf muscle actin<sup>20</sup> and myosin,<sup>21</sup> pig brain tubulin,<sup>22</sup> and TNP.25/BSA<sup>23</sup> were prepared according to described methods. Mouse maxillary renin was a gift from Dr P.A. Corvol, INSERM, U36, Paris. Human RBC spectrin was a gift from Dr E. Lazarides, Caltech, Pasadena, CA.

**Antibodies.** Goat antibodies to human IgA, IgM, and IgG isolated by passage of antisera over immunoadsorbents were purchased from Biosys, Compiègne, France. They were coupled to *Escherichia coli*- $\beta$ -galactosidase (donated by Dr Ullman, Institut Pasteur, Paris), according to the procedures previously described by Avrameas et al.<sup>24</sup>

**Reagents.** Gelatin was purchased from Prolabo, Paris. Tween 20 was purchased from E. Merck, Darmstadt, FRG. "Luxon" microtitration plates with 96 400  $\mu$ L flat-bottomed wells were obtained from C.M.L., Nemours, France. O-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) was purchased from Sigma Chemical Co.

**Establishment of lymphoid cell lines.** Lymphocytes were obtained from heparinized sterile venous blood, bone marrow aspirates after informed consent from the patients, or biopsies, and isolated by Ficoll-Hypaque density gradient centrifugation. The

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cells were then infected with the B 95-8 strain of Epstein-Barr virus, as previously described.<sup>25,26</sup>

Under the same conditions, the cells were incubated in parallel without the addition of EBV. The cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% CO<sub>2</sub>, and changed twice weekly. After a seven-day incubation, the supernatant media were harvested and centrifuged for antibody secretion analysis. Polyspecific Ig-producing cell lines have been cloned and subcloned by limiting dilution in wells containing  $\gamma$ -irradiated human fibroblasts.

Intracytoplasmic immunoglobulins were detected by indirect immunofluorescence on fixed cells with fluorescein conjugated monospecific antibodies (or F(ab')<sub>2</sub> fragments) to human  $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\kappa$ , and  $\lambda$  chains (Biosys). IgG, IgA, and IgM secreted in the cell culture supernatants were determined and quantified using a sandwich enzyme-linked immunosorbent assay as already described,<sup>27</sup> with Dako monospecific antisera.

**Screening of the culture supernatants for natural antibody activity by enzyme-linked immunosorbent assay.** Luxon flat-bottomed plates were coated with 100  $\mu$ L of various antigens diluted in 0.1 mol/L carbonate-bicarbonate buffer, pH 9.5, for one hour at 37°C and one night at 4°C. The concentrations of the different antigen solutions used for coating were: TNP.25/BSA, 1  $\mu$ g/mL; DNA, 50  $\mu$ g/mL; G.A.T, myosin, renin, spectrin, 5  $\mu$ g/mL; transferrin, thyroglobulin, actin, myoglobin, 10  $\mu$ g/mL; tubulin, 3  $\mu$ g/mL; albumin, 0.4  $\mu$ g/mL; and peroxidase, 20  $\mu$ g/mL. After washing, supernatants (diluted 1/5) were added and allowed to incubate. Plates were washed again and  $\beta$ -galactosidase-labeled-goat anti-IgA, IgM, or IgG antibodies were added. After a further washing the enzyme substrate was added. Details of the technique have been already described.<sup>2</sup> The values obtained with the culture medium under the same conditions were considered as the background level (OD  $\leq$  0.010). As a positive control, a serum pool obtained from 800 normal human donors was used at four dilutions (1/75, 1/150, 1/300, and 1/600).

**Screening of the culture supernatants for antibody to tissue and viral antigens.** Autoantibodies directed against tissue antigens were detected by indirect immunofluorescence on fixed cultured human fibroblasts and on unfixed cryostat sections of various animal and human organs. Anti-viral antibodies were detected in the supernatants by indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition, and/or neutralization tests.<sup>26</sup> The antigens used were herpes simplex virus type 1, cytomegalovirus, varicella-zoster virus, EBV Viral Capsid Antigens (VCA), rubella and measles viruses, and respiratory syncytial virus.

**Isolation and characterization of monoclonal IgM.** The IgM of some of the supernatants expressing anti TNP-activity were isolated by using an E.2.4. DNP-lysine Sepharose adsorbent equilibrated with 0.2 mol/L H<sub>3</sub>BO<sub>3</sub>-NaCl buffer, pH 8, according to the procedure described by Jaffe et al.<sup>28</sup> Furthermore, IgM were isolated by molecular sieving on S-200 Sephacryl. Five milliliters of the 50% ammonium sulfate saturated supernatants were applied to an S-200 Sephacryl gel column (80 cm  $\times$  2 cm) at room temperature, equilibrated with 5 mmol/L phosphate buffer, pH 8.<sup>6</sup> The specificities of the isolated monoclonal IgM were examined using a previously described competitive enzyme-immunoassay-procedure.<sup>2</sup> Briefly, the 50% fixation point of the monoclonal IgM bound to the antigen immobilized on the plate was established using the ELISA described above. The IgM was then incubated at this concentration with increasing concentrations of soluble competing antigens (1 pmol/mL to 5 nmol/mL) and the inhibition of IgM fixation to the plates was determined.

## RESULTS

Forty-two lymphoid cell lines derived from various individuals and cloned were analyzed (Table 1). All these LCLs, but none of the 3 T cell lines used as controls, secreted Igs: 18 secreted IgM, 10 IgG, and 14 IgA. The light chains were mostly of the kappa type (11 of 14 cases). The levels of Ig secretion ranged from 1.8 to 25  $\mu$ g/mL. No significant differences were noted in the isotypes obtained in relation to the classification of the donors.

The LCLs were tested on a panel of self and non-self antigens (Ags). Among the 18 lines secreting IgM, seven were positive with one or more Ags; of the 14 secreting IgA, six reacted with one or more Ags, whereas only one of the ten secreting IgG was positive. Thus, 33% of the Ig-secreting cell lines (n = 14) were found to have autoantibody function (Table 2). Most of these clones originated from malignant or benign dysglobulinemias (11 of 14). All 14 antibody-positive lines reacted with TNP. The next most frequently represented specificities, in order, were: spectrin (n = 12); cytoskeleton proteins (n = 10); myoglobin (n = 8); and thyroglobulin (n = 8); followed by: GAT (n = 5); albumin (n = 4); and DNA (n = 4), whereas peroxidase (n = 2), transferrin (n = 2), and renin (n = 3) were only slightly represented. The vast majority of these 14 antibody-positive lines reacted with more than five antigens. From Table 2, it also appears

**Table 1. Lymphoid Cell Lines Examined (42 LCLs + 3 T Cell Lines as Controls)**

	Donors	No.	Origin of Cells	Isotypes Secreted		
				IgM	IgG	IgA
Group I	Fetus, cord blood	2	Blood	1		1
Group II	Healthy individuals	9	Blood	4	2	3
Group III	Acute viral infections	5	Blood	2	2	1
Group IV	Lupus	1	Blood		1	
Group V	Cancer, leukemia	8	[Blood (n = 6) Bone marrow (n = 1) Spleen (n = 1)]	3	1	4
Group VI	Myeloma, Waldenström	10	[Blood (n = 7) Bone marrow (n = 3)]	5	3	2
Group VII	Benign dysglobulinemia	7	[Blood (n = 4) Bone marrow (n = 2) Lymph node biopsy (n = 1)]	3	1	3
Control T cell lines		3	Blood	—	—	—
	Molt-4, JM, H9					

**Table 2. Specificities of Antibody Positive Lines**

Cell Clones Group of Donor Isotypes	D12 II $\mu\kappa$	F16 VII $\mu\kappa$	R31 VI $\mu\kappa$	R36 VI $\mu\kappa$	R38 VII $\mu\kappa$	G19 VI $\mu\lambda$	M29 VI $\mu\lambda$	T41 IV $\gamma\kappa$	A4 VII $\alpha\kappa$	F14 VI $\alpha\kappa$	G17 VII $\alpha\kappa$	J22 V $\alpha\kappa$	M28 VII $\alpha\kappa$	R34 VII $\alpha\lambda$
<b>Tested by ELISA*</b>														
TNP	++	++	+++	+++	+++	+++	++	+	+++	+++	+++	+++	+++	+++
DNA						+++				+++		+++	++	
GAT				ND		+++				++	++	+++		++
Actin	+	++	+++	+++	+++	+++	+	+++				++		
Myosin	+	++	+++	++	++	+++	+++	++				++		
Tubulin	+	++	+++	+++	+	+++	++	++			+++	+++		
Albumin			+++		+					++		+++		
Renin				ND	ND	ND		+++	+			++		
Spectrin	++	++		++	++	+++	+	+	++	++	++	+++	++	
Transferrin				ND	ND	++						++		
Thyroglobulin			+++		+	+++			++	+		+	+	+
Myoglobin	+	++	+	++	+++	+++		+++				++		
Peroxidase	+				+			ND				ND	ND	—
Tested by immunofluorescence		Th	Th	SM	—	SM	SM	—	Th	Nu	—	Nu	—	—

Abbreviations: Th, positive on thyroid tissue; SM, positive on smooth muscle; Nu, positive on nuclei of various tissues; —, negative with all tissues examined; ND, not done; blank is negative.

\* +, 0.030 ≤ OD < 0.070; ++, 0.070 ≤ OD < 0.150; +++, 0.150 ≤ OD < 2 corresponding to OD of the ELISA performed as defined in Materials and Methods.

that antibody activity is associated with IgM, as well as IgG and IgA. No correlation was found between an isotype and a given antibody specificity: IgM, IgG, or IgA could react with any of the antigens on the panel. However, the IgMs seemed to recognize more Ags, since the average of the number of proteins reacting with IgM was seven, whereas for IgA it was only five. More than half of the cell lines had positive immunofluorescent reactions on frozen tissue sections (thyroid, smooth muscle, nuclei).

Supernatants from all the 42 Ig-secreting cell lines were tested for antiviral specificities. Among them, four were found to possess an antiviral activity, against herpes simplex virus (two, IgG), cytomegalovirus (one, IgM) and varicella-zoster virus (one, IgG). None of the cell lines secreting antiviral monoclonal antibodies reacted with any of the Ags of the panel.

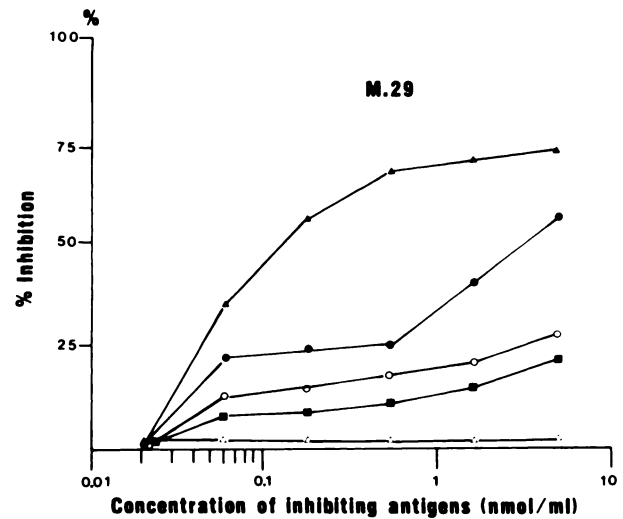
The antibody specificity of the supernatants was examined early after the establishment of the cell lines, and all the clones derived from these cultures by subcloning were also analyzed. Most of the positive clones preserved their antibody secreting function throughout a 6-month period of continuous culture. For three lines, however, the antibody specificity disappeared after 20 to 30 passages (10 to 15 weeks). Thus two lines (J22 and M28) initially secreting IgAK, on subsequent passages, ceased to produce IgA and simultaneously did not react with Ags. The third line (T41) secreted only the IgG isotype and reacted with six Ags, and particularly strongly with renin. After 2 months of culture, the secreted IgG reacted only with actin and renin; whereas after 4 months, the secreted IgG did not possess any antibody activity.

The monoclonal IgMs from the supernatants of clones D12, F16, and R31 were isolated by passage on a DNP-Sepharose immunoabsorbent, while that of M29 was not bound on the immunoabsorbent and was isolated only by Sephacryl. In all cases, the isolated IgMs bore the same

specificities as the initial supernatants. Furthermore, no antibody activity was detected in all the effluents of the DNP-Sepharose columns. Inhibition experiments undertaken to further examine their specificities showed that an IgM antibody which bound primarily to a given antigen was preferentially inhibited by this antigen, while other antigens could inhibit or not the binding. Representative results of these inhibition experiments, obtained with M29 monoclonal IgM antibodies, are given in Fig 1 (compare Fig 1 with Table 2).

**DISCUSSION**

In the present study we have prepared human B cell clones after infecting human lymphocytes with Epstein-Barr virus.



**Fig 1. Inhibition of binding of an IgM monoclonal antibody (M29 of Table 2) on plates coated with myosin, by different concentrations of various antigens. ▲, myosin; ●, TNP.25/BSA; ○, tubulin; ■, actin; △, DNA or BSA.**

We have established 42 lymphoblastoid cell lines, originating from healthy individuals or patients with various diseases, among them leukemia, myeloma, and dysglobulinemia. The lines were subcloned and the supernatants of all these cell cultures were examined to determine their immunoglobulin isotypes present and antibody specificities using a panel of self and non-self antigens. All of them secreted Igs and no clear differences were found according to the classification of the individuals.

The results obtained show that the majority of clones produced IgM as has been recently published by Chan et al<sup>29</sup> who confirmed the high frequency of EBV-transformable IgM-secreting cells. In our study, it is interesting to note that a proportion of these clones were found to secrete IgA and IgG. When the clones were examined for antibody function, a high proportion of them (33%) were found to react with one or more antigens on the panel. A broad range of antibodies, reacting with three to four antigens or reacting with almost all the antigens on the panel was observed. Half of the antibodies also reacted with cellular antigens present on tissue sections. Thus, taking into consideration our previous results with mice, it can be concluded that murine monoclonal antibodies produced by clones established by cell hybridization as well as human monoclonal antibodies synthesized by clones established after EBV infection possess similar specificities. A polyspecificity frequently found in this study was the binding of antibodies to cytoskeleton proteins, as has also been reported in healthy young individuals.<sup>30</sup> In contrast, however, to the results obtained with mice,<sup>4,6,7</sup> in the present study, polyspecific antibody function was also found to be expressed by IgA and in rare cases by IgG isotypes. Thus the main difference noted between murine and human natural antibodies seems not to be related to their specificities but rather to their isotypes. The difference noted between human and mouse clones could be due to the different tissue origin used to prepare the cell lines. In mice, clones were established from splenocytes, whereas human lines were mostly derived from circulating lymphocytes. These differences, however, could also be due to the different methods used to produce the clones. There is much evidence suggesting that the reactions observed with super-

natants and isolated immunoglobulins reveal true antibody specificities, since all the supernatants from Burkitt's lymphoma cell lines were always negative, and clones secreting IgM, IgG, or IgA and prepared from an individual could be either positive or negative.

In previous studies we have noted that human myeloma immunoglobulins belonging to either the IgG, IgA, or IgM isotype could possess polyspecific antibody functions.<sup>3,19</sup> In the present study, we have found that the majority of the clones secreting polyspecific antibodies were obtained from the patients with Waldenström's disease, benign dysglobulinemia or lymphoproliferative diseases in general (11 cases). The reactive isotype, however, was sometimes different from that of the monoclonal Ig found in the serum of the patient (data not shown). The different isotype obtained in vitro probably reflects the difficulty of establishing a lymphoblastoid cell line by direct outgrowth of the malignant cell. It seems most likely that the EBV genome, rather than the malignant process, renders the cell proliferative in vitro. In one previous case,<sup>31</sup> however, a malignant monoclonal B cell line had been established from a patient with Waldenström's macroglobulinemia: it secreted an immunoglobulin of the same isotype and antibody reactivity as that of the in vivo monoclonal protein.

The results obtained in the present study indicate that immunoglobulins secreted by human B cell clones obtained after EBV immortalization may sometimes bear polyspecific autoantibody functions similar to those found in normal human polyclonal antibodies, in human monoclonal paraproteins and in natural monoclonal antibodies synthesized by murine or rat clones obtained from physiologically normal animals.

Furthermore, our results support the concept that auto-reactive B cells and autoantibodies are not necessarily linked with immunopathological situations, but are rather involved in the normal function of the immune system.

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