

THE NATURE OF THE IMMUNOGLOBULIN G ON THE  
SURFACE OF B LYMPHOCYTES IN  
CHRONIC LYMPHOCYTIC LEUKEMIA\*

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Chronic lymphocytic leukemia (CLL)<sup>1</sup> appears to represent a clonal proliferation of B lymphocytes in that the abnormal cells found in blood and tissues have on their surfaces immunoglobulin (Ig) molecules bearing the same idiotype and light chain type. The expression of more than one heavy chain class is known to be compatible with monoclonality (1). The isotypes found most commonly are IgM and IgD, and such cells presumably reflect a stage of differentiation of normal B lymphocytes.

The presence of IgG on such cells has been reported but has often been considered to be of serum origin, possibly at times representing autoantibodies or immune complexes bound to Fcγ receptors (2). Incubation of the cells at 37°C was then shown to remove such extrinsic IgG, and this has been adopted as a precaution before analysis of surface Ig (3). More recently, however, as detection methods have increased in sensitivity, it has been found that surface IgG is present together with IgM and IgD in some cases of CLL where the intrinsic nature of all classes is suggested by observation of only a single light chain type. In a detailed study of CLL surface isotypes using a sensitive rosette test and observing precautions to remove extrinsic IgG, it was found that out of 24 patients expressing IgM plus IgD on the cell surface, 18 also expressed IgG (4). Similarly, in our smaller study using immunofluorescence, five out of eight patients with surface IgM plus IgD also expressed IgG (5). To investigate the nature of this IgG, anti-idiotypic antibody was raised against the surface IgM on the neoplastic B lymphocytes of the five patients with CLL. By immunosorption studies it has been shown for all five cases that cellular IgM and IgD are idiotypic, but that the IgG is not.

#### Materials and Methods

*Preparation of Cell Lysates.* Peripheral blood lymphocytes from patients with CLL were isolated by gradient centrifugation on Ficoll-Hypaque (Pharmacia Inc., Upsala, Sweden) and washed as described previously (5). The cells were incubated for 2 h at 37°C to remove extrinsic Ig and washed with warm medium followed by cold phosphate-buffered saline (PBS). Cells were then lysed by suspension at 10<sup>8</sup>/ml in PBS containing iodoacetamide (0.5 M), to which an equal volume of PBS containing 1% Nonidet P-40 (NP-40) with soybean trypsin inhibitor (20

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<sup>1</sup> *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

$\mu\text{g/ml}$ ) had been added. After standing at  $4^{\circ}\text{C}$  for 15 min, the nuclei were removed by centrifugation at  $23,000\text{ g}$  for 1 h and the supernatant fraction collected. Such cell lysates did not precipitate further for  $\sim 24\text{ h}$  at  $4^{\circ}\text{C}$  and were used within this period.

*Preparation of Anti-idiotypic Antibodies.* This was carried out as described previously (5). Briefly, for each patient  $\text{Fab}\mu$  obtained by digestion of cell surface  $\text{IgM}$  with papain was isolated on an immunosorbent column to which sheep anti-human  $\text{Fd}\mu$  had been coupled. Immune complexes were formed on the column by passing through sheep anti-human  $\text{Fab}\mu$ , to occupy free determinants on the bound antigen. The antigen and fluid phase antibody were subsequently eluted with  $0.5\text{ M NH}_3$  and  $1.0\text{ M KSCN}$ , and immediately transferred back to neutral buffer by passage through Sephadex G25 so that they recombined to form immune complexes. The complexes were used to immunize two sheep per patient with primary and booster injections each containing  $1\text{--}10\ \mu\text{g Fab}\mu$  given 4 wk apart. After preparation of the  $\text{IgG}$  fraction from the serum, antibody against the constant regions of the  $\text{Fab}\mu$  was removed by immunosorption.

*Immunosorbents.* Solid-phase immunosorbents were prepared by coupling antibody to Sepharose 4B-CL by the use of cyanogen bromide (6). The  $\text{IgG}$  fraction containing anti-idiotypic antibody ( $20\text{ mg}$ ) specific for each patient was coupled to  $2\text{ ml}$  of Sepharose 4B-CL to prepare an idio-type-specific immunosorbent. Normal sheep  $\text{IgG}$ , previously absorbed with human globulins, was similarly coupled to Sepharose 4B-CL to provide a control immunosorbent. Cell lysates ( $2\text{ ml}$ ) obtained from  $10^8$  cells were passed through each of the immunosorbents ( $2\text{ ml}$ ) packed into  $5\text{-ml}$  plastic syringes. The lysates were washed through with a solution of assay buffer and bovine hemoglobin ( $1\text{ mg/ml}$ ) described previously (5) containing  $0.5\%$  NP-40 and soybean trypsin inhibitor ( $20\ \mu\text{g/ml}$ ), until  $8\text{ ml}$  was collected from each.

*Radioimmunoassay (RIA).* RIA for  $\text{IgM}$  and  $\text{IgD}$  were carried out as described previously (5). RIA for  $\text{IgG}$  was established by the use of sheep anti-human  $\text{Fd}\gamma$  coupled to Sephadex G-25 as solid-phase antibody with  $\text{Fab}\gamma$  as the radiolabeled antigen. Specificity was shown by  $<0.5\%$  interference in the assay by  $\text{IgM}$ ,  $\kappa$ , or  $\lambda$  light chains. All RIA on cell lysates were carried out in the presence of  $0.5\%$  NP-40, hemoglobin, and soybean trypsin inhibitor, as used in immunosorbent separations.

## Results

The five patients chosen for study were considered typical of CLL. All had high peripheral leukocyte counts, consisting predominantly of small to medium lymphocytes with the surface  $\text{Ig}$  isotypes shown in Table I. Only one light chain type was found for each patient. The capacity of such cells to secrete small amounts of pentameric  $\text{IgM}$ , and, in the case of patients War and Wat,  $\text{IgD}$ , has been described previously (5).

The RIA results obtained for the cell lysates are shown in Table II, revealing that  $\mu$  is the predominant heavy chain class for all five patients. This includes both surface membrane and intracellular  $\text{IgM}$ . The levels of  $\text{IgD}$  and  $\text{IgG}$  found in lysates were low, with  $\text{IgG}$  exceeding  $\text{IgD}$  for all patients.

TABLE I  
*Surface Ig Isotypes Expressed on Peripheral Blood Lymphocytes  
of CLL Patients*

Patient	Lymphocytes in blood	Surface $\text{Ig}^*$ isotypes
	$\times 10^7/\text{ml}$	
War	18	$\text{IgMDG}\lambda$
Wat	2.9	$\text{IgMDG}\lambda$
Jen	11	$\text{IgMDG}\kappa$
Han	5.0	$\text{IgMDG}\kappa$ (weak)
Hal	11	$\text{IgMDG}\kappa$

\* In all preparations of peripheral blood lymphocytes examined,  $>95\%$  of the cells showed the surface isotypes indicated.

TABLE II  
*Binding of the Three Ig Classes Found in CLL Cell Lysates by Specific or Nonspecific Immunosorbents*

Patient	IgM			IgD			IgG		
	Lysate	Percent through control	Percent through anti-idiotypic	Lysate	Percent through control	Percent through anti-idiotypic	Lysate	Percent through control	Percent through anti-idiotypic
	<i>ng</i>			<i>ng</i>			<i>ng</i>		
War	4,400	121	3	97	109	7	132	71	89
Wat	5,200	114	2	202	131	2	326	110	87
Jen	5,120	79	2	42	114	11	171	98	81
Han	2,880	100	4	44	96	15	216	100	100
Hal	1,140	123	8	24	74	0	168	96	86
Hal*			85			81			101

\* These figures refer to the levels of the three Ig isotypes obtained from Hal cell lysate that passed through immunosorbent carrying anti-Jen idiotype antibody.

The results of passage of the cell lysates through either the specific anti-idiotypic or the control immunosorbents are also shown in Table II. For all five patients, removal of IgM by anti-idiotypic was almost complete, whereas recovery from the control was 79–123%. Similar results were obtained for IgD with errors being larger due to the small amounts of IgD present. For IgG, however, 81–100% passed through the anti-idiotypic column with 71–110% passing through the control, clearly demonstrating the non idiotypic nature of the IgG.

As a further demonstration of the specificity of the anti-idiotypic immunosorbent, Table II shows the results of passing lysate from the cells of one patient (Hal) through an immunosorbent carrying anti-idiotypic directed against another patient (Jen) of the same surface Ig isotype. The majority of all three Ig isotypes were not retarded.

### Discussion

The significance of the presence of IgG on the surface of B lymphocytes in CLL has been difficult to assess largely because serum IgG, either alone or in immune complexes, can bind to cells with Fc $\gamma$  receptors. Studies using F(ab')<sub>2</sub> fractions from anti- $\gamma$  antibodies have suggested that the presence of IgG on CLL cells is a comparatively rare event and that IgM and IgD are the common cell surface isotypes (7). However, as detection methods have increased in sensitivity, the presence of IgG together with IgM and IgD has again been reported on CLL lymphocytes (4, 8) even when conditions have been chosen to exclude extrinsic IgG and only one light chain type has been found.

In humans, it has generally been thought that the immature B lymphocyte expressing only IgM matures into one expressing IgM plus IgD, and that further differentiation may give rise to cells expressing IgM and IgG (9), such cells having been identified in lymphoma (10). Whether or not there is a transitional stage where the cell expresses IgM, IgD, and IgG is debatable. Studies of proliferative responses of normal B lymphocytes to anti-Ig treatment have suggested that the three isotypes may be present together on some cells (11), and it is of interest to know if CLL cells represent such a population arrested at this stage of differentiation.

In the present study the cells of five patients with morphologically typical CLL were examined by the use of anti-idiotypic antibody. All five had been reported as having surface IgM, IgD, and IgG detectable by immunofluorescence. The intrinsic

origin of all three classes had been suggested by the detection of only one light chain type. Specific RIA was used to show the presence of IgM, IgD, and IgG in cell lysates, and it was found that the amounts of IgD and IgG were comparable and much smaller than IgM. However, the idiotypic nature of the IgM and IgD and the nonidiotypic nature of the IgG were clearly shown by immunosorption studies using anti-idiotypic antibody specific for each patient. If all the IgG detected in the lysates arose from extrinsic material bound to the cell surface, one would expect both  $\kappa$  and  $\lambda$  light chains to have been detected by immunofluorescence. However, the quantity of second light chain type associated with extrinsic IgG is apt to be small compared with the light chain type on all intrinsic plus some extrinsic Ig, and might not yield fluorescence significantly in excess of incidental background.

We shall continue to search for idiotypically positive cell surface IgG in cases of CLL also expressing IgM and IgD in the expectation that this sometimes occurs. However, our uniform failure to find it in the first cases examined suggests that its occurrence is much rarer than recent reports would suggest.

### Summary

The nature of the immunoglobulin (Ig) G found associated with the neoplastic B lymphocytes in chronic lymphocytic leukemia that also express IgM and IgD has been investigated by absorption studies using anti-idiotypic antibodies raised against cell surface IgM from five patients. In all five cases, although cellular IgM and IgD behaved as idiotypic, the IgG did not. Thus the IgG frequently found associated with lymphocytes at this stage of differentiation is likely, at least in many cases, to be of extrinsic origin.

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