PARTIAL CHARACTERIZATION OF THE SHIFT FROM IgG TO IgA SYNTHESIS IN THE CLONAL DIFFERENTIATION OF HUMAN LEUKEMIC BONE MARROW-DERIVED LYMPHOCYTES*

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With rare exception chronic lymphocytic leukemia (CLL)[1] lymphocytes are of bone marrow-derived lymphocyte lineage. The majority of studies indicate that CLL lymphocytes represent an early stage of B-cell differentiation in which cells bear surface immunoglubulin (SIg) usually IgM and IgD (1–3). Intracellular immunoglobulin (CIg) is not readily detected utilizing immunofluorescence techniques (4). As differentiation proceeds, however, SIg is lost in favor of CIg and lymphoid cells assume the morphologic appearance of plasma cells. The sequence of events which occur with the maturation of a single clone of IgM-and IgD-bearing lymphocytes has recently been described by FU et al. (5, 6). Such clones would appear to progressively lose SIgD in favor of SIgM before synthesis of IgM by plasma cells with little if any SIg.

Since IgG is the major gene product of the total B-cell population it is reasonable to postulate that a transition to IgG expression must take place at some point in the differentiation sequence. That this occurs within the sphere of syndromes which we recognize as CLL is suggested by the finding of a monoclonal serum IgG in a minor population of individuals with CLL who have IgG as the predominant SIg (7). To date, however, an IgM to IgG switch mechanism has only been documented at the plasma cell level (8–10). A similar transition involving IgG and IgA synthesis would appear to be a late differentiation event not described in B lymphocytes which has previously been documented in single clones of plasma cells (11, 12).

These studies were prompted by the discovery of an unusual CLL patient (Tun) with a dual population of lymphocytes bearing either IgG(κ) or IgA(κ) associated with a single monoclonal serum IgG(κ) protein. Idiotypic antibodies specific for the serum IgG(κ) were prepared which detected this determinant on the surface membrane and the cytoplasm of both the IgG(κ) and IgA(κ) cell populations thereby suggesting a common clonal origin. Terminal differentiation

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[1] *Abbreviations used in this paper:* CIg, intracellular Ig; CLL, chronic lymphocytic leukemia; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; SIg, surface Ig.

of the IgG(κ)-bearing cell population was evidenced by the presence of typical IgG(κ)-staining plasma cells. Double-labeling experiments utilizing rhodamine- and fluorescein-conjugated IgG and IgA antisera indicated that a switch from IgG to IgA synthesis apparently involving the V_H gene had taken place within this single clone.

Materials and Methods

Source of Material. Patient Tun was a 54-yr old man with a 3-yr history of CLL whose leukocyte counts were consistently greater than 100,000/mm^3 when studied. His serum contained a single IgG(κ) monoclonal protein with a concentration of 30 mg/ml. An IgA monoclonal protein was not detected and no free light chains were present in serum or urine.

Non-Ig Surface Markers. The presence of spontaneous sheep red blood cell rosetting and the C'3 receptor were evaluated as described by Baxley et al. (13).

Fluorescent-Conjugated Antisera. Monospecific antisera to human IgG, IgA, and κ- chains were prepared in rabbits and conjugated with tetramethyl rhodamine isothiocyanate as described previously (11). The resulting conjugates were characterized according to Wood et al. (14), and Cebra and Goldstein (15). The reagents had OD_{280}/D_{515} ratios of approximately 2 and were used at protein concentrations of 700 μg/ml. Fluorescein isothiocyanate-conjugated goat antisera to human IgG, IgA, IgM, IgD, κ, and λ chains and rabbit IgG were purchased from Meloy Laboratories Inc., Springfield, Va. These reagents had OD_{280}/OD_{495} ratios of approximately 2 and were used at protein concentrations of 200-500 μg/ml.

Each fluorescent antiserum was tested for monospecificity by immunodiffusion, immunoelectrophoresis, and the cytoplasmic staining of a panel of fixed plasma cells of known Ig antigenic composition. Specificity of the observed fluorescent reactions was assessed by appropriate blocking and absorption experiments. All antisera were routinely centrifuged before use in order to remove aggregates.

Detection of SIg. Living lymphocytes were isolated from peripheral blood on Ficoll-Hypaque gradients (2.4 vol of 9% Ficoll to 1 vol of 33% Hypaque). Monocyte contamination was evaluated by peroxidase and neutral red staining. After washing twice in Hanks' balanced salt solution (HBSS) 10^6 cells were incubated for 0.5 h at 4°C with 50 μl of the appropriate dilution of heavy or light chain-specific fluorescent-conjugated antiserum. The cell pellet was washed three times in phosphate-buffered saline (PBS), resuspended in glycerine-PBS on a glass slide, and at least 200 cells were observed immediately in a Leitz Ortholux microscope with an Opak-Fluor vertical illuminator (E. Leitz, Inc., Rockleigh, N. J.). The same procedure was used for double-staining experiments in which 10^6 cells were stained sequentially with antisera to two heavy and/or light chains conjugated to different fluorochromes (rhodamine and fluorescein).

Lymphocytes were stripped of their SIg by incubation for 0.5 h at 37°C with 2.5% crystalline trypsin in Gibco minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (FCS). The cells were examined for SIg immediately thereafter and at 6 h. Cell suspensions were also cultured for 72 h at 37°C under 10% CO_2 in MEM and 10% FCS. SIg was determined from samples of the cell cultures initially and at 24-h intervals.

Cytoplasmic Ig Staining. Fresh lymphocytes isolated from Ficoll-Hypaque gradients were stained with a single fluorochrome or sequentially with double fluorochromes as described previously (11). Combined staining of the surface membrane followed by cytoplasmic staining was performed according to the method of Pernis (16).

Preparation of Idiotypic Antibodies Against Tun IgG(κ). Plasma obtained by plasmapharesis was clotted by the addition of $CaCl_2$ (1 ml of 10% solution/100 ml plasma). The serum was added to half a volume of saturated NH_4SO_4 and the γ-globulin fraction was reprecipitated three times. Excess NH_4SO_4 was removed by overnight dialysis against 0.15 M cold saline and the γ-globulin fraction was placed on a Sephadex G-200 column in 0.3 M saline. The monoclonal IgG(κ) peak was identified, isolated, and lyophilized. The purity of the monoclonal IgG(κ) preparation was verified by immunoelectrophoresis and immunodiffusion. Three albino rabbits were immunized weekly with 1 mg each of purified antigen in complete Freund's adjuvant injected into multiple subcutaneous sites and the rabbit with the highest titer of idiotypic antibodies was selected. Antibodies to nonidiotypic determinants were removed by the sequential incubation of 2 ml of antiserum with 500 mg of glutaral-

dehyde-insolubilized pooled normal human Igs at room temperature for 0.5 h followed by centrifugation. Usually four adsorptions were necessary to render the antiserum specific for the idiotypic determinant. The specificity of the idiotypic antiserum was determined in agar gel by the detection of a precipitin band with Tun IgG(κ) when no reaction was obtained with pooled normal human globulins or a panel of known myeloma proteins and Bence Jones proteins. We also used a more sensitive indicator for the differentiation of nonidiotypic from idiotypic antibodies consisting of indirect immunofluorescence with a panel of frozen and fresh CLL lymphocytes of known SIg antigenic composition and frozen pooled normal human peripheral blood lymphocytes. Absorption and dilution of the idiotypic antiserum was carried out until when tested in this system, no SIg fluorescence was obtained with CLL cells expressing IgG, IgM, IgD, κ, and λ chains and the pool of normal lymphocytes expressing all Ig chains.

Lymphocytes harvested on Ficoll-Hypaque gradients were frozen for use 3-6 wk later when the idiotypic antiserum was prepared. Freezing was accomplished in a liquid nitrogen tank at −290°C (Linde system; Linde Division, Union Carbide, New York). 1-ml vials of CLL lymphocytes containing 10^{-7} cells/ml in HBSS with 40% FCS and 10% dimethylsulfoxide have been frozen in this manner for long periods of time. At 6 mo, greater than 90% viability by trypan blue exclusion is usual.

Indirect Immunofluorescence Demonstration of Idiotypic SIg. 10^6 cells were incubated with 50 μl of the unconjugated test antiserum at 4°C for 0.5 h. The cell pellet was washed three times in PBS and reincubated at 4°C for 0.5 h with 50 μl of a 1:25 dilution of the goat antirabbit IgG antiserum. The pellet was washed three times in PBS, mounted in PBS-glycerine, and observed in a fluorescence microscope.

Photography. All cell photographs were taken with a Reichert automatic camera attachment (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) using Agfa ASA 500 film with 10–20 s exposure times.

Results

Characteristics of Tun Ficoll-Hypaque Gradient Cell Population. Cells isolated from Ficoll-Hypaque gradients consisted almost entirely of mononuclear cells (less than 1% granulocytes). When stained for either peroxidase or neutral red, markers for cells of the nonlymphocytic series, less than 1% of the cells were found to be positive. It would appear therefore that the Tun mononuclear cell population was an almost pure population of lymphoid cells. Morphologically the lymphoid population was quite pleomorphic and consisted of 26% small lymphocytes, 62% large intermediate cells, and 12% plasmacytoid cells (6% frank plasma cells).

When examined for T-cell markers, only 5% of the mononuclear cells formed rosettes with sheep red blood cells. This figure was increased to 10% with prior sheep red cell treatment with neuraminadase. On the other hand the C'3 receptor was detected on 74% of cells and surface Ig was found on 96%, thus confirming the B-cell lineage of the proliferation.

SIg Detected by Immunofluorescence. Using fluorescein-labeled antisera the percentage of the cell population staining for the various Ig chains is listed in Table I. The high percentage of cells expressing IgA is in sharp contrast to the very low incidence of IgA-bearing cells in a series of 30 consecutive CLL patients studied with the identical reagents in this laboratory (17) and similar reports by other investigators (7). IgA staining was quite striking with most cells exhibiting more than five spots (Fig. 1). Almost equal numbers of cells stained for IgG and IgA and 93% stained for κ-chains. The percentage of cells staining for IgM and IgD was negligible and again is contrary to our general experience utilizing these reagents where 80% of CLL patients expressed IgM, IgD, or both on the surface

TABLE I
SIg and CIg Staining of TUN Cells

Fluorescent reagent*	Individual positive cells	
	Surface	Cytoplasm
	%	%
Single label		
IgG	44	30
IgA	32	17
IgG + IgA	76	53
κ	93	80
λ	<5	0
IgM	<5	0
IgD	<5	0
Double label‡		
IgG + κ	40	—
IgA + κ	30	—
IgG + IgA	<2	<1

* Either fluorescein or rhodamine.
‡ Both present in single cells.

membrane. The percentage of κ-bearing cells was high, exceeding the combined frequencies of the various heavy chain determinants and thus raising the possibility that certain cells expressed only light chain determinants. After stripping of SIg with trypsin and 72 h in short-term culture, Tun CLL cells continued to express IgA, IgG, and κ-chains in the same frequency suggesting again that their presence on the membrane was not artifactual.

Sequential SIg staining with IgA and IgG antisera conjugated with opposite fluorochromes clearly demonstrated two separate cell populations, one expressing IgG(κ) and the other IgA(κ) (Table I). When cells were stained sequentially with conjugated anti-IgA or anti-IgG and anti-κ of the opposite fluorochrome, double staining was observed and when redistribution occurred, a mixed or contiguous pattern of polar capping was apparent. An occasional lymphocyte (less than 2% on repeated experiments) displayed surface spots for both IgG and IgA. Staining by fluorescent IgG or IgA antisera was not blocked by prior incubation with unconjugated anti-κ antiserum but was completely blocked by preincubation with the corresponding unlabeled IgG or IgA antiserum.

Cytoplasmic Fluorescent Staining. A significant number of Tun CLL cells stained for cytoplasmic Ig. In our series, significant cytoplasmic Ig staining was only found in CLL patients with associated monoclonal IgG serum proteins. Approximately equal numbers of cells stained for IgG and IgA, the sum of which was significantly less than the percentage of cells staining for κ-chains (Table I). In a typical experiment 30% of cells stained for IgG and 17% stained for IgA. Double-labeling studies again defined the presence of a dual population of cells with cytoplasmic IgG(κ) or IgA(κ) (Fig. 2). Double staining for both IgG and IgA was noted in a small percentage of cells (<1%). Preincubation of smears with unlabeled IgG or IgA antiserum abolished cytoplasmic Ig staining with the corresponding fluorescent-labeled antiserum.

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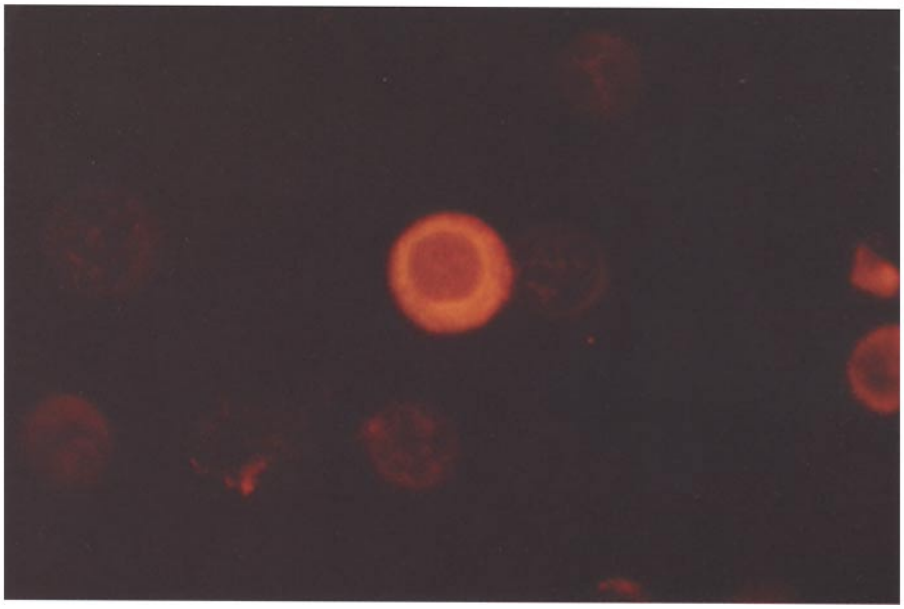

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FIG. 1. Surface staining of Tun peripheral blood cells with rhodamine-conjugated IgA antiserum.

FIG. 2. Intracellular cytoplasmic staining of Tun peripheral blood cells with rhodamine-conjugated IgA antiserum.

Surface and Cytoplasmic Idiotypic Determinants. The antiserum prepared from the monoclonal Tun IgG(κ) reacted strongly by immunodiffusion with Tun IgG(κ), other IgG myeloma proteins, and pooled normal human globulins. After

absorption with insoluble pooled normal human Igs it reacted only with Tun IgG(κ).

When tested by indirect surface immunofluorescence the idiotypic antiserum did not react with other CLL cell populations expressing IgM, IgD, IgA, κ, and λ chains. In this group there were three populations expressing IgG(κ) associated with the corresponding IgG(κ) serum monoclonal protein. In each instance no surface fluorescent staining was observed. A pool of peripheral blood lymphocytes obtained from 10 normal healthy donors of all ages also gave no surface fluorescence with the idiotypic reagent.

In contrast, 84% of Tun lymphocyte surfaces reacted strongly with the rabbit idiotypic antiserum to a dilution of 1:160 (Fig. 3) indicating the presence of the idiotype on both the IgG- and IgA-bearing cell populations. Preincubation of this antiserum with Tun Ig(κ) protein abolished this reaction. Blocking with goat anti-IgG and goat anti-IgA antiserum before indirect immunofluorescence reduced the number of cells staining for the idiotype as anticipated from the relative frequencies of IgG- and IgA-bearing cells (Table II). A goat antihuman Ig antiserum totally blocked the idiotypic antiserum except for minimal residual staining in a small percentage of cells. Prior absorption of the idiotypic antiserum with Tun living lymphocytes completely abolished subsequent staining.

The idiotypic antiserum also detected the idiotypic determinant in the

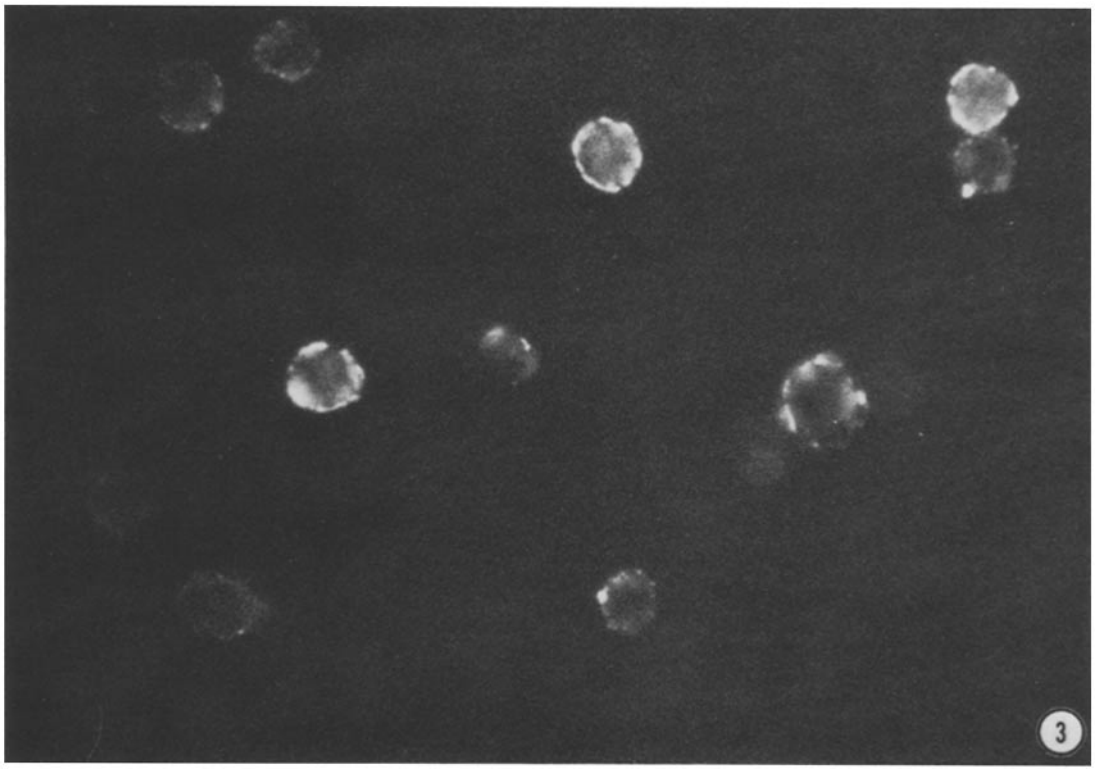

FIG. 3. Surface staining of Tun peripheral blood cells with rabbit idiotypic antiserum (indirect immunofluorescence).

TABLE II
Surface and Cytoplasmic Indirect Fluorescent Staining with TUN Idiotypic Antiserum

Prior Blocking or absorption	Positive cells	
	Surface	Cytoplasm
	%	%
Preincubation		
Control (saline)	84	45
Anti-IgG*	32	19
Anti-IgA*	50	34
Antihuman Igs	10‡	—
Absorption		
TUN IgG(κ)	0	0

* Goat source.
‡ Faint staining.

cytoplasm of 45% of Tun cells (Table II). The staining was generally moderate when compared to the usual fluorescent staining of plasma cells. Some cells, however, were brightly stained and 2–3% had the classic morphologic appearance of plasma cells. Preincubation of cells with an unlabeled goat IgA or IgG antiserum decreased the percentage of cells subsequently staining for the idiotypic determinant in proportion to the precentage of cells staining for cytoplasmic IgA or IgG. Absorption with Tun IgG(κ) completely abolished staining with the idiotypic reagent.

In order to document the presence of the idiotypic determinant on the surface of the population of cells with cytoplasmic IgA, living cells were stained for the presence of the idiotypic determinant by indirect immunofluorescence utilizing the fluorescein-conjugated goat antirabbit reagent followed by staining of the fixed smeared cells with the IgA rhodamine-labeled reagent. In several experiments an average of 80% of cells exhibited green fluorescent surface spots. The cytoplasm of 20% of the idiotypic SIg-positive cells also stained red with the IgA rhodamine reagent thus indicating the presence of the idiotypic marker on the surface of the cell population that contained intracellular IgA. The presence of the idiotype on the IgA-bearing cell population is consistent with a common clonal origin for the IgG and IgA cell populations.

SIg and CIg Characterization of Single Cells. The relationship of SIg to CIg in the cell population was evaluated by the sequential staining of surface membrane and cytoplasm with IgG and IgA reagents conjugated to opposite fluorochromes. These experiments documented the presence of subpopulations of cells within the IgG- and IgA-bearing populations defined by their SIg and CIg characteristics. Those cells which bore IgG on their surface either had no cytoplasmic IgG or stained moderately for cytoplasmic IgG. Approximately 7% of those cells with cytoplasmic IgG had the characteristic morphologic appearance of plasma cells and stained brightly (Fig. 4). There was a clear diminution of surface IgG at the plasma cell level. The same progression was noted amongst cells bearing IgA. 14% of the total cell population had surface IgA without demonstrable cytoplasmic

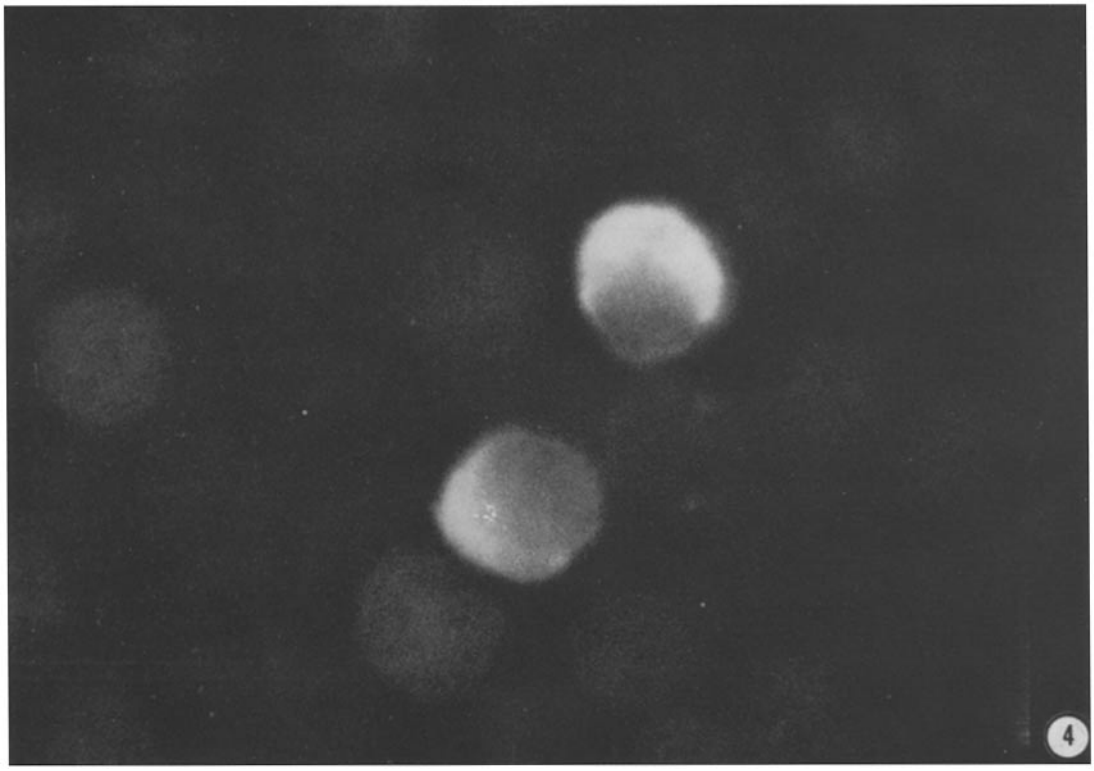

FIG. 4. Intracellular cytoplasmic staining of Tun peripheral blood plasma cells with fluorescein-conjugated IgG antiserum.

IgA and 13% had both surface IgA and cytoplasmic IgA. However, only 1% of cells had only cytoplasmic IgA without surface IgA and although cytoplasmic IgA staining was bright in many instances no IgA-containing plasma cells were seen. These findings suggest a failure of terminal differentiation within the IgA-bearing population and correlate with the absence of a demonstrable serum monoclonal IgA(κ). Of particular note was the complete absence of cells with cytoplasmic IgG that bore IgA on the surface and the presence of a small population (2%) of cells with cytoplasmic IgA that had IgG detectable on the cell surface.

Discussion

The two major Igs detected on the surface of leukemic lymphocytes by most investigators have been IgM and IgD (1–3). Wernet et al. have shown that when a serum monoclonal IgM is present it shares idiotypic specificity with lymphocyte SIgM (18). Recently Fu et al. have shown that when IgM and IgD are present on the same lymphocyte membrane they share idiotypic specificity with serum monoclonal IgM (5, 6). These observations have suggested that the CLL cell population is the source of the serum monoclonal protein and that both IgM and IgD probably function as lymphocyte membrane receptor proteins. Some CLL cells express IgG as the major SIg and occasionally the corresponding monoclonal

IgG is present in the serum (7). It has been assumed that the IgG-bearing lymphocyte is the source of the serum monoclonal protein and these clones represent a subpopulation of B cells which have differentiated to a stage of IgG synthesis and secretion. Confirming this view, shared idiotypic specificity between SIgG and a serum monoclonal IgG in a single clone has recently been documented (19). In contrast, IgA is not a major SIg of CLL lymphocytes. With our reagents IgA was found in high percentage in only 2 of 30 individuals studied and it was in fact the significant number of brightly staining IgA-bearing cells which focused our attention on the unusual Tun proliferation. The paucity of serum monoclonal IgA proteins associated with CLL has been amply documented and correlates with its absence on the membrane and in the cytoplasm of the vast majority of CLL cells (7). The minor role of IgA in this circumstance may indeed correlate with the relatively small percentage of the normal B-cell pool engaged in serum IgA synthesis. On the other hand it is likely that the usual CLL lymphocyte reflects an early phase of normal human B-cell differentiation in which SIgM and SIgD are most often expressed and that IgG and IgA expression occur as differentiation proceeds.

In the present study we have demonstrated that the idiotypic determinant of the serum monoclonal IgG(κ) is present both on the membrane and in the cytoplasm of a dual population of CLL cells; one expressing IgG(κ) and the other IgA(κ). The implications of this finding are threefold. First, it confirms that this CLL cell population is the source of the serum IgG(κ) protein. Second, shared idiotypic specificity is consistent with the expansion of a single clone rather than separate IgA(κ) and IgG(κ) clones. Third, it defines a switch between IgG and IgA synthesis within this single clone analogous to what has been described previously in myeloma plasma cells (11, 12).

Combined staining of the SIg and CIg of single cells characterized a progression of development within the IgG-bearing population beginning with IgG-bearing cells without demonstrable cytoplasmic IgG, terminating in plasma cells with high density cytoplasmic IgG. Within the IgA-bearing population the same profile was detected with the exception of terminally differentiated IgA-containing plasma cells. The presence of small numbers of cells with surface IgA and surface IgG or surface IgG and cytoplasmic IgA, and the complete absence of IgG-containing cells with surface IgA is most consistent with a progression from IgG to IgA synthesis rather than vice versa. Additional evidence for this direction of switching has previously been suggested from studies of ontogeny in the human fetus (20) and immunosuppression in mice after the administration of anti-μ antibodies (21).

In recent years, considerable evidence has accumulated to support the view expressed by Wang and others (22) that two genes (V and C) appear to regulate the synthesis of an individual Ig polypeptide chain and that in the process of differentiation certain B-cell clones undergo a switch in Ig-chain synthesis characterized at the genetic level by a change in the C_H gene without an apparent change in the C_L, V_H, or V_L genes. The demonstration of shared idiotypic specificity between the IgG- and IgA-bearing cell populations described in this study suggests structural similarities between the Fab portions of the IgG and IgA molecules. As in similar cases this is consistent with an alteration in the V_H

gene determining a switchover from the synthesis of the constant regions of the γ-heavy-chain to that of the α-heavy-chain.

According to present concepts SIgM and SIgD are thought to represent membrane receptor protein of B lymphocytes present early in the differentiation sequence. As differentiation proceeds SIgM and SIgD are lost and there is progressive maturation of B lymphocytes to plasma cells (or plasmacytoid lymphocytes) synthesizing IgM, IgG, or IgA. The transitions from IgM to IgG synthesis and from IgG to IgA synthesis in individual clones have been described and appear to be either intermediate or late differentiation events. The data presented in this report suggest that it may be possible to detect a transition from IgG to IgA synthesis in certain B-cell CLL clones before complete differentiation to morphologically recognizable Ig-synthesizing plasma cells. Such clones appear to represent a level of B-cell differentation which still encompasses both the B lymphocyte and the Ig-synthesizing plasma cell.

Summary

An unusual B-cell proliferation was noted in an individual (Tun) which was characterized by the presence of two separate populations of chronic lymphocytic leukemia (CLL) cells staining on the surface and in the cytoplasm for either IgG(κ) or IgA(κ). Utilizing an idiotypic antiserum prepared from the associated serum monoclonal IgG(κ) protein the idiotype was detected on the surface and in the cytoplasm of both the IgG- and IgA-bearing cell populations. These observations are consistent with a common clonal origin and a switch mechanism involving IgG and IgA synthesis.

Sequential-labeling of Surface Ig and intracellular Ig with antisera conjugated to opposite fluorochromes documented the progressive maturation of the IgG-bearing cell population to recognizable plasma cells and the failure of terminal differentiation of the IgA-bearing cell population at a level before morphologically distinct plasma cells. The distribution and pattern of surface and cytoplasmic IgG and IgA staining in individual cells suggest that the direction of switching is from IgG to IgA synthesis. The demonstration of shared idiotypic specificity between the IgG- and IgA-bearing populations is consistent with a transition in Ig heavy chain synthesis resulting from an alteration in the C_H gene. It is concluded that certain CLL clones may manifest a switch from IgG to IgA synthesis at a level of B-cell differentiation which encompasses both the B lymphocyte and the Ig-synthesizing plasma cell.

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