

ANTI-IDIOTYPE SERA RAISED  
AGAINST SURFACE IMMUNOGLOBULIN OF HUMAN  
NEOPLASTIC LYMPHOCYTES\*

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Present concepts of the maturation of a B-lymphocytic clone involve progressive changes in cellular morphology and mode of immunoglobulin (Ig) production. The ancestral small lymphocyte synthesizes Ig for insertion into its plasma membrane, while those of its descendants destined to produce humoral antibody show first a superimposed synthesis of Ig for export and finally a heavy export with little or no surface Ig apparent (1-4). In line with this apparent dichotomy of the surface and export pathways, turnover data make it unlikely that the material for export ever has a surface phase (3, 4). It is a central contention of the clonal selection theory that throughout this maturational process the variable (V)<sup>1</sup> regions of the Ig heavy and light chains, responsible for antibody activity and idiotypic antigenic specificity (5), remain unchanged. Experimental evidence supports this concept (6-8).

Neoplasms appear to arise at various stages of B-cell maturation, developing with a restricted range of morphologies and exhibiting modes of Ig synthesis broadly in line with their normal analogues (Fig. 1). The Ig products of a single tumor might be expected to bear a uniform set of idiotypic determinants as a monoclonal hallmark. This is well established for exported Ig, in fact such products, appearing as serum monoclonal proteins, led to the first description of idiotypic specificity (9). Idiotypes on surface Ig have recently been demonstrated for those neoplasms exhibiting both surface and exported Ig. Here anti-idiotype sera raised against the exported Ig have reacted specifically with the homologous tumor cell surfaces (10-15). In one such case mice immunized against idiotypic determinants on the exported Ig of a syngeneic myeloma were thereby afforded a measure of specific protection against tumor challenge (10), presumably due to cytotoxic reactions mediated via the surface idiotypic determinants.

The nonexporting neoplasms to the left of Fig. 1 provide a situation where the tumor Ig appears in extracellular fluid in only minute quantities arising from turnover on the membrane. There is thus a problem in harvesting the Ig and obtaining antisera to its presumed idiotypic determinants. However, the thera-

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<sup>1</sup> Abbreviations used in this paper: C, constant; CLL, chronic lymphocytic leukemia; V, variable.

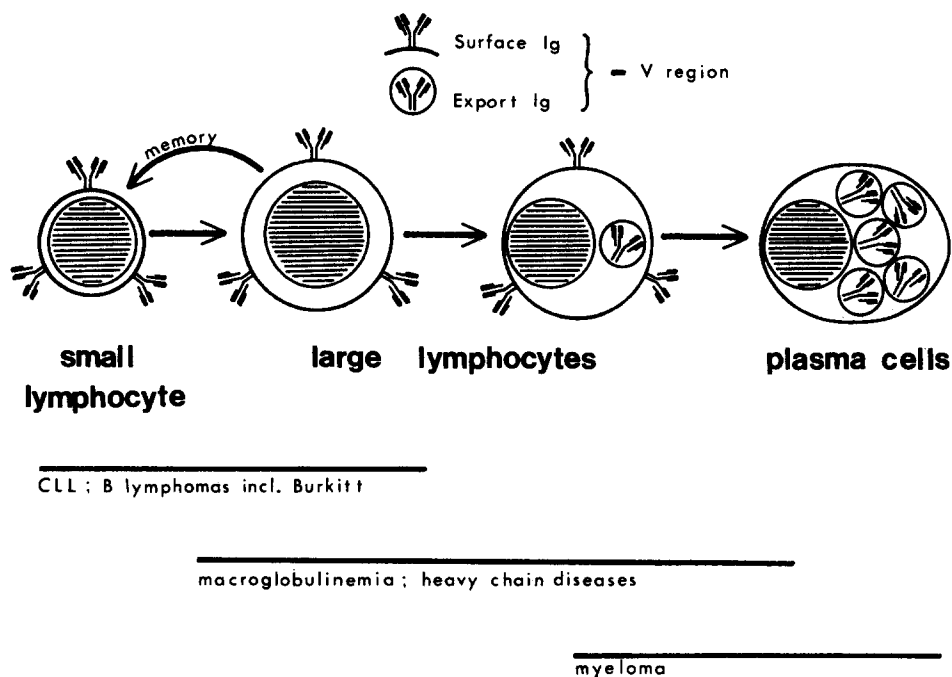


FIG. 1. The physiological transformation of a B-lymphoid clone and its possible neoplastic degenerations. Neoplasms can apparently arise at various stages along the path of differentiation; the horizontal lines depict the ranges of cell morphologies and modes of Ig production commonly seen. Variations in the normal clone can occur due to the content of rough endoplasmic reticulum, indicating an export function, varying to some extent independently of cell size. Additional complexities occur among the neoplasms without negating the overall validity of the scheme.

peutic potential of such antisera appears much more attractive than in the case of exporting neoplasms, because there is little tumor Ig to form an extracellular barrier.

Recently we were able to define idiotypic determinants on the surface IgM of nonexporting guinea pig leukemic lymphocytes (16). The antiserum had been raised by a procedure adaptable to routine use, entailing enzymatic removal of Fab from the surface Ig (17, 18), its recovery on immunosorbent particles, and immunization of animals with the immunosorbent-Fab complex (19). The solid phase of the immunosorbent was microcrystalline cellulose, chosen because its particles were largely in a phagocytizable size range (1-10  $\mu\text{m}$ ). Antibody coupled to the cellulose was specific for the constant regions of Fab and had been raised in the same species (sheep) as was to provide the anti-idiotypic serum. In this way the definitive immunization utilized particles with no foreign configurations apart from the tumor Fab and the cellulose matrix.

We now report the raising of anti-idiotypic sera against surface Ig from two cases of a nonexporting human neoplasm, chronic lymphocytic leukemia (CLL).

### Materials and Methods

*Immunochemical Reagents.* IgG, Fab $\gamma$ , IgM, and Fab $\mu$  were prepared from normal sera by conventional methods (17).  $\kappa$ - and  $\lambda$ -light chains were prepared from urinary Bence Jones pro-

teins. A sample of myeloma IgD was a gift from Dr. K. J. Dorrington. Antisera to these proteins were raised in sheep by multiple subcutaneous injections of emulsions made with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). For use in direct immunofluorescence, fluorescein conjugates of antibody-containing IgG from the antisera were prepared, absorbed, and tested for specificity by methods previously described (17).

*Lymphocytes.* Venous blood was collected in heparinized bottles. Lymphocytes were separated and washed as previously described (17); it is important to note the incubation for 1 h at 37°C which is interpolated into the washing procedures so as to promote removal of plasma IgG from the cell surfaces. The lymphocytes from Patients 1 and 2 are described in detail below. Lymphocytes for use as controls were obtained from five healthy laboratory workers and from two further patients with CLL.

*Fab From Surface Ig.* Fab fragments from surface Ig molecules on leukemic lymphocytes were obtained in solution by subjecting the living cells at  $10^6$ /ml to surface proteolysis by papain (0.5 mg/ml, 30 min, 37°C) (17, 18). The cells were removed from the digest by centrifugation, and the papain was inactivated by adding iodoacetamide to 5 mM. Amounts of Fab $\gamma$  and Fab $\mu$  released were estimated by solid-phase radioimmunoassays (20). Each assay had been rendered specific for its individual class of Fab by appropriate absorptions of the antibodies.

Fab was separated from the cell-surface digest by immunosorption onto particles of microcrystalline cellulose (Merck A. G., Inc., Darmstadt, W. Germany) to which sheep antibody to Fab $\gamma$  had been coupled by the cyanogen bromide method (21). This immunosorbent is able to bind all classes of Fab due to its activity against their constituent light chains. After a preliminary blank absorption with cellulose-normal IgG, the immunosorbent-digest suspension, 0.05% vol/vol in terms of hydrated particles, was stirred for 24 h at 4°C. The particles were then separated by centrifugation and washed four times in phosphate-buffered saline, pH 7.4.

*Preparation of Antibodies to Idiotypes (16).* Washed cellulose immunosorbent laden with Fab from tumor surface Ig was suspended at 1% vol/vol in phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories). The emulsion was used to immunize two sheep for each patient, using multiple subcutaneous injections. Primary and secondary injections were given 6 wk apart. Each course of injections was accompanied by an intravenous (i.v.) injection of 30-ml sheep antiserum directed against the constant (C) regions on the predominant class of surface Ig; in the case of Patient 1 this was sheep antiserum to IgD, raised against a myeloma protein of this class, and in the case of Patient 2 it was sheep antiserum to human normal Fab $\mu$ . The passive antiserum is designed to suppress the antibody response to the C regions on the tumor Fab and enhance the response to the V regions (19).

The sheep were bled from the jugular vein 1, 2, and 3 wk after the booster injection. An IgG-containing fraction was precipitated from the serum with 1.6 M ammonium sulfate, and IgG $_2$  (the electrophoretically slower of the two ovine IgG subclasses [22]) was then obtained by chromatography on diethylaminoethylcellulose (Whatman DE32) in 0.02 M sodium phosphate buffer, pH 7.0. These IgG $_2$  fractions were immunoelectrophoretically pure, contained most of the anti-idiotypic activity, and were used as the source of anti-idiotypic for all experiments reported in this paper.

Absorptions were necessary for the IgG $_2$  from anti-idiotypic (Patient 1) but not anti-idiotypic (Patient 2). Activity against normal human cell surfaces, detected by indirect immunofluorescence, was removed by three absorptions at 50% vol/vol with red cells of blood group O and three absorptions with allogeneic leukemic lymphocytes at the same concentration (obtained by leukapheresis from a patient with prolymphocytic leukemia, and kindly supplied by Dr. A. Okos). Activity against Ig C regions, detected by Ouchterlony precipitation, was removed by passage through a column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) coupled to globulins precipitated from human serum with 1.6 M ammonium sulfate. The antibody preparations were finally adjusted to 4 mg IgG $_2$ /ml in phosphate-buffered saline, pH 7.4.

*Characterization of Anti-idiotypic Sera by Immunofluorescence and Inhibition of Lymphocyte Migration.* Immunofluorescence was by the indirect method. Washed cells at  $10^7$ /ml in Eagle's medium containing 25% human AB serum were mixed with an equal volume of IgG $_2$  solution (anti-idiotypic or control) and allowed to react for 30 min at 4°C. The cells were then washed three times in cold phosphate-buffered saline and treated with fluorescein-conjugated antiglobulin reagent for 30 min at 4°C. Finally, they were washed three times in saline containing 1% bovine serum albumin and 20 mM sodium azide, resuspended in a small volume of this medium, and examined using a Leitz Orthoplan microscope equipped with a Ploem incident illuminator and 200

W mercury lamp. The use of human AB serum in the initial incubation reduced background staining occurring with sheep normal IgG<sub>2</sub>, while the addition of azide to the final wash solutions prevented capping and pinocytosis (23).

The ability of human leukemic lymphocytes to migrate from capillary tubes (24) afforded the opportunity for assessing antibodies to their surface components by inhibition of migration (25, 16). Duplicate samples of cells were allowed to migrate from glass capillary tubes (1 mm internal diameter) into medium (Eagle's, plus 1% nonessential amino acids, 10% fetal calf serum, and 100 i.u./ml of both penicillin and streptomycin) containing varying concentrations of IgG<sub>2</sub> from either anti-idiotypic or normal sheep serum. After migration had proceeded for 16 h at 37°C the relative areas covered were estimated by projecting the cell patterns onto paper, cutting out the shadowed areas, and weighing.

*Patients.* Patient 1 (Pic) is a 75-yr-old man who presented in August 1973 with a tender enlarged spleen. There were slightly enlarged soft nodes in axillae and groins, Hb 16 g/100 ml, leukocytes 34,000/ $\mu$ l, platelets 160,000/ $\mu$ l. More than 80% of the leukocytes were small lymphocytes which on electron microscopy revealed a large proportion of electron-transparent chromatin, numerous mitochondria but few other organelles, and occasional traces of rough endoplasmic reticulum. Fluorescence microscopy revealed surface IgD and IgM, at frequencies indicating that some cells were exhibiting both, with the light chain class restricted to  $\lambda$  (Table I). During the ensuing 7 mo the patient's leukocyte count rose to 120,000/ $\mu$ l and his spleen enlarged from 2 cm below the costal margin to 10 cm. At this stage 100 ml venous blood was collected for lymphocyte processing. Treatment with chlorambucil (6 mg/day) reduced the leukocyte count to 10,000/ $\mu$ l and splenomegaly to 3 cm. 9 mo after stopping treatment his spleen had enlarged to 10 cm and was causing discomfort, while his leukocyte count was 160,000/ $\mu$ l. He was treated with further chlorambucil (8 mg/day) together with leukapheresis, reducing his leukocyte count to 6,000/ $\mu$ l and his splenomegaly to 6 cm.

Patient 2 (Eve) is a 66-yr-old woman who presented in July 1973 with an enlarged lymph node in the left submandibular region. Her Hb was 12.5 g/100 ml, leukocyte count 68,000/ $\mu$ l, and platelets 170,000/ $\mu$ l. The blood film showed over 90% small lymphocytes, including many smear cells, and marrow examination was characteristic of CLL. The cells on electron microscopy were typical small lymphocytes with scanty cytoplasm, few organelles, and no visible rough endoplasmic reticulum. Fluorescent microscopy revealed a surface Ig characteristic of B cells: there was staining for IgM and more faintly for IgD with  $\kappa$ -light chains. Again the frequencies of occurrence of the heavy chain classes suggested that some cells were exhibiting both (Table I). After 18 mo the leukocyte count was 130,000/ $\mu$ l and 150 ml venous blood was taken for lymphocyte processing. 2½ yr after diagnosis the patient remained asymptomatic apart from some concern about the mass in her neck, but her Hb was now 0.9 g/100 ml and leukocyte count 240,000/ $\mu$ l.

## Results

*Isolation of Surface Ig.* Freshly taken peripheral blood lymphocytes from both patients were washed and exposed to papain as described in Materials and Methods. This procedure cleaves the surface IgM to release Fab $\mu$ , which survives the digestion antigenically intact (17). The amounts of Fab $\mu$  recovered are shown in Table II. That recovered from a control culture in the absence of papain is regarded as arising from IgM released by normal turnover on the membrane and by trauma incidental to dispersing the cells. The increment associated with exposure of the cells to papain probably arises from IgM cleaved in situ on the cell membrane (18).

The cell surface IgD is expected to have the same idiotypes as the IgM (12), so that Fab $\delta$  released from the cells can be used along with the Fab $\mu$  for the raising of anti-idiotypic sera. Unfortunately at the time these studies on material destined for use as immunogen were carried out we were unable to quantify the Fab $\delta$  in the digest. A subsequent digestion of cells from Patient 1, using thiol-free papain at 0.5 mg/ml for 30 min at 37°C, yielded 70 ng Fab $\mu$  and 6 ng Fab $\delta$  per 10<sup>7</sup> cells. The reason for the low yield of Fab $\delta$  is being investigated.

TABLE I  
*Surface Immunoglobulin on Patients' Lymphocytes*

	Percent of blood lymphocytes positive for				
	$\gamma$	$\mu$	$\delta$	$\kappa$	$\lambda$
Patient 1	5	29	78	4	56
Patient 2	0	69	31	60	0

Testing was by direct immunofluorescence using conjugates of sheep IgG antibodies specific for the indicated Ig polypeptide chains. The small numbers of cells from Patient 1 staining for  $\gamma$  and  $\kappa$  are thought to reflect some residual contamination with plasma IgG.

For Patient 1 the staining for  $\delta$  was stronger than for  $\mu$ , while the reverse was the case for Patient 2.

Cell surface IgG was observed irregularly in the two present cases of CLL and probably represents residual plasma IgG attached to Fc $\gamma$  receptors (26, 27). It would follow that the Fab $\gamma$  found in the digest for Patient 2 is polyclonal and a perturbing factor in raising antibodies to the idiotypes of intrinsic tumor Ig. It was therefore removed by an immunosorbent specific for its Fd $\gamma$  region; it can be seen from Table II that >90% of the Fab $\gamma$  was removed for an accompanying loss of 26% of the Fab $\mu$ . This procedure was not available when processing digest from Patient 1.

Fab was now purified from the digests by immunosorption onto microcrystalline cellulose and the cellulose-Fab complex used to raise anti-idiotypic sera in sheep (see Materials and Methods). An assay of the digest from Patient 2 after immunosorption indicated that 66% of the Fab $\mu$  had been bound, so that the total dose of Fab $\mu$  given to each sheep was 7.5  $\mu$ g, derived from approximately  $6 \times 10^9$  lymphocytes. For Patient 1 the doses of Fab $\mu$  were probably of a similar magnitude, being derived from  $2.5 \times 10^9$  lymphocytes for each animal immunized.

*Reactivity of Anti-idiotypic Preparations with Leukemic Cells.* By indirect immunofluorescence anti-idiotypic (Patient 1) stained more than 80% of the blood lymphocytes from this patient. Most of the cells showed a faint circumferential ring with sparse superimposed bright spots. Cells from Patient 2, from another case (Hor) of CLL, and from five normal subjects all failed to stain.

Similarly anti-idiotypic (Patient 2) stained more than 80% of the blood lymphocytes from Patient 2, the predominant pattern being one of circumferential spots. Again the control preparations were all negative: cells from Patient 1, from another case (Bee) of CLL, and from five normal subjects.

The migrations of leukemic cells in the presence of antibody preparations, relative to their migrations in the presence of sheep normal IgG<sub>2</sub>, are set out in Table III. Despite some irregularities in the ratios it is clear that only in the presence of the homologous anti-idiotypic is migration clearly inhibited, with this inhibition diluting out with the antibody.

*Confirmation of Molecular Target.* To prove that the specific reactivity of anti-idiotypic (Patient 1) was associated with an antigenic target on the cell surface Ig, we took advantage of the 'capping' phenomenon in which two sets of antigenic determinants cap simultaneously if present on the same molecules but otherwise independently (23).

TABLE II  
*Immunoglobulin Obtained from Lymphocytic Surfaces*

	Ig (ng Fab/10 <sup>7</sup> cells) detected in supernates after			
	Culture in absence of papain		Culture in presence of papain	
	Fab $\mu$	Fab $\gamma$	Fab $\mu$	Fab $\gamma$
Patient 1	9.2	ND	48	ND
Patient 2	10.1	3.0	27	14
Patient 2, after removal of Fab $\gamma$			20	<1

Estimations were by radioimmunoassay. ND, not done.

The cells were cultured for 30 min at 37°C with or without papain at 0.5 mg/ml (see Materials and Methods). The solutions were then chilled and the cells removed by centrifuging. The supernate from the papain digest was alkylated so as to inactivate the papain. The supernate from the control culture was subjected to a 30-min digestion by papain, followed by alkylation, so as to yield similar molecular forms of Ig for comparison by the assay.

The removal of Fab $\gamma$  from the papain digest from Patient 2 was accomplished by adding, to a concentration of 0.4% vol/vol, an immunosorbent consisting of anti-Fd $\gamma$  coupled to microcrystalline cellulose. After stirring for 24 h at 4°C the immunosorbent was removed by centrifuging.

In the first experiment lymphocytes from Patient 1 were exposed to polyspecific anti-Ig serum under capping conditions (cells at  $2 \times 10^7$ /ml in 50% rabbit anti-human Fab' $\gamma$  in Eagle's medium at 37°C for 30 min). This resulted in the surface Ig being completely capped or internalized, as judged by immediate subsequent staining of the chilled and washed cells by fluorescein-anti-IgD at 4°C; there were only occasional cells with caps, the remainder being unstained. When, in place of anti-IgD, the cells were allowed to react with anti-idiotypic (IgG<sub>2</sub> at 1 mg/ml in 25% human AB serum at 4°C for 30 min) followed by fluorescein-anti-sheep IgG as indicator, the staining pattern was the same as seen with fluorescein-anti-IgD. Thus, there was nothing to suggest the presence of non-Ig material on the cell surfaces reacting with the anti-idiotypic preparation.

A second experiment demonstrated that the anti-idiotypic preparation reacted with all the surface Ig molecules, in that capping by anti-idiotypic involved all antigens reactive with antisera to Ig C regions. Capping had to be carried out by the sandwich method. Cells at  $2 \times 10^7$ /ml were allowed first to react with anti-idiotypic (IgG<sub>2</sub> at 1 mg/ml in 25% human Ab serum) for 30 min at 4°C and were then washed and exposed to 50% rabbit anti-sheep IgG serum (not itself reactive with the cells) for 30 min at 37°C. Subsequent staining of the chilled and washed cells by fluorescein-anti-IgD or anti-Fab' $\gamma$  indicated that the surface Ig of most cells had been completely internalized, with only occasional cells retaining it in caps. The cells of Patient 2 did not survive the manipulations sufficiently well enough to permit these experiments to be applied to them.

### Discussion

We draw two main conclusions from the experiments reported here. Firstly, immunoglobulin idiotypes are demonstrable on human B-lymphocytic neoplasms which synthesize Ig only for insertion into the surface membrane.

TABLE III  
*Migration of Leukemic Lymphocytes in Presence of Anti-Idiotype*

Anti-idiotypic against cells from	Migrating cells from	Relative migration at IgG concentrations (mg/ml) of					
		4	2	1	0.5	0.25	0.125
Patient 1	Patient 1	0.11	0.23	0.55	0.42		
	Patient 2	1.2	1.2	0.73	1.3		
	Patient Hor	1.5	1.1	1.2	1.5		
Patient 2	Patient 2			0.58	0.56	0.74	0.78
	Patient Bee			0.85	1.3	1.05	

Relative migration is defined as the ratio of the area covered by cells in the presence of IgG<sub>2</sub> from anti-idiotypic serum to the area covered by the same cells in the presence of IgG<sub>2</sub> from normal sheep serum. The IgG<sub>2</sub> from normal serum had been subjected to the same absorption procedures as had the antibody IgG<sub>2</sub>.

The nonhomologous cells against which anti-idiotypes were tested were determined simply by availability at the time.

Secondly, the raising of antisera to these idiotypes presents no serious technical problems.

The evidence that our antisera are indeed directed against Ig idiotypic determinants resides in (a) the method of preparation whereby tumor Fab is presented as immunogen, with any antibody response to the constant regions either suppressed or absorbed; (b) their specificity for the homologous tumor cells; (c) the coincident capping of surface Ig and the targets of the antisera.

Although the immunization schedule is still at an early stage of refinement, success was achieved with the first two human cases to which it was applied after its feasibility was demonstrated in an animal leukemia (16). So practical difficulties do not appear to loom large, and we are hopeful that considerable improvement in the quality of the antisera will be possible. We are reinvestigating the immunization schedule with regard to the nature of the solid phase, the route of immunization, and the value of passive antiserum in specifically suppressing the response to the C regions.

An extension of our approach to the obtaining of anti-idiotypic sera against the surface Ig of B lymphomas with no obvious leukemic phase should be dependent only upon obtaining sufficient cells in a dispersed form: from effusions, by dispersing solid tumor, or by the vigorous harvesting of small leukemic over-spills from peripheral blood.

We have preferred to isolate surface Ig by the action of papain on intact cells, rather than by harvesting culture supernates or disrupting plasma membranes by agents such as detergent. Papain appears to give a high yield of Fab $\mu$  from surface IgM, although the yield of Fab $\delta$  from surface IgD has still to be defined. The other methods mentioned should yield whole Ig molecules, with a greater proportion of determinants irrelevant for our purposes. Ig shed spontaneously from the cell surface might even have additional membrane constituents attached to it (28). The alternative methods also have no obvious quantitative advantages. The potential yield of Ig upon simple harvesting of culture fluid is apt to be less than implied by the 30-min figures in Table II, as these undoubt-

edly include a contribution from trauma on dispersing the cells. Disrupting the cells by detergent or other means will yield a lot of intracellular material to render immunosorption more difficult. Nor is it likely to give access to much more cellular Ig, as most of this is on the surface in nonexporting cells (29).

The anti-idiotypic sera are of potential diagnostic and prognostic value. Having been raised against cells taken before the tumor is suppressed by treatment they could be useful in identifying neoplastic cells in blood or marrow, thereby predicting clinical relapse. Such an antiserum has already helped to identify sublines of the guinea pig L2C lymphocytic leukemia (30).

The therapeutic potential of the antisera is a more difficult question. Mice immunized by a conventional schedule against the idiotypes on a purified myeloma protein exhibited some resistance to inoculations of the corresponding plasmacytoma (10). However, in the only relevant study available to date there is no evidence that surface Ig idiotypes can act as a 'tumor-specific transplantation antigen' in the usual sense; a subline of the guinea pig L2C leukemia lacking Ia antigen but retaining surface IgM (and idiotypes) afforded no protection against itself or other idiotypic-bearing sublines when whole cells were used as immunogen in syngeneic animals (30). Clearly the situation we describe, in which xenogenic anti-idiotypic serum is made available in almost arbitrarily large quantities, is different from the preceding two. We are investigating the therapeutic possibilities in the L2C leukemia and in certain human cases. It is probably important to note that the best known pathways whereby antibodies kill cells, complement-mediated and K-cell-mediated cytotoxicities, do not always work across species barriers. Thus, it is desirable to identify the class of Ig in which the predominant anti-idiotypic activity resides and then to ascertain whether this class is capable of collaborating with the complement and K cells of the species to be treated. Other experimentally defined effects of antibodies against surface antigens on lymphoid or neoplastic clones might also prove therapeutically relevant: invoking of suppression by T cells (31), stimulation (32), and synergism with cytotoxic drugs (33).

### Summary

The idiotypic determinants of surface immunoglobulins on B-cell lymphomas and lymphocytic leukemias represent tumor-specific antigens, individually unique for each tumor. As such they have both diagnostic and therapeutic potential, particularly for those neoplasms with no serum monoclonal immunoglobulin arising from synthesis of the protein for export. We describe the raising in animals of anti-idiotypic sera directed against two examples of a nonexporting neoplasm, human chronic lymphocytic leukemia. The procedure involves exposing the cells to papain so as to remove the Fab fragments (containing the idiotypic determinants) from the surface immunoglobulin, recovering the Fab on cellulose immunosorbent particles, and immunizing animals with the immunosorbent-Fab complex.

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