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Immunology

Rescue of immunoglobulin secretion from human neoplastic lymphoid cells by somatic cell hybridization

(mouse–human hybrids/hybridomas/human lymphomas/chronic lymphocytic leukemia/B-cell differentiation)

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ABSTRACT **B leukemia cells from four different patients were hybridized with a mouse myeloma cell line with polyethylene glycol as a fusing agent. The original leukemia cells all expressed immunoglobulin on their surface, but failed to secrete it. Over 200 different human–mouse somatic cell hybrids were obtained; 57% of them secreted human immunoglobulin in large amounts. Human immunoglobulin secretion can be a stable property of these hybrid cells over months of continuous culture. In each case the human immunoglobulin secreted was restricted to the light chain type expressed by the parental B leukemia cell. In addition, these hybrid cells secreted the original mouse myeloma protein and a variety of mixed human–mouse immunoglobulin molecules.**

It is now clear that most of the malignant lymphoproliferative diseases that occur in *adult* humans are derived from B cells (1, 2). That is, they are composed of monoclonal populations of cells engaged in the synthesis of immunoglobulin. In some of these diseases, i.e., multiple myeloma and Waldenstrom macroglobulinemia, there is extensive secretion of immunoglobulin by the malignant cells. However, in the majority of adult lymphomas, immunoglobulin is synthesized and inserted into the cell membrane, but not secreted. Morphologically, these human lymphomas appear to represent a variety of different types of lymphoid cells, from the small lymphocyte of chronic lymphocytic leukemia to the larger "poorly differentiated" lymphocyte of follicular lymphoma to the very large lymphocyte of "diffuse histiocytic lymphoma" (3–5). These diseases are thus relatively pure populations of B cells that may be arrested at various stages of differentiation. They provide unique sources of cell populations for the study of human B cell maturation and differentiation.

The question addressed by the present study is whether nonsecreting human B cells can be caused to become secretors of immunoglobulin when provided with the secretory apparatus present in a fully differentiated plasma cell. We performed somatic cell hybridization between mouse myeloma cells and human neoplastic B cells and found that a high proportion of the hybrids secreted human immunoglobulin which was restricted to the same light chain type found on the surface of the parental B cell. In addition, these hybrids secreted the original mouse myeloma protein and a number of different mouse–human mixed molecules.

MATERIALS AND METHODS

Human Malignant Lymphoid Cells. Blood samples were obtained from four different patients with lymphoproliferative disease: two with chronic lymphocytic leukemia and two with lymphosarcoma cell leukemia. All of the patients had circulating lymphocyte levels of greater than 50,000/mm^3. None of these patients had detectable paraproteins (M-components) in their serum, but they all had diseases characterized by a neoplastic proliferation of B lymphoid cells (see below). The leukemic cells were isolated by the Ficoll–Hypaque sedimentation technique (6) and used for immunofluorescence or for somatic cell hybridization, either fresh or after storage in 10% dimethylsulfoxide in a liquid N_2 freezer.

Somatic Cell Hybridization. The IgG (κ)-secreting mouse myeloma cell line P3-X63-Ag8, an 8-azaguanine-resistant mutant of MOPC-21 described by Kohler and Milstein (7), was used for hybridization with human lymphoid cells. Polyethylene glycol (PEG, Baker, 1540) was used as a fusing agent as described (7, 8). Hybridization was performed at room temperature. Typically, 5×10^7 human leukemic cells and 2×10^7 mouse myeloma cells were centrifuged into a common pellet and washed twice in protein-free Dulbecco's modified Eagle's medium. The pellet was gently resuspended in 2 ml of 40% PEG and centrifuged for 6 min in the presence of the PEG, gradually bringing the speed of centrifugation up to $400 \times g$. The PEG was removed and the pellet was slowly resuspended in 12 ml of protein-free modified Eagle's medium. The cells were again pelleted, suspended in 50 ml of modified Eagle's medium containing 15% fetal calf serum and hypoxanthine/aminopterin/thymidine (9), and dispensed into 96-well tissue culture plates (Linbro). After 2–4 weeks colonies of hybrid clones became evident. Subcloning was performed by dilution into wells containing normal mouse spleen cells as feeders.

For biosynthetic labeling of secreted material, 10^6 cells were incubated for 18 hr in 0.1–1.0 ml of leucine-free minimal Eagle's medium containing hypoxanthine, thymidine, and 10 μCi of L-[^{14}C]leucine (New England Nuclear, 294 Ci/mol). Labeled proteins in culture supernatants were separated from free [^{14}C]leucine by centrifugation through wet-packed Sephadex G-25 fine.

Immunochemical Reagents and Procedures. Our preparation of Fab_2' fragments of purified goat antibodies to human κ and λ light chains and their use for direct cell membrane immunofluorescence has been described in detail (10).

Goat anti-MOPC-21 and anti-mouse Fab antisera were prepared by immunization with the purified MOPC-21 myeloma protein and a Fab fragment of purified mouse anti-Dnp antibody, respectively. These antisera were obtained as a gift from J. Haimovich and Y. Bergman.

A radioimmunoassay similar to that described by Sigal (11) was used for detection of immunoglobulin secretion by cells in culture. Goat antisera diluted 1/5000 in 0.01 M KH_2PO_2, pH 7.4/0.14 M NaCl (P_i/NaCl) or Fab_2' fragments of purified goat antibodies at a concentration of 2 μg/ml in P_i/NaCl were used

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Abbreviations: P_i/NaCl, 0.01 M KH_2PO_2, pH 7.4/0.14 M NaCl; NaDodSO$_4$, sodium dodecyl sulfate; PEG, polyethylene glycol.

Table 1. Radioimmunoassays for secreted immunoglobulins

Assay	Coating antibody	^{125}I-labeled ligand	Standard competitor
Human κ	Fab_2' of purified anti-human κ antibody	Fab_2' from pooled normal human IgG	Normal human IgG
Human λ	Fab_2' of purified anti-human λ antibody	Fab_2' from pooled human IgG	Normal human IgG
Human μ	Goat anti-human μ antiserum	Human macroglobulin	Human macroglobulin
Mouse κ	Goat anti-mouse Fab antiserum	MOPC-21	MOPC-21
Mouse γ	Goat anti-MOPC-21 absorbed on mouse $\alpha\kappa$ Sepharose	MOPC-21	MOPC-21

to coat the surface of flexible vinyl microtiter plates (Cooke 1-220-24B) for 3 hr at 20°. The plates were then washed with 5% fetal calf serum in P_i/NaCl, and an appropriate ^{125}I-labeled ligand was added to the wells in the presence of various concentrations of unlabeled standard competitors or culture supernatants. The plates were incubated a minimum of another 3 hr at 20°, then washed with 5% fetal calf serum in P_i/NaCl and cut with a hot wire device. The wells were dropped into tubes for γ-counting. The coating antibodies, labeled ligands, and unlabeled competitors for each specific assay are listed in Table 1.

Biosynthetically labeled, secreted immunoglobulin was analyzed by sequential immunoabsoption and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. Sepharose-bound (12) anti-human κ and anti-MOPC-21 were prepared from the Fab_2' of purified anti-human κ and the globulin fraction on anti-MOPC-21, respectively. The anti-MOPC-21 globulin was absorbed on human IgG-Sepharose prior to use, to remove any possible crossreaction with human determinants. ^{14}C-labeled culture supernatants were passed over these two columns in a sequential fashion, and the columns were extensively washed with 1% Nonidet P-40 in P_i/NaCl. Bound radioactivity was then eluted by boiling the column packing for 2 min in 1% $NaDodSO_4$.

The resulting eluates were analyzed by $NaDodSO_4$ gel electrophoresis (13) in slab gels both in 5% polyacrylamide under nonreducing conditions and in 10% polyacrylamide after addition of 2-mercaptoethanol to the samples. Gels were dried and fluorographed as described by Bonner and Laskey (14).

RESULTS

Human Ig production by hybrids

The human leukemic cells used in these studies all contained surface Ig which was restricted to a single light chain type, either κ or λ, in each case (Table 2). However, no Ig secretion by these cells was evident, either in the serum of the patients or in short-term culture *in vitro*.

A series of somatic cell hybrids was constructed between the mouse myeloma cell line P3-X63-Ag8 and the human malignant B lymphoid cells. When vigorous growth became apparent, the culture supernatants were assayed for human κ and λ light chains, human μ heavy chain, and mouse γ and κ chains by radioimmunoassay. The results of a single assay of a group of supernatants for human κ light chain are shown in Fig. 1. All hybrids produced both heavy and light mouse Ig chains. In addition, as shown in Fig. 1, a number of them produced human Ig. These positive supernatants contained between 1 and 10 μg of human Ig per ml. The actual level of Ig in the supernatant varied as a function of the cell density and exhaustion of the medium and, in some cases, reached 200 μg/ml. All of the human κ-secreting hybrids shown in Fig. 1 were derived from patients M and S, whereas the negative hybrid shown, W1F7, was derived from patient W. This particular hybrid fluid was positive for human λ at a level of 1 μg/ml and, conversely, all of the fluids from M and S hybrids were totally negative for human λ. In each instance the human light chain secreted by the hybrid was an exact reflection of the surface Ig light chain type of the original human cell. This point is demonstrated in greater detail in Table 2. A total of 67 individual mouse–human hybrid clones were isolated from the cells of four patients, and 38 of them (57%) produced human Ig. All pro-

Table 2. Human immunoglobulin secretion by somatic cell hybrids

Patients	Disease	Cell surface Ig light chain type	Hybrids isolated	Producers of human Ig κ	λ	μ
M	CLL*	κ	27	19	0	17
S	CLL	κ	21	14	0	13
H	LSCL†	κ	200‡	200	0	ND§
W	LSCL	λ	11	0	4	4

* Chronic lymphocytic leukemia.
† Lymphosarcoma cell leukemia.
‡ Not isolated as single clones. Each well growing more than 10 clones.
§ Not done.

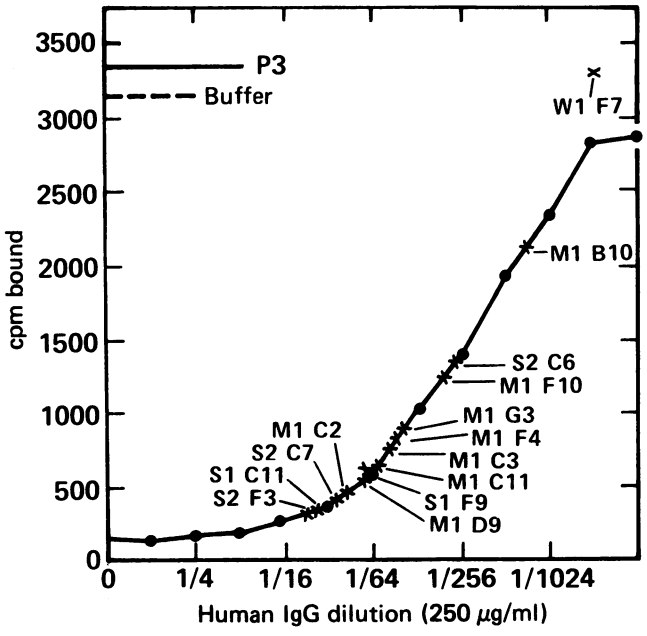

FIG. 1. Radioimmunoassay for human κ chains by inhibition of radioligand binding. The wells of vinyl microtiter plates were coated with Fab_2' of purified goat anti-human κ. The binding of ^{125}I-labeled human Fab_2' (maximum of 15% of input cpm) was inhibited by increasing concentrations of unlabeled normal human IgG. Culture fluid from the P3 mouse myeloma cell line gave no inhibition, indicating no crossreaction between the anti-human κ reagent and mouse κ determinants. Fourteen different hybridoma fluids gave different degrees of inhibition, some of them equivalent to 10 μg of human Ig per ml.

Table 3. Time course of appearance of human Ig-secreting hybrids

Weeks	Patient M	Patient S	Patient W	Total	Percent
2	0/6	0/5	0/5	0/16	0
3	0/1	0/5	0/1	0/3	0
4	9/10	10/10	1/4	20/24	83
5	5/5	0/0	0/0	5/5	100
6	4/4	0/0	3/3	7/7	100
7	1/1	3/4	0/0	4/5	80
8	2/3	0/0	0/0	2/3	67

duced mouse Ig as well. In addition, another 200 hybrids isolated from patient H produced human Ig, but the percentage of these hybrids that produced human Ig was not determined because they were not cloned. In each case, the light chain type of the rescued human Ig was the same as that found on the surface of the human input cell. Since the human input cell populations were all monoclonal, we presume that the individual hybrids derived from a single patient all secreted the identical monoclonal Ig, but this remains to be demonstrated formally. The individual fluids were also examined for human μ heavy chains, and most (34/38) were positive. The four hybrids that were negative for human μ may have lost the production of human heavy chain entirely or may have produced an alternate human heavy chain.

An interesting observation was that hybrids that grew early, within 3 weeks, were uniformly negative for human Ig secretion, but those appearing after 3 weeks were almost uniformly positive (Table 3). This rate of appearance was not related to intrinsic growth rate; once hybrid colonies first appeared, subsequent growth was rapid and indistinguishable from that of colonies that had appeared earlier. It is possible that chromosome segregation events (15) after hybridization determine initial growth rate as well as the capacity for human Ig production and secretion.

Analysis of products of a single hybrid

One hybrid, S_2F_3, a human κ,μ producer, was selected for further analysis of its secreted products. At the time of this analysis, S_2F_3 had been grown continuously for 4 months and still produced human beavy and light chains. This hybrid clone produced mouse κ, mouse γ, human κ, and human μ in a ratio of 3:3:3:1, respectively. Dilution subcloning of S_2F_3 was performed and the relative deficiency of human μ chain secretion was found to be a property of all the μ-secreting cells. In addition, subclones could be found that had lost the ability to secrete either or both of the human chains. The original S_2F_3 clone was biosynthetically labeled with [^{14}C]leucine and the secreted material was analyzed by sequential immunoabsorption and NaDodSO$_4$ gel electrophoresis. Two immunoabsorbant columns were prepared, one specific for both mouse γ and mouse κ, and another specific for human κ. These columns were first checked for specificity with ^{125}I-labeled MOPC-21 protein and human IgG (κ) myeloma protein. In each case less than 10% of the ^{125}I-labeled materials bound nonspecifically and more than 80% bound specifically. ^{14}C-Labeled proteins secreted by S_2F_3 were passed first over the anti-human κ column, to which 70% of the radioactivity bound, and then over the anti-mouse γ,κ column, to which approximately 50% of the remaining radioactivity bound.

Bound material was eluted in boiling NaDodSO$_4$ and analyzed by polyacrylamide gel electrophoresis in 5% acrylamide under nonreducing conditions (Fig. 2) and then in 10% acrylamide after reduction (Fig. 3). In Fig. 2, it can be seen that the major product of P3, the mouse myeloma cell, was an intact IgG molecule (identical to MOPC-21). Three minor products can

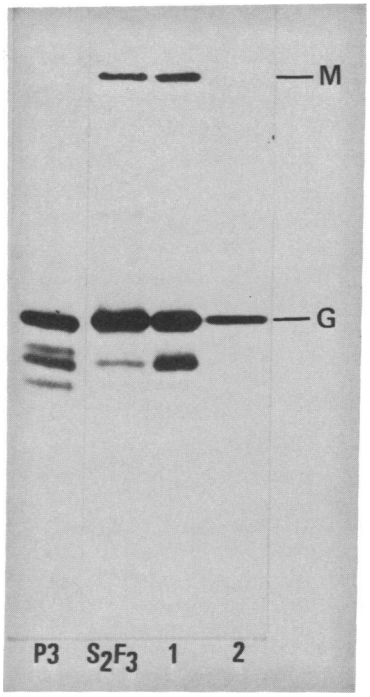

FIG. 2. NaDodSO$_4$/polyacrylamide gel electrophoresis of secreted products. [^{14}C]Leucine was incorporated into the secreted products of mouse myeloma cell P3 and into the hybridoma S_2F_3. Proteins were electrophoresed in 5% acrylamide slab gels. Sample 1 is the material secreted by S_2F_3 that bound to an anti-human κ immunoabsorbant. Sample 2 is the residual S_2F_3 material that subsequently bound to an anti-MOPC-21 immunoabsorbant. The positions of IgG and IgM are marked.

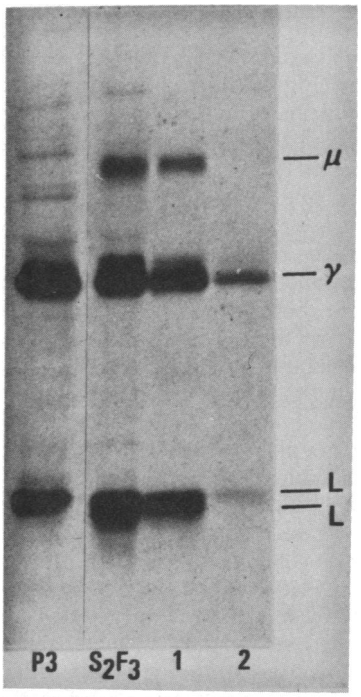

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis. Same samples as shown in Fig. 2, but samples were electrophoresed in 10% acrylamide after reduction in 2-mercaptoethanol. The positions of light, μ, and γ chains are marked.

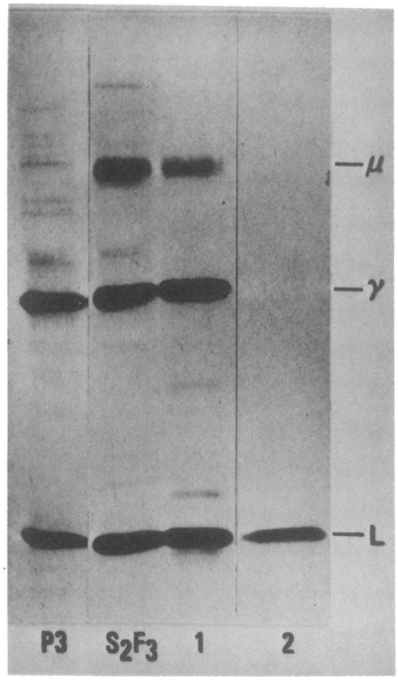

FIG. 4. NaDodSO4/polyacrylamide gel electrophoresis. Electrophoresis similar to that in Fig. 3, except the order of immunoabsorbant columns was reversed. Sample 1 contains the material secreted by S_2F_3 that bound to the anti-MOPC-21 column. Sample 2 is the residual material that subsequently bound to the anti-human κ column.

also be seen. Of these minor products, only the darkest, middle band contained Ig determinants, and it may represent a degradation product of MOPC-21. The hybrid S_2F_3 secreted a molecule identical in size to MOPC-21 and one identical to its smaller analog, but in addition secreted a high molecular weight molecule similar in mobility to an IgM pentamer (not entering the 5% gel). All of the high molecular weight product and most of the lower molecular weight products bound to the anti-human κ column (Fig. 2, sample 1) and thus contained at least one human κ light chain. Some of the lower molecular weight (7S) material failed to bind to the anti-human column, but subsequently bound to the anti-mouse column (Fig. 2, sample 2), and thus represented homologous MOPC-21 protein. Further analysis of these samples under reducing conditions is shown in Fig. 3. The hybrid secreted, in addition to the original MOPC-21 heavy and light chains, a μ chain and an additional light chain. Again, both of these new chains were contained in molecules bound by the anti-human κ column, and again most of the secreted mouse γ chains were likewise assembled in molecules binding to the anti-human κ column and thus contained at least one human κ light chain. Thus the hybrid cell secreted a mixture of Ig molecules, most of which contained both human and mouse chains. Further support for the secretion by the hybrid cells of molecules with recombined human and mouse chains was obtained by reversing the order of the immunoabsorbant columns (Fig. 4). Here, the material secreted by S_2F_3 was first passed over an anti-MOPC-21 column (with specificity both for mouse κ and mouse γ) to which more than 70% of the radioactivity bound. The material not binding to the anti-MOPC-21 was passed through the anti-human κ column to which the majority of the remaining radioactivity bound. The eluted materials are shown in Fig. 4. Again, the new μ chain can be seen in the material secreted by S_2F_3, and this time most of the molecules containing these μ chains as well as those containing the γ chains were bound to the anti-MOPC-21 column. The labeled material that did not bind to the anti-MOPC-21 column was composed mostly of free human chains, as can be seen by their binding to the anti-human κ column. On longer exposures of the gel in Fig. 4, a faint μ chain band could be seen in the material passing the anti-mouse column but binding to the anti-human column. Thus, there was low-level secretion of a totally human product composed of μ and κ chains. From this analysis, it is clear that the predominant molecule secreted by the S_2F_3 hybrid is a mixture containing two mouse γ chains, one mouse light chain, and one human light chain ($\kappa_M\gamma_M\gamma_M\kappa_H$). Other molecular species reasonably well demonstrated are $(\kappa_M\mu_H\mu_H\kappa_H)_5$, $(\kappa_H\mu_H\mu_H\kappa_H)_5$, and free κ_H. Many additional chain combinations are consistent with the data shown, but not unequivocally demonstrated by it.

DISCUSSION

The results presented here demonstrate that a human neoplastic B cell, which does not normally secrete Ig, can be caused to secrete large amounts of its Ig when it is hybridized to a mouse myeloma cell. Our results are analogous to those of Schwaber, who reported human Ig secretion by hybrids of mouse myeloma × human normal peripheral blood cells (16). In our case, the input human cells were an essentially homogeneous population of monoclonal B lymphocytes which we knew to be nonsecretors. As a result, the human Ig secreted by all the hybrids from a given patient was restricted to the homologous light chain type and presumably was derived from members of the same clone. The impetus for our experiments was to develop a general method for obtaining the monoclonal Ig of human neoplastic lymphocytes in quantities sufficient for the production of anti-idiotype antibodies against them (17). Such anti-idiotype reagents should prove extremely useful for identifying the cells of the malignant clone in a tumor-bearing patient. They should thus provide a sensitive approach to monitoring disease activity as well as a possible vehicle for the specific delivery of cytotoxic agents (18). Most of the Ig molecules secreted by these somatic cell hybrids were hybrid molecules composed of human and mouse Ig chains. Also, some completely human molecules as well as free human light chains were secreted. We are now preparing the desired anti-idiotype reagents against these secreted products.

In addition to the clinical applications described above, the experiments described here provide a possible approach for investigation of lymphocytic differentiation. Which lymphoid cells have differentiated to the point of immunoglobulin gene expression, given permissive conditions? This question may be reasonably approached by using somatic cell hybridization with myeloma cells. Certainly, fully differentiated plasma cells can continue to secrete their immunoglobulin product when they are fused with myeloma cells (19). Hybrids between myeloma cells and normal lymphoid cells have been described which secrete not only the original myeloma protein, but also normal immunoglobulins (16), or even specific antibodies (7). In these latter cases it is not yet established which type of normal cell is actually responsible for forming the antibody secreting hybrid, but presumably it is a cell that has differentiated at least to the point of commitment to the expression of single immunoglobulin V_H and V_L genes. One approach to this question would be to isolate various subpopulations of normal B cells bearing specific cell surface differentiation markers and to use these purified cell populations as hybridization partners with fully differentiated plasma cells. The alternative approach outlined here is to hybridize myeloma cells to homogeneous cell

populations from various types of lymphoid tumors. Because of the great variety of lymphoid tumor types available, the human lymphomas should provide an especially useful resource for these experiments.

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