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Immunology

Clonal evolution of myeloma cells leads to quantitative changes in immunoglobulin secretion and surface antigen expression

(somatic cell variants/fluorescence-activated cell sorter/H-2 histocompatibility antigens/surface immunoglobulin/cell differentiation)

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ABSTRACT **We report that a cloned population of tumor cells can rapidly produce variants that differ in their quantitative expression of surface proteins and in their rate of immunoglobulin secretion. A fresh clonal isolate of S107 myeloma cells possessing large amounts of surface IgA was continuously passaged *in vitro* for 2 years. During this period, fluorescence-activated cell sorter analysis indicated the development of subpopulations possessing decreased amounts of surface IgA. Cells from these variant subpopulations were isolated by first using the cell sorter to enrich for cells with decreased amounts of surface IgA and then cloning the selected population in soft agar. The 50 sublines that were isolated showed heritable differences in their levels of surface IgA and H-2 antigens and in their rates of myeloma protein secretion. Sublines having either large amounts, intermediate amounts, or absence of surface IgA also had corresponding large amounts, intermediate amounts, or absence of myeloma protein secretion. In contrast, a decrease or loss of surface Ig did not correlate with a decrease or loss of viral envelope glycoprotein gp71 and H-2 antigens. The variants did not resemble the phenotypes of less-differentiated normal lymphocyte populations of the B-cell lineage. The isolation and characterization of these variants allows us to explore the mechanisms and pathways of tumor cell differentiation as well as to study the regulation and function of cell surface proteins.**

Cell surface antigens can be used not only to separate morphologically similar lymphocytes into T and B cells but also to subdivide the cells into a large number of subclasses which differ in function and in developmental stage of differentiation (for review, see refs. 1–3). Thus, the development of antibody-secreting plasma cells, the end stage of B-cell differentiation, is preceded by several other phenotypically distinct cell populations. Precursor cells lacking surface immunoglobulin develop into surface Ig-positive B cells which, after antigenic stimulation, develop into lymphoblasts and plasmacytes. During the antigen-dependent differentiation, an increase in the secretion of immunoglobulin is accompanied by a decrease and eventual loss of surface immunoglobulin (4). Antigens coded for by the major histocompatibility complex as well as endogenous type C viral antigens may also have a physiological role in lymphocyte maturation because these antigens are differentially expressed during B-cell development (5–8).

Several tumors of B-lymphocyte lineage (e.g., myelomas, B-cell lymphomas) express various antigenic phenotypes, each resembling a particular stage of B-lymphocyte differentiation (9, 10). When adapted to culture and cloned, these tumor cells provide an ideal model for studying whether clonal evolution of variant tumor cells from a malignant cell is an inherent and fundamental part of tumor progression (11) and whether phenotypes of the variant tumor cells produced during this evolution resemble those of less-differentiated cells of the B-cell lineage (12).

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It has been reported that myeloma variants that had lost their ability to synthesize immunoglobulin expressed greatly reduced amounts of virus-specific antigens or had completely lost these antigenic determinants (13). In addition, H-2 antigen expression was also reduced in these variants.

We have studied the evolution of variants from a single well-differentiated myeloma cell. Using fluorescence-activated cell sorting in conjunction with soft agar cloning, we isolated variants that showed quantitative changes in immunoglobulin secretion, surface immunoglobulin, 71,000-dalton viral envelope glycoprotein (gp71), and H-2 antigens. These myeloma variants did not express phenotypes that resembled less-differentiated populations of the B-cell lineage. Changes in the expression of surface immunoglobulin and immunoglobulin secretion correlated perfectly in all variants. In contrast, a decrease or loss of surface immunoglobulin did not correlate with a decrease or loss of gp71 or H-2 antigens.

MATERIALS AND METHODS

Cells. The S107 myeloma line induced in BALB/c mice secretes an IgA (α, κ) that binds the hapten phosphocholine (14). The S107.3 subclone was kindly provided by Matthew Scharff (Albert Einstein College of Medicine, Bronx, NY). After repeated cloning we isolated subclone S107.3.7.1 which was used as the parental clone in this study. This cell line has a doubling time of about 15 hr, secretes large amounts of myeloma protein, and also displays a high density of surface immunoglobulin (15). Cells were grown in Dulbecco's modified Eagle's medium containing 20% heat-inactivated horse serum, glutamine, nonessential amino acids, penicillin, and streptomycin.

By using the following criteria we have found that, like many other murine myeloma lines (16), S107.3.7.1 releases type C retrovirus: electron microscopic visualization of type C particles, [^{3}H]uridine incorporation and positive reverse transcriptase activity in sucrose gradient-purified fractions corresponding to a buoyant density of 1.16–1.18 g/cm^3, and positive cell surface immunofluorescence upon treatment with heterologous antisera specific for gp71 component of Rauscher murine leukemia virus.

Antisera. Rabbit antisera to IgA heavy chain (H chains) were prepared by subcutaneous immunization with approximately 1 mg of purified (17) S107 myeloma protein emulsified in complete Freund's adjuvant, followed by monthly subcutaneous injections of the protein in incomplete Freund's adjuvant. The anti-κ activity was then removed by extensive absorption of this antiserum on Sepharose 4B coupled with CBPC-112 (μ, κ) and MOPC 21 (γ_1, κ) myeloma proteins. Rabbit anti-κ light chain (L chain) antisera were obtained by immunization with MPC-31 (γ_{2b}, κ), GPC 8 (γ_{2a}, κ), and MOPC 21 (γ_1, κ) mye-

Abbreviations: H chain, heavy chain; L chain, light chain; gp71, 71,000-dalton viral surface glycoprotein.

loma proteins, absorption of the antiserum on a CBPC-112-Sepharose column, and subsequent elution. The specificities of the anti-IgA and anti-κ antisera were confirmed by testing these antisera against a battery of different myeloma proteins in Ouchterlony double-diffusion gels.

Goat antiserum raised against Rauscher leukemia virus gp71 (lot 775-85) was kindly provided by the Office of Program Resources and Logistics, National Cancer Institute. Bourguignon *et al.* (18) have reported that this antiserum reacts with a single protein on the surface of BW5147 lymphoma cells.

Anti-H-2K^d (C3H-SW/A anti-Meth A) and anti-H-2D^d (BALB/g anti-Meth A) antisera were the generous gift of Frank Lilly (Albert Einstein College of Medicine).

All fluorescein-conjugated secondary antisera were specific for purified IgG fractions and did not react by themselves with the surface of the S107 myeloma cell lines.

Cell Sorting and Quantitation of Surface Antigen. For cell surface analysis, indirect fluorescent staining of the myeloma cells was done at 0°C in culture medium containing 0.1% sodium azide as described (19). Control staining, in which the cells were incubated with only the secondary antibody, was performed for each experiment. The results of this staining were identical to those obtained with preimmune serum. Ten thousand stained myeloma cells were then individually analyzed on a fluorescence-activated cell sorter (FACS-2, Becton-Dickinson, Mountain View, CA), by using the 488-nm laser line, and histograms were collected in which the number of cells was plotted as a function of fluorescence intensity (20).

In order to sort for cells that expressed decreased amounts of IgA H chain on their surface, the parental population was stained with rabbit anti-IgA H chain antiserum and then with fluorescein-conjugated goat anti-rabbit IgG antiserum. The 10% of this cell population that possessed the least amount of IgA-specific surface fluorescence were then isolated from the rest of the population at 0°C with the cell sorter (21). The selected subpopulations were reanalyzed immediately after the sorting and also 2 weeks after passage in suspension culture.

Cell Cycle Analysis. The myeloma cells were stained for DNA content with the fluorochrome mithramycin as described (22, 23). A total of 10,000 myeloma cells were individually analyzed in each sample with the cell sorter by using the 457.9-nm laser line to obtain the DNA cell cycle histograms.

Cloning and Isolation of Myeloma Sublines. Myeloma cells were cloned in soft agar by using a modification (15) of a method described by Coffino *et al.* (24). Individual colonies were picked with a micropipette and expanded in liquid suspension cultures.

Radioimmunoassay for Measurement of S107 Idiotype Secretion. Antisera raised in A/He mice against purified S107 myeloma protein (17) were passed through a column of Sepharose coupled with MOPC 47A (α, κ) and then adsorbed onto a column of Sepharose coupled with S107 myeloma protein. After extensive washing, ^{125}I, chloramine-T, and sodium metabisulfite were added, and the purified ^{125}I-labeled idiotype-specific antibody was acid eluted into neutralizing buffer. Radioimmunoassays were performed by a modification of solid-phase assays (25) for determining the amount of S107 idiotype secreted into the culture supernatant. Polyvinyl microtiter plates were coated with a dilution S107 ascites sufficient to bind 4000 cpm of the ^{125}I-labeled anti-idiotypic antiserum per well. Plates were then washed, incubated for 2 hr with 1% bovine serum albumin in phosphate-buffered saline, and washed again. Ten microliters of a dilution of culture supernatants (or control amounts of S107 myeloma protein) and 100 μl of the labeled anti-idiotypic antibody were added to each well and incubated overnight at 4°C. Then the unbound labeled antibody was removed by suction and the washed plates were cut into individual wells, which were then counted in a gamma counter. Cells of each of the sublines were washed and adjusted to a concentration of 3×10^5 cells per ml. After 20 hr of culture at 37°C, the cells were spun out and the supernatants were tested for myeloma protein by the radioimmunoassay.

RESULTS

Alterations of Surface Ig During Passage of S107 Cells. The amount of cell surface IgA H chain on a continuously passaged S107.3.7.1 myeloma cell line decreased as a function of time after cloning. This is illustrated in the fluorescence histograms (Fig. 1). The freshly isolated and recloned cell population initially had high levels of surface IgA but after 16 months of serial *in vitro* passage there was a bimodal distribution in which 30% of the cells possessed an intermediate level of surface IgA. After 30 months, >90% of the cell population had intermediate levels of surface IgA. A similar progressive shift in the surface expression from high surface IgA to intermediate IgA was observed for two independent passages of the original S107.3.7.1.

Enrichment for Cells with Decreased Levels of Surface IgA. The S107.3.7.1 cell line that had been continuously passaged *in vitro* for 20 months was analyzed for IgA H chain expression by using indirect fluorescent labeling and the cell sorter was then used to isolate cells possessing low levels of surface IgA H chain. The isolation consisted of three serial separations (over a 6-week period) in which the 10% of the population that possessed the least amount of H chain-specific fluorescence were selected during each sort. Because immediately after each separation the isolated population was shown

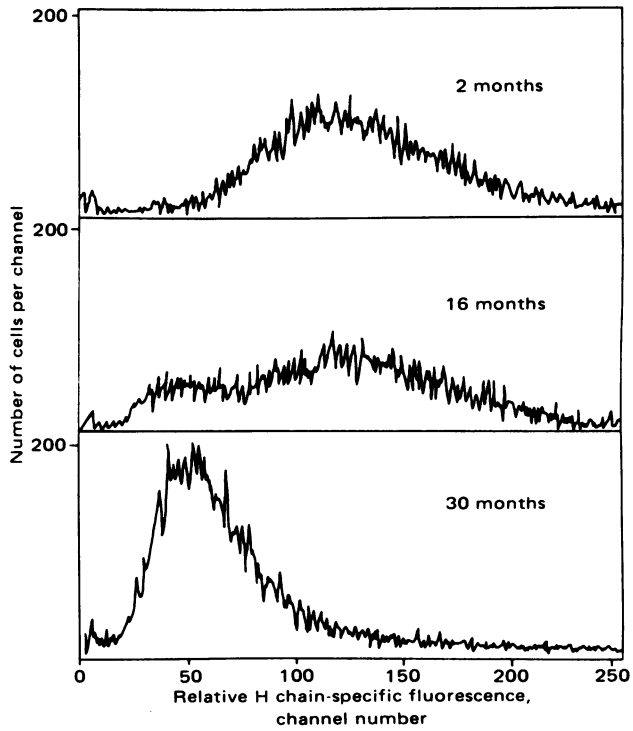

FIG. 1. Changes in surface IgA H chain for freshly isolated and recloned S107.3.7.1 myeloma cells during continuous passage in liquid suspension cultures. After 2 months, 16 months, and 30 months of passage, the cells were stained for IgA H chain-specific fluorescence (primary stain with a 1:50 dilution of rabbit anti-IgA H chain antiserum and secondary stain with a 1:40 dilution of fluorescein-conjugated goat anti-rabbit IgG) and then 10,000 myeloma cells from each sample were analyzed with the cell sorter. The negative staining control (incubation with the secondary antibody alone) gave a median channel value of 15.

to have a purity of >90% of dull fluorescent cells, the cell sorter had effectively isolated those cells that at the time of analysis possessed low levels of surface immunoglobulin. In order to determine whether the selected cells would maintain their low levels of surface IgA, each sorted population was reanalyzed after it had been passaged continuously in culture for 2 weeks. Reanalysis of the cells after the first separation indicated that there had only been a slight enrichment of cells with lower surface IgA because the fluorescence histogram of the sorted population had redistributed itself only slightly to the left of the original paternal population. However, reanalysis after the second separation demonstrated a more substantial enrichment for cells with heritably lower surface IgA and, after the third separation, the cells clearly possessed little or no IgA H chain on their surface (Fig. 2). More than 45% of the cells of the population obtained from the third separation had the same background fluorescence as was displayed by control cells stained with secondary antibody alone, and the remaining cells had intermediate levels of surface IgA H chain.

Isolation and Characterization of Sublines Differing in Their Levels of Surface IgA. Myeloma cells that had been serially sorted three times were cloned in soft agar and 35 sublines were randomly isolated. In addition, 15 clones were randomly isolated from the continuously passaged parental S107.3.7.1 line. Each of these 50 sublines (given the prefix J) were analyzed for their levels of surface IgA H chain. The sublines fell into three major groupings with respect to surface IgA profiles (Fig. 3): high surface IgA with median channel values ranging from 85 to 137 (e.g., JP29 isolated from the parental line), intermediate surface IgA with median channel values ranging from 43 to 71 (e.g., JS1 isolated from the sorted population), or negative for surface IgA with median channel values ranging from 11 to 23 (e.g., JS2 isolated from the sorted population). Within these major groupings there was some heterogeneity in surface immunoglobulin expression such that some sublines repeatedly showed slightly higher surface immunoglobulin than did others. The sublines isolated from the

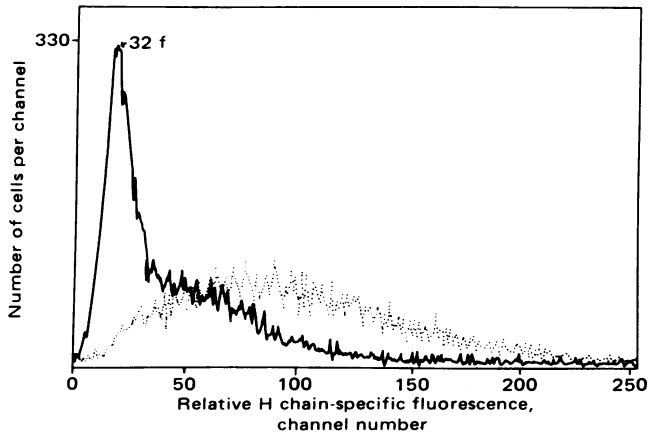

FIG. 2. Enrichment for cells with low levels of surface IgA by repeated serial selection using the cell sorter. S107.3.7.1 myeloma cells that had been continuously passaged *in vitro* for 20 months were stained for IgA H chain-specific fluorescence. The cell sorter was calibrated to deflect the 10% of the stained population that possessed the least amount of fluorescence. This deflected population was grown for 2 weeks in culture and then subjected to two more cycles of cell sorter selection as described in the text. After 2 more weeks of *in vitro* passage, the unsorted parental population (dotted lines) and the cells that had been sorted three times (solid line) were stained for IgA H chain-specific fluorescence and 10,000 myeloma cells from each sample were analyzed with the cell sorter. The negative staining control (incubation with fluorescein-labeled goat anti-rabbit IgG alone) gave a median channel value of 17.

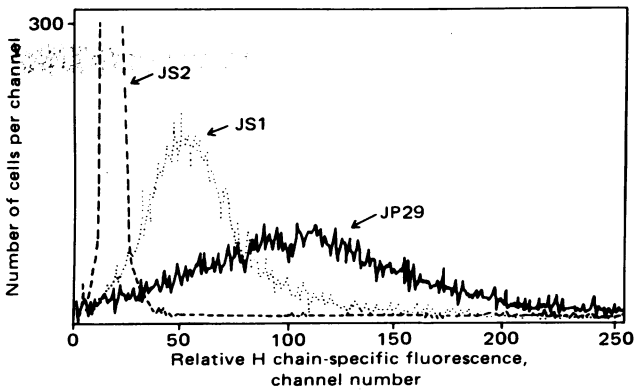

FIG. 3. Differences in intensity of IgA H chain-specific surface fluorescence among three freshly sublines of S107 myeloma cells. A freshly recloned isolate of the parental S107.3.7.1 cell line (JP29, solid line) and two clonal isolates from the thrice-sorted population (JS1, dotted line; JS2, dashed line) were stained for IgA H chain-specific fluorescence (primary stain with a 1:50 dilution of rabbit anti-IgA H chain antiserum and a secondary stain with a 1:40 dilution of fluorescein-labeled goat anti-rabbit IgG). Ten thousand myeloma cells from each population were then analyzed with the cell sorter. The negative staining control (incubation with the secondary antibody alone) gave a median channel value of 15.

sorted population possessed significantly lower levels of surface immunoglobulin than did those sublines that were recloned isolates of the parental S107.3.7.1 (Fig. 4A). Among the 35 sublines isolated from the sorted population there were 20 sublines lacking surface H chains, 14 sublines with intermediate amounts of surface H chains, and only 1 subline with high surface H chains, whereas among the 15 sublines isolated from the unsorted parental population, there were 11 sublines expressing high levels and only 4 sublines with intermediate levels of surface IgA H chains.

The characteristic large amount, intermediate amount, or absence of surface IgA H chain found on each subline, as well as the heterogeneity within each group of sublines, remained stable during 4 months of continuous passage. Reanalysis of the sublines after 6 months, however, showed that three of the sublines that had been high for surface Ig now possessed a bimodal distribution of cells with high and intermediate levels of surface IgA H chains. Reanalysis at this time also demonstrated that two other sublines that had possessed intermediate levels of surface Ig now displayed a bimodal distribution of cells with intermediate levels and absence of surface IgA.

We then analyzed the sublines isolated from the sorted and parental populations for their levels of L chain expression. Fluorescence analysis indicated that those sublines having high and intermediate levels of H chain also had corresponding high and intermediate levels of L chain. Of the 20 sublines that were negative for H chain, 9 sublines also lacked surface L chain whereas the remaining 11 sublines had intermediate levels of surface L chain. None of the 15 sublines randomly isolated from the parental unsorted population that had been passed continuously for 20 months showed expression of L chains only on the cell surface. Sorter analysis of the parental population showed that the frequency of cells with only L chains was <1%.

DNA cell cycle analysis demonstrated that all of the sublines except one were near tetraploid. The one exception was an octaploid subline possessing a high surface immunoglobulin level.

Relationship among Surface IgA, Myeloma Protein Secretion, H-2 Antigens, and gp71 Antigens. The various isolated sublines were tested for rates of secretion of the S107 idiotype by radioimmunoassay. There was a direct correlation between

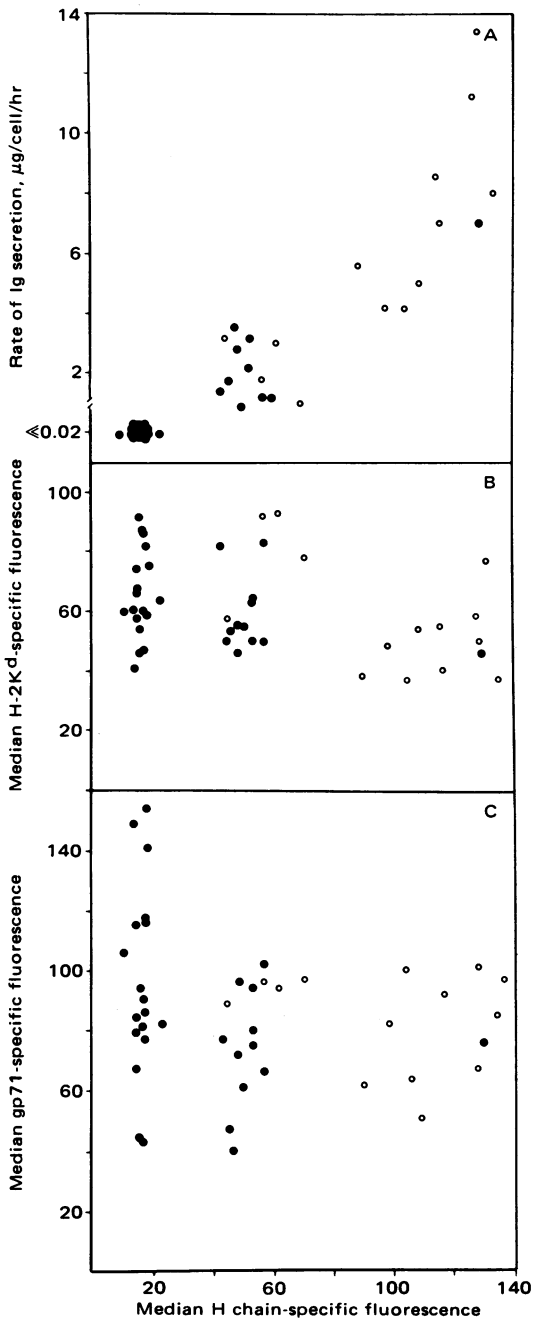

FIG. 4. Comparisons of levels of surface IgA H chain expression and levels of myeloma protein secretion (*A*), H-2K^d antigen expression (*B*), or gp71 antigen expression (*C*). Recloned isolates from the parental S107.3.7.1 cell line (○) and clonal isolates from the thrice-sorted population (●) were analyzed for their amounts of cell surface antigens and myeloma protein secretion. Staining for surface IgA-, H-2K-, or gp71-specific fluorescence was done by incubation with unlabeled primary antibodies (rabbit anti-IgA H-chain, mouse anti-H2K^d, or goat anti-Rauscher leukemia virus gp71) followed by incubation with fluorescein-labeled secondary antibodies (goat anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG). Ten thousand cells from each sample were then individually analyzed by using the cell sorter, and the median channel of fluorescence was recorded. In order to evaluate myeloma protein secretion, each subline was washed and adjusted to a concentration of 3×10^5 cells per ml. After 20 hr of growth, the cells were spun out and the cell-free supernatants were tested for levels of secreted myeloma protein idiotype by using a radioimmunoassay as described in the text.

the rate of secretion of myeloma idiotype and the amount of surface IgA H chain expressed on each subline (Fig. 4*A*). Thus, sublines classified as having large amounts, intermediate amounts, or absence of surface IgA also secreted corresponding amounts of myeloma protein. Because the assay was idiotype-specific, the 11 sublines expressing L chain only were detected as negative for secretion.

Other cell surface antigens were quantitated on the sublines in order to investigate if the levels of IgA H chain were related to amounts of H-2K^d and H-2D^d as well as of gp71. The individual sublines showed considerable variation in amounts of H-2K^d and H-2D^d on their surface, and repeated analysis over a 6-month period showed that the different levels of H-2 antigen expression were heritably stable. There was no correlation between the expression of IgA H chain and the expression of either H-2K^d (Fig. 4*B*) or H-2D^d (data not shown) on the surface of the tumor cells. The relative amounts of H-2K^d and H-2D^d surface antigen expressed correlated in most sublines but not in all of them.

The analysis of gp71 surface antigen expression also showed heterogeneity among the different S107 sublines (Fig. 4*C*). In contrast to the heritably stable phenotypes of H chain, L chain, H-2K^d, and H-2D^d expression on the sublines, the gp71 antigen levels showed nonheritable phenotypic variations with as much as 5-fold change (increase or decrease) in surface antigen when assayed in exponentially growing cultures over a period of 2 weeks.

Among the group of sublines lacking surface H chain, there was no correlation between L chain expression and expression of H-2K^d, H-2D^d, or gp71.

DISCUSSION

We have isolated a number of myeloma variants which showed heritable quantitative changes in expression of cell surface antigens. The emergence of such variants from a cloned population of myeloma cells that had been passaged in culture for an extended period of time was identified by using the fluorescence-activated cell sorter. The distribution of the cells in the fluorescence histogram changed with time due to the development of variant cells, and cell sorting followed by soft-agar cloning allowed for the enrichment and isolation of these variants.

During normal B-cell differentiation, less-mature cells express surface immunoglobulin in the absence of secretion, and with the onset of secretory function there is a decrease in the level of surface immunoglobulin (4). Subsequent to antigen or mitogen stimulation, B cells express gp71 on their surface (7, 8). Our observations in this study indicate that these regular differentiation pathways need not be followed by malignant B cells as they "dedifferentiate" during tumor progression. A decrease in surface immunoglobulin among the myeloma cell variants was tightly linked to a corresponding decrease in the amount of secretion. This correlation was observed for myeloma cell variants regardless of whether they were selected on the basis of low amounts of secretion, as reported (15), or on the basis of low amounts of membrane-bound immunoglobulin as in this study. Furthermore, myeloma variants that showed neither secretory nor membrane-bound immunoglobulin still expressed gp71 on their surface.

Although changes in the amounts of secreted and plasma membrane-associated immunoglobulin were tightly linked, a decrease in immunoglobulin expression did not correlate with a decrease of H-2 or viral surface antigens, in contrast to earlier observations on a smaller number of myeloma cell variants (13). We have previously shown that a small decrease in the amounts of tumor-specific idiotypic surface antigens can make myeloma

cells resistant to anti-idiotypic immunity (15). Thus, changes in the amount, rather than the complete loss, of idiotypic and possibly other tumor-specific antigens could be a frequent and rapid way for malignant cells to escape tumor-specific immunity (26–28). Because T cell-mediated cytotoxicity against tumor-specific antigens of malignant cells may depend on the simultaneous recognition of H-2 antigens (29), the independent regulation of these two types of surface antigens might also play a role in tumor escape. During immunosurveillance, tumor cells with decreased amounts of H-2 antigen might become relatively or absolutely resistant to T cell-mediated immunity in spite of the persistance of the tumor-specific antigens. Others have reported that different myeloma cells can have different levels of H-2 antigen (26, 30), and we find in this study that, during the clonal evolution of myeloma cells, variants are produced that have heritably different amounts of H-2.

The molecular basis of immunoglobulin synthesis, assembly, glycolysation, and secretion has been studied with myeloma variants (31, 32). However, little is known about how normal or malignant B cells or plasma cells regulate their amounts of secreted or membrane-bound immunoglobulin. We have isolated myeloma variants which differ heritably in their amount of immunoglobulin expression. These variants fell into three groups—high, intermediate, and negative—according to the amount of surface H chain expression. The development of variants with intermediate levels of surface immunoglobulin was observed in several independently carried clones of myeloma cells that originally expressed a high amount of surface immunoglobulin. Even without cell sorter enrichment, substantial percentages (up to 30%) of the cells in the parental populations showed intermediate levels of surface immunoglobulin. In contrast, the frequency of variant cells expressing L chain only was <1% in these populations. This indicated a significant difference between the frequency of variants that had intermediate levels of H chain expression and the frequency of those that had lost H chain expression completely but maintained their ability to produce L chain. This is of particular interest because the latter type of variant has been found to be generated within different myeloma lines at a rate of approximately 10^{-3} per cell per generation (33).

One explanation for the different levels of H chain expression among the myeloma variants consistent with all of the data is that our parental myeloma cells may have more than one functional copy of the H and L chain genes. Our myeloma lines, like many other myelomas, have a near-tetraploid DNA content and thus may have two functional copies of the immunoglobulin genes (taking into account gene inactivation by allelic exclusion). During polyploid formation in female mammalian cells, the additional copies of the functioning X chromosome can remain active (34). Furthermore, in systems in which two functional copies of a structural gene are known to be present, such as the adenine phosphoribosyltransferase locus in Chinese hamster cells, loss of the gene product due to mutation occurs in two discrete steps with an intermediate level of gene expression (35, 36). It is clear from our study that changes in other surface markers such as gp71 and histocompatibility antigens need not be correlated with changes in immunoglobulin expression. This indicates that our variants might be useful tools for studies on the regulation of expression of individual surface proteins.

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