Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7297–7301, December 1980
Cell Biology

Cell cycle and the differential expression of HLA-A,B and HLA-DR antigens on human B lymphoid cells

(synchronous culture/mitosis/monoclonal antibody/immunofluorescence/fluorescence-activated cell sorter)

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Communicated by Helen M. Ranney, September 2, 1980

ABSTRACT **Monoclonal antibodies specific to HLA antigens and the fluorescence-activated cell sorter were used to analyze the changes in the density of human histocompatibility antigens HLA-A,B and HLA-DR on the surface of synchronously growing WI-L2 cells (a human B cell line) progressing through the cell cycle. The WI-L2 cells were synchronized by density-dependent arrest in G_1, and samples from G_0, G_1, late S and late G_2 phases were used to determine the frequency distribution of cell volume, DNA content, and the relative amounts of cell surface HLA antigens; the observed density changes were calculated from these values. The HLA-A,B density remained nearly constant throughout the cell cycle, whereas the HLA-DR density increased sharply at the G_2–M stage. These results suggest a cell cycle-dependent differential control of the expression of these two sets of histocompatibility antigens on B cells.**

The progression of cells through the cell cycle is associated with an orderly series of events, some of which control the expression of cell surface macromolecules. Characterization of these molecules should aid in our understanding of the regulatory mechanisms operating in normal and malignant cells because the cell periphery is one of the principal loci of cell growth control (1). Moreover, the expression of major histocompatibility complex genes (2) in the cell cycle is of considerable interest because the products of these genes mediate cell–cell interactions and are involved in various immunological phenomena (3–5) related to cell proliferation, cell killing, and various regulatory activities.

The availability of monoclonal antibodies to HLA antigens and the development of flow cytofluorometric quantitation of the antibody bound to single cells have provided optimal technical conditions for the analysis of cell cycle-dependent HLA expression (6, 7). In this study we have used these approaches to investigate the relationship between the cell cycle and the expression of HLA-A,B, and HLA-DR (8) antigens by WI-L2 cells [a diploid human B lymphoblastoid cell line (9, 10)] synchronized by density-dependent arrest in G_1 (11). These antigens are membrane-bound glycoproteins consisting of two polypeptide chains and display serological polymorphism controlled by the major histocompatibility complex (2, 12, 13) genes. HLA-A,B,C antigens are expressed on all nucleated cells; HLA-DR antigens have a restricted tissue distribution and are primarily associated with cells involved in immune functions (12, 13).

Results presented here indicate that there is a cell cycle phase-dependent expression of HLA-DR antigens, the magnitude of expression being lowest at the quiescent state and highest at the G_2 phase, whereas the HLA-A,B antigens maintain a relatively constant surface density throughout the entire cell cycle.

MATERIALS AND METHODS

Cell Line. The diploid human B lymphoid cell line WI-L2 (HLA-A1, A2, B5, B17, DRw4, DRw7, IgG.Fc receptor-negative), which synthesizes and secretes IgG and IgM with κ type light chains (9, 10), was grown in minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, Na pyruvate, vitamins, and glutamine (11).

HLA Monoclonal Antibodies and Polyclonal Anti-Mouse Ig Xenoantisera. Monoclonal antibodies (MoAb) were obtained from clones derived from the fusion of murine myeloma cells (P3-X63-Ag) with splenocytes from mice immunized with HLA bearing cells (14). MoAb 28 (15) is directed against framework antigenic determinants expressed on the heavy chain of HLA-A,B antigens because: (*a*) in the presence of rabbit complement it is cytolytic to cells carrying HLA-A,B antigens but not to the cultured human B lymphoid Daudi cells which lack HLA-A,B,C antigens and β_2-microglobulin (β_2-μ) (12); (*b*) its cytolytic activity is prevented by coating target cells with $F(ab)_2$ fragments from "specific" antihuman β_2-μ xenoantiserum; (*c*) it does not react with purified human β_2-μ in a radioimmunoassay; and (*d*) it immunoprecipitates M_r 45,000 and 12,000 components from intrinsically labeled glycoproteins purified from human lymphoid cells. These glycoprotein components have the M_rs characteristic of the heavy chain and of β_2-μ of the HLA-A,B antigenic molecular complex.

MoAb 513 (16) is directed against an HLA-DR antigen framework determinant because: (*a*) it reacts with cultured B lymphoid cells and peripheral blood B lymphocytes but not with cultured human T lymphoid cells or purified peripheral T lymphocytes as detected by a complement-dependent microcytotoxicity test (17); (*b*) the reaction with B lymphoid cells is prevented by coating the target cells with $F(ab)_2$ fragments from "specific" anti-HLA-DR xenoantisera; and (*c*) in reactions with intrinsically labeled B lymphoid cell lysates, it immunoprecipitates glycoproteins that have the M_rs characteristic of the α and β chains of HLA-DR antigens.

Rabbit anti-mouse Ig fluorescein isothiocyanate-labeled antiserum was purchased from Miles–Yeda. It was used at a protein concentration of 60 μg/ml (fluorescein/protein ratio, 3.5). The antiserum was extensively absorbed with WI-L2 cells to remove background binding to human lymphoid cells.

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Abbreviations: MoAb, monoclonal antibodies; β_2-μ, β_2-microglobulin; FACS, fluorescence-activated cell sorter; CV, coefficient of variation.

Cell Synchronization. WI-L2 cells were synchronized as described (11). Briefly, cultures were maintained at stationary phase (G_0) for a few days until the rate of DNA synthesis ([^{3}H]thymidine incorporation per viable cell) was approximately 2% of maximum. The cells were seeded into fresh prewarmed medium at a concentration of 2×10^5 cells per ml. WI-L2 cells uniformly enter G_1, proceed through the cell cycle in a synchronous manner, and double the number of cells within 22–24 hr (11). The phase durations are 8 hr at G_1, 8 hr at S, 4–5 hr at G_2, and 1–1½ hr at mitosis. Cells representing each of the above phases were collected at the following intervals for this study: G_0 (before the start of the cycle), mid-G_1, late S, and late G_2.

Frequency Distribution of Cell DNA Content. The cells were stained by the fluorescent Feulgen method for determination of relative cell DNA content with the fluorescence-activated cell sorter (FACS) according to the methods described previously (18). The proportions of cells in different growth phases were obtained by computer analysis of frequency distributions. The FACS instrument (designed and built at the University of California at San Diego, La Jolla) and the computer program used in these experiments were similar to the ones originally reported by the group at Los Alamos and Lawrence Livermore Laboratories (7, 19).

Determination of Cell Volume. The frequency distribution of volume was obtained from a Coulter Counter (Coulter Electronics, Hialeah, FL, Model ZB1,P64) fitted with a cell size analyzer. This volume was calibrated against three standard spherical beads (5, 10, and 18 μm in diameter; Coulter Electronics) and the frequency distributions were used to derive the mean (±SD) volume for calculation of the cell surface area. The cells in growth medium were gently triturated and diluted in phosphate-buffered saline containing 10% (vol/vol) fetal calf serum for simultaneous counting and sizing in the Coulter apparatus.

Frequency Distribution of Cells Differing in Amounts of Cell Surface Antigen. The relative amount of cell surface antigen was determined by first coating WI-L2 cells with saturating amounts of specific MoAb followed by reincubating the carefully washed and coated cells with an excess of rabbit anti-mouse Ig conjugated with fluorescein isothiocyanate. The procedure for calibrating the saturating concentrations of the two antibodies and the quantitation of relative amounts of cell surface antigen per cell with the FACS instrument have been described (20).

Freshly washed WI-L2 cells were resuspended (1×10^6 cells per 0.1 ml) in cold phosphate-buffered saline and incubated with 100 μl of an appropriate dilution of the MoAb in an ice bath for 30 min. Then the cells were washed twice on a cushion of heat-inactivated fetal calf serum and resuspended with 100 μl of dilute fluorescein isothiocyanate-conjugated anti-mouse second antibody. After an additional 30 min in an ice bath, the cells were washed once with phosphate-buffered saline and resuspended to 1×10^5 cells per ml. The specific cell immunofluorescence was expressed in relative fluorescence units or channel numbers (20, 21). The mean (±SD) fluorescence value calculated from this distribution represents the mean cell surface antigen content. In order to obtain antigen density, the mean value of cell antigen content was divided by the mean cell surface area of the cell population at that growth phase.

RESULTS

Volume and DNA Content of WI-L2 Cells Moving Through the Cell Cycle. Upon prolonged incubation at stationary growth conditions, the WI-L2 cell culture reaches a quiescent state; at this stage, the cell volume as well as all synthetic capacities are at their minima (11). Upon dilution in prewarmed growth medium, the cells immediately enter the G_1 phase in a synchronized manner, and the synchrony is well maintained for at least one cell cycle as shown by the increase in cell volume and cell DNA content. The mean cell volume (Fig. 1) increased sharply between G_0 and G_1 (623 μm^3 to 720 μm^3) and between S and G_2 (1092 to 1248). The cell surface

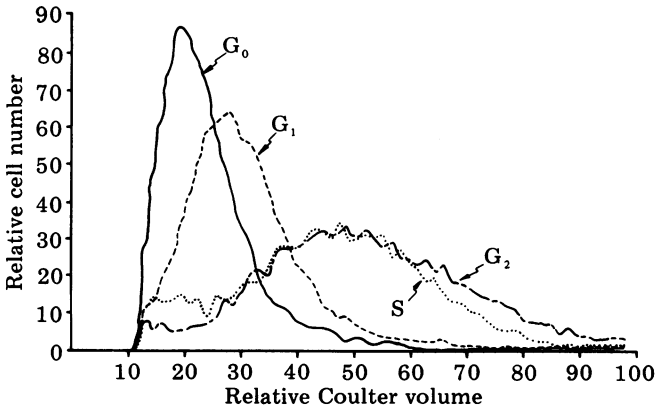
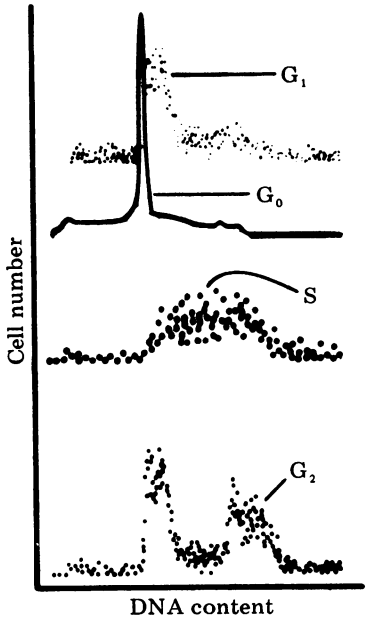


FIG. 1. Frequency distribution of the Coulter volume of WI-L2 cells during synchronized growth. An equal number of cells (4000) from each cell cycle phase sampled were processed in the Coulter Counter with a cell size analyzer. The relative mean volumes were: G_0, 16 ± 7; G_1, 29 ± 8; S, 47 ± 16; and G_2, 53 ± 18.



	G_1	S	G_2
0 time	78	22	—
Mid-G_1	83	17	—
Late S	5	80	15
Late G_2	44	15	41

FIG. 2. Frequency distribution of DNA content of synchronized WI-L2 cells as determined by FACS. Cells were stained with acriflavine dye by the fluorescent Feulgen method (18). An equal number of cells (2000) from each cell cycle phase were analyzed. The relative fluorescence in channel number (abscissa) represents DNA content. Aliquots of cells were taken at G_0, mid-G_1, late S, and late G_2 to determine the frequency distribution of cell DNA content. The distributions at four sampling points in the cell cycle are given from top to bottom; the accompanying table presents the relative proportions of cells at different phases of the cycle in each of these sampling times.

area increased from 332 μm^2 at G_0 to a premitotic value of 560 μm^2, a nearly 60% increase compared to the doubling of cell volume. The coefficient of variation (CV) in cell volume was in the order of 30% at all phases, whereas for the frequency distribution of DNA content at the beginning the CV was only 4% and increased slightly with cycle progression.

The profile of cell DNA content indicates that nearly 80% of the cells were synchronized for their entry into G_1 (Fig. 2). Although at late S there were a few cells still remaining at G_1 and a few had already progressed into G_2, the contribution of these cells outlying the principal synchronized population is rather small because the CV remained nearly the same during the entire generation time. The sampling at late G_2 showed that about equal numbers (40%) of cells were at G_1 and G_2 and 15% were at S phase; synchrony was lost to a certain degree in the 20 hr that had passed from the onset of the culture. However, we chose the sampling time in late G_2 when the population of cells at S was at a minimum such that an effective measurement of the mean antigen content at G_2 could be made.

Density of HLA Antigens on WI-L2 Cells Moving Through Cell Cycle. WI-L2 cells sampled from the synchronized cultures were treated with an excess of HLA-A,B and HLA-DR MoAb and then stained with saturating concentrations of rabbit anti-mouse Ig antisera. The mean antigen content of the surface-labeled cells was derived from the FACS analysis of the frequency distribution of fluorescence produced by the stained cells. The dead cells, which accumulate due to prolonged culture incubation at the stationary phase, were on the order of 5% of total cells; these cells could be excluded from the scale because they usually were 3–5 times brighter than the specifically stained fluorescent viable cells.

The immunofluorescence due to the MoAb binding of HLA-A,B and HLA-DR cell surface antigens is shown as a frequency distribution in Fig. 3. The two distributions depicted in each figure represent the antibody activities at G_0 and late G_2 and illustrate the extreme differences in cell surface antigen content in the cell cycle traverse. For example, in Fig. 3 *Left*, the two frequency distributions of cell fluorescence are nearly normal, and the mean fluorescence of the late G_2 cell (i.e., the HLA-A,B antigen content) is 34% higher than that of cells at G_0. The kinetics of the 34% increase of the mean fluorescence from the initial to the final phase of the cell cycle are shown in the *Inset*. Here the mean fluorescence at each phase is plotted against cell volume to indicate the phase dependence in the cell cycle. It is only during the early part of the cell cycle that the HLA-A,B antigens increase in accordance with cell volume expansion and there is no further increase in HLA-A,B content although the volume expands again rapidly between late S and the premitotic stage.

In contrast, the cell surface HLA-DR antigens increase almost linearly with cell volume (Fig. 3 *Right Inset*)—the surface HLA-DR content doubles with the cell volume. The frequency distribution of cell fluorescence for HLA-DR antigen at late G_2 is not a normal distribution and the heterogeneity has probably resulted from the varied HLA-DR content of cells lying outside the principal synchronous population (Figs. 1 and 2). Thus, the mean HLA-DR content at late G_2 as shown in Fig. 3 *Right Inset* would be higher if all cells in the culture were at this phase.

The mean antigen density at each cell cycle phase as calculated from the mean fluorescence values (Fig. 3) and the cell surface area at a given phase. In order to compare the main

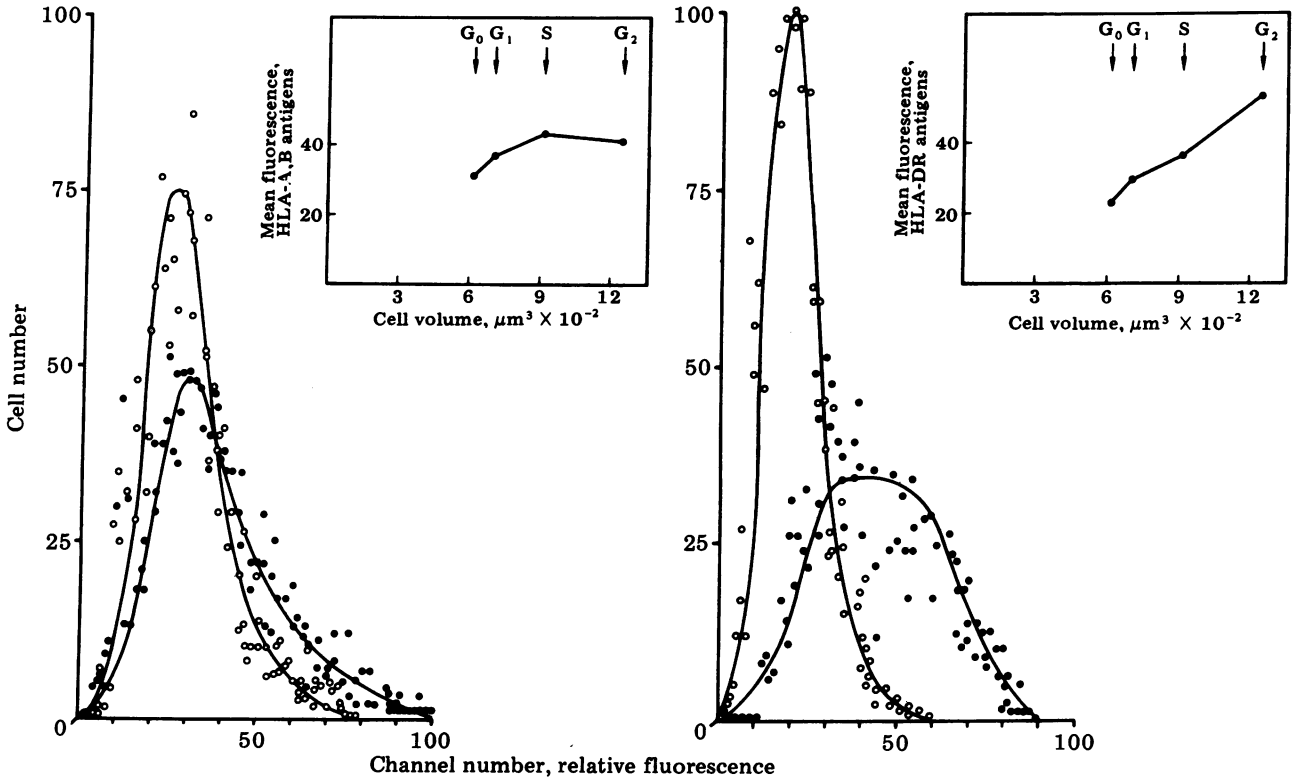


FIG. 3. Cell surface antigen expression by synchronized WI-L2 cells. WI-L2 cells sampled at G_0, mid-G_1, late S, and late G_2 were treated separately with MoAb specific for HLA-A,B (*Left*, MoAb 28) and for HLA-DR (*Right*, MoAb 513) antigens and then subjected to direct immunofluorescence staining with rabbit anti-mouse Ig conjugated with fluorescein isothiocyanate. Stained cells (2000) were passed through the FACS to obtain the frequency distribution of cell fluorescence. Two such distributions for antibody against HLA-A,B (*Left*) and HLA-DR (*Right*) are shown for cells at G_0 (○) and G_2 (●). The intensity scale (abscissa) was adjusted to exclude the high fluorescence of dead cells. The statistical variables were determined from all data points shown. The smooth lines are drawn for a simpler visualization of the distributions. (*Insets*) Mean cell fluorescence values at each cell cycle phase plotted against respective mean cell volumes.

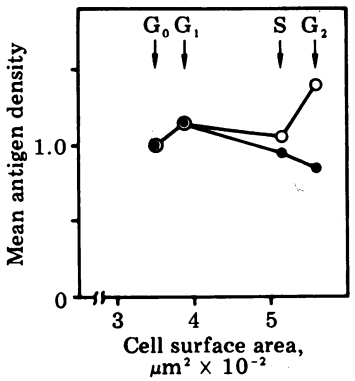


FIG. 4. Fluctuation of antigen density in the WI-L2 cell cycle. The antigen density values were obtained by dividing the mean fluorescence (representing the average antibody binding to surface antigens) by cell surface area at respective cell cycle phases. These values were normalized relative to the values obtained at G_0 (set equal to 1) and are plotted against the mean cell surface area of the respective phase. ●, HLA-A,B antigens; ○, HLA-DR antigens.

features of HLA-A,B and HLA-DR antigen expression, the mean antigen densities thus obtained were normalized to the values at G_0 (set equal to 1) and were plotted against the progressive increase in cell surface area (Fig. 4). The fluctuations of HLA-A,B antigen density were small through the entire cell cycle. The HLA-DR antigen density also did not fluctuate appreciably from G_0 to the end of S phase; however, unlike the HLA-A,B determinants, with HLA-DR there was a rapid increase in density at the premitotic stage.

DISCUSSION

The MoAb specific to the HLA framework antigenic determinants and the sensitive quantitation provided by the FACS were used in this study to demonstrate the differential expression of HLA-A,B and HLA-DR antigens on B lymphoid cells moving through the cell cycle. Whereas the cell surface density of HLA-A,B remained constant, that of HLA-DR increased on cells moving from S phase to G_2–M phase.

Previous studies using conventional reagents and techniques such as alloantisera, immunofluorescence methods, quantitative absorption, and complement-dependent cytotoxicity have had conflicting results on the expression of HLA-A,B antigens and their murine counterpart, H-2 antigen, on synchronously growing cell populations (22–24). No data are available on the HLA-DR antigens or their counterparts in other animal species. The homogeneity of MoAb in detecting a given specificity in all cells provides a high degree of sensitivity and, when used in saturating concentration, provides a good estimate of the relative quantitative change in cell surface antigen expression during passage through the cell cycle.

By means of various tests (15), we have been able to establish that MoAb 28 used in our experiments recognizes a framework antigenic determinant expressed on the heavy chain of HLA-A,B molecules. Similarly, MoAb 513 detects a framework antigenic determinant of HLA-DR molecules (16). The ability to synchronize the WI-L2 cell by density-dependent arrest in G_1 eliminates the use of drugs that may alter the cell surface antigen expression. The degree of synchrony achieved by this method has been reasonably good. The frequency distributions of Coulter volume and cell DNA content indicate that approximately 80% of the cells could be made to enter the G_1 phase from the resting state within a brief incubation period (Figs. 1 and 2), although the extent of synchrony is progressively reduced as the cell cycle progresses. The values for relative HLA antigen density were obtained (Fig. 4) by calculating the mean cell surface area from the Coulter volume (Fig. 1) and the mean cell fluorescence due to the monoclonal antibody binding in the same cell cycle stage (Fig. 3). Comparison of the relative quantitative changes in the density of HLA-A,B and HLA-DR antigens clearly shows a significant difference in the regulating aspect of antigen expression prior to cell division (Fig. 4).

Until late S, both antigens seem to be expressed at similar rates such that the surface densities of the two antigens remain nearly the same relative to their densities at the start of the cycle (Fig. 3 *Inset*). In G_2, HLA-DR antigen density increases at an accelerated rate, whereas HLA-A,B antigen density remains unchanged. The HLA-DR antigen density increase is approximately 50% over the resting state, and probably slightly higher, because the value represents only an estimated 40% of cells at late G_2 whereas the rest of the cells (44%) have traversed into G_1 of the next cycle at the time the sample is taken (Fig. 2). The exact timing of the HLA-DR antigen expression at the cell cycle terminal point is not certain because the culture becames less synchronous at this stage.

The constancy of the HLA-A,B antigen density in growing lymphoid cells is contrasted by the relative increase of this antigen and β_2-μ when cell growth is inhibited by interferon (25–27). We have treated WI-L2 synchronous cultures at G_1 with human fibroblast interferon (1500 international units per ml; gift from L. Kronenberg) and detected a lengthening of the S + G_2 phase and a concomitant increase in HLA-A,B antigen density; however, the HLA-DR antigen expression remained at the observed G_1 level and there was no characteristic antigen density increase at G_2 compared to the control cultures (unpublished data). Growth, or lack of growth, produces opposite effects in the HLA-A,B and HLA-DR antigen expression; the HLA-DR expression is positively correlated with cell growth whereas inhibition of cell division has a positive feedback effect on the HLA-A,B antigen expression. It appears that the expression of the structural genes for β_2-μ, HLA-A,B, and the α and β subunits of HLA-DR operates under a common framework of regulation of the cell cycle. It is known that the immunoglobulin molecules are synthesized and secreted by WI-L2 almost exclusively during the late G_1 and S phases (28). Hence, we suspect that the rapid appearance of HLA-DR antigens in G_2 is probably made possible by the prior accumulation, during G_1 to S phase, of nascent molecules which could be translocated to the surface at the G_2 stage.

WI-L2 cells treated with inhibitors of protein synthesis at late S expressed as much HLA-DR antigen as did the control cells nearing mitosis 4–5 hr later, whereas inhibitors added at any earlier time reduced HLA-DR expression on the cell surface within 2-hr (data not shown). Therefore, the decision for an accelerated HLA-DR expression at G_2 was probably made several hours prior to the nascent HLA-DR molecular translocation to the cell surface. The exact mechanism of the molecular nature of this decision is not clear at this time.

Note Added in Proof. MoAb 28 is now designated Q1/28 (15) and MoAb 513 is Q5/13 (16).

We thank Drs. Melvin Cohn and Ralph A. Reisfeld for valuable criticisms and Mr. Jeffrey S. Frisbie for excellent technical assistance. This work was supported by National Institutes of Health Biochemical Genetics Program Project GM17702-10 (O.W.J. and S.S.), AI13154, CA16069, CA24329 (S.F.), Postdoctoral Fellowship 1F32 CA06415-01 (M.C.G.), and an American Heart Association Established Investigatorship Award (S.F.).

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