VARIATION IN SUSCEPTIBILITY OF A HUMAN LYMPHOID CELL LINE TO IMMUNE LYSIS DURING THE CELL CYCLE

LACK OF CORRELATION WITH ANTIGEN DENSITY AND COMPLEMENT BINDING*

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The progression of cells through the mitotic cycle is characterized not only by ordered changes in cellular functions concerned with DNA synthesis and mitosis, but also by numerous physical and chemical alterations in other properties of the cell. Many cell cycle-dependent changes in the external cell membrane have been described including gross alterations in physical structure (1, 2) and fluctuations in synthesis of enzymes and cell products such as immunoglobulins (3, 4). Changes in the structure of the cell membrane are of particular interest because alterations in the properties of the cell surface are believed to contribute to the disordered proliferations which characterize malignant cells.

Previous work has shown that the human cultured cell line WI-L2 does not vary during its growth cycle in susceptibility to lysis in the cytotoxic test with complement and antibodies directed against the antigens of the major histocompatibility locus in man (HL-A) (5). On the other hand, the susceptibility of certain cell lines to lysis induced by antibody directed against membrane antigens and complement, changes during the growth cycle. This phenomenon has been demonstrated for several cell lines including the murine tumor lines YCAB (6, 7), YAC (8), L 1210 (9), and Chinese hamster lung cells (10). In each of these cell lines the times of maximal and minimal susceptibility to complement-mediated lytic damage corresponded to the G1 and S phases, respectively. In the case of murine tumor cells, the expression of cell surface histocompatibility antigens, as evidenced by the ability of cells to quantitatively absorb alloantisera directed against histocompatibility antigen was most pronounced during the period of maximal lytic susceptibility. However, additional studies of the murine tumor cell line YCAB did not support the conten-

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tion that these variations in antigenic expression led to an increased ability to activate the complement system in free solution or on the cell surface since complement activation and late complement component binding to the cell surface remained constant throughout the cell cycle (7, 11). In the present study we have examined a human cell line (RPMI 8866) which also exhibits variability in cytolytic susceptibility during the cell cycle but which does not show significant variation in the cell surface expression of histocompatibility antigens. These data suggest that variables other than antigenic expression, antibody binding, and complement activation are responsible for the variation in susceptibility of complement-mediated damage during certain phases of the cell cycle.

Materials and Methods

Cultured Human Lymphoid Cell Line.—Cultured human lymphoid cells RPMI 8866 (HL-A 2, 3, 7, 12) derived from a donor with myelogenous leukemia (12) were grown in suspension in medium RPMI 1640 with 10% fetal calf serum. Cell concentration and viability were determined by trypan blue exclusion and use of a hemacytometer.

HL-A Alloantisera.—Antisera were obtained from the National Institute of Allergy and Infectious Diseases serum bank and from the laboratories of Doctors R. Ceppellini, R. Payne, and P. I. Terasaki. All sera had been frozen and thawed several times.

Rabbit Antihuman Lymphocyte Serum.—This antiserum was produced as previously described (13) and had a titer of 1:16,000 against cultured human lymphoid cells in the microlymphocytotoxic test.

Rabbit Natural Antihuman Lymphocyte Antibodies.—Rabbit IgM antihuman lymphocyte antibodies were isolated from fresh normal rabbit serum by sequential ammonium sulfate fractionation and gel filtration on Sephadex G-200 (14).

Complement.—Rabbit complement was a pool of nine rabbit sera selected after individual blood samples proved nontoxic to peripheral lymphocytes from four randomly chosen subjects in the cytotoxic assay. This complement was absorbed with cultured human lymphoid cells in the presence of EDTA (final concentration 10 mM) at 4°C for 60 min (15). Guinea pig complement was a pool of eight guinea pig sera. A pool of fresh sera derived from five healthy donors with no previous history of pregnancy or blood transfusion served as the human complement source. Complement pools were stored at −70°C in small samples and were used only once after thawing. Human complement components C4, C3, and C8, purified from human serum (16, 17), were radiolabeled with 125I by the chloramine-T method (18) without loss of activity. The specific radioactivity of C4, C3, and C8 was, respectively, 2.2 × 106, 2.2 × 106, and 4.5 × 106 cpm/µg; these values correspond to 0.22, 0.22, and 0.45 μCi/µg.

Cell Synchronization.—RPMI 8866 cells were synchronized as previously described (19). Briefly, cultures were seeded at 2 × 105 cells/ml; when the DNA synthesis rate, as judged by incorporation of tritiated thymidine, was approximately 2−10% of the maximum (G0 phase of cell growth), resting cells were harvested and resuspended in fresh, 37°C medium. Cell number and course of DNA synthesis were then ascertained in order to identify the G1 and S phases.

Cytotoxic Assay.—This assay was performed as a dye exclusion test (15). When cultured human lymphoid cells were the target cells, absorbed rabbit complement, guinea pig complement, or human complement were used. When peripheral human lymphocytes were the targets, undiluted fresh rabbit serum was the complement source.

Quantitation of Cell-Bound Radiolabeled C4, C3, and C8.—At various stages of the growth cycle, 5 × 106 RPMI 8866 cells (washed three times with Hank's balanced salt solution
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[HBSS]\(^1\) were mixed with 20 µl of antisera (previously inactivated at 56°C for 30 min) and 40 µl of complement, the latter containing radiolabeled C4, C3, or C8. After 60 min of incubation at 37°C, cells were washed four times and analyzed for radioactivity in a well type scintillation counter. Reaction mixtures with heat-inactivated normal human serum in place of specific antisera were used to screen for nonspecific binding of complement components. Mol wt of 200,000, 185,000, and 150,000 were used to calculate the number of bound C4, C3, and C8 molecules, respectively.

Microabsorption Technique.—Quantitative microabsorption tests were performed as previously described (20). The results were expressed in terms of absorption dosage\(50\) (AD\(50\)), i.e., the number of cells required to reduce by 50% the cytotoxicity of an alloantisera used at a concentration twice that required to lyse 95% of selected target cells.

Isotopic Antiglobulin Test.—A rabbit antihuman IgG serum was fractionated by ion exchange chromatography on DEAE cellulose in 0.01 M phosphate buffer, pH 8. The IgG fraction was checked for specificity by immunoelectrophoresis against human IgG and labeled with \(^{125}\text{I}\) (New England Nuclear, Boston, Mass.) by the chloramine-T method (18) at a spec act of 0.9 mCi/µg of protein.

For the test, 2 × 10^5 RPMI 8866 cells in G\(_0\), G\(_1\), and S phases of the growth cycle were washed twice with HBSS and incubated with 20 µl of HL-A alloantisera for 60 min at room temperature in Beckman microtubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The cells were washed three times with HBSS, 10 µl of radiolabeled diluted antiglobulin reagent were added then, and the reaction mixtures again incubated for 60 min at room temperature. Next, the cells were washed three times and analyzed for radioactivity in a well type scintillation counter. Nonspecific binding of labeled antiglobulin reagent was determined from control reaction mixtures in which normal human serum was used. Specific binding, expressed in cpm, was calculated by subtracting the values for nonspecific uptake from the values for total bound radioactivity.

Extraction and Serologic Evaluation of Soluble HL-A Alloantigens.—Soluble HL-A alloantigens were extracted from cultured cells by the 3M KCl method (21). The specific antigenic activity of soluble HL-A antigens was evaluated as ability to specifically inhibit cytotoxicity of operationally monospecific HL-A alloantisera against selected target cells in the microcytotoxic test. Immunologic potency of soluble HL-A preparations was expressed as ID\(_{50}\) inhibition dosage (22), i.e., the minimum amount of soluble HL-A antigens necessary to specifically inhibit 50% of the activity of monospecific cytotoxic alloantisera. Immunologic specificity of the preparations was expressed in terms of specificity ratio (SR) (22, 23), i.e., the ratio between the degree to which antigens inhibit antisera directed against determinants absent from the cell source, as compared to those directed against determinants present on the cell source.

Determination of Cell Volume.—This determination was done as previously described (19).

Protein Concentration.—Protein was determined by a modification of the Lowry method (24); lysozyme was used as a standard.

Rosette Formation Test.—This was performed as previously described (25). Briefly, sheep erythrocytes coated with rabbit antisheep hemolysin (BBL, Bioquest Div., Cockeysville, Md.) (EA) and purified human C1, 4, 2 (EAC142\(^{h/u}\)), C1, 4, 2, 3 (EAC1-3\(^{h/u}\)), or C5-deficient mouse serum from strain B10.D2 old (EAC1-3\(^{m0}\)) were adjusted to 5 × 10^7 cells/ml in phosphate-buffered saline (PBS), pH 7.2. 200 µl of RPMI 8866 cell suspension (1 × 10^6 cells/ml HBSS) were incubated with 200 µl of EAC1-3\(^{h/u}\) or EAC1-3\(^{m0}\) and 200 µl PBS with 0.06 M EDTA in a vertical submersion rotator at 37°C for 10 min. At the end of the incubation, 250-300 lymphoid cells were counted; those having three or more EAC1-3 cells attached were considered rosette positive. EAC142\(^{h/u}\) and EA cells were included as controls.

\(^1\) Abbreviations used in this paper: AD\(_{50}\), absorption dosage\(_{50}\); HBSS, Hank’s balanced salt solution; PBS, phosphate-buffered solution; SR, specificity ratio.
RESULTS

Relationship between the Phase of the Growth Cycle and the Susceptibility of RPMI 8866 Cells to Immune Lysis in the Lymphocytotoxic Test.—To determine the relationship between the phase of the growth cycle and susceptibility to lysis mediated by HL-A alloantisera directed against antigens of the first and second segregant series, cells in synchronous growth were sampled in various phases of the growth cycle and incubated with HL-A alloantisera and rabbit complement. Fig. 1 shows that cells in the G1 phase were approximately two-fold less sensitive than those in the G0 or S phases of the growth cycle to the cytolytic potential of alloantisera directed against HL-A2. Similar results were obtained with alloantisera directed against HL-A7, a specificity of the second segregant series. Rabbit serum is known to contain natural antibodies directed against a polymorphic system of antigens present on human lymphoid cells (26, 27). To eliminate the possibility that the variation in the lysis obtained with HL-A alloantisera and rabbit complement was due only to changes in the expression of the antigenic determinants towards which these natural rabbit antibodies are directed, the cytotoxic test was repeated utilizing human and guinea pig sera, which do not contain antibodies directed against cultured human lymphoid cells, as the sources of complement. Although both of these complement sources were cytolytically less efficient than rabbit complement, the lytic pattern observed was identical to that found with rabbit complement and HL-A alloantisera (Fig. 1). In order to determine whether variable susceptibility to lysis applied also to other antigen systems, RPMI 8866 cells at various

![Fig. 1. Relationship between the growth cycle and susceptibility to lysis of lymphoid cells RPMI 8866 by HL-A alloantisera and complement. On the left, the growth curve and DNA synthesis of the cells is depicted. The small panels illustrate the susceptibility of RPMI 8866 cells to the lytic activity of anti-HL-A2 (upper panels) and anti-HL-A7 (lower panels) in conjunction with rabbit (A, D), human (B, E), and guinea pig (C, F) complement. The growth phase of the target cells is indicated by the corresponding symbol in the growth curve.](image_url)
phases of the growth cycle were reacted with rabbit IgM which contains the natural antibodies directed against human lymphocytes and human complement. Cells in the G₁ phase were again found to be least sensitive to cytotoxicity (Fig. 2).

These results indicate that RPMI 8866 cells change in susceptibility to immune lysis throughout their growth cycle, with cells in the G₁ phase being more resistant than cells in other portions of the cell cycle to the lytic activity of HL-A alloantisera and/or rabbit natural antibodies in conjunction with complement components supplied by either rabbit, human, or guinea pig serum.

Antibody-Binding Capacity of RPMI 8866 Cells during the Growth Cycle.—To analyze the possibility that variability in susceptibility to lysis was due to changes in the density of HL-A antigens during the cell cycle, we determined whether cells sampled from various phases of the growth cycle differed in their ability to bind HL-A alloantibodies. Two tests were utilized in these studies. First, cells sampled from various phases of the growth cycle were used in quantitative absorption tests with HL-A alloantisera. As indicated in Table I,

![Graph showing cytolytic activity of rabbit IgM and human complement towards RPMI 8866 cells in different stages of their growth cycle as indicated by the corresponding symbol in the growth curve depicted in Fig. 1.](image)

**Fig. 2. Cytolytic activity of rabbit IgM and human complement towards RPMI 8866 cells in different stages of their growth cycle as indicated by the corresponding symbol in the growth curve depicted in Fig. 1.**

**TABLE I**

| Phase of cell cycle | HL-A2 | HL-A3 | HL-A5 | HL-A7 | HL-A8 |
|---------------------|-------|-------|-------|-------|-------|
| G₀                  | 900   | 1,200 | —     | 600   | —     |
| G₁                  | 900   | 1,100 | —     | 700   | —     |
| S                   | 300   | 500   | —     | 250   | —     |

* Number of cells required to reduce by 50% the cytotoxicity of an alloantiserum.

† Indicates AD₅₀ values >50,000.
cells sampled from the G₀ and G₁ phases were equally able to absorb HL-A alloantisera directed against specificities of the first and second segregant series. Cells in the S phase had an approximately twofold higher absorbing capacity than those in the G₀ and G₁ phases. However, as RPMI 8866 cells have a two-to threefold larger volume during S than during G₀ or G₁, it is likely that the HL-A antigenic density is relatively constant throughout the cell cycle. To determine whether qualitative differences of HL-A antigens could be detected during the growth cycle, the curves relating percent absorption to number of cells needed for absorption in different stages of the growth cycle were compared. The absorption curves of cells in the G₀ or G₁ phases were superimposable. The absorption curve obtained with cells in the S phase was also superimposable on those obtained for G₀ and G₁ cells when the values for this curve were multiplied by a constant factor (2.9 for HL-A2, 2.1 for HL-A3, and 2.6 for HL-A7). As discussed by Ohanian et al. (28), these findings constitute presumptive evidence that the antigens being compared are very similar or identical.

In the second test, an isotopic antiglobulin assay was used to quantitate the antigenic density of cells sampled in various phases of the cell cycle. In this test cells were incubated with HL-A alloantisera, washed, and then incubated with radiolabeled rabbit antihuman IgG antibodies. Specific binding of the second radioactively labeled antibody is a function of the number of bound HL-A antibodies and thus, indirectly, of the number of accessible antigenic sites. As was observed in the quantitative absorption assay, G₀ and G₁ cells exhibited similar binding capacities while cells sampled during the S phase absorbed two to three times more antibody (Fig. 3). For reasons already discussed, this difference probably reflects changes in cell volume during the growth cycle.

To further assess the number and distribution of HL-A antigens during the cell cycle, HL-A antigens were extracted in 3 M KCl from RPMI 8866 cells.
TABLE II

Solubilization of HL-A Antigens from Cultured Lymphoid Cells RPMI 8866 in Different Stages of Cell Growth

| Phase of cell cycle | ID₅₀* units/10⁶ cells (X10⁻³) |
|---------------------|-------------------------------|
|                     | HL-A2 | HL-A3 | HL-A7 |
| G₀                  | 207   | 207   | 267   |
| G₁                  | 250   | 250   | 295   |
| S                   | 245   | 245   | 295   |

* Amount of antigen (expressed as µg/µl) required for a 50% reduction of the cytotoxicity activity of an alloantiserum. This ID₅₀ value arbitrarily represents one unit of activity.

Fig. 4. Binding of [¹²⁵I]radiolabeled complement components by RPMI 8866 cells sensitized with either HL-A alloantisera, rabbit antihuman lymphocyte serum or rabbit IgM and incubated with serum containing C4, C3, or C8. Cells were sampled from cultures in G₀ (hatched-), G₁ (open-), and S (closed-) phases. No difference was detected in the total amount of HL-A antigenic activity solubilized throughout the growth cycle, as shown in Table II, although twice as much protein was solubilized from cells in the G₀ phase than from cells in the G₁ or S phases. This finding suggests that larger amounts of contaminating proteins are solubilized from cells in the G₀ phase.

Binding of Complement by Sensitized RPMI 8866 Cells.—In order to determine if the ability of RPMI 8866 cells to bind complement varied during the growth cycle, cells sampled in various phases of the cycle were sensitized with HL-A alloantisera, rabbit IgM natural antibody or rabbit ALS and mixed with rabbit, human, or guinea pig complement containing radiolabeled human C3, C4, or C8. After incubation the cells were washed and the specifically bound radioactivity was determined. As shown in Fig. 4, cells sampled in the S phase generally exhibited a two- to threefold greater ability to bind C4, C3, and C8 after complement activation, than cells in the G₀ and G₁ phases of the growth
cycle. As discussed earlier, this probably reflects the larger volume of cells in the S phase. No significant difference was found between the four antisera in the amounts of radiolabeled C4 and C3 bound to the cells (Fig. 4), after incubation with human complement containing labeled components. The amount of radiolabeled C8 bound was, however, greater with antihuman lymphocyte serum, a finding which correlates with the considerably greater cytolytic potential of this antiserum. Although only values for labeled components incorporated in human complement are shown, qualitatively identical results were obtained in comparable experiments with guinea pig and rabbit complement previously absorbed with human lymphoid cells.

On comparing binding of the various components present in human, guinea pig, or rabbit complement, no significant difference in binding of C3 and C4 was observed when these components were incorporated in the three complement sources. The binding of C8, however, was considerably greater when incorporated in rabbit sera (Fig. 5). This finding correlates with the more efficient cytolytic potential of rabbit complement. Although only values for S phase and for one antiserum (Stockenberg, anti-HL-A2) are shown in Fig. 5, comparable results were obtained with G0 and G1 cells and with the other three antisera.

**Influence of the Cell Cycle on the Pathway of C Activation.**—We have recently shown that some HL-A alloantisera activate the classical pathway, others the alternate pathway, and a third group both pathways of human complement (29). In order to determine whether the pathway of complement activation changes during the cell cycle, the lymphocytotoxic test was performed with RPMI 8866 cells sampled at various phases of the cell cycle with an alloantisera (Bizot, anti-HL-A2) which mediates lysis through the alternate pathway. Human serum in which the alternate pathway had been selectively inactivated by destruction of C3PA was employed as the complement source. If a change in the pathway of activation of complement occurred, one would expect the cytotoxic activity to become evident since the complement source contained
HL-A alloantisera have been shown to activate the classical pathway of the rabbit complement system (14). In studies which are not shown, inhibition of this pathway by selective depletion of calcium in the reaction mixture prevented lysis of cells sampled in G0, G1, or S and sensitized with HL-A alloantibodies.

Expression of Other Cell Surface Markers during the Growth Cycle.—To determine whether the expression of cell surface markers other than HL-A antigens changed during the growth cycle of RPMI 8866 cells, the reactivity of receptors for C3b and for degradation products of C3, presumably C3d, was examined. Reactivity of RPMI 8866 cells with sensitized sheep erythrocytes coated with isolated human C1, C4, C2, and C3 did not change during the cell cycle as evidenced by percentage of rosettes formed. Reactivity with sensitized sheep erythrocytes coated with C5-deficient mouse serum also did not vary during the cell cycle. Thus, no variation in expression of receptors for C3b or C3d degradation products was detected during the cell cycle.
DISCUSSION

Our results indicate that the susceptibility of a human lymphoblastoid cell line, RPMI 8866, to complement-dependent lysis initiated by alloantibody directed against HL-A antigens or by heterologous antibody against membrane antigens varies with the phase of the cell cycle. The variation in susceptibility was approximately twofold with cells sampled in the G1 phase being less sensitive than cells examined in the G0 or S phases of the cell cycle. In an attempt to explain this variability in lytic susceptibility we utilized three independent tests to assess density of HL-A antigens on the surface of RPMI 8866 cells in synchronous growth. First, cells sampled from various phases of the growth cycle were used in quantitative absorption tests with HL-A alloantisera. Second, cells were reacted with HL-A alloantisera followed by radiolabeled antihuman IgG in an isotopic antiglobulin test, and third, HL-A antigens were extracted from RPMI 8866 cells at various stages of the growth cycle. These three independent tests fail to reveal any significant differences in antigenic density between the G0, G1, or S phases of the cycle when the (1.5-2 times) greater volume of S cells was taken into account. The ability of cells to interact with the complement system was also found not to vary during the course of the cell cycle. Thus, cells sampled in G0, G1, and S were equivalent in their ability after sensitization with various antisera to activate the complement system and to bind radiolabeled C3, C4, and C8 incorporated in the complement source. Further, studies revealed that the pathway of complement activation, whether alternate or classical depending on the source of complement employed, did not change during the cell cycle.

Although RPMI 8866 cells exhibit variable susceptibility to complement-mediated lysis during the cell cycle as was observed with murine tumor cells YCAB (6, 7) and L 1210 (9), these cells differ from the murine tumor cell lines both in the phase of the growth cycle during which they are least susceptible to lysis and in the lack of change in antigen density during the growth cycle. Thus, YCAB and L 1210 cells were found to be most sensitive to complement-mediated cytolytic damage initiated by H-2 antibodies in the G1 phase of the cell cycle when the quantitative expression of antigen as determined by absorption tests and extractibility was most pronounced. Taken together with the results of other investigators, these studies suggest that there is no direct correlation between susceptibility to lysis and the expression of membrane-associated antigens. The body of evidence thus far accumulated also suggests that variability in lytic susceptibility is not directly related to the ability of target cells to activate complement, to bind activated complement components or to sustain complement-dependent ultrastructural lesions (7, 11). In all probability, therefore, the differential susceptibility to lysis is due to changes in the properties of the cell membrane during the cell cycle. This is not unlikely in that many parameters of membrane function and structure are known to vary during cell growth, including turnover of membrane components (30),
fragility (1) and electrical potential (2, 31) of the cell membrane, formation of membrane associated enzymes (32, 33), synthesis of immunoglobulins (3, 4), and susceptibility to infection by virus (34) and to oncogenic transformation by chemical (35). Alternatively, it may be that the cell membrane possesses the capacity to repair complement-induced damage at certain intervals during the cell cycle.

The clinical relevance of these in vitro findings becomes apparent in planning immunotherapy regimen to combat neoplastic disease, e.g., when there is tumor cell growth while circulating antibodies are detected in a patient’s serum. The G1 phase of the cell cycle varies with the cell line; tumor cells with a long G1 phase have a far better chance of escaping antibody and complement action if their susceptibility to lysis varies as with RPMI 8866 cells. If this is indeed the case in the in vivo situation, the efficacy of immunotherapy regimens could be greatly increased by planning therapy accordingly.

SUMMARY

Cultured human lymphoid cells RPMI 8866 at different stages of their growth cycle vary in their susceptibility to lysis by rabbit, human, and guinea pig complement activated by HL-A antibodies or heterologous antibodies directed to membrane antigens; cells in G1 phase are the least sensitive to lysis. To investigate the cause of differential susceptibility of cells RPMI 8866 to lysis, the expression of HL-A determinants and the ability of cells to react with complement were investigated. No change was detected in the density of HL-A antigens on RPMI 8866 cells in synchronous growth as determined by quantitative microabsorption assays, isotopic antiglobulin tests and yields of soluble HL-A antigens. Cells did not vary during the growth cycle in their ability to interact with complement components and in their capacity to activate the complement system through the classical or alternate pathway. These data suggest that variability in lytic susceptibility is due to changes in the structure of the cell membrane or in its ability to repair complement induced damage at certain intervals during the cell cycle. Therefore, this cell line constitutes a useful model to investigate the final steps of the cytolytic reaction.

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