INTERACTION OF HISTOCOMPATIBILITY (HL-A) ANTIBODIES AND COMPLEMENT WITH SYNCHRONIZED HUMAN LYMPHOID CELLS IN CONTINUOUS CULTURE*

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HL-A antigens are gene products of the major histocompatibility locus in man (1). These cell surface antigens are the human counterparts of the H-2 (2,3), AgB (4), CHL-A (5), and DL-A (6) antigen systems in mice, rats, chimpanzees, and dogs, respectively. In the past few years there have been considerable efforts to elucidate the genetic and chemical characteristics of the various HL-A antigens largely because of their crucial roles in transplantation immunity. Although the singular capacity of this system to regulate the survival of tissue grafts has recently been questioned (7), there is no doubt that the HL-A system is a controlling factor of the allograft rejection process.

Aside from the importance of histocompatibility antigens in transplantation, interest in the biology of this system stems from studies which suggest that there is a relationship between particular histocompatibility antigens and the immune response to certain antigens as well as the capacity to resist disease (8). Thus, inbred strains of mice differing only at the H-2 locus vary markedly in the incidence of spontaneous tumors and in their susceptibility to tumor induction by various agents (9, 10). The counterpart of this observation in human pathology may be the statistical correlation which prevails between certain histocompatibility antigens and several forms of neoplastic and autoimmune diseases (10).

Since HL-A antigens are located on the surfaces of cells, they provide excellent markers with which to study various membrane properties and functions such as cytoarchitecture, permeability, metabolism, and cell recognition. Human lymphoid cells are particularly useful for these studies because they can be maintained in continuous culture and their growth can be synchronized.

In this project we have investigated HL-A antigenic expression on the surfaces of synchronized human lymphoid cells during different phases of their
growth cycle as judged by sensitivity to lysis in the complement-dependent cytotoxic test, ability to activate the complement effector system, and quantitative absorption studies of HL-A alloantisera.

**Materials and Methods**

**Human Lymphoid Cell Line.**—The WI-L2 cell line which was originally derived from splenic lymphocytes of a patient with hereditary spherocytic anemia (11) was used in these experiments. The HL-A phenotype is HL-A1, 2, 5, Tr (W 17) as determined by direct cytotoxic test and by absorption typing (12). Suspension cultures were grown and propagated in Eagle’s minimal essential medium (MEM,\(^1\) Autopow, Flow Laboratories, Inc., Rockville, Md.), supplemented with glutamine, nonessential amino acids, pyruvate, 10% fetal calf serum, penicillin, streptomycin, and Fungizone as described by Lerner and Hodge (13). Cell concentration and viability were determined by trypan blue exclusion using a hemacytometer.

**Synchronization of Cells.**—WI-L2 cells were synchronized by following the procedure described by Lerner and Hodge (13). Briefly, cultures were started at concentrations of 4 X 10\(^5\) cells/ml. The extent of DNA synthesis and cell viability were the criteria by which cell life cycles were followed. When the rate of DNA synthesis was approximately 2% of the maximum (Go or stationary phase of cell growth), the resting cells were harvested and resuspended in fresh, prewarmed (37°C) medium and the course of DNA synthesis ascertained to determine G1 and S phase, respectively.

**HL-A Alloantisera.**—The alloantisera Lewandowski 2-60-0-05-21-01 (anti-HL-A1), Gillespie 2-50-9-03-21-01 (anti-HL-A1), Pinquette 2-50-6-09-01 (anti-HL-A2), Stockenberg 1-04-0-07-06-01 (anti-HL-A2), McMullen 2-65-0-03-30-01 (anti-HL-A5), Peterson 1-08-9-01-08-01 (anti-HL-A12), and Viktor 1-01-9-07-17-04 (anti-HL-A5) which were utilized in this study were obtained from the serum bank at the National Institute of Allergy and Infectious Diseases and from the laboratories of Doctors R. Ceppellini, R. Payne, and P. I. Terasaki. All of the sera had been frozen and thawed several times.

**Rabbit Anti-Human Lymphocyte Sera.**—Sera were harvested from New Zealand rabbits immunized with washed cultured human lymphoid cells according to the method of Levey and Medawar (14). The sera had a titer of 1:16,000 against human lymphoid cells in the microlymphocytotoxic test. Before testing with cultured human lymphoid cells, the serum was inactivated by heating for 30 min at 56°C to destroy the natural rabbit antibodies directed against antigens present on human lymphoid cells (15).

**Complement.**—Rabbit complement was a pool of nine rabbits’ sera selected after their individual bleedings proved nontoxic in the cytotoxic assay with peripheral lymphocytes from four subjects of different ABO groups. A pool of fresh sera derived from five healthy donors with no previous history of pregnancy or blood transfusion served as the source of human complement. The complement pools were stored at −70°C in small samples and used only once after thawing. Purified human complement components C1, C2, C3, C4, C5, C6, C7, C8, and C9 were isolated from human serum by published methods (16-18);\(^2\)\(^3\). They were utilized for molecular hemolytic titrations (17-19). Preparations of C6, C7, and C8 were trace labeled with \(^{125}\)I by the chloramine-T method (20) without loss of activity and used for quantitative cell-binding studies. The specific radioactivity was, respectively, 8.1 X 10\(^5\), 0.4 X 10\(^6\), and 1 X 10\(^6\) cpm/μg of C6, C7, and C8, values which correspond to 0.92, 1.02, and 1.1 μCi/μg.

**Cytotoxic Assay.**—The slightly modified eosin method according to Mittal et al. (21) was

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1 Abbreviations used in this paper: AD\(_{50}\), 50\(^\circ\) absorption dosage; MEM, minimal essential medium.


used both with cultured human lymphoid cells (12) and with human peripheral lymphocytes. The reaction mixtures consisted of 1 μl of target cell suspension (2000 cells), 2 μl of antisera, and 3 μl of complement. When cultured human lymphoid cells were the target cells, human complement or a mixture of 1 vol of rabbit complement and 3 vol of human complement (hereafter abbreviated as rabbit complement) were used as indicated below. When peripheral human lymphocytes were the target cells, the source of complement was undiluted fresh rabbit serum.

Quantitation of Activation of the Complement System.—At various times in the growth of WI-L2 cells, samples of 5 × 10⁶ cells were withdrawn, washed three times in phosphate-buffered solution (0.005 M sodium phosphate buffer, 0.15 M NaCl, pH 7.1), and mixed with 20 μl of antisera which had been previously heated at 56°C for 30 min. After 10 min of incubation at 37°C, 40 μl of human complement was added and incubation continued for 60 min. Then the cells were sedimented by centrifugation at 13,000 g for 5 min in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.) and the supernatant was analyzed for residual C3, C4, and C8 complement components by specific stoichiometric titrations with the human complement intermediate complexes EACI, 4, 2, EACI, and EACI, 4, 2, 3, 5, 6, 7, respectively (17, 19). The results were expressed as the percent of the number of effective molecules remaining in serum samples similarly incubated with cells and heated normal serum in place of antibody.

Quantitation of Cell-Bound Radiolabeled C6, C7, and C8.—Binding of complement components was studied exactly as was activation of complement components except that radiolabeled C6, C7, and C8 were incorporated into the human serum complement source. After completion of incubation, cells were washed four times and analyzed for radioactivity in a well-type scintillation counter. Nonspecific binding of complement components was determined from reaction mixtures with heat-inactivated normal human serum in place of specific antisera. Specific binding was obtained by subtracting the values for nonspecific uptake from the total bound radioactivity. Nonspecific cellular binding of C6, C7, and C8 averaged 0.3-1.07% of the total radioactivity offered to the cells. Molecular weights of 125,000, 125,000, and 150,000 were used to calculate the number of bound C6, C7, and C8 molecules. In calculating these values, only the bound radiolabeled molecules were considered, since the concentration of unlabeled C6, C7, and C8 in the complement source was not known.

Microabsorption Technique.—Quantitative microabsorption test was performed as previously described (22). Briefly, 5 μl of HL-A alloantisera at a dilution corresponding to 1 cytotoxic unit was incubated in tubes with 5 μl of varying concentrations of the cell suspension. 1 cytotoxic unit is equal to twice the least amount of antiserum required to lyse 95% of the selected target cells. The material was mixed at 10-min intervals for a total incubation period of 60 min at room temperature. After incubation, the tubes were centrifuged for 5 min at 13,000 g in a Beckman microfuge. The supernatant fluid was transferred to fresh tubes and frozen at −20°C until it was tested for residual lymphocytotoxic activity against selected target cells. The 50% absorption dosage (AD₅₀) (the number of cells required to reduce by 50% the cytotoxicity of a selected alloantiserum) was used as the parameter for comparison of results from the various quantitative absorptions.

Determination of Cell Volume.—To compare the respective volumes of cells in phases of the growth cycle, a known number of WI-L2 cells were placed in cylindrical glass tubes (0.2 × 5 cm) and centrifuged at 450 g for 10 min at 4°C (23). The ratio of the respective cell volumes was considered equal to the height ratio of the packed cells.

RESULTS

Relationship between the Stage of the Cell Growth Cycle and the Susceptibility of WI-L2 Cells to Lysis in the Lymphocytotoxic Tests.—To investigate the relationship among phases of the growth cycles of WI-L2 cells and sensitivity to lysis mediated by anti-HL-A alloantibodies and complement, cells from log phase, G0, G1, and S phase cultures were tested with anti-HL-A1, 2, and 5 alloantisera and with rabbit serum as the complement source. Cells in different phases of their growth elicited concordant titers with the anti-HL-A alloantisera tested (Fig. 1). Similar results were obtained with cells from cultures in late resting phase (cell concentration of the culture $2.8 \times 10^6$ cells/ml).

Rabbit serum is known to contribute natural antibodies as well as complement components to the lymphocytotoxic reaction (15). In order to eliminate the possibility that cell cycle variability in the cell surface expression of the antigenic determinants against which the rabbit natural antibodies are directed might obscure a change in the expression of HL-A antigens, the cytotoxic test was performed with human complement which is not known to contain natural antibodies. Although the titer of HL-A alloantisera was lower when human complement replaced rabbit complement, the sensitivity of the cell membrane to the lytic action of anti-HL-A and human complement did not vary significantly during the cell cycle (Fig. 2).

Activation of the Complement System during the Cell Cycle by HL-A Antigens and HL-A Alloantisera.—Complement activation was appraised by measuring the amount of the third, fourth, and eighth complement components remaining after incubation of human serum (40 μl), as the complement source, with HL-A alloantisera (20 μl) and WI-L2 cultured lymphoid cells ($5 \times 10^6$) at various...
times in the growth cycle. The ratio between cells, antibody, and complement employed in these studies was chosen on the basis of preliminary experiments which indicated that fewer target cells produced little complement consumption. With larger numbers of target cells marked consumption of the early reacting complement components occurred in the absence of HL-A alloantisera. This “nonspecific” activation of the complement system could not be reduced by extensive absorption of the human complement with large numbers of cultured human lymphoid cells at 0°C; furthermore, it occurred with cells at all phases of the cell cycle.

The complement system was activated to a comparable extent at each phase of the growth cycle on addition of anti-lymphocyte serum to cells. Similar results were obtained throughout the cell cycle with each of the HL-A antisera tested. Fig. 3 shows representative results for the G1 and S phases of the growth cycle.

There were, however, differences between the antisera in ability to activate the complement system. The consumption of C4 was significantly greater in the rabbit anti-human lymphocyte serum and anti-HL-A1 alloantiserum than with anti-HL-A2 and anti-HL-A5. The pattern of consumption of complement components observed with anti-lymphocyte serum and anti-HL-A1 is characteristic of that observed on activation of the classical complement pathway. The pattern of component consumption observed with anti-HL-A2 and anti-HL-A5 is somewhat different; it may well be that these antisera activate the alternate as well as the classical pathways.

**Binding of Complement Components by Sensitized Cultured Lymphoid Cells.**—In order to determine if the ability of WI-L2 cells to bind late reacting complement components varied with the phases of the growth cycle, human serum
containing radiolabeled C6, C7, or C8 was added to cells which had been sensitized with anti-lymphocyte serum or HL-A alloantisera after varying periods of growth. The ability of each of the antisera to induce C6, C7, and C8 binding did not vary significantly during the cycle (Fig. 4). The various antisera differed in the extent of binding with anti-lymphocyte serum inducing approximately 10-fold greater binding of C6, C7, or C8 than anti-HL-A2 or anti-HL-A5. Anti-HL-A2 and anti-HL-A5 triggered comparable binding of late reacting complement components, approximately threefold greater than the baseline. Throughout the cell cycle, the binding of labeled complement components by cells sensitized with HL-A1 alloantisera did not exceed background values.

Quantitative Absorption Experiments with Anti-HL-A Alloantisera.—In order to gain additional quantitative data about the cell surface expression of HL-A antigens during the cell cycle, the absorbing capacities of cells at different phases of the cell cycle for anti-HL-A1, 2, and 5 alloantisera were investigated. Cells in S phase displayed the greatest absorbing capacity, whereas cells in the G1 phase had a slightly higher absorbing capacity than G0 cells (Table I). The specificity of the absorption was illustrated by the finding that in all phases of the cell cycle which were analyzed more than 50,000 cells/μl were necessary to absorb anti-HL-A12 alloantisera which were directed against determinants not present on WI-L2 cells. AD₉₀ value for HL-A2 specificity was significantly lower.
than those for HL-A1 and HL-A5, suggesting that a different number of antigenic determinants may be available to react with various alloantibodies.

The volume of the cells at different stages of the cell cycle was determined to ascertain whether variations with accompanying changes in surface area could account for the differences in $AD_{90}$ values observed between S and $G_1$ phases. The volume of cells in $G_1$ phase was 1.4-fold smaller than that of cells in S phase,

![Graph showing binding of $^{125}$I-radiolabeled late complement components by cultured human lymphoid cells WI-L2](image)

**Fig. 4.** Binding of $^{125}$I-radiolabeled late complement components by cultured human lymphoid cells WI-L2 sensitized with either rabbit anti-human lymphocyte serum (○—○), anti-HL-A2 alloantiserum (△—△), anti-HL-A5 alloantiserum (▽—▽), or anti-HL-A1 alloantiserum (□—□) and incubated with serum containing $^{125}$I[C6], $^{125}$I[C7], or $^{125}$I[C8]. The stippled area represents binding observed in the absence of antiserum. Samples taken at 38, 65, 2, and 18 h represent log, resting, $G_1$, and S phases, respectively.

**TABLE I**

Absorbing Capacity of Cultured Human Lymphoid Cells WI-L2
in Different Phases of the Growth Cycle

<table>
<thead>
<tr>
<th>Phase of cell cycle</th>
<th>Time after release from Go</th>
<th>$AD_{90}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HL-A1</td>
</tr>
<tr>
<td>$G_0$</td>
<td>—</td>
<td>1750</td>
</tr>
<tr>
<td>$G_1$</td>
<td>2</td>
<td>1600</td>
</tr>
<tr>
<td>S</td>
<td>12</td>
<td>700</td>
</tr>
</tbody>
</table>

which is consistent with the mentioned differences in $AD_{90}$ values, especially since the cells in S phase have considerably more microvilli and thus more irregular and larger areas than cells in $G_1$ phase.

**DISCUSSION**

The results of this study indicate that there is no significant variation throughout the cell growth cycle in (a) the sensitivity of cultured WI-L2 lymph-

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oid cells to HL-A antibody-mediated lysis in the cytotoxic test, (b) the extent of activation of the complement system, (c) the degree to which labeled complement components bind to cells, and (d) the ability of these cells to absorb HL-A alloantibodies. In addition, there was no significant difference in the titers of cytotoxic HL-A alloantisera tested with cultured cells at various phases of their cell growth cycle when a mixture of rabbit and human sera was employed as the cytolytic reagent. Since rabbit serum is known to contribute natural antibodies directed against determinants present on lymphoid cells, as well as complement components (15), this finding indicates that there is no detectable change throughout the growth cycle in either those antigens against which the natural rabbit antibodies are directed or in the HL-A determinants as far as their availability to combine with antibodies and to activate complement is concerned.

It has recently become clear that there are two pathways of activation of the terminal portion of the complement sequence (24–26). One mechanism of activation, termed the classical pathway, involves C1, C4, and C2 whereas an unknown number of serum factors are involved in the alternate pathway; both pathways converge at the step where C3 becomes involved. Activation of the classical pathway is reflected by the marked consumption of the early components C1, C4, and C2, while little depletion of these components is seen upon activation of the alternate pathway (26).  

The reaction patterns of rabbit anti-human lymphocyte sera and anti-HL-A alloantisera used in the present study were typically those of complement component consumption after activation of the classical pathway. The anti-HL-A2 and anti-HL-A5 antisera which were examined appeared to activate both pathways. Although comparison of the extents of activation of complement by the various antisera is difficult because different mechanisms of activation were apparently involved, all antisera appeared to elicit very similar levels of C3 and C8 consumption. The complement activation studies thus did not correlate well with the results of cytotoxic assays and of binding studies involving C6, C7, and C8 which indicated that HL-A2 and HL-A5 alloantisera were more potent than anti-HL-A1 alloantisera. Killing of target cells in the cytotoxic test also failed to correlate with the extent to which the complement sequence was activated in previous studies of murine Moloney virus-induced tumor cells sensitized with anti-Moloney virus antibody (27). Although sensitivity to complement-dependent lymphocytotoxicity during the cell cycle varied greatly in these studies, with the greatest killing of target cells occurring in the G1 phase, the extent of activation of the complement system by the sensitized cells was essentially constant throughout the cell growth cycle.

These data emphasize the complexity of the cytotoxic reaction, which is the final result of a series of interactions among antigenic determinants, antibody,

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complement components, and the cell membrane. These variables influence the ultimate result of the reaction at different levels. The contribution of antigenic determinants is controlled by their density and distribution; if the fluid mosaic model of membrane structure recently proposed by Singer and Nicolson (28) proves to be correct, the diffusion of antigens within the cell membrane may cause additional variability. On the other hand, the reactivity of antibodies is determined by their affinity for antigen, by their ability to activate the complement sequence, and probably by their concentration in the reaction mixture. It has already been emphasized that the requirement of complement varies among HL-A specificities since false negative reactions (CYNAP, i.e. cytotoxic negative, absorption positive) occur most frequently with the HL-A specificity 4b (29). When rabbit serum is the source of complement, the influence of natural rabbit antibodies directed against determinants present on human lymphoid cells also has to be considered; these antibodies not only possess different specificity and titer, but the determinants against which they are directed are expressed in variable amounts on human lymphocytes from different subjects, and spatial relationships between these determinants and HL-A antigens may also influence cytotoxicity. The pathway of complement activated by the immune reaction may well determine the outcome of the cytolytic reaction. Studies with several types of cells indicate that the alternate pathway has poor cytolytic potential. Thus, an antigen-antibody complex on the cell surface which activates only the alternate pathway might well be noncytotoxic. In order to achieve cytolysis, complement activation must be sustained and proceed through the reaction of the ninth component; furthermore, the terminal steps, from C5 through C9, must occur on the surface of the target cell. Failure of any components to react for any reason or absence of a binding site for a late component preclude lysis.

In the course of this study, it was observed that early reacting complement components were reduced in human complement after incubation with cultured WI-L2 cells in the absence of HL-A antibodies. This type of activation is analogous to that induced by immune complexes. While natural antibodies directed against constituents of the cell membrane of cultured cells (30, 31) might be responsible for complement activation, we were unable to diminish the consumption of early reacting components significantly by extensive absorption of the human complement source with cultured lymphoid cells. Alternatively, it is possible that the membranes of lymphoid cells perpetuated in culture have characteristics which trigger activation of complement. In fact, peripheral lymphocytes do not activate the complement sequence when incubated with human complement in the absence of HL-A antibodies.
The cell surface expression of histocompatibility antigens during the cell cycle has been investigated in murine tumor cells (32) and in human cells derived from Burkitt lymphoma (33); results different from those reached in this experiment have been obtained. In these studies with malignant cells, the cell surface expression of histocompatibility antigens varies during the cell cycle, being maximal in G1 phase on both human and murine tumor cells. It seems worthwhile to emphasize that results comparable to these were obtained also in this laboratory when some techniques employed in the present study were utilized to investigate the cell surface expression of H-2 antigens at different stages of growth of murine tumor cells. This observation underlines the real discrepancy of the results obtained with malignant murine and human cells vs. those found in the present study with cultured human cells (WI-L2) derived from a donor free of malignancy. The different results obtained with WI-L2 cells as compared with those found with other cultured lymphoid cells studied may indeed reflect the different sources of the cells used. Thus, tumor cells were utilized exclusively in those studies where histocompatibility antigens were preferentially expressed in G1 phase, while in the present investigation indicating no difference of the cell surface expression of these antigens throughout the growth cycle, the cultured cells were derived from a donor free of malignancy.

Several lines of evidence suggest a relationship between malignancy and the expression of histocompatibility antigens, and qualitative and quantitative changes of histocompatibility antigens during the course of malignancy have been documented both in man and mice. H-2 antigens decreased in H-2 homozygous murine tumor cells (34, 35) and an irreversible loss of antigens controlled from one of the two H-2 alleles was observed in H-2 heterozygous tumors (36). Furthermore, in TL leukemia cells an inverse relationship existed between the quantitative expression of tumor-specific cell surface antigens and normal H-2 antigens; the phenotypic expression of TL antigens reduced the demonstrable amount of certain H-2 antigens on the cell surface (3), while the decrease of TL antigens caused by a modulation phenomenon evoked a compensatory increase in H-2 antigen expression (38). Parallel observations have been made in man; decrease or loss of HL-A antigens have been reported during some malignancies (39-41), whereas in other cases there has been increased reactivity of leukemic cells with some anti-HL-A alloantisera, probably reflecting heightened expression of HL-A antigens (10). Finally, changes in the HL-A profiles of some patients with acute leukemia paralleled the activity of the disease as new HL-A antigens appeared during the acute phase of the disease, disappeared during remission, and reappeared during relapse (42). It is pertinent to recall the different effects on the expression of histocompatibility antigens observed on tumor cells and cells derived from a donor free of malignancy after treatment with inhibitors of macromolecular synthesis. Actinomycin D, an inhibitor of macro-

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molecular synthesis, enhanced the cell surface expression of histocompatibility antigens on murine tumor cells (43), but had no detectable effect on cells from a nonmalignant donor. Still too few data are available to answer the question as to whether the malignant process modifies the regulatory mechanism of the synthesis of histocompatibility antigens or only affects their expression on cell surfaces.

The lack of any detectable quantitative variation of histocompatibility antigens in cells derived from a donor free of malignancy contrasts with the variable expression of other antigens such as blood groups H (44) and AB (45) on cultured cells. The constancy of histocompatibility antigen expression throughout the growth cycle of cultured cells suggests that these cell surface markers are an essential part of membrane cytoarchitecture or play a critical role for the normal function of the cell membrane. This view is reinforced by the persistence of histocompatibility antigens on murine (46) and human lymphoid cells (47) even after long-term culture, although other antigens are lost (48, 49). Similarly, human diploid fibroblasts, which have a finite life-span in vitro, change metabolically, functionally, and morphologically during senescence, but their surface expression of HL-A antigens remains practically unchanged throughout their in vitro lifetime. 1112

SUMMARY

The interaction of histocompatibility (HL-A) antibodies and complement with synchronized human lymphoid cells in continuous culture has been investigated. The sensitivity of cultured lymphoid cells to HL-A antibody-mediated lysis in the cytotoxic test, the extent of activation of the complement system, the degree to which labeled complement components are bound, and the ability of these cells to absorb HL-A alloantibodies do not vary significantly during the cell growth cycle. The constancy of histocompatibility antigen expression throughout the growth cycle of cultured cells suggests that these cell surface markers are an essential part of membrane cytoarchitecture and could well play a critical role in determining the normal function of the cell membrane.

REFERENCES


