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Immunology

Enhanced representation of HL-A antigens on human lymphocytes after mitogenesis induced by phytohemagglutinin or Epstein–Barr virus

(alloantibodies/cell surface/histocompatibility/malignancy/transformation)

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ABSTRACT **The amount of HL-A antigens present on the peripheral blood lymphocytes of a single human donor was increased about 11-fold after stimulation with phytohemagglutinin and 36-fold after transformation with Epstein–Barr virus. This increase applied to all four HL-A specificities of these cells. The response to phytohemagglutinin was dependent on dose and was first observed at 12 hr of incubation. Measurements of the amount of surface membranes by geometry, by radioiodinatable surface proteins, and by 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) assay all indicated that the enhanced representation of HL-A antigens after stimulation by phytohemagglutinin or transformation by Epstein–Barr virus must be due to an increase in the density of these antigens on the cell surface.**

HL-A antigens are serologically defined cell surface glycoproteins which are major determinants of histocompatibility. Their antigenic structure is prescribed by genes in the "*LA*" and "*Four*" loci at each end of a complex of genes (called the major histocompatibility complex) governing several processes of immune function. The antigens of these two loci are composed of a 44,000 dalton glycopeptide and noncovalently associated β_2-microglobulin, a 12,000 dalton peptide. Because of several structural resemblances of HL-A antigens to immunoglobulins, including the sequence homology of β_2-microglobulin to the C_H3 domain (in constant region of heavy chain) of IgG, the descent of HL-A and immunoglobulin genes from a primordial immunogene has been proposed (1). In this hypothesis, after gene duplication the membrane-bound HL-A antigens would have evolved in an autorecognition or immune surveillance function, while immunoglobulins evolved as a defense against invading microorganisms. Reported changes in the expression of HL-A antigens in malignancy (2–4), in continuous cell line cultures (5–9), and in immune deficiency diseases (10), are consistent with a role of HL-A antigens in recognition phenomena. However, possible qualitative or quantitative alterations of HL-A antigens under various circumstances required further evaluation under defined conditions. We have, therefore, examined the quantitative and qualitative changes in HL-A antigens during transformation of normal cells with phytohemagglutinin (PHA) or Epstein–Barr virus (EBV).

MATERIALS AND METHODS

Cells. All of the cell types used in this study were derived from one individual (RH). Peripheral blood lymphocytes (PBL's) were prepared by the Ficoll-Hypaque, density gradient centrifugation method (11). Phytohemagglutinin-stimulated lymphocytes (PHAL's) were generated by culture of PBL's with phytohemagglutinin (Difco, PHA-P) for variable periods of time and PHA concentration. These cells were cultured at 2×10^6 cells per ml in RPMI 1640 medium with 10% fetal calf serum, penicillin, and streptomycin (ABS), and a 5% CO_2 environment.

The RH-1 lymphoblast cell line was established by transformation of normal PBL's. Epstein–Barr virus was obtained in the Millipore filter-sterilized supernatant solution of a culture of the B95-8 marmoset lymphoblast cell line (12). For 3 days prior to harvesting the virus, the culture had been maintained at 32° to enhance virus production. Five milliliters of the supernatant solution was added to 5 ml of PBL's (at 2×10^6 cells per ml in RPMI 1640 culture medium with 10% fetal calf serum). After a 1-hr adsorption of the virus, the cells were pelleted by centrifugation and resuspended in 5 ml of fresh medium. One half of this medium (excluding settled cells) was changed with fresh medium every 2 days. After 28 days the cell line had been transformed as evidenced by its increased growth rate, variable morphology, and clumping. It has been maintained in culture for 1 year. The cells were always fed 2 days before an assay. This line is a B cell line as judged by lysis with anti-immunoglobulin antisera and the presence of a B cell specific differentiation antigen.*

Quantitative Absorption Assay. RH cells were histotyped by Dr. Thomas Fuller of Massachusetts General Hospital to be HL-A3, W-28; HL-A7, HL-A27. Alloantisera used to assay these specificities included: BC (HL-A3) and DAL (HL-A27), the gift of Dr. Bernard Amos; MWS (W-28), the gift of Dr. Thomas Fuller, and SAN (Sanderson, HL-A7), the gift of Dr. Arnold Sanderson. Two anti-HL-A1 antisera were used in a control for nonspecific absorption: RAY (Raymond, NIH no. 1-01-9-07-17-05) and DES (Desande, NIH no. 1-01-8-08-13-54), obtained from the Division of Transplantation Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

For quantitative absorption assays, freshly prepared or cultured cells were washed three times in dextrose–gelatin–Veronal buffer, pH 7.1 (Gibco, DGV) counted in a hemacytometer with a phase contrast microscope, and tested for viability by exclusion of trypan blue dye. After a 1:2 serial dilution in DGV in a V-bottom microtiter plate (Cooke no. 220-25A), aliquots (5 μl) of cells were transferred to a second plate for incubation at 4° with a constant 5 μl aliquot of specific antisera (diluted to twice that amount required for a 5 μl aliquot to yield 80% lysis in a standard cytotoxic assay). After 1 hour, the cells were pelleted by centrifugation and 5 μl of the supernatant solution was transferred to a third plate

Abbreviations: EBV, Epstein–Barr virus; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; PHAL, phytohemagglutinin-stimulated peripheral blood lymphocytes.

* R. E. Humphreys, manuscript in preparation.

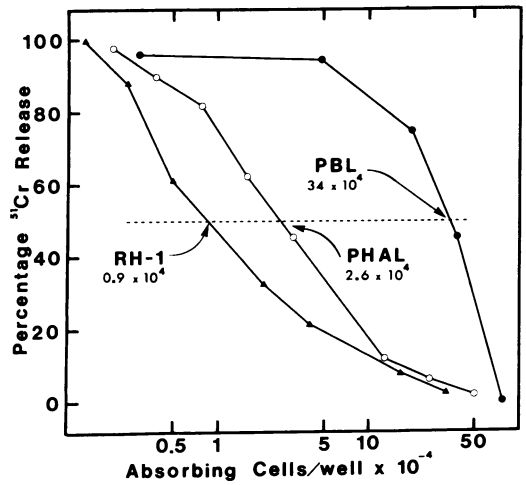

FIG. 1. Comparison of absorptive capacity of peripheral blood (PBL), phytohemagglutinin-stimulated (PHAL), and EBV-transformed (RH-1) lymphocytes for BC alloantisera (HL-A3). PHAL's were cultured for 48 hr with 10 μg of PHA per ml. Absorption endpoints are indicated (see *text* for explanation).

to assay its residual cytotoxic activity. Five μl of ^{51}Cr-labeled PBL's (RH) at 2 × 10^6 cells per ml were added, followed by a 30-min incubation at room temperature, and the addition of 5 μl of rabbit complement. The cells were incubated at 37° for 45 min and 150 μl of cold phosphate-buffered saline (PBS) was added. After centrifugation, 100 μl of the supernatant solution was counted for released ^{51}Cr. Controls were included to measure (*1*) lysis with rabbit complement alone, (*2*) the maximum antibody-specific lysis, and (*3*) the maximum ^{51}Cr release by repeated freezing and thawing of the cells. The complement control was 15% of the freeze–thaw value while maximum antibody-specific lysis was 90% of that total. The antibody-specific lysis has been adjusted to a 100% scale. The "absorption endpoint" is defined to be that number of cells per well which decreased the antibody-specific lysis by 50% (13, 14). All endpoints are calculated from an average of at least three separate assays for each experiment. The experimental error in this assay is about 25% of the observed value. Nonspecific absorption was measured by incubating RH cells for 1 hr at 4° with RAY or DES alloantisera, followed by an assay of the residual cytotoxic activity of the sera against an HL-A1 target (CT).

Estimations of Surface Area. The cell surface areas of PBL's, 48 hr-PHAL's, and RH-1 cells were determined by three methods: (*1*) The average geometric surface area of 200 cells was calculated from two diameter measurements, made at right angles to each other, against a calibrated ocular vernier in a phase contrast microscope.

(*2*) Cells were radioiodinated by the lactoperoxidase method (15). Labeled cells (10^4 cells) were combined with 10 μg of IM-1 cultured lymphoblast cell membranes as carrier, dissolved in buffered 0.1% sodium dodecyl sulfate, and electrophoresed in a sodium dodecyl sulfate–polyacrylamide gel (16). Radioiodine counts integrated from 1 mm slices of the gel were taken to be representative of the total amount of exposed protein of each cell type. (*3*) The activity of membrane-associated 5′-nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) in PBL's, PHAL's, and RH-1 cells was determined (17). Cells were incubated with 5′-[^{32}P]AMP in the presence of β-glycerophosphate and tartrate. The cleavage product, inorganic [^{32}P]phosphate, was counted in Aquasol (New England Nuclear) on a Beckman model LS-230 liquid scintillation counter. The 5′-nucleotidase activity reported for each cell type is the average of at least three determinations and is expressed as μmol/hr per 10^8 lymphocytes.

Table 1. Absorption of anti-HL-A alloantisera with PBL, PHAL, and RH-1 cells

Specificity	Serum	Absorption endpoints (cells/well × 10^{-4}) PBL	PHAL	RH-1	Ratio of absorption endpoints PBL/PHAL	PBL/RH-1
HL-A3	BC	34	2.6	0.9	13	38
W-28	MWS	14	1.5	0.8	9	18
HL-A7	SAN	15	1.2	0.4	12	38
HL-A27	DAL	21	1.8	0.4	11	52
				Averages	*11**	*36**

* These values correspond to an average 6-fold increase in HL-A expression per unit surface area (Table 2) for PHAL's relative to PBL's and an average 13-fold increase in HL-A antigen density on RH-1 cells relative to PBL's.

RESULTS

Absorption of alloantisera by PBL's, PHAL's, and RH-1 cells

The relative absorptive capacities of PBL's, PHAL's (after 48 hr culture with 10 μg/ml of PHA), and RH-1 cells for one alloantiserum, BC (anti-HL-A3) are illustrated in Fig. 1. The end points for 50% absorption were 34 × 10^4, 2.6 × 10^4, and 0.9 × 10^4 cells per well, respectively. On a per cell basis, the antibody absorption was 13 times as much by PHAL's as by PBL's, and 38 times as much by RH-1 cultured lymphoblasts as by PBL's. Similar data were obtained for the three other HL-A antigens of these cells and are summarized in Table 1.

To test whether transformed cells absorbed antisera in a nonspecific manner, the absorption of two anti-HL-A1 sera by PBL's, PHAL's, and RH-1 cells (not possessing the HL-A1 antigen) was examined. RAY was not absorbed at all by any of these three cell populations. The absorption endpoints for DES were: PBL = 12 × 10^4 cells, PHAL = 7 × 10^4 cells, RH-1 = 1.8 × 10^4 cells. On a per cell basis, nonspecific antibody absorption was 1.7 times as much by PHAL's as by PBL's, and 6.7 times as much by RH-1 cells as by PHA's. The absorption of DES by PBL's and the relatively small increases seen in PHAL's and RH-1's (especially as compared to the increase in surface area, see below) could be attributed either to cross-reaction of the anti-HL-A1 in DES with one of the specificities on RH cells or to the presence of a second non-HL-A cytotoxic antibody in DES directed against a determinant shared by RH cells and CT cells (the HL-A1 target). In any case the large absorption increase with RH-specific alloantisera cannot be attributed to nonspecific effects.

HL-A expression related to the dose of PHA

To ascertain that the increase in HL-A antigens on cells incubated with PHA for 48 hr is related to the presence and concentration of PHA, the response to variable doses of the mitogen was studied. Zero, 1, 10, or 67 μg/ml of PHA was added to separate cultures of PBL's at 2 × 10^6 cells per ml for 48 hr. The alloantiserum absorbing capacity of these cell populations for BC (anti-HL-A3) is shown in Fig. 2 as a function of the concentration of PHA. A minimal increase in

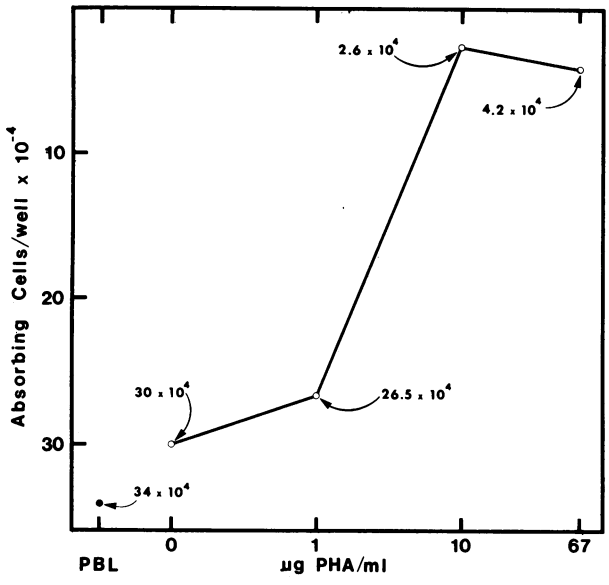

FIG. 2. Effect of variable PHA dosage on the proliferation of HL-A antigens in 48 hr PHAL's. PBL: normal peripheral blood lymphocytes. All other points represent the absorption endpoints of cell populations cultured for 48 hr with the indicated dosages of PHA.

the absorptive capacity was seen with cells incubated for 48 hr without PHA or with 1 µg/ml of PHA. PHA at 10 µg/ml (about the same concentration needed to stimulate thymidine incorporation maximally) stimulated the greatest expression of HL-A3. No marked inhibition of the response at higher mitogen concentration was observed. Similar dose-dependent results were obtained for the three other HL-A antigens of these cells.

Time course of PHA-induced increase in HL-A expression

The alloantisera absorbing capacity of PHA-stimulated lymphocytes was investigated as a function of time of incubation with 10 µg/ml of PHA. Data for BC (anti-HL-A3) are presented in Fig. 3. No increased expression of HL-A antigens was observed after 2 or 6 hr of incubation. At 12–24 hr of PHA stimulation, a 3-fold increase in HL-A expression had occurred, followed at 48 hr with a further increase (to 13-fold) in HL-A antigens per cell. PHA stimulation was also

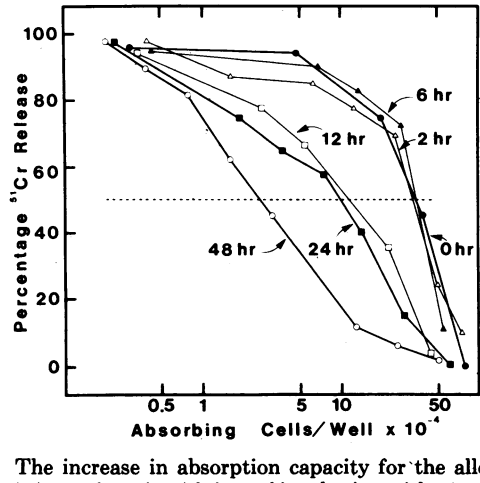

FIG. 3. The increase in absorption capacity for the alloantisera BC (HL-A3) as a function of time of incubation with 10 µg of PHA per ml of culture media.

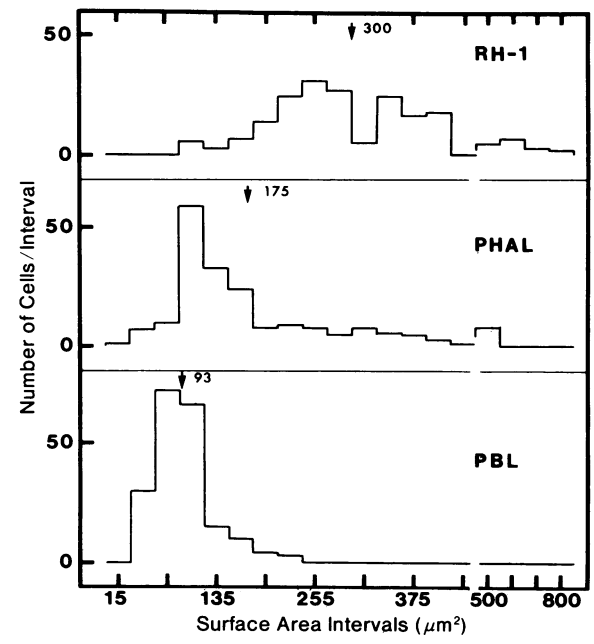

FIG. 4. Distribution of cell sizes in peripheral blood (PBL), phytohemagglutinin-stimulated (PHAL), and EBV-transformed (RH-1) lymphocyte populations. The geometric mean of the surface area for each cell type is indicated.

found to induce a similar pattern in the increase of the expression of the specificities W-28, HL-A7, and HL-A27. A synchronous variation in HL-A and H-2 expression with the growth cycle of culture lymphoblasts has been reported (13, 18, 19), varying over a 3-fold range. PHA appears to evoke a similar cell surface change in the expression of HL-A antigens, although of larger magnitude.

Estimation of relative cell surface area

To evaluate whether the increased absorptive capacity of PHAL's and RH-1 cells reflected a higher site density of HL-A antigens, cell surface area was estimated by three independent parameters: (*1*) mean geometric surface area, (2) radioiodinatable surface protein, and (3) 5′-nucleotidase activity.

Geometric surface areas were calculated for 200 cells of each cell type. Distribution graphs of the surface area of these cells are presented in Fig. 4. The mean geometric surface area were: PBL's, 93 µm²; PHAL's, 175 µm², and RH-1 cells, 299 µm².

To obtain relative cell surface area estimates more reflective of membrane convolutions, two additional characteristics were examined. The relative amount of lactoperoxidase-radioiodinatable surface protein was determined. The iodine incorporation of PHAL's was 2.3 times that of PBL's, com-

Table 2. Comparison of surface area parameters

	Mean geometric surface area		Radioiodine incorporation		5′-nucleotidase activity	
Cell	µm²	ratio	cpm/10⁴ cells	ratio	µmol/hr per 10⁸ cells	ratio
PBL	93	1.0	2.6 x 10⁵	1.0	1.05	1.0
PHAL	175	1.9	6.1 x 10⁵	2.3	1.59	1.5
RH-1	299	3.2	(nd)	(nd)	2.58	2.5

nd = not done.

pared to a 1.9-fold increase in the mean geometric surface area. The activity of 5′-nucleotidase, an enzyme whose activity is limited to membranes and which has been used as a membrane marker (17, 20), was also determined (Table 2). A 1.5-fold increase in activity per PHAL and a 2.5-fold increase in activity per RH-1 cell was observed.

The approximate nature of these three estimations of relative surface area is apparent. Taken together, however, they indicate that the mean surface area of PHAL's is twice that of PBL's, while the mean surface area of the RH-1 cell is about three times that of PBL's.

DISCUSSION

These experiments demonstrate that mitogenic stimulation with PHA or transformation with Epstein–Barr virus of normal peripheral blood lymphocytes results in an increased representation of HL-A antigens. On a per cell basis, PHAL's had about 11 times as much exposed HL-A as PBL's, while EBV-transformed cells had about 36-fold greater expression of antigen. The increased expression induced by PHA, a dose-dependent phenomenon, began at 12 hr, an early stage of mitogenesis. Three independent estimates of cell surface area showed PHAL's to have twice the surface area of PBL's, and RH-1 cells to have a three-times-larger surface area. These experiments show that the increased expression of HL-A antigens on PHAL's and RH-1 cells is not accompanied by a similar large increase in cell surface area. It appears, therefore, that the site density of HL-A antigens is much greater on PHA-stimulated or EBV-transformed lymphocytes than on the average normal peripheral blood lymphocyte. This fact is reflected in the large amount of HL-A antigens which have been isolated from EBV-transformed cultured human lymphocytes as compared to human spleens (21).

Subpopulations of PBL's were not examined in this study. The standard of comparison, peripheral blood lymphocytes, is a heterogeneous population of T, B, and null cells (22). PHA at early times stimulates predominantly T cells [although it may also stimulate human B cells and null cells at a later time (23, 24)], and the RH-1 line is a homogeneous B cell line. These experiments compared the increases in HL-A antigens and cell surface area, averaged over these cell samples. The results indicate that, averaged across an entire cell population, HL-A site density increases on PHAL's and RH-1 cells.

If some cells in the heterogeneous sample stimulated by PHA did not express an increased amount of HL-A antigen, then others may have shown a greater increase than revealed in the average value. This view is supported by the observation that the RH-1 line, a homogeneous B cell population, has a 36-fold increase in HL-A antigens per cell. Techniques requiring fewer cells are necessary to examine subpopulations of lymphocytes. In addition to the possibility that mitogenesis induced by PHA or EBV increases the site density of HL-A, the possibility that these stimuli may select a small subpopulation with an already enhanced representation of HL-A antigens at the cell surface must be explored. This question may be particularly important to examine in the case of transformation because the efficiency of transformation of PBL's by EBV is extremely low. The question may therefore be correspondingly difficult to answer.

It seems clear, whatever the mechanism, that lymphocytes with widely different representations of HL-A can be obtained. If HL-A antigens are mediators of intercellular recognition, an increased density of HL-A antigens may imply heightened potential for contact with other cells or soluble factors. Submembranal events which are dependent on a critical concentration of HL-A antigens at the surface, perhaps related to some role in immune responses, may occur more readily upon cell contact after the density of HL-A molecules has increased. Whether or not the site density differences in HL-A antigens reflect some function or simply reflect generalized cell surface hyperactivity in stimulated cells remains to be explored.

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