

Human Erythrocyte Antigens

REGULATION OF EXPRESSION OF A NOVEL ERYTHROCYTE SURFACE ANTIGEN BY THE INHIBITOR LUTHERAN *In(Lu)* GENE

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ABSTRACT Our study describes a novel human erythrocyte protein antigen, the expression of which is regulated by the rare Lutheran inhibitor *In(Lu)* gene. We have produced a monoclonal antibody (A3D8) that bound strongly to erythrocytes from subjects with Lutheran phenotypes Lu(a+b+), Lu(a+b-), and Lu(a-b+) but bound negligibly to erythrocytes from subjects with the dominant form of Lu(a-b-) phenotype, reflecting inheritance of the *In(Lu)* gene. Importantly, erythrocytes from an individual with the recessive form of Lu(a-b-) phenotype (i.e., absence of the *In(Lu)* gene and absence of genes encoding for Lutheran antigens) showed reactivity with A3D8 antibody comparable to that seen with Lu(a+) or Lu(b+) erythrocytes. A3D8 antigen activity was also found on all leukocytes and in serum and plasma; this activity also appeared to be regulated by the *In(Lu)* gene in serum, plasma, and on a subset of leukocytes. Thus, we have identified a human erythrocyte protein whose expression is modified by the *In(Lu)* gene. This knowledge that such an antigen exists on erythrocytes and in normal plasma should allow further studies into the molecular genetics of the *In(Lu)* gene and into the functional and structural significance of the A3D8 antigen.

INTRODUCTION

Lutheran antigens were the first erythrocyte blood group system shown to be controlled by an independent dominant inhibitor gene (1). This Lutheran inhibitory *In(Lu)*¹ gene is an autosomal gene, not linked

to the Lutheran loci, which in the heterozygote, suppresses almost totally the expression of Lutheran antigens (2-7). Partial penetrance of the gene is also believed to account for some phenotypes with weak expression of Lutheran antigens, which is also inherited as a dominant trait (4, 8). The *In(Lu)* gene is also known to reduce expression of other erythrocyte antigens, such as Auberger (Au^a), P₁, and i (9, 10). The mechanism for the action of the *In(Lu)* gene remains unknown; it has been postulated that it may turn off production of a precursor or backbone molecule required for expression of the Lu, Au^a, P₁, and i antigens or that the *In(Lu)* gene may code for an enzyme that modifies the substrate of the Lu, Au^a, P₁, and i gene products, thus reducing or preventing expression of these antigens (11). Both Lu^a and Lu^b antigens have been shown to vary in strength from one family to another, such that a heterozygote for Lu^b in one family may express as much antigen as a homozygote in another family (4, 8, 12). This phenomenon is also thought by some to reflect variants of the *In(Lu)* gene. Little progress has been made in understanding the action of the *In(Lu)* gene because both the gene product and its substrate(s) have remained unidentified. In this study, we report the identification via the use of murine monoclonal antibody (A3D8) of a novel erythrocyte cell surface protein whose expression is modified by the human *In(Lu)* gene. Knowles et al. (13) have recently reported two monoclonal antibodies that identify antigens, different from those described in this paper, also inhibited by the *In(Lu)* gene.

METHODS

Production of monoclonal antibody. The A3D8 cell line was established by fusion of BALB/c spleen cells from animals immunized with circulating malignant human Sezary T cells with the P3 × 63/Ag8 BALB/c myeloma cell line

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¹ Abbreviations used in this paper: [Au^a], Auberger; Ch, Chido; Fy, Duffy; In, inhibitor; K₀, Kell null; Jk, Kidd; Le, Lewis; Lu, Lutheran; Rg, Rodgers.

(14). The A3D8 antibody was initially chosen for further investigation after supernatant was found to react with a number of T cell lines (HUT 78, HUT 102, HSB-2) but not with Burkitt's B cell lines (Raji, EB-3, Daudi). Antibody A3D8 was subsequently found to react with human erythrocytes as well as with selected lymphoid cell lines. Ouchterlony immunodiffusion analysis revealed the A3D8 antibody heavy chain isotype to be IgM. The A3D8 cell line was cloned three times by limiting dilution and has been stable in tissue culture and in passage as an ascites tumor for >50 passages.

Assays to measure cell surface A3D8 antibody reactivity. Erythrocytes or leukocytes were incubated 30 min at 4°C with dilutions of A3D8 ascitic fluid or control IgG1 or IgM murine myeloma protein, washed in RPMI 1640 media supplemented with 2% bovine serum albumin (BSA) and 0.1% sodium azide, followed by incubation for 30 min at 4°C with affinity pure fluorescein-labeled goat anti-mouse IgG, F/P-6.0, heavy + light chain specific (TAGO, Inc., Burlingame, CA). Cells were washed twice in phosphate-buffered saline (PBS) with 2% BSA and 0.1% sodium azide, resuspended in PBS, and analyzed for percent labeled cells and relative fluorescence intensity by flow cytofluorography on an Ortho 50H cytofluorograph (Ortho Diagnostics, Raritan, NJ). In some cases, ¹²⁵I-labeled affinity purified F(ab)₂ anti-mouse Ig antibodies were used to detect specific binding of A3D8 antibody to erythrocytes or leukocyte subsets as previously described (15). In order to determine the presence of A3D8 antigen in various human tissues, 4-μm acetone-fixed frozen sections of various human tissues were prepared and stained with monoclonal antibody A3D8 as previously described (16).

Analysis and distribution of A3D8 antigen. Purified mononuclear cell suspensions were obtained from heparinized venous blood via Hypaque-Ficoll density centrifugation (17). Peripheral blood T lymphocytes were isolated by formation of rosettes (ER) with neuraminidase-treated sheep erythrocytes, followed again by Hypaque-Ficoll density centrifugation (18). Monocytes were identified by the presence of ingested latex particles after being incubated for 1 h at 37°C with latex particles. B lymphocytes were identified by the presence of surface Ig using fluorescein-labeled F(ab)₂ goat anti-polyvalent human Ig (Cappel Laboratories, Cochranville, PA). Polymorphonuclear cells were purified using 3% dextran sedimentation followed by hypotonic lysis of erythrocytes (19). Erythrocytes were obtained from heparinized blood by centrifugation and repeated washing to remove the buffy coat. Alternatively, erythrocytes were used as provided by commercial suppliers of blood bank erythrocyte panels. Phenotypically rare erythrocytes were obtained from the Duke University Medical Center rare cell bank or were the generous gift of W. L. Marsh, New York Blood Center, and were tested after being frozen and thawed, using routine blood banking techniques. Thymic tissue was obtained from patients undergoing cardiovascular surgery. Thymus cell suspensions were obtained by gently teasing cells from stromal tissue, followed by Hypaque-Ficoll density centrifugation. Other human tissues were obtained during routine autopsies of patients dying from nonmalignant causes. Cell lines were acquired and maintained as previously described (19).

Analysis of A3D8 antigen sensitivity to enzymatic degradation. Type O positive peripheral blood lymphocytes and erythrocytes were treated with trypsin (type III, Sigma Chemical Co., St. Louis, MO) at concentrations ranging from 0.1 to 4 mg/ml or with neuraminidase (clostridium perfringens, type IV, Sigma Chemical Co.) at concentrations from

0.1 to 5 IU/ml for 45 min at 37°C. The cells were washed three times in RPMI 1640 media supplemented with 20% fetal calf serum (Gibco Laboratories, Grand Island, NY) and then assayed in the indirect fluorescent assay by cytofluorography.

Blocking of A3D8 antibody binding to erythrocytes by Lutheran alloantisera. Group O erythrocytes were obtained from normal donors or commercial sources as described above. Lu(a+b+) cells were incubated for 30 min at 20°C with saturating dilutions of Dade DUA 12 anti-Lu^a serum, and Lu(a-b+) cells were incubated for 30 min at 20°C with either Ortho LUB 106 anti-Lu^b serum or anti-Lu3 serum (kindly provided by W. L. Marsh, New York Blood Center). Binding of alloantisera was confirmed by subsequent incubation of erythrocytes with fluorescein-conjugated affinity-purified goat anti-human IgG (TAGO, Inc.) and cell sorter analysis. Aliquots of cells were also stained as described above using normal human serum as a control. After incubation 45 min at 4°C the erythrocytes were washed twice and incubated with either subsaturating amounts of A3D8 antibody (ascitic fluid at a final dilution of 1:4,000) or nonerythrocyte-binding murine hybridoma IgM antibody as a control.

Blocking of A3D8 antibody binding to erythrocytes by whole serum or plasma. Group O erythrocytes were obtained from normal donors. A3D8 ascites fluid was diluted 1:10 in RPMI 1640 media supplemented with 2% BSA and 0.1% sodium azide. Aliquots of this stock solution were then further diluted to final concentrations of 1:1,000, 1:2,000, and 1:4,000 in either RPMI 1640 medium, serum, or plasma. The final concentrations of serum or plasma at these dilutions were 90, 95, and 97.5%, respectively. A nonerythrocyte-binding hybridoma IgM antibody was similarly diluted as a control. Indirect fluorescent staining was then carried out as described above, and both the percentage of positive cells and their relative fluorescence intensity were analyzed by flow cytofluorography. It should be pointed out that this series of experiments utilized the incubation of serum or plasma with erythrocytes in the presence of antibody A3D8. In contrast, blocking of antibody A3D8 with various types of antisera (see previous section) utilized preincubation of erythrocytes with various alloantisera followed by several washes and then the addition of A3D8 antibody.

Analysis of radioimmunoassay (RIA) and cytofluorographic data. RIA data were expressed as Δ counts per minute as determined by the equation: Δcpm = cpm experimental - cpm control.

To compare cytofluorograph data from various experiments, samples were analyzed both for the percentage of fluorescent cells and for the channel of mean fluorescence i.e., the channel at which 50% of cells were brighter and 50% duller or negative.

Measurements of relative fluorescence were obtained by dividing the length of the three-phase log scale used by the cytofluorograph by 512 (the number of channels) to obtain a millimeter/channel measurement of 0.51 mm on standard 3 cycle semilogarithmic paper. The mean fluorescent channel for each sample was multiplied by 0.51 mm, and the resulting number measured along the log scale to obtain the mean relative fluorescence of the sample. Data were analyzed using the Student's *t* test.

RESULTS

Reduced expression of A3D8 antigen in the presence of the In(Lu) gene. A3D8 antigen was on the

cell surface of all circulating lymphocytes, monocytes, polymorphonuclear leukocytes, and erythrocytes from all normal Lu(a+) or Lu(b+) donors tested. Regardless of ABO blood group or Rh phenotype, erythrocytes from a large panel of normal donors were all strongly positive when assayed for A3D8 antibody reactivity (Table I). A3D8 antibody also reacted normally with cells with the following rare phenotypes: Bombay (O_h), Le(a-b-), adult i, P₂ (P₁-) Fy(a-b-), U-negative, Jk(a-b-), Ch(a-), Rg(a-), Vel negative, Au(a-), K₀, and McLeod phenotype K:-9 (Table I). Erythrocytes lacking other antigens known to be shared by erythrocytes and leukocytes, namely Cost, York, Bennett-Goodspeed, and McCoy, also reacted normally with A3D8 antibody (data not shown). The only erythrocytes that showed a significant decrease in A3D8 expression were those of the dominant Lu(a-b-) phenotype caused by the *In(Lu)* gene (1) (Table I and Fig. 1). All erythrocyte samples from four donors with dominant Lu(a-b-) cells showed marked reduction in reactivity with A3D8 antibody.

Erythrocytes from all adult donors expressing Lu^a and/or Lu^b antigens bound A3D8 antibody with a mean relative fluorescence of 10.7±0.4 (±SEM) while dominant Lu(a-b-) erythrocytes bound A3D8 antibody weakly and had mean relative fluorescence of

TABLE I
Distribution of A3D8 Reactivity with Erythrocytes of Various Known Phenotypes

Phenotype	n	MRF*	SEM
Dominant Lu(a-b-)	4	2.5	±0.4
Lu(a-b+)	47	10.8	±0.4
Lu(a+b-)	1	9	—
Lu(a+b+)	2	11	1
Recessive Lu(a-b-)	1	13	—
Cord	6†	16.9	±2.5
Adult i (I neg)	1	10	—
O _h	1	10.5	—
P ₂ (P ₁ neg)	5	12.6	±1.5
Tj (a-)	1	12.5	—
Le(a-b-)	4	9.3	±1.4
U neg.	1	14	—
K ₀	1	12.5	—
K:-9	1	10	—
Jk (a-b-)	1	7.4	—
Fy (a-b-)	5	9.3	±1.6
Au (a-)	1	13	—

* Mean relative fluorescence (MRF), i.e., the fluorescence intensity at which 50% of cells were brighter and 50% were duller.

† Three samples were commercially provided pooled group O cord blood cells.

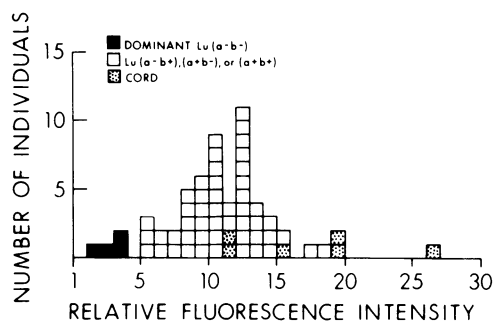


FIGURE 1 Distribution of degree of A3D8 reactivity of erythrocytes of various known phenotypes. Samples of erythrocytes from cord blood, adults with normal Lutheran antigens (a+ and/or b+), and four subjects with the dominant Lu(a-b-) phenotype were tested via indirect immunofluorescence for A3D8 reactivity. Both adult erythrocytes with Lu(a+) or Lu(b+) antigens and cord erythrocytes showed a broad range of reactivity. Normal adult cells had a fluorescence intensity of ≥5 and ≤19, while cord erythrocytes had a fluorescence intensity of between 11 and 27. All dominant Lu(a-b-) erythrocytes had fluorescence intensities <4.

only 2.5±0.4 (Table I and Fig. 1). This represented a fivefold increase in binding of A3D8 antibody to Lu(a+) or Lu(b+) cells compared with dominant Lu(a-b-) cells ($P < 0.0005$). Within the Lu(a+) and/or (b+) population, reactivity with A3D8 antibody, as measured by fluorescence, showed a broad range, from 5.6 to 18 fluorescent units. This distribution of strength of expression of A3D8 antigen is similar in magnitude to the distribution of strength of Lutheran antigens demonstrated by Greenwalt et al. using anti-Lu^a and anti-Lu^b alloantisera (12). Of great interest was the observation that erythrocytes from a donor with the recessive Lu(a-b-) negative phenotype, caused by the presence of two amorphic or null Lutheran alleles (in the absence of expression of the *In(Lu)* gene [20]) bound A3D8 antibody normally (Table I and Fig. 2). Erythrocytes negative for Au-berger and P₁ antigens—antigens known to be weakly expressed or absent on dominant Lu(a-b-) erythrocytes—also bound A3D8 antibody normally (Table I). Cord blood erythrocytes, which are known to express decreased amounts of Lu^a and Lu^b antigens, and to express large amounts of the i antigen, bound A3D8 antibody in normal or increased amounts compared with adult erythrocytes (Fig. 1). Adult i (I negative) cells, which also have increased amounts of i antigen, bound normal amounts of A3D8 antibody compared with adult I cells (Table I). When A3D8 antibody was titrated against cord blood and adult erythrocytes by indirect immunofluorescence, the maximum percentage of both types of cells were labeled by dilutions of

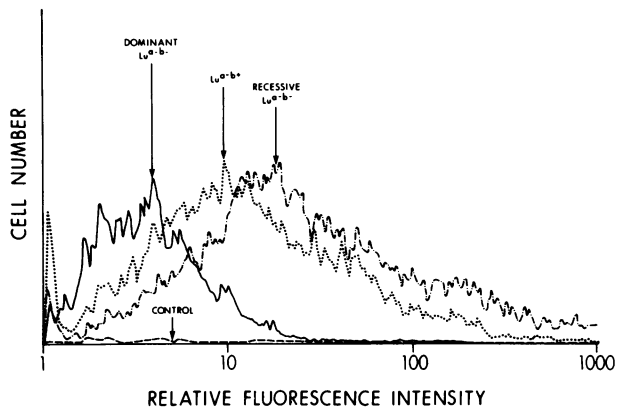


FIGURE 2 Reactivity of various Lutheran phenotypes with A3D8 antibody. Samples of erythrocytes from single donors with known Lutheran phenotypes were stained with A3D8 antibody and fluorescein-conjugated goat anti-mouse antibody, then analyzed by flow cytofluorography for distribution and intensity of fluorescent staining on a triphasic log scale. The $\text{Lu}(a-b^+)$ cells ($\cdots\cdots$) had a mean relative fluorescence (MRF) after staining with A3D8 of 10, while the recessive-type $\text{Lu}(a-b^-)$ cells ($-\cdots-$) had an MRF of 20. The dominant-type $\text{Lu}(a-b^-)$ cells ($---$) had an MRF of only 1, as 59% of the cells were in channel 1, with no detectable fluorescence, as seen by the leftward position of the dominant $\text{Lu}(a-b^-)$ curve. All three cell samples gave superimposable control curves when stained with nonreactive mouse IgM myeloma protein ($----$).

A3D8 ascitic fluid up to a final concentration of 1:4,000.

The lymphocytes and monocytes of two unrelated dominant-type $\text{Lu}(a-b^-)$ donors, D.J. and M.C., were also tested for binding of A3D8 antibody. Both subjects had increased A3D8⁻ populations of lymphocytes (56% of D.J. vs. 15% for the normal $\text{Lu}(b^+)$ control in one instance, 26% for M.C. vs. 13% for the normal $\text{Lu}(b^+)$ control in the other; Fig. 3). However, lymphocytes from both $\text{Lu}(a-b^-)$ donors that did react with A3D8 antibody did so with a distribution and relative fluorescence comparable to $\text{Lu}(b^+)$ subjects (Fig. 3C, D). In contrast, monocytes from these dominant $\text{Lu}(a-b^-)$ donors showed a cytofluorographic pattern not seen using monocytes from 11 $\text{Lu}(b^+)$ donors. While monocytes from all 11 $\text{Lu}(b^+)$ subjects exhibited uniform strong reactivity with A3D8 antibody, monocytes from the $\text{Lu}(a-b^-)$ donors contained two populations—one, representing 40% of the monocytes, reacting with A3D8 very weakly, and a second population, also representing ~40% of the monocytes, which bound A3D8 strongly (Fig. 3E, F). Monocytes from the $\text{Lu}(a-b^-)$ donors also included a large population of A3D8⁻ cells (15% of monocytes), while 95% of monocytes from all $\text{Lu}(b^+)$ subjects were strongly A3D8⁺ (Fig. 3E, F).

Several examples of the regulator Rh null phenotype were also tested for A3D8 reactivity. When these cells were thawed and transported for testing with A3D8 antibody, results fell within the low-normal range or at the upper end of the dominant $\text{Lu}(a-b^-)$ range (data not shown). However, when these cells were tested promptly after thawing, using an agglutination technique, A3D8 antibody was normally reactive with both amorphous and regulator type Rh null cells, as well as with a large panel of cells of other phenotypes; only the dominant $\text{Lu}(a-b^-)$ cells were nonreactive (W. L. Marsh, New York Blood Center. Personal communication). Agglutination assays were performed with dilutions of A3D8 ascites fluid of 1:80,000. Thus, given the normal results in the agglutination assay with multiple Rh null samples, the relatively low degree of immunofluorescent staining of Rh null cells in our hands probably reflects the fragility of these cells when exposed to varied temperatures and transportation times. Fragility of Rh null cells and reduced *in vivo* half-life of Rh null cells have previously been well documented (21).

Characterization of A3D8 antigen. A3D8 antigen showed marked sensitivity to trypsin digestion. Erythrocyte suspensions pretreated with 0.1 mg/ml trypsin showed decrease in A3D8 expression (Fig. 4), and treatment of erythrocytes with 1 mg/ml of trypsin abolished all specific binding of A3D8 antibody. Identical results were obtained when peripheral blood lymphocytes were pretreated with trypsin before being stained with A3D8 antibody (Fig. 4). In contrast, neuraminidase treatment of $\text{Lu}(b^+)$ erythrocytes or lymphocytes did not decrease A3D8 antibody binding.

Distribution of A3D8 antigen. A3D8 antigen was also present on cell types other than erythrocytes. Circulating B and T lymphocytes strongly bound antibody A3D8, as did monocytes and polymorphonuclear leukocytes. Antibody A3D8 also reacted strongly with many malignant lymphocyte cell lines, including HUT 78 and HUT 102 (Sezary T cell), HSB-2 (T-ALL) and the normal B cell lines SB, B958, and YT4E. However, all Burkitt's lymphoma B cell lines thus far tested (EB-3, Raji, and Daudi) did not bind A3D8. Circulating leukemic cells from patients with null ALL, T ALL, T and B CLL, polymphocytic leukemia, and hairy cell leukemia have thus far all been positive for A3D8 (data not shown).

In addition, A3D8 antigen activity was found circulating in serum and plasma from $\text{Lu}(b^+)$ subjects. When group O $\text{Lu}(b^+)$ erythrocytes were incubated with nonsaturating amounts of A3D8 antibody in the presence of autologous or allogeneic serum or plasma, serum or plasma invariably produced inhibition of A3D8 binding (Fig. 5). Normal serum or plasma from

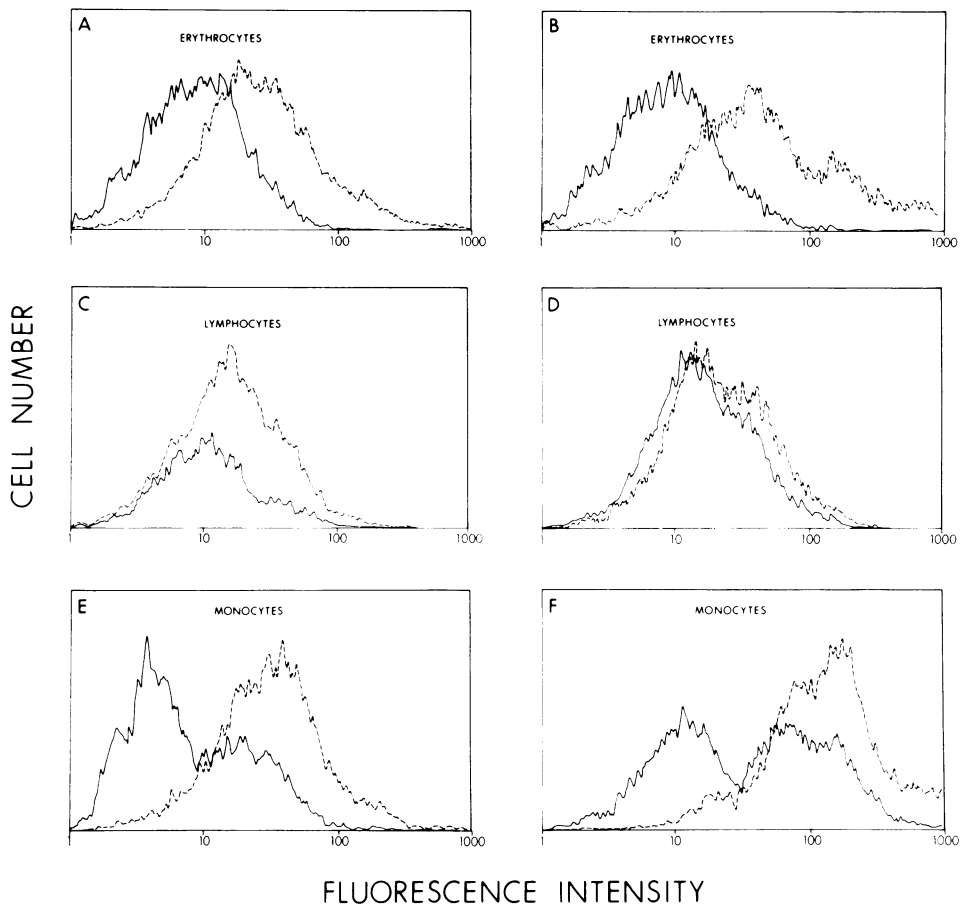


FIGURE 3 Reactivity of A3D8 antibody with erythrocytes, lymphocytes, and monocytes from dominant Lu(a-b-) donors. In each graph, results of staining cells from Lu(a-b-) donors are expressed as a solid line, while results obtained with cells from Lu(b+) donors are expressed with a dashed line. Graphs A, C, E, and B, D, F show A3D8 reactivity with cells from Lu(a-b-) donors D.J. and M.C., respectively. Erythrocytes and a subset of monocytes from both Lu(a-b-) donors showed decreased reactivity, compared with cells from Lu(b+) donors. In graphs C and D, lymphocytes from donors D.J. and M.C., respectively, are compared to lymphocytes from Lu(b+) subjects.

five Lu(a+) or Lu(b+) ABH secretor or nonsecretor donors produced inhibition of a nonsaturating dilution of A3D8 binding of $67 \pm 6\%$ (mean \pm SEM). In contrast, the presence of plasma from two dominant-type Lu(a-b-) donors (DJ and MC) produced a mean inhibition of A3D8 binding to allogeneic Lu(b+) erythrocytes of only $33 \pm 9\%$ ($P < 0.05$ compared to Lu(b+) plasma; Fig. 5). At lower dilutions of A3D8 ascites (1:8,000), both Lu(b+) and Lu(a-b-) plasma produced $>90\%$ inhibition of A3D8 binding (Fig. 5). Serum from normal rabbits, whose erythrocytes do not express A3D8, produced no inhibition of A3D8 binding to human erythrocytes. Also, human plasma from an se/se donor (i.e., a nonsecretor of ABH blood group

antigens) was able to maximally inhibit A3D8 antibody binding. Finally, incubation at 4°C of dominant-type Lu(a-b-) cells in allogeneic Lu(b+) plasma failed to increase A3D8 antigen expression on dominant Lu(a-b-) erythrocytes. To investigate the possibility that Lu(b+) serum and plasma blocking activity might be due to low levels of circulating anti-mouse Ig or anti-idiotypic antibody, the presence of human immunoglobulin on erythrocytes in the above experiments was sought by indirect immunofluorescence and flow cytofluorography. We were unable to demonstrate any erythrocyte bound human immunoglobulin under conditions in which there was maximal inhibition of binding of A3D8 antibody.

A3D8 antibody reacted with most lymphoid cells, including those from lymph nodes, spleen, and thymus. Antibody A3D8 reacted equally in B and T cell areas of lymph nodes but in the thymus was selectively expressed only by thymic medullary cells and not by cortical thymocytes. Squamous epithelial cells of skin, cornea, and conjunctiva bound antibody A3D8, but liver, pancreas, thyroid, adrenal, and pituitary cells did not, although Kuppfer cells of the liver brightly stained with A3D8 antibody. Brain also showed bright staining for A3D8 on neuronal bodies in both cerebellum and medulla.

Phylogenetic distribution of A3D8 antigen. A3D8 antibody bound to mononuclear cells and erythrocytes of higher primates, including gorillas, chimpanzees, orangutans, and Gibbon apes. A3D8 antibody did not bind, however, to the cells of all Old World Monkeys tested (Rhesus, pig-tail, stump-tail), and to the erythrocytes of other mammals, including sheep, cows, and rabbits. Two to four animals of each species were tested.

DISCUSSION

Our study describes a novel human erythrocyte cell surface antigen different from any other erythrocyte or leukocyte antigen previously identified with either alloantisera or monoclonal antibodies (13, 22-26). Moreover, we have shown that the expression of this antigen (as defined by monoclonal antibody A3D8) on erythrocytes and in plasma is regulated by the rare Lutheran inhibitor or *In(Lu)* gene. The *In(Lu)* gene has been shown to down regulate the expression of a

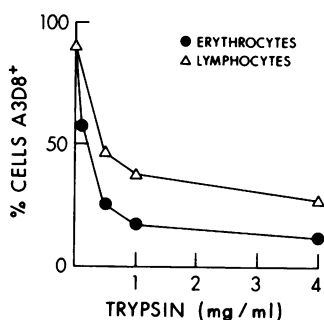


FIGURE 4 Sensitivity of A3D8 antigen to trypsin degradation. Erythrocytes and lymphocytes from group O Lu(b+) donors were incubated with varying dilutions of trypsin at 37°C for 45 min, washed in RPMI + 20% fetal calf serum, and assayed for A3D8 reactivity by indirect immunofluorescence. Final concentrations of trypsin as low as 0.1 mg/ml markedly decreased A3D8 reactivity, and increasing concentrations of trypsin produced decreasing degrees of A3D8 reactivity.

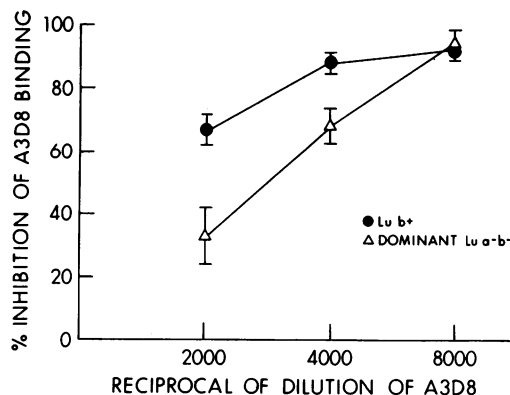


FIGURE 5 Inhibition of A3D8 binding to erythrocytes by human serum and plasma. Group O Lu(b+) erythrocytes were reacted with varying concentrations of A3D8 antibody diluted in either RPMI + 2% bovine serum albumin, human serum, plasma from Lu(b+) donors, or plasma from dominant Lu(a-b-) subjects. Cells were then stained with fluorescein-conjugated goat anti-mouse Ig and analyzed by flow cytometry. Results are expressed as percent inhibition, by serum or plasma, of A3D8 binding to Lu(b+) erythrocytes compared with reactivity of A3D8 antibody in RPMI alone with erythrocytes. Normal serum or plasma from Lu(b+) subjects produced 67±6% inhibition (mean±SEM) of reactivity with A3D8 at a dilution of 1:2,000, while plasma from dominant Lu(a-b-) donors produced only 33±9% inhibition of A3D8 binding at the same dilution.

number of erythrocyte antigens including Lutheran, Auberger, i, and P₁ (9, 10). While the polysaccharide structures of the P₁ and i antigens are fairly well understood (11, 27), the biochemical natures of the Lutheran and Auberger antigens are not; the mechanism whereby the dominant *In(Lu)* gene down regulates the expression of all of these antigens has yet to be determined. The identification of the A3D8 antigen as a protein antigen whose expression on erythrocytes, a subset of monocytes, and in plasma is markedly reduced by the *In(Lu)* gene provides a new means by which this problem can now be approached.

Although trypsin sensitivity does not prove that A3D8 antibody identifies a protein antigen, other evidence supports this conclusion. Chromosomal mapping studies, using human-hamster hybrids, demonstrate that A3D8 antigen is present in cells whose human chromosome content is limited to the short arm of chromosome 11 (28). This type of chromosomal mapping technique, however, does not exclude the possibility that the human chromosome 11 encodes for both a protein and a transferase that generates on that protein a carbohydrate epitope recognized by A3D8 antibody; alternatively the human chromosome 11 could encode for a transferase that can generate the A3D8 antigen by acting on a hamster-produced sub-

strate. Nevertheless, radioimmunoprecipitations using both A3D8 antibody and an IgG₂ murine monoclonal antibody against the same antigen show that this antigen is an 80,000-D protein.²

A3D8 antigen is clearly not identical to any of the known antigens already identified as being suppressed by the *In(Lu)* gene. Because it is present in normal amounts on recessive-type Lu(a-b-) cells, which are homozygous for an amorphic or null Lutheran allele (20), A3D8 is not a Lutheran antigen, such as Lu^a, Lu^b, or Lu⁶. Other data that support this conclusion include strong expression of A3D8 antigen on cord blood erythrocytes, which express Lutheran antigens weakly, and our observation that Lu(a+b-), Lu(a-b+), and Lu(a+b+) cells bound A3D8 antibody equally well (Table I). A3D8 antigen is also not identical to antigens identified by previously described monoclonal antibodies. Knowles and colleagues (13) have described two murine monoclonal antibodies, H86 and M447, which react with erythrocytes from all adults except those that express the *In(Lu)* gene. However, the antigen detected by these antibodies is absent on cord cells and is protease resistant. Thus, antibodies H86 and M447 appear to identify another erythrocyte antigen inhibited by the *In(Lu)* gene but with a different tissue distribution and biochemical nature than the antigen identified by A3D8 antibody. However, we cannot rule out that the H87, M447, and A3D8 antibodies define different epitopes on the same molecule. Our phylogenetic data indicate that A3D8 antibody reactivity is absent from primate (e.g., Rhesus monkey) erythrocytes known to have i antigen on their surfaces (29); thus A3D8 antibody does not identify the i antigen.

While A3D8 antigen is clearly not one of the Lutheran allotypic antigens currently recognized by routine blood banking procedures, it is related to the Lutheran, P₁, Auberger, and i antigens via the action of the *In(Lu)* gene. The *In(Lu)* gene does not completely prevent formation of Lutheran antigens. When these are looked for by sensitive adsorption and elution methods rather than by agglutination, dominant Lu(a-b-) cells can be shown to have small amounts of Lutheran antigens (30). Similarly, the A3D8 antigen is markedly reduced but not abolished by the *In(Lu)* gene, as A3D8 antibody did react weakly with dominant Lu(a-b-) erythrocytes (Table I and Figs. 1 and 2). Also, A3D8 antigen shows a broad range of strength of expression, as do both Lu^a and Lu^b antigens (12). Thus A3D8 antigen could be the precursor molecule

of Lutheran antigens, the strength of expression of which is regulated by the *In(Lu)* gene and its variants. Since neither the Lutheran gene products nor the Lutheran antigens have been isolated, this theory remains to be proven.

The A3D8 antigen is strongly expressed on a variety of leukocytes, whereas Lutheran antigens have never been shown on leukocytes by adsorption/elution techniques (31) or by mixed erythrocyte-leukocyte agglutination (32). Of particular interest was the observation that in individuals with the dominant Lu(a-b-) erythrocyte phenotype, some cells (i.e., lymphocytes and a subset of monocytes) bound A3D8 antibody in amounts comparable to leukocytes of Lu(a+) or Lu(b+) subjects (Fig. 3). Similarly, normal cortical thymocytes from Lu(b+) subjects did not bind A3D8 antibody, whereas medullary thymocytes were strongly A3D8⁺. These data suggest that select tissues as well as discrete stages of differentiation within a particular cell type exist where the *In(Lu)* gene may not be expressed in dominant Lu(a-b-) subjects (i.e., lymphocytes and a subset of monocytes) and may be expressed in Lu(b+) subjects (i.e., cortical thymocytes). However, we cannot rule out the possibility that expression of A3D8 antigen is regulated in erythrocytes by the *In(Lu)* gene but in other tissues may be regulated by mechanisms unrelated to the *In(Lu)* gene.

Our finding of A3D8 antibody blocking activity in serum and plasma, and that the level of blocking activity is reduced in the presence of the *In(Lu)* gene, is of particular interest and may represent an entirely new observation in the area of soluble blood group antigens. To date, no Lutheran-related antigen nor *In(Lu)* gene controlled antigen (with the exception of i[29]) has been found in plasma. Because there has been some controversy as to whether the secretor gene, which determines the presence of A, B, H, and Lewis substances in saliva, also affects their presence in serum (33, 34), we tested both Lu(b+) non-secretors (se/se) and a dominant Lu(a-b-) secretor (M.C.). We found that the presence of plasma blocking activity of A3D8 antibody is not dependent on ABH secretor status, but is dependent on the absence of the *In(Lu)* gene. We have been able to selectively remove A3D8 blocking activity from serum by passage of serum over an A3D8-sepharose 4B affinity column but not by passage of serum over a normal mouse serum-sepharose 4B affinity column (Palker, T., M. Telen, and B. Haynes. Unpublished observations.). These data suggest that the serum and plasma factor with blocking activity for A3D8 antibody is a circulating antigen specifically recognized by monoclonal antibody A3D8. This observation should aid in isolating and biochemically defining the A3D8 antigen.

² Haynes, B. F., M. J. Telen, and T. J. Palker. Manuscript in preparation.

Thus, we have used hybridoma technology to identify a novel human erythrocyte antigen whose expression is regulated by the *In(Lu)* gene. The new knowledge that such an antigen exists on most mature hematopoietic cells and in normal plasma, coupled with this monoclonal antibody probe, should allow further studies into the molecular genetics of the *In(Lu)* gene and into the functional and structural significance of the A3D8 molecule.

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