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Inactivation of Kell blood group antigens by 2-aminoethylisothiouronium bromide

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SUMMARY. Human red cells incubated with a solution containing 6% 2-aminoethylisothiouronium bromide (AET) lose activity of antigens that are part of, or related to, the Kell blood group system. However, Kx antigen is not inactivated. Studies on a wide range of other blood-group antigens show no other evidence of changes and AET appears to react specifically with red-cell membrane structures that have Kell activity. The AET procedure produces an artificial K_0 red cell that can be used in blood group serology, and allows easy recognition of antibodies that are associated with the Kell system.

AET has been used by other workers to produce a red cell that has many serological and biochemical characteristics of a PNH cell. Our studies on red cells from PNH patients have not shown any changes in Kell blood-group antigens.

Treatment of human red cells with the compound 2-aminoethylisothiouronium bromide (AET) modifies the cell membrane and greatly enhances sensitivity of the cells to the lytic action of complement (Sirchia *et al*, 1965; Sirchia & Dacie, 1967). The membrane lesion that is induced by AET closely resembles that which occurs naturally in paroxysmal nocturnal haemoglobinuria (PNH) (Sirchia & Ferrone, 1972). AET treatment has been used to reproduce *in vitro* a PNH-like cellular defect and such cells have been extensively studied (Canellos *et al*, 1970; De Sandre *et al*, 1970; Ferrone *et al*, 1972; Burapakulsolsri *et al*, 1979).

The Kell blood group system has greatly increased in its recognized complexity during the past few years and presently comprises 22 antigens that are part of, or related to, the system (Issitt & Issitt, 1975; Marsh, 1975; Sabo *et al*, 1979; Yamaguchi *et al*, 1979). Kell is a system of major clinical importance and immunization to Kell-system antigens is a fairly common sequel to blood transfusion (Kornstad & Heistö, 1957; Giblett, 1961). In addition, the Kell group has an important role in maintenance of red-cell membrane integrity, for cells of the

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McLeod phenotype in this system have abnormal morphology, and individuals of this blood type have a permanent haemolytic state (Wimer *et al.* 1977; Symmans *et al.* 1979).

We have found that red cells incubated with AET undergo specific inactivation of antigens in the Kell blood group, but antigens from a wide range of other blood groups are not affected. The finding contributes information to several areas of Kell blood-group involvement, and provides a practical procedure that facilitates laboratory investigation of Kell-related problems. This report presents data from serological aspects of this investigation.

MATERIALS AND METHODS

Serological tests were made by standard procedures using 3–5% red cell suspensions and allowing 30 min incubation times. Antiglobulin reagents were of commercial origin. Blood-group antisera and red-cell samples were from our own reference collections; some were received through the SCARF international exchange programme. Most red-cell samples were stored frozen in liquid nitrogen. Antibody elution from sensitized red cells was made using a modification of Rubin's (1963) ether method. AET was obtained from Calbiochem, Behring Corp., California. It has a molecular weight of 281 Daltons and is stable in the dry state at room temperature.

Preparation of AET solution. Following our initial observations in the Kell system, preliminary studies were made into the effect of variation in AET concentration, pH, reaction time and temperature. They showed that 6% AET dissolved in distilled water, and adjusted to pH 8·0 by addition of 5 N NaOH, was optimum. One volume of washed packed red cells was mixed with 4 volumes of this solution and incubated at 37°C for 20 min. The reaction was stopped by washing the cells in phosphate-buffered saline at pH 7·0. The first wash fluids usually showed haemolysis, and washing was continued until the supernatant fluid was clear (four to six washes). Treated cells were kept refrigerated and used for up to 24 h. The alkaline AET solution should be prepared immediately before use.

RESULTS

Inactivation of Kell antigens

Appropriate red cells have been tested in parallel studies with AET-treated and untreated cells, using IgG Kell antisera by the indirect antiglobulin test. The following Kell-system antigens are inactivated by AET: K, k, Kp^a, Kp^b, Kp^c, Js^a, Js^b, K17(Wk^a), K11, $U1^a$, K12, K13, K14, K18, K19, K20, K22 and Ku. In the case of K, k and Kp^b many examples of the antibodies have been used. Other specificities have been studied with duplicate antisera, except for Kp^c, K17, $U1^a$, K13 and K22, where only one example of each antibody was available. Antibody absorption and elution studies using anti-k, anti-Kp^b, anti-Js^b and anti-Ku show that while untreated reactive red cells react strongly and yield potent eluates, the same cells treated with AET do not react; eluates prepared from them are inactive.

The Kx (K15) antigen is related to the Kell system although it is not a product of the Kell gene (Marsh *et al.* 1975b). To study the effect of AET on Kx antigen, an anti-KL serum,

containing anti-Kx and anti-Km, was absorbed with K_0 (Kx positive) cells and an eluate prepared from them. Tests with this eluate against AET-treated and untreated cells of common Kell type showed that Kx was not inactivated by AET, but, on the contrary, treated cells appeared to have enhanced activity. Table I shows results obtained in these experiments using anti-Kx. In further studies, monospecific anti-Km present in the serum of a non-CGD McLeod-type man who had been immunized by blood transfusion, did not react with AET-treated red cells.

Other antigens on AET-treated red cells

To investigate the specificity of AET inactivation of red cell antigens appropriate cells have been tested, before and after treatment, with antibodies to the following blood-group antigens: A, A_1, B, H, I, i, Le^a, Le^b, Sd^a, Sd^x, Sp_1(Pr), M, N, S, s, U, P_1, 'Tj^a', D, C, c, C^W, E, e, Rh29, Lu^a, Lu^b, Lu3, Fy^a, Fy^b, Fy3, Jk^a, Jk^b, Jk3, Xg^a, Co^a, Co^b, En^a, Wr^a, Wr^b, Di^a, Di^b, At^a, Cs^a, Lan, Ch^a, Rg^a, Vel and Yt^a. In all cases AET-treated cells reacted as strongly as did untreated cells.

The Gerbich (Ge) antigen has an association with the Kell system, for some Ge-negative cells have weak Kell antigens (Muller *et al*, 1973). The nature of the association is unknown. Direct tests with three anti-Ge sera gave strong reactions with AET-treated cells and in a titration study the scores of treated and untreated cells were the same (Table II). In further titration studies using anti-Fy^a, anti-D, anti-M, anti-Lu3, anti-Di^b and anti-Jk^a, the results given by treated and untreated cells were indistinguishable. Table II shows representative results obtained from titration studies with various antibodies against AET-treated red cells. In antibody absorption and elution studies, using anti-Fy^a and anti-U, potent eluates were obtained from AET-treated red cells. AET did not induce formation of new blood group antigenic configurations in the systems tested; treated Fy(a−) red cells did not become Fy(a+), Jk(a−) red cells did not become Jk(a+), and D-negative red cells did not become D-positive.

Table I. Results obtained from titrating anti-Kx against AET-treated and untreated red cells

Test cells	Anti-Kx Reciprocals of dilutions 1	2	4	8	Total score
K−k+ untreated	5	0	0	0	5
K−k+ AET-treated	7	6	5	0	18
K_0 untreated	7	5	4	0	16
K_0 AET-treated	7	5	5	0	17

Tests by antiglobulin method. Scoring system of Marsh (1972).

Table II. Results of titrating blood group alloantibodies against untreated and AET-treated red cells. These data are representative of many studies; appropriate red cells were used for each antibody specificity.

	Reciprocals of antibody dilutions								
Test cells	1	2	4	8	16	32	64	128	Total score
Anti-Fy[a]									
Untreated	9	8	5	4	2	1	0	0	29
AET-treated	11	7	7	3	2	0	0	0	30
Anti-D									
Untreated	12	12	11	10	8	0	0	0	53
AET-treated	12	12	10	10	8	6	0	0	58
Anti-Ge									
Untreated	10	10	9	7	4	0	0	0	40
AET-treated	9	9	8	5	4	0	0	0	35
Anti-K									
Untreated	11	10	10	7	5	2	0	0	45
AET-treated	0	0	0	0	0	0	0	0	0
Anti-Kp[b]									
Untreated	10	10	8	7	6	4	2	0	47
AET-treated	0	0	0	0	0	0	0	0	0
Anti-Js[b]									
Untreated	12	10	10	10	8	6	4	1	61
AET-treated	0	0	0	0	0	0	0	0	0

Tests by antiglobulin method.

AET treatment of K_o and McLeod phenotype red cells

K_o red cells, which lack all antigens of the Kell system except Kx (Chown *et al*, 1957; Marsh *et al*, 1975b), do not become reactive with anti-K, anti-Kp[b], anti-Js[b] or anti-Ku after treatment with AET. However, the cells retain unchanged their strong reactivity with anti-Kx. McLeod-phenotype red cells have weak Kell antigens (Allen *et al*, 1961). AET-treated McLeod-type red cells do not react with anti-k, anti-Kp[b] or anti-Js[b] either by direct testing or by an antibody absorption and elution procedure. The treated cells do not react with anti-KL.

Autoimmune antibodies with specificity in the Kell system

In rare cases of IgG autoimmune haemolytic anaemia (AIHA) the autoantibody has specificity for an antigen in the Kell system (Seyfried *et al*, 1972; Beck *et al*, 1979; Marsh *et al*, 1979a). We have tested four examples of auto-anti-K and two examples of auto-anti-Kp[b] against AET-treated red cells. In each case activity of the definitive red cell antigen was

eliminated. In addition, we studied two patients whose blood contained an autoantibody that reacted with all random and most selected bloods, but did not react with K_o cells. In both cases, specificity for a known high-incidence antigen in the Kell group has been excluded. Serological details of one of these patients have been reported (Marsh *et al*, 1979b). Neither of these autoantibodies reacted with AET-treated red cells of common Kell type.

Non-specific agglutination

Red cells treated with AET may give weak non-specific reactions in the antiglobulin test when polyspecific antiglobulin reagents are used. Tests with fresh sera from 30 random blood donors showed no agglutination of saline-suspended AET-treated group O red cells, but varied reactions scoring up to 2 or 3 (Marsh, 1972) in the indirect antiglobulin test. No reactions were obtained when monospecific anti-IgG antiglobulin reagent was used. Weak reactions with anti-C3 antiglobulin reagent indicate that the phenomenon is caused by complement that binds to the membrane of treated red cells. Treated cells do not react with lectins prepared from *Arachis hypogea, Salvia sclarea, Salvia horminum, Phaseolus limensis*, or with the BSII lectin prepared from *Bandeiraea simplicifolia* seeds. Weak variable reactions were obtained with some preparations of *Glycine soja* lectin.

As a precaution against non-specific agglutination, most tests in this investigation using the antiglobulin method were made with anti-IgG reagent. In addition, confirmatory tests have been made with antibodies representative of the ABO, Rh, MN, Duffy and Kidd blood groups that were isolated by absorption and elution from reactive red cells. All of our data show that Kell antigens are inactivated by AET, but antigens of other blood group systems are unchanged.

PNH red cells

The observation by other workers that AET induces a PNH-like lesion on red cells prompted an investigation to determine whether Kell antigens are modified on red cells from patients with PNH. Clinical colleagues have kindly supplied us with blood samples and we have studied nine patients with PNH. Each patient was of common Kell phenotype. In titration studies using anti-k, anti-Kp^b, anti-Js^b and anti-Ku, scores comparable to positive controls were obtained. No evidence of a mixed red-cell population was found in any of these studies. Red cells from three patients were treated with AET. Subsequent tests showed that Kell-system blood-group antigens were inactivated. These patients had transfusion histories that ranged from a few transfusions to several hundred. The serum of six of the nine cases contained anti-K.

Cellular morphological changes caused by AET

Red cells treated with 6% AET show some fragmentation when studied by light microscopy, but no evidence of acanthocytosis or other gross changes in morphology.

DISCUSSION

2-Aminoethylisothiouronium bromide has been widely used to produce *in vitro* red cells that have PNH-like activity. Such cells have abnormal sensitivity to the lytic action of complement, and react as do PNH cells in the acidified serum and sucrose haemolysis tests (Sirchia *et al*, 1965; Ferrone *et al*, 1972). The cells also show the reduced acetylcholinesterase activity that is characteristic of the cell-membrane defect in PNH (Sirchia *et al*, 1966). However, the membrane change that is induced by AET is not the same as that present in PNH for Kell antigens of AET-treated cells are inactive, but Kell antigens of PNH red cells are not.

Lewis *et al* (1971) have compared the morphology of PNH red cells with the morphology of red cells treated with AET, using scanning and transmission electron microscopy. AET treatment induced spherical shape changes with surface craters and pits, while natural PNH cells showed electron-dense material scattered over the cell with fine surface pitting. They concluded that the morphological changes seen in both cells were similar but not identical. We have found that 6% AET does not induce gross changes in red-cell morphology, when viewed by light microscopy, and gives no evidence of the acanthocytic changes that are characteristic of the McLeod Kell variant.

The mechanism by which AET affects cell-membrane characteristics, either in creating a PNH-like defect or in inactivating Kell-system antigens, is unknown. Nor is it known whether the two defects are related, or are unrelated coincidental occurrences.

From the blood group viewpoint, the finding that Kell-system antigens are specifically inactivated by AET makes several theoretical and practical contributions. Four sets of antigens, K–k, Kp^a–Kp^b–Kp^c, Js^a–Js^b and K17–K11, and one low-incidence antigen, Ul^a, are known to be controlled by the Kell gene. However, a number of high-incidence antibodies have been described that react with random bloods but not with K_0 cells, and have all other serological characteristics typical of Kell antibodies (Issitt & Issitt, 1975; Marsh, 1975; Sabo *et al*, 1979). Proof that the antigens are products of the Kell gene has not, thus far, been possible. K12, K13, K14, K18, K19, K20 (Km) and K22 are all different high-incidence antigens of this kind. But the finding that these antigens are inactivated by AET is evidence that they have a close biochemical relationship with Kell and makes it unlikely that they are not part of the Kell system. Genetic proof by pedigree analysis that these antigens are controlled by the Kell gene awaits the finding of antibodies that define hypothetical low-incidence antigens that are produced by alleles. Here again the AET procedure may help, for if a 'new' low-incidence antigen is inactivated by AET it is probably part of the Kell complex.

From the practical immunohaematology viewpoint the AET procedure provides an artificial K_0 red cell that can be used to determine whether antibodies to high-incidence antigens have specificity in the Kell system. Furthermore, it is often difficult to determine whether antisera such as anti-k, anti-Kp^b or anti-Js^b contain other alloantibodies. Tests against a panel of AET-treated red cells will prevent activity of the Kell antibody and allow other alloantibodies to be recognized. Table III shows results from investigation of a serum containing anti-Kp^b and anti-Fy^a, that illustrates this application.

The studies on specific Kell antibodies found in autoimmune situations show that in these

Table III. Studies on a multispecific antiserum utilizing AET-treated and untreated red cells

Panel cell phenotype				Test results	
Kp^a	Kp^b	Fy^a	Fy^b	Untreated red cells	AET-treated red cells
0	+	+	+	+	+
0	+	0	+	+	0
0	+	+	+	+	+
0	+	0	+	+	0
+	+	+	0	+	+
+	0	0	+	0	0

Tests by antiglobulin method. The serum contains anti-Kp^b and anti-Fy^a.

cases, also, the reactive red-cell antigen is inactivated by AET. Similarly, the autoantibodies that react with all bloods except those of K_o type, are non-reactive with AET-treated red cells. The specificity of some of these antibodies may be anti-Ku, but it is also possible that they define other, as yet unknown, high-incidence Kell-related antigens. However, it must be concluded that these autoantibodies and antigens all have a close serological and biochemical relationship to the Kell system.

Although Kx antigen is associated with the Kell blood group it is not a product of a gene at the Kell locus. Xk, the locus that controls Kx synthesis, is X-linked (Marsh *et al*, 1975a; Marsh, 1975, 1978). Other studies have suggested that Kx substance is a precursor utilized in the normal Kell biosynthetic pathway (Marsh *et al*, 1975b). The AET data are not in disagreement with this hypothesis, for they show that Kx is not inactivated by AET, but treated red cells of common Kell type have enhanced reactivity with anti-Kx.

The weak Kell antigens present on red cells of McLeod phenotype differ qualitatively from the Kell antigens present on normal red cells (Marsh *et al*, 1975b). However, McLeod-type Kell antigens are also inactivated by AET. The observation that anti-Km (K20) does not react with AET-treated red cells indicates that Km is a part of the Kell complex, and supports the conclusion of White *et al* (1980) who considered Km to be a product of all Kell genes utilizing normal Kx-positive precursor.

Considering the known serological and genetic information on the Kell blood group, together with data from our present investigation, we think it likely that AET specifically inactivates antigens that are products of the gene, or genes, at the Kell locus. Conversely, an antigen that is not inactivated by AET is probably not coded for by a gene at the Kell locus. Biochemical studies directed towards elucidating the mechanism by which AET inactivates antigens of the Kell blood group are in progress.

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