Characterization of the Blood Group Kell (K1) Antigen With a Human Monoclonal Antibody

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A human monoclonal anti-Kell (K1) antibody secreted by an Epstein-Barr virus (EBV)-transformed B-cell line was used for binding studies and immunopurification of the K1 blood group antigen. The ^{125}I-labeled antibody bound to 4 to 5 × 10^3 and 2.5 to 3 × 10^3 antigenic sites on K1K1 and K1K2 erythrocytes, respectively, with an affinity constant of 5 × 10^8 mol/L^{-1}. Immunoprecipitation analysis showed that the K1 antigen is carried by a 93 Kd glycoprotein containing several cysteine residues, and approximately six *N*-glycosidically linked sugar chains but no detectable *O*-linked sugar. A minor labeled component of 32 Kd was also immunoprecipitated from K1K1 RBCs but the 93- and 32-Kd components were absent from K2K2 and Kell null erythrocytes. Under nonreducing conditions, three bands were detected at 200 (weak), 120, and 93 Kd. We suggest that the 120-Kd component represents a heterodimer of the 93- and 32-Kd proteins covalently linked by disulfide bridge(s). The 93-Kd glycoprotein is a transmembrane component which interacts with the membrane skeleton but is distinct from band 3 as shown by one-dimensional peptide mapping. The site density of K1 antigen blood group on Gerbich-negative RBCs (Ge:-2,-3) was threefold lower than on K1K1 erythrocytes, but the qualitative properties of the 93-Kd component were not modified.

THE KELL BLOOD system is of great medical interest since the Kell (K1) antigen is a strong immunogen in humans.[1,2] and can be responsible for fetomaternal immunization.[3] In addition, variant cells with a severe reduction of Kell antigens (McLeod phenotype) exhibit biochemical and hematologic abnormalities known as the McLeod syndrome.[4] The Kell system is a complex system in which 23 antigens have now been identified.[5]

The Kell antigens are inactivated by heating,[6] by chemical treatment with 0.2 mol/L dithiothreitol (DTT),[7] 2-aminoethylisothiouronium bromide,[8] or by simultaneous treatment with DTT and papain,[6] indicating that a protein component containing disulfide bonds is required for Kell antigenic activity. Recently, immunoprecipitation studies showed that the K1, K2, K7, K14, K22, and K23 antigens are carried by a 93-Kd glycoprotein of human erythrocytes.[9-12] This component forms oligomers with other RBC membrane proteins which, however, are not yet identified.[9,10] Independent observations showed that some Gerbich-negative RBCs have a reduced expression of the Kell antigens.[13] To investigate the molecular nature of the Kell (K1) antigen and its relationship with the Gerbich blood group antigens further, we used a human anti-K1 monoclonal antibody (MoAb) obtained from Epstein-Barr virus (EBV)-transformed B-lymphoid cells[14] for binding studies and immunopurification.

MATERIALS AND METHODS

Materials. ^{125}INa (625 MBq/μg iodine) was from the Radiochemical Centre (Amersham, Bucks, England). Protein A-Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden). Iodo-gen (1,3,4,6-tetrachloro-3α-6α-diphenylglycoluril) was from Pierce (Rockford, IL). Lactoperoxidase from bovine milk (60 U/mg) and DL-dithiothreitol (DTT) were obtained from Sigma Chemical (St Louis). Murine monoclonal antiglycophorin C (MR4-130), human polyclonal anti-K1 (serum n8730954; titer: 16), and anti-Ge:3 (Ped serum; titer: 8) were from our institute. Another polyclonal anti-K1 (titer: 32) was from Ortho Diagnostic System (Raritan, NJ), and anti-Ge:2 (Rir and C.E. sera; titers: 4) were obtained from Dr W. Dahr (Heidelberg, FRG).

Enzymatic and chemical treatments of RBCs. RBCs from K1K1, K1K2, and K2K2 donors, all phenotyped as Gerbich-positive (Ge:+2,+3) were collected at the Centre National de Transfusion Sanguine (Paris). Kell null or Ko cells (donor Rou) lacking all Kell antigens except Kx (K15) and K1-positive, Ge:−2,−3 RBCs lacking the blood group Ge antigens (donors Ped and Per) were obtained from the Centre National de Référence pour les Groupes Sangiuns (Paris) and stored in liquid nitrogen until used. Papain treatment was performed according to the instructions of the manufacturer (Diagnostic Transfusion, Marnes la Coquette, France). Chemical treatment of RBCs with DTT (0.1 or 0.2 mol/L), iodoacetamide (0.1 mol/L) and with papain plus DTT (ZZAP treatment) was performed according to published procedures.[6,7,11]

Purification and quantitative binding of MoAb to human RBCs. Human anti-Kell (K1) MoAb MID11G4 (IgG1, γ^{14}) was purified by Protein A-Sepharose chromatography,[15] and the IgG were ^{125}I-labeled by the Iodo-gen procedure[16] with a specific activity of 24 KBq/μg protein. Binding studies were performed at 37°C in polypropylene microcentrifuge tubes using 5 × 10^7 cells as described previously.[15] Unbound antibody was washed out according to the method of Briles et al.[17] The number of K1 epitopes on each erythrocyte and the antibody binding affinity were estimated by Scatchard plot analysis.[18]

Competitive binding assay. Binding of ^{125}I-labeled anti-K1 antibody to K1K2 RBCs was investigated with and without inhibitory antibody (unlabeled MoAbs or polyclonal antibodies) according to Hughes-Jones et al[19] (Fig 1). Unbound antibodies were washed out as described above. The 100% binding was estimated from control experiments performed with phosphate-buffered saline (PBS) containing 1% (wt/vol) bovine serum albumin (BSA) instead of competitor.

Solubilization of the K1 antigen by detergents. The K1 antigen content of Triton shells, prepared from K1-positive RBCs coated

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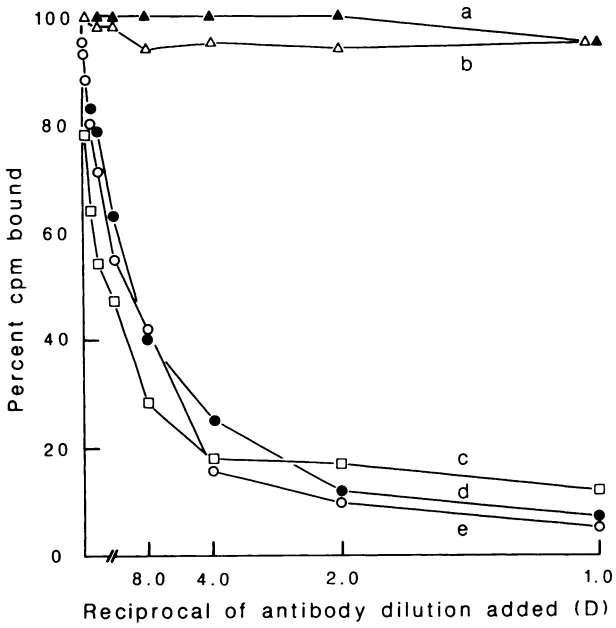

Fig 1. Competitive binding assay. K1K2 erythrocytes (2×10^7) were incubated for one hour at room temperature with 25 ng (0.6 KBq) of ^{125}I-labeled anti-K1 MoAb and the competitive antibody in a final volume of 100 μL. The amount of inhibitory antibody added is indicated as the reciprocal of the dilution. Anti-Ge:3, Ped serum or anti-Ge:2, Rir and C.E. sera tested separately (a,▲); ascitic fluid from the monoclonal antiglycophorin C, initial dilution 1:50 (b,△); unlabeled anti-K1 MoAb, 8 μg/mL concentration (c,□); anti-K1, serum n 8730954 (d,●); and anti-K 1 serum from Ortho (e,○).

with the labeled MoAb, was estimated as previously described[15] after extraction with increasing amounts of Triton X-100 (Boehringer Mannheim, West Germany).

Immunoprecipitation and purification of the K1-active protein. Five hundred microliters K1-positive cells was labeled with ^{125}I by the lactoperoxidase procedure,[20] washed and mixed with 2 mL unlabeled RBCs. The washed membranes[21] were incubated overnight at 4°C with the purified monoclonal anti-K1 (50 μg/mL membranes). The immune complexes were solubilized in 0.2% (wt/vol) sodium deoxycholate, 1% (wt/vol) Triton X-100, purified on Protein A-Sepharose[15] and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[22] For preparative purposes, the membranes from one blood unit of K1K1 or K1K2 individuals were incubated overnight at 4°C with a saturating amount of the purified MoAb. Immune complexes were isolated on Protein A-Sepharose and separated under reducing conditions by preparative SDS-PAGE (7% separating gel, 3 mm thick). The 93-Kd protein was electroeluted in 50 mmol/L ammonium bicarbonate, 0.1% (wt/vol) SDS by Protean II electroeluter (BioRad, Richmond, CA) and was further purified by precipitation with 5 vol cold acetone and dried in a vacuum speed centrifuge (Savant Instruments, New York).

Peptide mapping. Proteins were visualized by Coomassie blue staining[23] and ^{125}I-labeled within the gel slice according to the method of Elder et al.[24] Extensively washed gel slices were incubated overnight with 50 μg trypsin treated with DCC (Serva, Heidelberg, FRG) in 1 mL 50 mmol/L ammonium bicarbonate. Soluble peptides were recovered in the supernatant, dried, and analyzed by thin-layer chromatography on high-performance thin-layer chromatography (HPTLC) silica gel plates (Merck, Darmstadt, FRG).

Amino-acid and carbohydrate analyses. Aliquots containing 50 pmol purified protein were hydrolyzed in 6 N HCl containing 0.2% phenol at 110°C in sealed evacuated tubes for 20 hours. The hydrolysates were analyzed with a Beckman 7300 autoanalyser (postcolumn ninhydrin detection). Cysteine residues were determined as cysteic acid after performic oxidation. Carbohydrate analysis was performed by gas-liquid chromatography as described previously.[25]

RESULTS

Biologic activity and binding properties of the human anti-K1 MoAb. The purified MoAb agglutinated untreated K1K1 and K1K2 RBCs at low concentration (0.15 nmol/L), whereas K2K2 or Kell null were not agglutinated even at 9.5 nmol/L concentration. Agglutination of K1-positive cells from Ge:−2,−3 (donor Ped) was only observed with concentrations ≥0.6 nmol/L. The agglutination of K1 RBCs (Ge:+2,+3 or Ge:−2,−3) was not modified by treatment with papain, 0.1 mol/L DTT or 0.1 mol/L iodoacetamide. Sequential treatment of RBCs with papain and then with DTT (0.1 mol/L) did not alter the K1 epitope. However, incubation with DTT (0.1 mol/L) followed by papain or with the ZZAP reagent destroyed the K1 antigen. The agglutination of K1 erythrocytes was also abolished after sequential treatment with 0.1 mol/L DTT and iodoacetamide or with 0.2 mol/L DTT.

After ^{125}I labeling, the biologic activity of the MoAb was not modified. Scatchard analysis indicated that there were 4 to 5×10^3 and 2.5 to 3×10^3 K1 epitopes on K1K1 and K1K2 RBCs, respectively, whereas Ped erythrocytes carried only 1.2 to 1.5×10^3 antigen sites per cell. No significant binding occurred to K2K2 or Kell null RBCs. Whatever the phenotype of the RBCs used, the functional affinity was 5×10^8 mol/L.$^{-1}$

Competitive binding assay. Because expression of the K1 epitope is decreased on Ge:−2,−3 erythrocytes,[13] we tested for a relationship between the K1 and Ge epitopes by competition experiments with anti-K1 and polyclonal anti-Ge sera (Fig 1). A preliminary control showed that the binding of ^{125}I-labeled anti-K1 MoAb to K1K2, Ge-positive RBCs was almost completely inhibited by the unlabeled monoclonal anti-K1 and by two human anti-K1 polyclonal antibodies, demonstrating that the polyclonal antibodies and the human MoAb anti-K1 compete for an identical or closely located epitope. The anti-Ge antibodies (anti-Ge 3 or anti-Ge 2), which bound to glycophorins C and D,[26] did not prevent binding of the labeled anti-K1 antibody to these erythrocytes, suggesting that the K1 and Ge:2 or Ge:3 epitopes are not in close association. Similarly, a murine MoAb MR4-130[27] which binds to the *N*-terminal domain of glycophorin C[28] had no inhibitory effect.

Binding of the Kell protein to the membrane skeleton. K1-positive RBCs were coated with the ^{125}I-labeled MoAb, and the immune complexes were disrupted from the membrane with detergents. Experiments performed with Triton X-100 alone indicated that the K1-active component remained firmly bound to the membrane skeleton since only 10% to 20% of the immune complexes were solubilized with 0.1% to 1% of the nonionic detergent and ~30% could be recovered in the supernatant with a 5% detergent concentration (data not shown). However, two to four times more

immune complexes could be solubilized by addition of 0.2% sodium deoxycholate. Because prior binding of ligands to RBCs could affect the interaction of membrane components with the RBC skeleton,[29] we attempted to quantify the immunoprecipitates obtained from detergent lysates of ^{125}I-labeled cells. Only a fraction of the Kell protein was immunoprecipitated from 0.1% to 1% Triton X-100 lysates since a significant amount (at least 50%) of the K1 protein remained attached to the detergent insoluble fraction and could be partially solubilized after addition of 0.2% sodium deoxycholate. These results indicated that the Kell protein interacts firmly with the membrane skeleton. However, a potentiation of this interaction by prior antibody binding to the K1 component could not be excluded since immunoprecipitation experiments performed with detergent lysates are only semiquantitative.

Immunoprecipitation of the K1 protein. The immune complexes obtained from ^{125}I-labeled RBCs were analyzed by SDS-PAGE. After Coomassie blue staining, a major component of 93-Kd was specifically isolated from K1K1 RBCs, both under reducing and non-reducing conditions (data not shown). The 93-Kd component is a glycoprotein that could be stained with the Schiff reagent after oxidation in 2% periodic acid for 16 hours (not shown). In addition, this molecule carries external tyrosine residues which were labeled with ^{125}I on intact cells by the lactoperoxidase procedure (Fig 2). Under reducing conditions, a sharp band at 93 Kd and a weakly labeled band of 32 Kd were found with all erythrocyte samples (Fig 2, lanes 2, 3, and 5). No radioactivity could be immunoprecipitated from K2K2 RBCs (Fig 2, lane 6). As expected from the serologic results, no band was detectable in experiments performed with RBCs sequentially treated with DTT and iodoacetamide (Fig 2, lane 4). Under nonreducing conditions, the radiolabeled 93-Kd component, immunoprecipitated from native RBCs, migrated as a diffuse band. In addition, two other radioactive bands of apparent molecular weight (mol wt) 120 and 200 Kd (weak) were detected on the gels (Fig 2, lane 7). A similar pattern was observed with iodoacetamide-treated erythrocytes (Fig 2, lane 8) and with Ge:−2,−3 erythrocytes (Fig 2, lane 10), but the 120-Kd component was predominant in the former cells.

In agreement with previous reports,[9,10,12] these data indicated that the 93-Kd glycoprotein interacts, through interchain disulfide bonds, with other membrane protein(s), possibly the 32-Kd component we identified. The 32-Kd component is different from the unusual glycoprotein (mol wt 24 to 26 Kd) which is present on Ge:−2,−3 erythrocytes that are also deficient in GPC and GPD. Furthermore, immunoblotting of the immune complexes and subsequent staining with a rabbit antibody specific for the C-terminal portion of glycophorins C and D[28] were negative, again suggesting that the 32-Kd molecule is not glycophorin C (data not shown).

Purification and properties of the Kell protein. Because the 93-Kd component comigrates with band 3 in SDS-

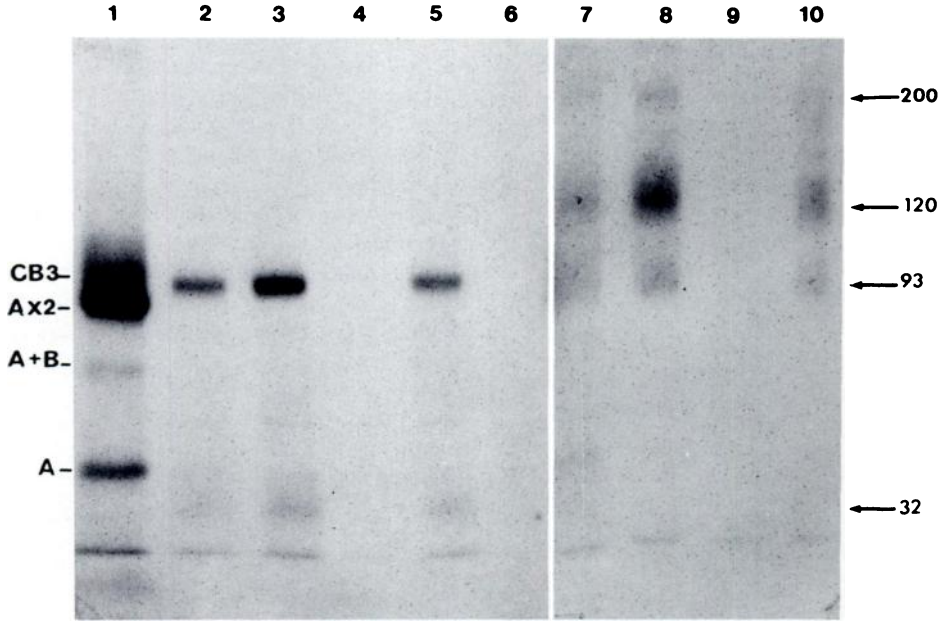

Fig 2. SDS-PAGE of immunoprecipitates obtained from ^{125}I-labeled RBCs was performed in a 7% separating gel in a discontinuous buffer system.[22] The gel was dried and exposed for four days at −80°C to Hyperfilm (Amersham) with intensifying screen (Dupont de Nemours). Lane 1: Membranes from K1K2 RBCs. Lanes 2 through 4: Immune complexes obtained from native, iodoacetamide-treated, and DTT- and iodoacetamide-treated K1-positive, Ge-positive cells, respectively. Lanes 5 and 6: Immune complexes from K1-positive, Ge-negative (Ge:−2,−3) and K2K2, Ge-positive labeled erythrocytes, respectively. Samples in lanes 1 through 6 were run under reducing conditions. Immune complexes in lanes 7 through 10 correspond to those in lanes 2 through 5, respectively, but were run under nonreducing conditions. Arrows and numbers on right refer to the apparent molecular mass ($\times 10^{-3}$) of the bands identified on the gels. Symbols on the left are as follows: Coomassie band 3 (CB3), glycophorin A dimer (A × 2), heterodimer of glycophorin A and B (A + B), and glycophorin A monomer (A).

PAGE, a structural comparison of the two molecules was performed by one-dimensional tryptic map analysis (Fig 3). We showed that the major iodopeptides released from the two ^{125}I-labeled proteins migrated differently on HPTLC, indicating that the 93-Kd protein is different from the band 3 anion transporter.

About 0.5 to 1 nmol 93-Kd protein was obtained from one blood unit of a K1K2 and a K1K1 individual, respectively. The material was pure, as judged from Coomassie blue and silver staining. Although small differences were observed, predominantly in the amount of glycine residues, the amino-acid compositions of the two preparations were similar and indicated the presence of approximately eight cysteine residues per molecule (Table 1). Carbohydrate analysis showed that the K1 glycoprotein contained 12% (wt/wt) sugars, most likely of the *N*-glycan type. No significant amounts of neuraminic acid or *N*-acetylgalactosamine could be detected, suggesting the absence of *O*-glycans. From the molar ratio of sugars, we calculated that the 93-Kd glycoprotein might carry approximately six *N*-glycosidically linked oligosaccharide chains of the biantennary type. Determination of the *N*-terminal sequence of the protein was unsuccessful, suggesting that the *N*-terminus was blocked.

DISCUSSION

Anti-K1 MoAb M1D11G4 gave results in agglutination tests and binding studies similar to those of polyclonal anti-K1 reagents.[6,7,11,30,31] This MoAb binds to an antigenic structure whose conformation is maintained by disulphide bonds.[6,8] That free sulfydryl groups are involved in the binding site appeared unlikely since biologic activity of the anti-K1 MoAb was not affected by prior treatment of K1-positive RBCs with 0.1 mol/L iodoacetamide. It was also apparent that the K1 antigen carries a proteolytic cleavage site which is unmasked after extensive reduction, possibly by disruption of an intrachain disulphide bond or by removal of a thiol-containing component attached to the Kell antigen by a disulfide linkage.

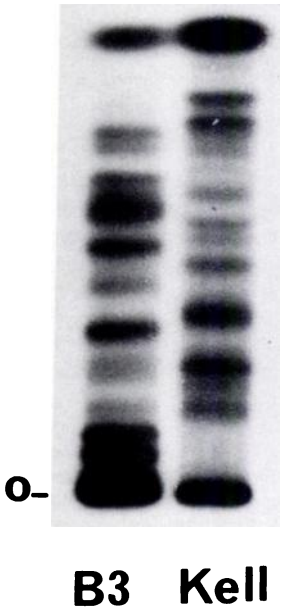

Fig 3. One-dimensional peptide map of band 3 and the 93-Kd components. Tryptic peptides were prepared as described in the Materials and Methods section. About 30 × 10^3 cpm were applied on the HPTLC silica gel plate and developed in an alkaline solvent (chloroform:methanol:ammonium hydroxide; 2:2:1, by volume) until the solvent front reached the top of the plate (~30 minutes). The air-dried plate was exposed to x-ray film at −80°C for six hours (Hyperfilm).

Table 1. Amino-Acid and Sugar Compositions of 93-Kd Protein Immunochemically Purified From K1K1 and K1K2 RBCs

Amino Acids or Sugars	K1K1	K1K2
Asx	7.72	8.12
Thr	4.90	4.89
Ser	6.96	7.92
Glx	12.65	12.42
Pro	6.17	5.28
Gly	9.88	15.26
Ala	8.67	7.04
Cys-SO3H	1.19	1.07
Val	4.90	5.67
Met	0.35	0.42
Ile	2.38	3.42
Leu	11.96	11.15
Tyr	2.21	2.03
Phe	3.32	4.40
His	4.33	2.05
Lys	5.81	3.91
Arg	6.42	4.98
Man	ND	3.0
Fuc	ND	1.1
Gal	ND	2.5
GlcNAc	ND	3.2
GalNAc	ND	0
NeuAc	ND	0

ND, not determined.

Amino-acid and sugar compositions (mol % and molar ratio, respectively). Numbers for sugars were based on three mannose residues.

About four times more MoAb than common K1-positive RBCs of the Ge-positive (Ge:+2,+3) phenotype was required to agglutinate K1-positive RBCs from donor Ped (Ge:−2,−3). However, the K1 epitope from Ge-negative RBCs (Ped) was not qualitatively different from that of Ge-positive erythrocytes, as deduced from the pattern of agglutination observed following enzymatic and chemical treatments.

Weakened expression of the Kell antigens is also a characteristic of "Leach" erythrocytes but not of Ge:−2,+3 RBCs.[32] On a structural basis, Leach erythrocytes lack glycophorins C and D as well as the abnormal glycoprotein that is present on Ge:−2,−3 and Ge:−2,+3 RBCs.[32,33] How the presence of the Ge:3 antigens prevents quantitative depression of Kell antigens is not now clear, since competitive binding assays indicated that K1 and Ge:3 epitopes are not related. In addition, the results from agglutination tests with enzymatically and chemically treated erythrocytes showed that the K1 antigen of Ge:−2,−3 RBCs was not qualitatively altered.

In agreement with other investigators,[9-11] our results showed that the K1 antigen is localized on a 93-Kd glycoprotein. The molecule contained cysteine residues, probably involved in its biologic activity, and carried approximately six *N*-glycans but no *O*-glycans. The Kell glycoprotein, like glycophorins C and D and Rh polypeptides,[15,34-36] interacts with the RBC membrane skeleton and therefore belongs to the class of transmembrane proteins. Such protein–protein associations may play an important role in membrane func-

tion since certain variant RBCs with abnormal expression or total lack of these antigens (McLeod, Leach, Rh null, and Rh mod phenotypes) exhibit biochemical and morphologic alterations.[4,32] This is not, at least for the Kell system, related to the antigens themselves, since Ko erythrocytes which lack all Kell antigens have a normal viability and shape. Ko cells, however, have a strong expression of the K15 (or Kx) antigen which is the product of an X-linked gene encoding a 37-Kd RBC surface protein.[37] When the K15 antigen is absent, as in McLeod erythrocytes, the Kell antigens are severely depressed and the cells exhibit a number of hematologic and biochemical abnormalities known as the McLeod syndrome.[4]

The-32 Kd component could represent a unique protein interacting with the 93-Kd component, and the 120-Kd component identified in non reducing conditions corresponds to a heterodimer of these two proteins linked by a disulfide bridge. The 32-Kd molecule may be identical to the Kx protein (37 Kd), recently identified by Redman et al.[37] This would correlate with the possibility that the Kell and Kx antigens represent two subunits associated by an interchain disulfide bond.[38] Alternatively, the 32-Kd species could be a degradation product, but this hypothesis seems unlikely since this component was absent when the immune complexes were analyzed under nonreducing conditions (Fig 3, lanes 7 through 10). Further investigations will be required to clarify the molecular relationship—certainly critical for normal cell function (described above, McLeod syndrome)—between the Kell (93 Kd) and Kx (37 Kd) proteins.

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