Rapid Papers

(Pages 333–352)

Biochem. J. (1978) **173**, 333–336
Printed in Great Britain

Blood-Group-I Activity Associated with Band 3, the Major Intrinsic Membrane Protein of Human Erythrocytes

By ROBERT A. CHILDS,* TEN FEIZI,* MINORU FUKUDA† and SEN-ITIROH HAKOMORI†
**Division of Communicable Diseases, Clinical Research Centre, Harrow, Middx., U.K., and †Division of Biochemical Oncology, Fred Hutchinson Cancer Research Centre, University of Washington, Seattle, WA 98104, U.S.A.*

(*Received* 23 *March* 1978)

Affinity chromatography of radioiodinated solubilized erythrocyte stroma and of radioiodinated purified Band 3 on an anti-(blood-group I)-adsorbent column showed blood-group-I activity associated with a subpopulation of Band 3. The specificity of binding was confirmed by inhibition with known blood-group substances in radioimmunoassays.

Band 3, the major intrinsic protein of the erythrocyte membrane, has attracted much interest as it is believed to be involved in the transport of small molecules across the membrane (reviewed by Marchesi *et al.*, 1976). It contains approx. 5–8% carbohydrate and migrates as a diffuse band on SDS/polyacrylamide-gel electrophoresis with an apparent mol.wt. of 90000–100000. Band 3 contains populations of molecules with differing binding characteristics towards concanavalin A and *Lens culinaris* and *Ricinus communis* lectins, suggesting heterogeneity in its carbohydrate chains (Findlay, 1974). The present report describes the occurrence of blood-group-I antigenic activity (Race & Sanger, 1975) associated with a subpopulation of this protein.

Experimental

Preparation and iodination of erythrocyte stroma and of Band 3 protein

Erythrocyte stroma was prepared by the method of Dodge *et al.* (1963) from fresh defibrinated blood from a blood-group-O adult. Solubilization of the stroma suspended in 50mM-Tris/HCl, pH8.2, at a protein concentration of 2mg/ml [determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard] was achieved by adding Empigen B.B. (Allbright and Wilson, Whitehaven, Cumbria, U.K.) to a final concentration of 2% (v/v) (for 60min at 0°C) and SDS to a final concentration of 1% (w/v) (for 15min at 20°C). The solubilized stroma (400μg of protein) was labelled with carrier-free ^{125}I (The Radiochemical Centre, Amersham, Bucks., U.K.) by the chloramine-T method (Greenwood *et al.*, 1963). Radioactivity was measured in a Nuclear Enterprises 1600 gamma counter. The labelled stroma had 2×10^6 c.p.m./μg of protein; 53% of these counts were precipitable in ice-cold 10% (w/v) trichloroacetic acid.

Band 3 was isolated from erythrocyte stroma of fresh human blood by a novel three-step procedure (Fukuda *et al.*, 1978) involving: (1) treatment of the stroma with 0.1mM-EDTA, pH8.0, to remove spectrin and Band 5 followed by extraction with lithium 3,5-di-iodosalicylate, which removes most of the remaining extrinsic proteins; (2) chromatography on an activated-thiol Sepharose column to separate Band 3 from the sialoglycopeptides and Band 7; (3) gel filtration on Sepharose 6B. The purified Band 3 was extracted twice with chloroform/methanol (2:1, v/v) and reconstituted in 100mM-Tris/HCl, pH8.2, containing 2% SDS at 37°C for 30min followed by 80°C for 3min. It was dialysed against 10mM-Tris/HCl, pH8.2, containing 0.2% SDS and labelled with ^{125}I, as described above. The labelled Band 3 had 2.5×10^6 c.p.m./μg of protein; 91% of these counts were precipitable in 10% trichloroacetic acid.

Affinity chromatography of solubilized iodinated stroma and Band 3

Iodinated solubilized stroma or Band 3 (200μg of protein containing 4×10^8 and 5.1×10^8 c.p.m. respectively) in 6ml of equilibration buffer (10mM-Tris/HCl, pH8.2, containing 1% Empigen, 0.02% SDS, 0.1% bovine serum albumin and 0.02% sodium azide) was passed through an anti-(blood-group I) or a control column. The former contained 50mg of antibody (a macroglobulin) isolated from the serum of a patient (Low) and coupled to 5ml of Sepharose 2B as described by Feizi *et al.* (1975), The control adsorbent contained 50mg of a Waldenström

Abbreviation used: SDS, sodium dodecyl sulphate.

macroglobulin without anti-(blood-group I) activity. Adsorption was carried out at 4°C (flow rate, 2ml/h); unbound material was removed by washing with equilibration buffer at 4°C (flow rate, 40ml/h); bound material was eluted in two steps by washing with (1) equilibration buffer at 37°C and (2) equilibration buffer containing 1M-NaI at 4°C. The NaI eluate was immediately dialysed against running tap water for 2h followed by 100vol. of equilibration buffer at 4°C for 18h.

Polyacrylamide-gel electrophoresis

Samples for electrophoresis were made up to 3% (w/v) SDS, 2M-urea, 2.5% (v/v) 2-mercaptoethanol, 5% (w/v) sucrose and 0.1mg of Bromophenol Blue/ml in 10mM-Tris/HCl buffer, pH6.8, and incubated at 37°C for 30min. Electrophoresis was performed at 100V in 7.5% (w/v) polyacrylamide slab gels containing 0.1% SDS and 0.5M-urea by the system of Laemmli (1970). The gels were then frozen at −70°C and cut into 1mm slices with a Mickle gel slicer (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) and radioactivity was counted directly.

Radioimmunoassays

Binding curves were obtained by a double-antibody method (Lecomte & Feizi, 1975; Wood *et al.*, 1978) with an anti-(blood-group I) serum (from patient Step) and ^{125}I-labelled Band 3 [37°C eluate from the anti-(blood-group I) column] or a ^{125}I-labelled blood-group-I-active sheep stomach glycoprotein (S). Inhibition of binding assays was performed with the following inhibitors: (*a*) Band 3, (*b*) glycoprotein S, (*c*) a blood-group-B-active (blood-group-I-inactive) ovarian-cyst glycoprotein Beach (donated by Dr. E. A. Kabat, Columbia Medical Center, New York, NY, U.S.A.) and (*d*) a blood-group-I-active glycolipid purified from bovine erythrocytes (Niemann *et al.*, 1978). For the assay, the glycolipid was complexed with cholesterol and phosphatidylcholine, as described by Feizi *et al.* (1978).

Results and Discussion

The affinity-chromatography profiles of the solubilized ^{125}I-labelled blood-group O erythrocyte stroma on the anti-(blood-group-I) column and the control column were markedly different. Elution at 37°C or in the presence of 1M-NaI produced radioactivity peaks from the anti-(blood-group I) column, but not from the control column (Fig. 1*a*). On SDS/polyacrylamide-gel electrophoresis the radioactivity profile of the original stroma showed three major peaks with apparent mol.wts. of approx. 200000, 100000 and less than 20000 (Fig. 1*b*). The first peak could represent spectrin (Fairbanks *et al.*, 1971), the second corresponds to Band 3 and the third, which migrated near the Bromophenol Blue dye marker, could well represent uptake of ^{125}I by membrane lipids (Butters & Hughes, 1975). The two eluates from the anti-(blood-group I) column showed radioactivity peaks in the area corresponding to Band 3 (Figs. 1*c* and 1*d*). In addition, considerable radioactivity was present at the origin and in the region of the dye marker. The latter could represent iodinated membrane glycolipids with blood-group-I activities retained by the anti-(blood-group I) column. Erythrocyte glycolipids with blood-group-I activities have been described by Watanabe *et al.* (1975), Gardas (1976), Koscielak *et al.* (1976) and Feizi *et al.* (1978).

Affinity chromatography of purified ^{125}I-labelled Band 3 on the anti-(blood-group I) column yielded an eluate at 37°C that amounted to 3% of the total trichloroacetatic acid-precipitable radioactivity applied. Elution in the presence of 1M-NaI yielded only 0.2% of the total radioactivity applied. Eluates from the control column yielded only 0.2 and 0.1% of the total radioactivity applied. The 3% recovery may be an underestimate of the proportion of Band 3 molecules carrying blood-group-I antigenic determinants, since only molecules with multiple antigenic determinants but not single determinants bind to an anti-(blood-group I) adsorbent (T. Feizi & D. A. Zopf, unpublished observations). The radioactivity profiles of the original labelled Band 3, the unretained fraction and the two eluted fractions were similar. In each instance, the major band was diffuse with an apparent mol.wt. of about 100000 (Figs. 1*e*–1*h*). Considerable radioactivity remained near the origin and two minor bands were observed: one at an apparent mol.wt. of 65000 and the other near the dye front. The high radioactivity at the origin of the polyacrylamide gels of the stroma eluates and of the isolated Band 3 and its fractions could represent aggregated Band 3 (Fairbanks *et al.*, 1971). Although the minor bands contain a trivial proportion of the total radioactivity recovered from the gels, their presence in all the Band 3 fractions requires further investigation. It is likely that they represent products of partial degradation of Band 3 protein (Cabantchik & Rothstein, 1974). The minor band running with the dye front could, however, represent glycolipids with an unusually tight association with Band 3, which spans the erythrocyte lipid bilayer (Bretscher, 1971; Jenkins & Tanner, 1975; Steck *et al.*, 1976).

The blood-group-I activity associated with Band 3 was confirmed by the binding of the ^{125}I-labelled 37°C eluate from the anti-(blood-group I) column (Low) to a second anti-(blood-group I) antibody (Step) in radioimmunoassays (Fig. 2*a*). This binding was inhibitible by unlabelled Band 3, and by the blood-group-I-active glycoprotein and glycolipid, but not

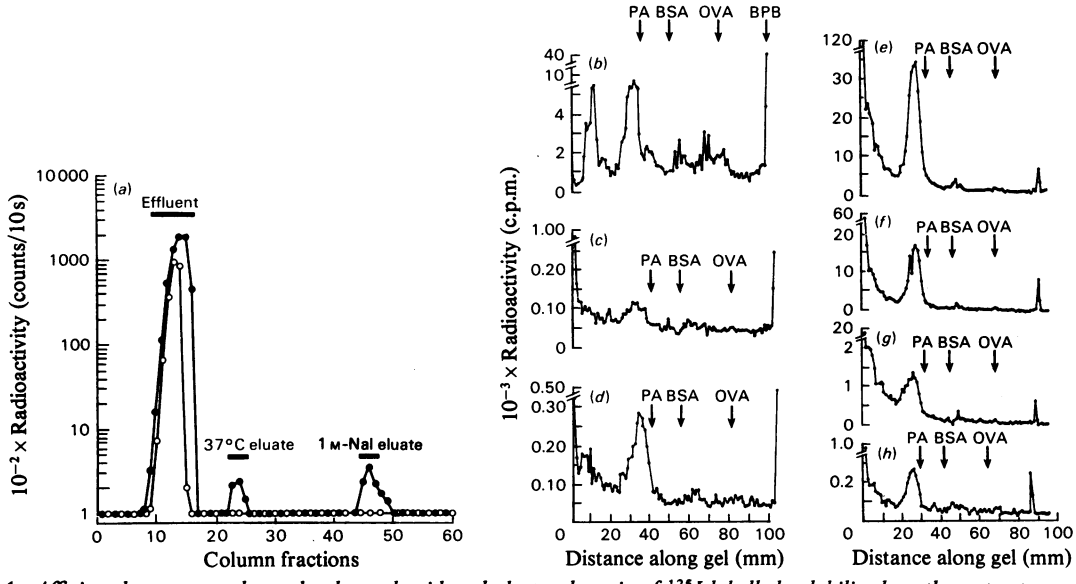

Fig. 1. *Affinity chromatography and polyacrylamide-gel electrophoresis of ^{125}I-labelled solubilized erythrocyte stroma and of isolated Band 3*

(*a*) Iodinated solubilized stroma containing 200μg of protein and 4×10^8c.p.m. was passed through an anti-(blood-group I) column (●) as described in the text, or a control macroglobulin column lacking anti-(blood-group I) specificity (○). Fraction volumes were: 0.5ml for unbound fractions, 10ml for washing fractions and 1ml for the 37°C and the NaI eluates; radioactivity was measured on 10μl samples. (*b–h*) Polyacrylamide-gel electrophoresis of iodinated solubilized stroma, of iodinated isolated Band 3 and of their fractions obtained after passing through the anti-(blood-group I) column. Electrophoresis was performed as described in the text; migration was from left to right. (*b–d*) Original solubilized stroma, 37°C eluate and NaI eluate respectively; (*e–h*) original Band 3, unretained fraction, 37°C eluate and NaI eluate respectively. Molecular-weight markers were: PA, phosphorylase *a* (94000); BSA, bovine serum albumin (68000); OVA, ovalbumin (43000). BPB, Bromophenol Blue.

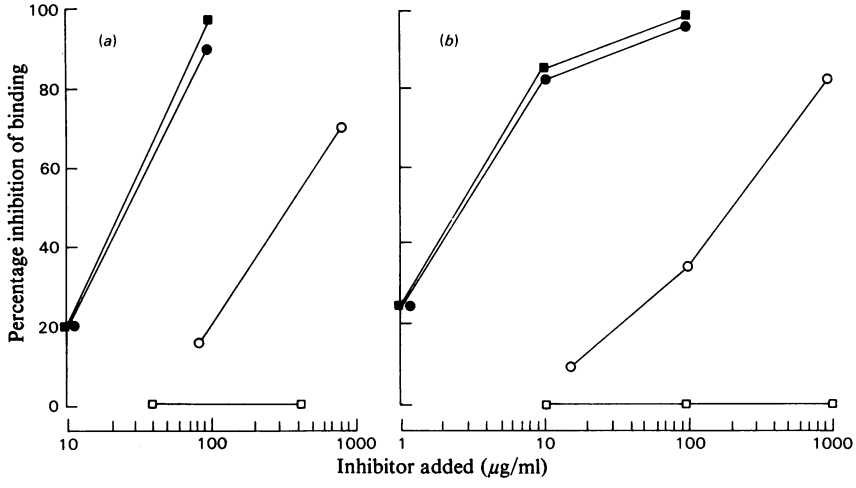

Fig. 2. *Inhibition of binding assays with the anti(blood-group I) serum of patient Step*

Inhibition of binding assays were performed with a dilution of anti-(blood-group I) serum that gave approx. 20% binding to the radioiodinated antigen. (*a*) Inhibition of binding of anti-(blood-group I) antibody (Step) at 1:100 dilution to the ^{125}I-labelled 37°C eluate of Band 3; (*b*) inhibition of binding of anti-(blood-group I) antibody (Step) at 1:2000 dilution to ^{125}I-labelled glycoprotein S. Symbols for unlabelled inhibitors: ■, glycoprotein S; ●, blood-group-I-active glycolipid; ○, original preparation of Band 3; □, blood-group-B-active (I-inactive) ovarian-cyst glycoprotein.

by the blood-group-B-active glycoprotein (Fig. 2*a*). In additional radioimmunoassays (Fig. 2*b*), unlabelled Band 3 was shown to inhibit the binding of anti-(blood-group I) antibody (Step) to the blood-group-I-active glycoprotein S.

These data show that, among the solubilized erythrocyte proteins that are iodinatable, blood-group-I activity is clearly associated with Band 3. Blood-group-I antigens are known to be associated with precursors of the blood-group-ABH antigens (Feizi *et al.*, 1971). The present studies raise the intriguing question as to whether other blood-group activities are associated with Band 3 and whether these and the precursor-type oligosaccharide chains are in some way related to the function of this protein. A branched oligosaccharide chain containing fucose on a blood-group-precursor-like structure, which would be predicted to have blood-group activity (Feizi *et al.*, 1971), has recently been isolated from Band 3 by Osawa (1978).

M. F. and S. H. were supported by The National Cancer Institute, Grant CA 19224.

References

Butters, T. D. & Hughes, R. C. (1975) *Biochem. J.* **150**, 59–69

Bretscher, M. S. (1971) *J. Mol. Biol.* **59**, 351–357

Cabantchik, Z. I. & Rothstein, A. (1974) *J. Membr. Biol.* **15**, 227–248

Dodge, J. T., Mitchell, C. & Hanahan, D. J. (1963) *Arch. Biochem. Biophys.* **100**, 119–130

Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617

Feizi, T., Kabat, E. A., Vicari, G., Anderson, B. & Marsh, W. L. (1971) *J. Immunol.* **106**, 1578–1592

Feizi, T., Cederqvist, L. L. & Childs, R. (1975) *Br. J. Haematol.* **30**, 489–497

Feizi, T., Childs, R. A., Hakomori, S.-I. & Powell, M. E. (1978) *Biochem. J.* **173**, 245–254

Findlay, J. B. C. (1974) *J. Biol. Chem.* **249**, 4398–4403

Fukuda, M., Eshdat, Y., Tarone, G. & Marchesi, V. T. (1978) *J. Biol. Chem.* in the press

Gardas, A. (1976) *Eur. J. Biochem.* **68**, 185–191

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123

Jenkins, R. E. & Tanner, M. J. A. (1975) *Biochem. J.* **147**, 393–399

Koscielak, J., Miller-Podraza, H., Krause, R. & Piasek, A. (1976) *Eur. J. Biochem.* **71**, 9–18

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Lecomte, J. & Feizi, T. (1975) *Clin. Exp. Immunol.* **20**, 287–302

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Marchesi, V. T., Furthmayr, H. & Tomita, M. (1976) *Annu. Rev. Biochem.* **45**, 667–698

Niemann, H., Watanabe, K., Hakomori, S., Childs, R. & Feizi, T. (1978) *Biochem. Biophys. Res. Commun.* in the press

Osawa, T. (1978) *Proc. Int. Symp. Glycoconjugates 4th* in the press

Race, R. R. & Sanger, R. (1975) *Blood Groups in Man*, 6th edn., pp. 327–335, Blackwell Scientific Publications, Oxford

Steck, T. L., Ramos, B. & Strapazon, E. (1976) *Biochemistry* **15**, 1154–1161

Watanabe, K., Laine, R. A. & Hakomori, S. (1975) *Biochemistry* **14**, 2725–2733

Wood, E., Lecomte, J., Childs, R. & Feizi, T. (1978) *Proc. Int. Symp. Glycoconjugates 4th* in the press