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Dissection of the human erythrocyte spectrin molecule using monoclonal antibodies

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One IgM and three IgG monoclonal antibodies specific to band 1 of human erythrocyte spectrin have been characterised. The antigenic sites of the IgG antibodies have been identified and mapped by radioimmune labelling of tryptic fragments of spectrin fractionated by SDS slab gel electrophoresis and blotted onto nitrocellulose filters. The binding site of one of these antibodies has also been directly visualised in the electron microscope after low-angle shadowing of the antibody-spectrin dimer complex, and lies at that end of the dimer which is responsible for tetramer formation.
Key words: erythrocyte/spectrin/monoclonal antibody

Introduction

Spectrin is the major structural protein of human erythrocyte membranes and is intimately involved in the control of red cell shape and deformability. When eluted from erythrocyte membranes at 37°C, spectrin is found as an $\alpha\beta$ heterodimer (Ralston *et al.*, 1977) consisting of one copy of band 1 (mol. wt. 240 000) and one copy of band 2 (mol. wt. 220 000) (nomenclature of Steck, 1974). The heterodimer has been shown to be an extended molecule of length 97 nm, composed of two strands lying side by side and running the entire length of the molecule (Shotton *et al.*, 1979). In 0°C extracts, however, spectrin is found predominantly in the form of a 960 000 dalton tetramer (Ralston, 1975) composed of two heterodimers associated head to head (Shotton *et al.*, 1979; Tyler *et al.*, 1979).

Spectrin is known to associate directly with at least three other major protein components of the erythrocyte membrane. Ankyrin (band 2.1) binds to band 2 at sites ~20 nm either side of the mid-point of the spectrin tetramer (Tyler *et al.*, 1979) and serves to link spectrin molecules to the major integral membrane protein of the membrane, the anion channel (band 3). In addition, band 4.1 and actin (band 5) both bind to sites very close to the ends of the spectrin tetramers, crosslinking them into an extensive two-dimensional protein meshwork, the erythrocyte membrane skeleton, which laminates the cytoplasmic surface of the lipid bilayer and which imparts to the cell membrane its extraordinary strength and elastic properties (Tyler *et al.*, 1979, 1980; Cohen *et al.*, 1980).

Very little is known about the substructure of either of the spectrin polypeptide chains, although Morrow *et al.* (1980) and Speicher *et al.* (1980) have shown by limited proteolytic digestion that each chain may be cleaved into a small number of discrete, large and structurally stable domains which are relatively resistant to tryptic attack and which uniquely contain individual binding sites for other polypeptides.

Since one prerequisite for a complete understanding of how spectrin functions in the erythrocyte must be a detailed knowledge of the structure of this enormous molecule, we have undertaken an immunological dissection of the molecule using monoclonal antibodies. Experiments are described here in which we have localised, at the chemical level, the binding sites on spectrin of three monoclonal anti-spectrin antibodies. In one case, the binding site has also been defined electron microscopically.

Results

Monoclonal antibody characterization

Four hybridoma clones secreting monoclonal anti-spectrin have been characterized, using the procedures introduced by Köhler and Milstein (1975). Two of these (clones 706 and 717) were obtained in fusions between spleen cells from spectrin-immunized mice and the SP2/0-Ag14 hybridoma cell line (Shulman *et al.*, 1978) while the remaining two (718/a and 718/b) were produced in a fusion with the X63-Ag8 myeloma line (Köhler and Milstein, 1975). The secretory products of these cell lines, labelled by incorporation of [^{14}C]leucine, have been analysed by SDS-polyacrylamide slab gel electrophoresis. As shown in Figure 1, the 717, 718/a, and 718/b cell lines all appear to secrete IgG, whereas clone 706 produces an IgM. Clone 718/a, in addition to secreting the specific anti-spectrin immunoglobulin light chain, continues to synthesise a small amount of the X63-Ag8 (MOPC-21) κ chain, which is the

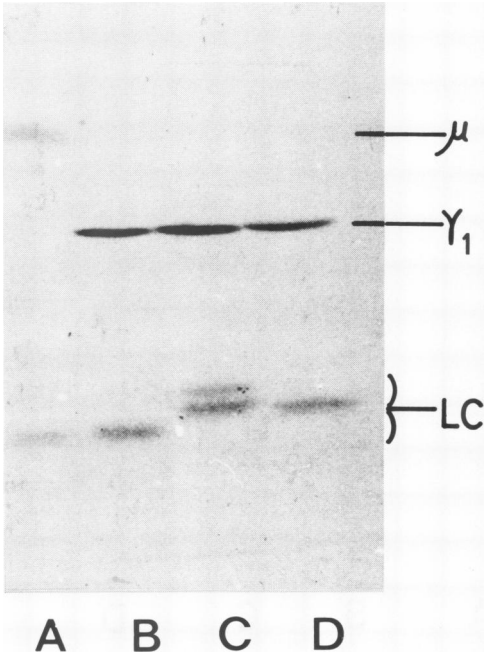

Fig. 1. Analysis by SDS gel electrophoresis and autoradiography of immunoglobulins secreted by four hybrid cell lines metabolically labelled with [^{14}C]leucine **(a)** 706, **(b)** 717, **(c)** 718/a, **(d)** 718/b. **(a)** and **(b)** were from Sp2/0-Ag14 fusions, whereas **(c)** and **(d)** were from an X63-Ag8 fusion.

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higher mol. wt. of the two light chain bands. Clone 718/b, however, does not express the X63-Ag8 κ chain.

Immunochemical identification of antigenic sites

All three monoclonal IgGs (717, 718/a and 718/b) have been shown to recognise antigenic determinants on band 1 by utilising purified band 1 in a solid phase radio-immune assay (Burke, 1981). In order to determine whether these antibody binding sites are unique to band 1, total red cell ghost protein was separated by analytical SDS-polyacrylamide slab gel electrophoresis and then blotted onto nitrocellulose filters (Bowen *et al.*, 1980). Strips from the filters were first individually labelled with one of the monoclonal antibodies and were then labelled with [^{125}I]rabbit anti-mouse IgG. Autoradiographs of these filters are shown in Figure 2. Clearly, each of the IgGs recognises a site found only on band 1. Under identical conditions the 706 IgM produces no labelled bands (results not shown). When this experiment was repeated using the direct gel labelling technique devised by Burridge (1976), where protein bands are labelled by overlaying the gel with antibody, the 717 and 718/a antibodies recognised band 1 as before, whereas the 718/b antibody failed to bind (results not shown). This result indicated to us that the 718/b antibody probably recognises some specific region of secondary structure in the band 1 polypeptide, since these two labelling techniques differ in that in the direct labelling method the SDS denatured polypeptide bands are fixed *in situ* before labelling, while in the blotting technique at least some degree of recovery of native three-dimensional conformation is possible during transfer of the unfixed polypeptides to the nitrocellulose filter (see Materials and methods).

In order to define further the locations of the antibody binding sites on the band 1 polypeptide, spectrin was briefly digested with trypsin and the fragments separated by SDS gel electrophoresis followed by blotting and labelling as before. The results are shown in Figure 3. The three IgGs showed a remarkably simple pattern of labelling of the complex array of digestion products. Furthermore, the 717 and 718/b antibodies showed identical patterns of labelling whereas that produced by the 718/a antibody appeared quite distinct from the other two.

Electron microscopy of spectrin-antibody complex

The 718/a antibody has been purified from ascites fluid and used to label spectrin heterodimers prior to low-angle shadowing and electron microscopy. As shown in Figure 4, this antibody labels a single site very close to one terminus of the heterodimer. Similar labelling of tetramers (not shown) indicated that the antibody binds very close to the dimer-dimer association region, since no tetramers were found to be terminally labelled but a small number were identified with an antibody molecule situated centrally.

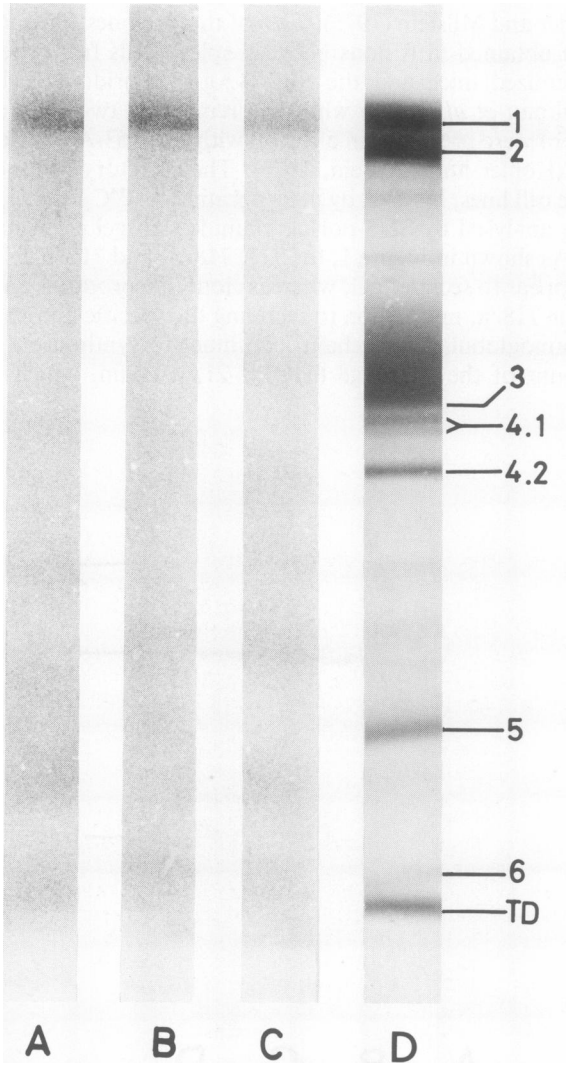

Fig. 2. Determination of antibody specificity. **(a)** to **(c)** Autoradiographs of erythrocyte membrane proteins fractionated on SDS polyacrylamide gels, blotted onto nitrocellulose filter strips and labelled with **(a)** 717 antibody, **(b)** 718/a, **(c)** 718/b. **(d)** Unblotted gel strip stained with Coomassie brilliant blue.

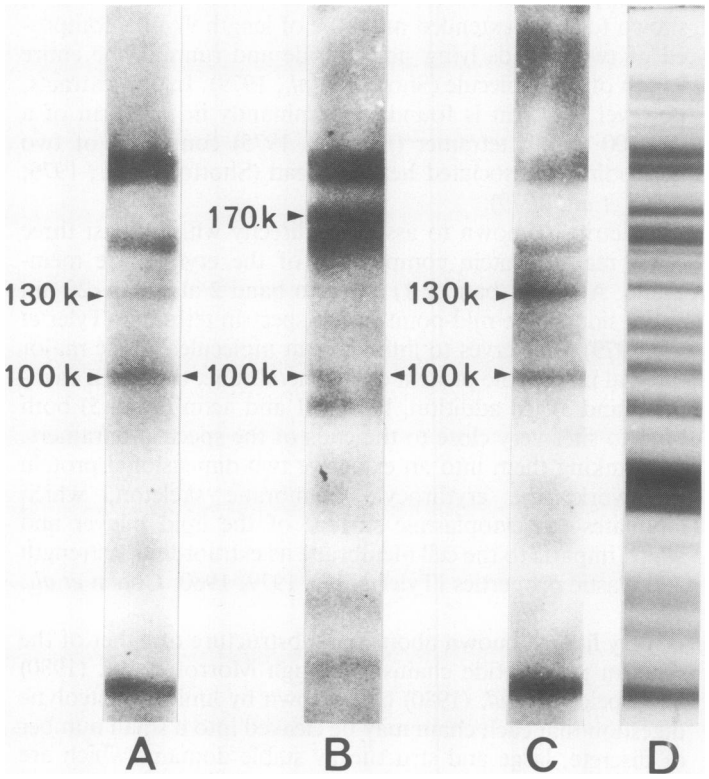

Fig. 3. Localisation of the antibody binding sites on band 1 fragments. **(a)** to **(c)** Autoradiographs of a tryptic digest of spectrin fractionated on a polyacrylamide gel, blotted onto a nitrocellulose filter and labelled with **(a)** 717 antibody, **(b)** 718/a, **(c)** 718/b. **(d)** Unblotted gel strip stained with Coomassie brilliant blue.

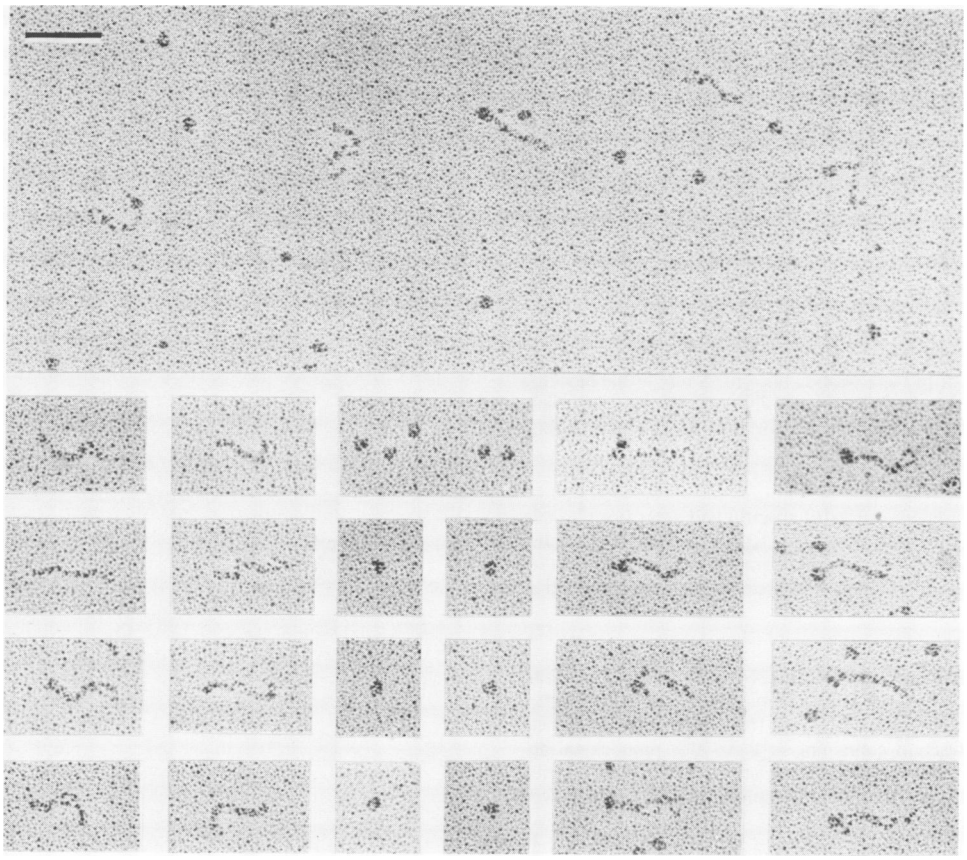

Fig. 4. Electron microscopic localisation of the 718/a antibody binding site on spectrin heterodimers. **Main field**: 718/a antibody plus spectrin heterodimers. **Left hand two columns**: individual spectrin heterodimers. **Centre pair of columns**: 718/a antibody molecules. **Right hand two columns**: individual heterodimers each with a single antibody bound at one terminus. All magnifications x 100 000. Bar = 100 nm.

Discussion

The specificity of these monoclonal antibodies, raised against whole human erythrocyte spectrin, for band 1 alone proves quite conclusively that band 1 is antigenically distinct from band 2, a finding which has not been at all obvious in previous reports in which polyclonal antisera have been utilised (Bjerrum *et al.*, 1975; Ziparo *et al.*, 1978). Whether band 1 is more immunogenic than band 2 is as yet uncertain in view of our limited results, although Sheetz *et al.* (1976) have provided some evidence that this may indeed be the case.

The direct visualization, by electron microscopy after low-angle shadowing, of a monoclonal IgG antibody molecule bound to its unique antigenic-specific site on an isolated spectrin molecule demonstrates the general usefulness of this procedure for the physical location of antigenic sites on large macromolecules, and hence for the correlation of structural and chemical data.

We have shown that it is feasible to dissect immunologically a large protein molecule such as spectrin using monoclonal antibodies, and have demonstrated that the protein blotting technique provides a means of defining the chemical location of the antibody binding sites with considerable accuracy, since the same antibodies may be used in conjunction with several different proteolytic enzymes. We now wish to extend these studies to more carefully defined digests of spectrin (Speicher *et al.*, 1980) and to produce a range of monoclonal anti-spectrin antibodies with which both to map and to isolate specific functional segments from both polypeptide chains of this important membrane skeletal protein.

Materials and methods

Spectrin preparation

Spectrin was prepared by low ionic strength extraction from erythrocyte ghosts, and purified by gel filtration on Sepharose CL 4B, as detailed by Shotton *et al.* (1979).

Monoclonal antibody production

Monoclonal antibodies were obtained using the procedures introduced by Köhler and Milstein (1975). Balb/c mice were immunised i.p. with 50 μg purified human spectrin emulsified with Freund's complete adjuvant (Gibco). One month later the mice were boosted with a similar dose in incomplete adjuvant. Three days after this boost their spleens were removed and the dissociated spleen cells (10^8) fused with 10^7 myeloma cells (either X63-Ag8 or Sp2/0-Ag14) using polyethylene glycol 1500 (BDH) essentially as described by Galfre *et al.* (1977). After fusion, the cells, in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% new born calf serum (hybridoma medium), were distributed in 1 ml aliquots in 2 x 24 well Costar trays. 5 x 10^5 mouse peritoneal macrophages were also added to each well.

After a 24-h incubation, 1 ml of hybridoma medium supplemented with hypoxanthine, aminopterin, and thymidine (HAT) was added to each well. 1 ml of medium was replaced with 1 ml of fresh HAT medium on the following 2 days and then at 2-day intervals. After 2–3 weeks each well was tested for anti-spectrin activity by means of a solid phase radio-immune assay (Catt and Tregear, 1967) in flexible PVC microtitration plates (Cooke). Cells from anti-spectrin positive wells were cloned by plating at low density in soft agar (Cotton *et al.*, 1973). Clones were picked out of the agar at random, grown up in individual Costar tray wells, and then retested. This cloning procedure was repeated twice for each hybrid cell line obtained.

Characterization of monoclonal antibodies

To analyse the secreted products from cloned hybridomas, cells were grown for 18 h at a concentration of 10^6 cells/ml in leucine-free DMEM (Gibco) supplemented with 5% dialysed foetal calf serum and 5 μCi/ml [^{14}C]leucine (Amersham). Culture supernatants were analysed by electrophoresis on SDS 10% polyacrylamide slab gels (Laemmli, 1970) followed by autoradiography.

Specificities of the antibodies were determined by labelling of spectrin bands blotted from SDS gels onto nitrocellulose filters. Total red blood ghost proteins were separated by SDS-polyacrylamide slab gel electrophoresis (7.5% polyacrylamide) and then blotted onto a sheet of nitrocellulose exactly as described by Bowen *et al.* (1980) following a 3-h immersion of the gel in 4 M urea, 50 mM NaCl, 2 mM EDTA, 0.1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.0. Immediately after transfer, all remaining protein binding sites on the filter were quenched to reduce non-specific binding of antibody by incubation at room temperature in a blocking solution consisting of phosphate buffered saline (PBS), pH 7.6, containing 1% gelatin and 0.05% Nonidet P-40 (BDH). Identical longitudinal strips from the blocked filter were cut and separately immersed in ~50 ml of cell culture supernatants containing monoclonal antibody and incubated for 2 h at 37°C. The strips were given three 5-min washes in blocking solution and then overlaid with [^{125}I]rabbit anti-mouse IgG, prepared from the unlabelled antibody (Miles) by chloramine-T iodination, at ~2 x 10^5 c.p.m./cm^2. Incubation with labelled antibody was for 2 h at room temperature. The labelled strips were washed once for 5 min in the blocking solution and then three times for 5 min each in PBS. Finally, they were thoroughly air dried and autoradiographed for 6–8 h at −70°C using presensitized film and an intensifying screen.

Labelling of spectrin fragments

A crude 0°C low ionic strength extract of spectrin and actin from human erythrocyte ghosts was digested with trypsin for 10 min at room temperature as described by Anderson (1979). The tryptic fragments were then run out on SDS-polyacrylamide slab gels (7.5% polyacrylamide), blotted and labelled as described above.

Production of ascites fluid

Hybridoma cells (718/a) in serum-free DMEM were injected i.p. into pristane primed (Koprowski *et al.*, 1977) Balb/c mice (10^7 cells/mouse). After 2 weeks antibody-rich ascites fluid was obtained from the mice by peritoneal puncture. The bulk IgG fraction containing at least 90% monoclonal antibody, as judged by radio-immune assay and electrophoresis, was purified from cell-free ascites fluid by 40% ammonium sulphate precipitation followed by chromatography on DE52 (Whatman).

Antibody labelling of spectrin for low-angle shadowing

Spectrin heterodimers and monoclonal antibody 718/a, purified from ascites fluid, both at ~0.1 mg/ml, were mixed in PBS. The mixture was incubated for 18 h at 4°C, after which it was diluted x 10 with 155 mM ammonium acetate pH 7.4 containing 70% glycerol, sprayed onto the surface of freshly cleaved mica, and then dried and rotary shadowed as previously described (Shotton *et al.*, 1979). Replicas were picked up on bare 400 mesh copper grids and photographed in a Hitachi H-600 electron microscope operated at 75 kV under conventional conditions.

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References

Anderson,J.M. (1979) *J. Biol. Chem.*, **254**, 939-944.
Bjerrum,O.J., Bhakdi,S., Bog-Hansen,T.C., Knufferman,H., and Wallach, D.F.H. (1975) *Biochim. Biophys. Acta*, **406**, 489-504.
Bowen,B., Steinberg,J., Laemmli,U.K., and Weintraub,H. (1980) *Nucleic Acids Res.*, **8**, 1-20.
Burke,B.E. (1981) Ph.D. Thesis, University of London.
Burridge,K. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 4457-4461.
Catt,K., and Tregear,G.W. (1967) *Science (Wash.)*, **158**, 1570-1571.
Cohen,C., Tyler,J.M., and Branton,D. (1980) *Cell*, **21**, 875-883.
Cotton,R.G.H., Secher,D.S., and Milstein,C. (1973) *Eur. J. Immunol.*, **3**, 135-140.
Galfre,G., Howe,S.C., Milstein,C., Butcher,G.W., and Howard,J.C. (1977) *Nature*, **266**, 550-552.
Köhler,G., and Milstein,C. (1975) *Nature*, **256**, 495-497.
Koprowski,H., Gerhard,W., and Croce,C.M. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 2985-2988.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Morrow,J.S., Speicher,D.W., Knowles,W.J., Hsu,C.J., and Marchesi,V.T. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6592-6596.
Ralston,G. (1975) *Aust. J. Biol. Sci.*, **28**, 259-266.
Ralston,G., Dunbar,J., and White,M. (1977) *Biochim. Biophys. Acta*, **491**, 345-348.
Sheetz,M.P., Painter,R.G., and Singer,S.J. (1976) *Biochemistry (Wash.)*, **15**, 4486-4492.
Shotton,D.M., Burke,B.E., and Branton,D. (1979) *J. Mol. Biol.*, **131**, 303-329.
Shulman,M., Wilde,C.D., and Köhler,G. (1978) *Nature*, **276**, 269-270.
Speicher,D.W., Morrow,J.S., Knowles,W.J., and Marchesi,V.T. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 5673-5677.
Steck,T.L. (1974) *J. Cell Biol.*, **62**, 1-19.
Tyler,J.M., Hargreaves,W., and Branton,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 5192-5196.
Tyler,J.M., Reinhardt,B., and Branton,D. (1980) *J. Biol. Chem.*, **255**, 7034-7039.
Ziparo,E., Lemay,A., and Marchesi,V.T. (1978) *J. Cell Sci.*, **34**, 91-101.