Characterization of monoclonal antibodies that recognize band 4.5 polypeptides associated with nucleoside transport in pig erythrocytes

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Three monoclonal antibodies have been raised against partially purified band 4.5 polypeptides [Steck (1974) *J. Cell Biol.* **62**, 1–19] from pig erythrocyte membranes. The antibodies were capable of binding to both intact pig erythrocytes and protein-depleted membrane preparations and recognized detergent-solubilized polypeptides from adult and neonatal pig erythrocytes that were photolabelled with [G-³H]nitrobenzylthioinosine (NBMPR), a potent specific inhibitor of nucleoside transport. The antibodies did not recognize polypeptides from neonatal pig erythrocytes that were photolabelled with the glucose-transport inhibitor [³H]cytochalasin B. Reactivity with polypeptides of apparent M_r 64000 [10% (w/v) acrylamide gels] was demonstrated by Western-blot analysis. The antibodies recognized pig band 4.5 polypeptides after prolonged treatment with endoglycosidase F, a finding consistent with reactivity against polypeptide, rather than carbohydrate, determinants. Trypsin digestion of NBMPR-labelled protein-depleted pig erythrocyte membranes generated two labelled polypeptide fragments (M_r 43000 and 26000). Two of the antibodies recognized both fragments on Western blots, whereas the third bound to the larger, but not to the smaller, fragment. The antibodies had no significant effect on reversible binding of NBMPR to protein-depleted pig erythrocyte membranes and did not bind to NBMPR-labelled polypeptides in human, rabbit or mouse erythrocytes.

INTRODUCTION

Nitrobenzylthioinosine (NBMPR), a potent inhibitor of facilitated diffusion of nucleosides, binds reversibly, but with high affinity (K_d about 1 nM), to specific sites in the plasma membrane of erythrocytes. Covalent labelling of transport-inhibitory sites by photoactivation of site-bound [³H]NBMPR has enabled identification of the NBMPR-binding protein of human erythrocyte membranes as a glycosylated polypeptide(s) that migrates in the band 4.5 region (nomenclature of Steck, 1974) of electrophoretograms after separation of membrane proteins by SDS/polyacrylamide-gel electrophoresis (Wu *et al.*, 1983; Young *et al.*, 1983; Kwong *et al.*, 1986). Reconstitution of NBMPR binding and NBMPR-sensitive transport of uridine was demonstrated after incorporation of partially purified band 4.5 polypeptides into phospholipid vesicles (Tse *et al.*, 1985), confirming that nucleoside transport is mediated by band 4.5 polypeptides in human erythrocytes. The glucose transporter of human erythrocytes, identified by a number of different approaches, including photolabelling with the inhibitor [³H]cytochalasin B, is also a band 4.5 polypeptide (Carter-Su *et al.*, 1982). Chromatographic preparations of band 4.5 of human erythrocytes contain both nucleoside- and glucose-transporter activities (Tse

et al., 1985), and separation of these activities, which migrate on electrophoretograms with M_r values of 45000–66000, has not yet been achieved. Measurements of binding of radiolabelled cytochalasin B and NBMPR to human erythrocyte membranes indicate that 2.5×10^5 glucose-transport sites (Jung & Rampal, 1977) and 1×10^4 nucleoside-transport sites (Cass *et al.*, 1974) are present per erythrocyte.

Mature erythrocytes from the adult domestic pig do not metabolize extracellular glucose (Engelhardt & Ljubimova, 1930) and, lacking a functional glucose-transport system (Kim & McManus, 1971), utilize plasma inosine as a physiological source of metabolic energy (Young *et al.*, 1985, 1986; Ziedler *et al.*, 1985). The kinetic characteristics of nucleoside transport in erythrocytes from adult pigs are similar to those of the process in erythrocytes of humans and a number of other species (Jarvis *et al.*, 1982), and there are 5×10^3 NBMPR-binding sites per erythrocyte (Jarvis *et al.*, 1980). Membranes from adult pig erythrocytes have been used by Kwong *et al.* (1986) to prepare band 4.5 polypeptides free of the glucose transporter. The NBMPR-binding protein of pig erythrocytes is larger (M_r 64000) than that of human erythrocytes and migrates in SDS/polyacrylamide gels as a sharper band. The present study describes the first preparation and

Abbreviations used: NBMPR, nitrobenzylthioinosine; NBTGR, nitrobenzylthioguanosine; PMSF, phenylmethanesulphonyl fluoride; e.l.i.s.a., enzyme-linked immunosorbent assay.

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characterization of monoclonal antibodies that recognize the NBMPR-binding protein of pig erythrocytes.

EXPERIMENTAL

Materials

[G-³H]NBMPR (23 Ci/mmol) was purchased from Moravsek Biochemicals, Brea, CA, U.S.A. Nitrobenzylthioguanosine (NBTGR) was prepared as described previously (Paul *et al.*, 1975). Materials for cell culture, the BRL hybridoma screening kit and Freund's adjuvant were purchased from Gibco (Canada), Burlington, Ont., Canada. Goat anti-mouse IgG (whole molecule)-agarose, *p*-nitrophenyl phosphate, 2,6,10,14-tetramethylpentadecane (Pristane), trypsin (type III), polyoxyethylenesorbitan monolaurate (Tween 20), phenylmethanesulphonyl fluoride (PMSF) and glutaraldehyde were purchased from Sigma, St. Louis, MO, U.S.A. Goat anti-mouse immunoglobulins (IgA + IgG + IgM; H + L) and fluorescein-conjugated goat anti-mouse IgG (H + L) were from Cappel Division, Cooper Diagnostics, Cochranville, PA, U.S.A. Calf intestine alkaline phosphatase conjugated to goat anti-mouse IgG (H + L) was from Miles Scientific, Naperville, IL, U.S.A., and anti-mouse IgM (μ -chain-specific) conjugate were obtained from Boehringer-Mannheim Canada, Dorval, Que., Canada. Poly(ethylene glycol) 1450 (M_r 1300–1600) was from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A., and Pansorbin from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Endoglycosidase F was from NEN Products, Du Pont Canada, Lachine, Que., Canada.

Methods

Preparation of membranes and immunogen. Whole blood from adult pigs was collected at the Cheung Sha Wan Abattoir, Kowloon, Hong Kong. Heparin was used as an anti-coagulant. Erythrocytes were separated and washed three times with 20 vol. of a medium containing 140 mM-NaCl, 5 mM-KCl, 20 mM-Tris/HCl, pH 7.4 at 25 °C, 2 mM-MgCl₂ and 0.1 mM-EDTA (disodium salt). Haemoglobin-free erythrocyte membranes were prepared as described by Dodge *et al.* (1963), with 0.1 mM-PMSF present at the lysis stage. Erythrocyte membranes were stripped of their extrinsic proteins (Jarvis & Young, 1981) with 0.1 mM-EDTA, pH 11.2, and partially purified band 4.5 polypeptides were prepared as described elsewhere (Kwong *et al.*, 1986). Briefly, a mixture containing protein-depleted membranes (3 mg/ml in 5 mM-Tris/HCl, pH 7.4 at 4 °C, and 2 mM-dithiothreitol) was stirred (20 min, 4 °C) with an equal volume of detergent solution (92 mM-octyl glucoside in 5 mM-Tris/2 mM-dithiothreitol) and centrifuged (130 000 *g*, 4 °C, 1 h). The resulting supernatant was applied to a DEAE-cellulose ion-exchange column equilibrated with detergent solution (34 mM-octyl glucoside/5 mM-Tris/HCl/2 mM-dithiothreitol). Protein adsorbed to the column was eluted with a linear salt gradient (0–0.2 M-NaCl/34 mM-octyl glucoside/2 mM-dithiothreitol/5 mM-Tris/HCl). Fractions containing NBMPR-binding protein (0.08–0.11 M-NaCl) were pooled and dialysed (48 h) against four 2-litre changes of 50 mM-sodium phosphate/100 mM-NaCl/1 mM-EDTA, pH 7.4. Band 4.5 preparations were concentrated by

centrifugation (130 000 *g*, 1 h, 4 °C), and the resulting pellets were resuspended in 5 mM-phosphate, pH 8.0, freeze-dried and stored at –20 °C. Freeze-dried protein was rehydrated in distilled water (100 μ g/ml). Protein was determined by the method of Lowry *et al.* (1951), in the presence of 1% SDS, or as modified by Peterson (1977), with bovine serum albumin as a standard.

Production of monoclonal antibodies. A BALB/c female mouse (Health Sciences Animal Breeding Unit, University of Alberta) was injected intraperitoneally with 10 μ g of a band 4.5 preparation suspended in an equal volume of Freund's complete adjuvant and was boosted 58 and 88 days later with 10 μ g of a band 4.5 preparation suspended in an equal volume of Freund's incomplete adjuvant. At 3 days after the final injection, the mouse's spleen was disaggregated, and spleen cells were mixed with P3/NS1/1-Ag4-1 myeloma cells (Kohler *et al.*, 1976; Kohler & Milstein, 1976; Galfrè & Milstein, 1981) in a ratio of 4:1 and fused by using 50% (w/v) poly(ethylene glycol) 1450 as described previously (Oi & Herzenberg, 1980). Hybridomas were selected in growth medium supplemented with 1 μ M-azaserine and 100 μ M-hypoxanthine and were subsequently maintained in RPMI 1640 medium containing 20% heat-inactivated (56 °C, 30 min) calf serum. Hybridomas of interest were subcloned twice by limiting dilution and were free of *Mycoplasma*.

Antibody-containing supernatants were collected (800 *g*, 10 min) from 500 ml cultures after 2 weeks growth. Ascites fluids were produced by intraperitoneal injection of 1×10^6 hybridoma cells (harvested from actively proliferating suspension cultures), in 1.0 ml of phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-sodium phosphate/1.5 mM-potassium phosphate, pH 7.4) into BALB/c mice that had been primed 10–20 days previously with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane). Partially purified immunoglobulins, prepared by chromatography on hydroxyapatite (Stanker *et al.*, 1985), were pooled and the protein composition examined by SDS/polyacrylamide-gel electrophoresis. Scanning densitometry (Joyce-Loebl Chromoscan 3) of gels after staining with Coomassie Blue indicated that preparations were typically > 80% immunoglobulin.

Indirect immunoprecipitation of [³H]NBMPR-labelled polypeptides. Protein-depleted membranes of erythrocytes obtained from adult pigs (Gainers, Edmonton, Alberta, Canada) were photoaffinity labelled with [³H]NBMPR (Wu *et al.*, 1983; Gati *et al.*, 1986) with a free-radical scavenger (38.5 mM-dithiothreitol) included during exposure to u.v. light. Non-covalently bound [³H]NBMPR was removed by washing (34 000 *g*, 20 min, 4 °C) four times with a solution (30 μ M-PMSF in 50 mM-Tris/HCl, pH 7.9) containing 10 μ M-NBTGR. Typically, about 50% of ³H recovered after SDS/polyacrylamide-gel electrophoresis of photolabelled material was associated with band 4.5 polypeptides. About 20% of the ³H migrated in the region of the dye front, and the remaining 30% was associated with regions on either side of band 4.5, as shown in Fig. 3(a) below. Protein-depleted erythrocyte membranes from newborn piglets were photolabelled with [³H]cytochalasin B or [³H]NBMPR as described elsewhere (Jarvis *et al.*, 1985).

Photolabelled membrane suspensions (about 2 mg/ml in 50 mM-Tris, pH 7.9) were mixed with equal volumes of ice-cold 2% (w/v) Triton X-100 in 50 mM-Tris, pH 7.9, and, after stirring for 60 min at 4 °C, insoluble material was removed by centrifugation (100000 g, 4 °C, 45 min). Portions (50 µl) of solubilized photolabelled membranes were incubated (16 h, 4 °C) with 100 µl portions of cell-free supernatants from hybridoma cultures. Antigen-antibody complexes were precipitated by addition of 50 µl of Pansorbin (fixed *Staphylococcus aureus* cells in phosphate-buffered saline) or 100 µl of goat anti-mouse IgG (whole molecule)-agarose and incubation for 60 min at 4 °C. Precipitated material was separated by centrifugation (10000 g, 5 min, 4 °C), and 125 µl portions of supernatant fluids were reserved. The precipitates were washed three times (centrifugation at 10000 g, 2 min, 4 °C) with 1 ml portions of 0.5% Triton X-100 in 50 mM-Tris buffer, pH 7.9, at 4 °C. Radioactivity in the precipitates and supernatants was determined by liquid-scintillation counting in a Triton-based scintillation cocktail (Pande, 1976). The immunoprecipitated ³H was shown to be primarily band 4.5 polypeptides by extraction of Pansorbin pellets with 0.1 ml of gel sample buffer and analysis by SDS/polyacrylamide-gel electrophoresis. Over 70% of the extracted ³H migrated within a single broad band (M_r 64000), and the remaining ³H was diffusely spread throughout the gel. Antibodies 64C7 and 2B11 were used as negative controls for the indirect immunoprecipitation measurements. Antibody 64C7 (Jarvis *et al.*, 1985) recognizes band 4.5 polypeptides of human erythrocytes on Western blots, but does not bind to pig erythrocyte membranes, and antibody 2B11 (J. D. Craik, A. R. P. Paterson & C. E. Cass, unpublished work) recognizes band 3, but not band 4.5, on Western blots of SDS/polyacrylamide-gel electrophoretograms of pig erythrocyte membranes.

SDS/polyacrylamide-gel electrophoresis and electrophoretic blotting. SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970), on 10% or 12% (w/v) acrylamide gels. Migration distances of standard proteins were determined after staining with Coomassie Blue. Radioactivity from unstained lanes was measured by liquid-scintillation counting after swelling 2 mm slices for 24–48 h in 3% (v/v) Protosol in Econofluor (New England Nuclear) scintillation fluid. Recovery of radioactivity from gels was 80–85%. Western blots were performed by electrophoretic transfer of polypeptides from the polyacrylamide gel on to nitrocellulose membranes using a transfer buffer that consisted of 25 mM-Tris/192 mM-glycine (pH 8.3)/20% (v/v) methanol (Towbin *et al.*, 1979). Transfer was at 40 or 90 V for 12–16 h, and the transferred protein was detected by staining (5 min) with 0.1% (w/v) Amido Black in acetic acid/methanol/water (2:9:9, by vol.).

For analysis of immunoreactivity of proteins after electrophoretic transfer, non-specific binding was blocked by incubation (2 h, 22 °C, gentle agitation) of nitrocellulose with 3% (w/v) bovine serum albumin in phosphate-buffered saline, pH 7.4. Strips of nitrocellulose were incubated (overnight, 4 °C, gentle agitation) with 10 ml of culture supernatants containing the appropriate monoclonal antibodies and washed extensively with phosphate-buffered saline containing 0.05%

Tween 20 (PBS/Tween). The strips were next incubated (2 h, 22 °C, gentle agitation) with alkaline-phosphatase-conjugated antibodies [goat anti-mouse IgG (H+L) and goat anti-mouse IgM (µ-chain)] that had been diluted 1:1000 in 50 mM-Tris/150 mM-NaCl/5 mM-EDTA/0.05% (v/v) Nonidet P40/0.25% (w/v) bovine serum albumin and subsequently washed extensively with PBS/Tween and then with Tris/Mg buffer (0.1 M-Tris/0.5 mM-MgCl₂, pH 9.2). The nitrocellulose strips were stained (15–60 min) as described by Mason & Sammons (1978).

Enzymic cleavage of [³H]NBMPR-labelled polypeptides. Pig erythrocyte membranes, protein-depleted and photolabelled with [³H]NBMPR as described above, were washed and suspended in 5 mM-sodium phosphate, pH 8.0. Trypsin (type III) was added to give 3.3 or 10 µg of trypsin/mg of protein. After incubation for 15 min (4 °C) or 60 and 90 min (22 °C), soybean trypsin inhibitor (6.7 µg/mg) and bovine serum albumin (0.2%) were added to stop reactions.

For digestion with endoglycosidase F (Leinhard *et al.*, 1984), photolabelled membranes were treated (14 h, 37 °C) with 5.5 units of the enzyme/mg of protein in 100 mM-sodium phosphate/75 mM-β-mercaptoethanol/50 mM-EDTA/0.05% (w/v) SDS/0.5% (w/v) Triton X-100, pH 6.1. Enzyme digestion was terminated by the addition of an equal volume of SDS/polyacrylamide-gel sample buffer.

Immunofluorescence and e.l.i.s.a. Binding of antibodies to intact erythrocytes was determined by indirect immunofluorescence and analytical flow cytometry (Coulter EPICS V; Coulter Electronics, Hialeah, FL, U.S.A.) using goat anti-mouse IgG conjugated to fluorescein (Mannoni *et al.*, 1982). Binding of antibodies to erythrocyte membranes was determined by e.l.i.s.a. using protein-depleted membranes attached to microtitre plates by glutaraldehyde fixation (Stocker & Huesser, 1979; Kincade *et al.*, 1981). Microtitre plates were treated with bovine serum albumin to minimize non-specific binding of antibodies, and cell-free supernatants from hybridoma cultures were incubated overnight at 4 °C. Immunoglobulins that bound to erythrocyte membranes were detected with goat anti-mouse IgG and goat anti-mouse IgM antibodies conjugated to alkaline phosphatase. Phosphatase activity was detected with *p*-nitrophenyl phosphate and A_{405} was determined with an e.l.i.s.a. reader (Titertek Multiskan MC; Flow Laboratories, Mississauga, Ont., Canada).

Binding of NBMPR to protein-depleted ghost membranes in the presence of monoclonal antibodies. The effect of antibodies on equilibrium binding of [³H]NBMPR was assessed at a single non-saturating concentration of the latter. Protein-depleted membranes (about 40 µg/assay tube) were incubated (1 h, 22 °C) with 200 µl of purified monoclonal antibody in phosphate-buffered saline, after which 10 µl of water or 50 µM-NBTGR was added and the incubation continued for 30 min. [³H]NBMPR (20 µl, 2 nM) was then added and the resulting solutions were allowed to equilibrate for 30–60 min before the membranes were pelleted by centrifugation (10000 g, 30 min, 4 °C). Portions (200 µl) of the resulting supernatants were assayed for radioactivity by liquid-scintillation counting.

RESULTS AND DISCUSSION

Since erythrocytes from adult pigs do not transport glucose, Kwong *et al.* (1986) used this cell type to obtain preparations of band 4.5 polypeptides associated with nucleoside transport that are free of the glucose-transporter polypeptide. In the present study, partially purified band 4.5 polypeptides, prepared as described by Kwong *et al.* (1986), were used as the immunogen to obtain monoclonal antibodies directed against the NBMPR-binding protein of pig erythrocytes. In the absence of detergents, the preparation exhibited a 60-fold increase in specific activity (high-affinity binding of NBMPR) relative to that of erythrocyte ghost membranes (1420–1570 and 23 pmol of NBMPR/mg of protein respectively). Such preparations have been shown to impart uridine-uptake activity when reconstituted into soybean phospholipid vesicles (F. Y. P. Kwong, C. M. Tse & J. D. Young, unpublished work). Contaminating polypeptides present in the preparation used for immunization apparently included proteolytic fragments of band 3 (see below), since a number of antibodies recognizing band 3 were obtained.

The strategy employed for identification of antibodies directed against the NBMPR-binding component of pig erythrocytes involved screening of culture supernatants for antibodies capable of binding to (a) protein-depleted (unsealed) pig erythrocyte membranes (detected by e.i.s.a.) and (b) membrane proteins photolabelled with [³H]NBMPR (detected by the indirect immunoprecipitation assay illustrated in Fig. 1). Antibodies 11C4, 3E3 and 5E11 were selected for further study. Since these antibodies were originally detected in assays that employed unsealed or solubilized membranes, their ability to bind to intact cells was assessed in the experiments of Table 1. Immunofluorescence measurements demonstrated that all three antibodies bound to intact cells, indicating the presence of antigenic determinants at the external face of the pig erythrocyte membrane. Monoclonal antibodies raised against band 4.5 preparations of human erythrocytes (Allard & Leinhard, 1985; Boyle *et al.*, 1985) recognized deter-

Fig. 1. Reactivity of antibody 11C4 with [³H]NBMPR-labelled polypeptides from pig erythrocyte membranes

Photolabelled membranes were extracted with Triton X-100 and the soluble material was incubated with antibodies 11C4, 2B11 and 64C7. Indirect immunoprecipitation using goat anti-mouse IgG-agarose was performed as described in the Experimental section.

Table 1. Binding of monoclonal antibodies to intact pig erythrocytes

The percentage of pig erythrocytes labelled with antibody was determined by analytical flow cytometry after incubation of antibody-treated cells with goat anti-mouse IgG conjugated to fluorescein. About 10000 cells were monitored in each determination.

Antibody	Percentage of cells labelled
11C4	91
3E3	97
5E11	53
2B11	1
64C7	1

minants at the cytoplasmic face of the human erythrocyte membrane.

One (2B11) of several antibodies that recognized pig band 3 polypeptides on Western blots was also characterized. The preparation of band 4.5 polypeptides used as the immunogen evidently contained a band 3 fragment analogous to the M_r -55000 fragment (a membrane-associated domain) produced by endogenous proteinase action (Tarone *et al.*, 1979) or mild trypsin treatment of unsealed human erythrocyte membranes (Steck *et al.*, 1976). Intact band 3 was not seen on electrophoretograms of the immunogen. Antibody 2B11 recognized pig and human band 3, exhibited poor reactivity with mouse band 3 and did not recognize pig, human or mouse band 4.5 in electrophoretograms of solubilized protein-depleted membranes (results not shown).

Specificity of antibodies 11C4, 3E3 and 5E11 for the NBMPR-binding component of pig erythrocytes is

Table 2. Indirect immunoprecipitation of NBMPR-labelled material from detergent-solubilized membranes of pig erythrocytes

Erythrocyte membranes were protein-depleted, photolabelled with [³H]NBMPR, and solubilized with 2% Triton X-100 as described in the Experimental section. Membrane samples were incubated with cell-free portions of growth media from hybridoma cultures, and the radioactivity associated with antigen-antibody complexes was precipitated as described in the Experimental section by using goat anti-mouse IgG (whole molecule)-agarose.

Hybridoma	Immuno-globulin class	³ H remaining in solution (% of total)*	³ H-labelled band 4.5 precipitated (% of total)†
11C4	IgG	48.7	93.4
3E3	IgG	47.7	95.3
5E11	IgM	71.0	52.8
2B11	IgM	100.4	—
64C7	IgG	100.0	—

* Values are expressed as the percentages of total radioactivity present in reaction mixtures.

† Values are expressed as the percentages of photolabelled band 4.5 polypeptides precipitated; in this experiment 54.9% of total radioactivity was associated with band 4.5 polypeptides.

Table 3. Reactivity of antibodies with membranes of neonatal pig erythrocytes photolabelled with [³H]NBMPR or [³H]cytochalasin B

Membranes were photolabelled, solubilized in 1% Triton X-100, and the indirect immunoprecipitation assay was performed, as described in the Experimental section, by using goat anti-mouse IgG (whole molecule)-agarose to precipitate antibody-antigen complexes. Values are presented as percentages of radioactivity remaining in solution after immunoprecipitation.

Hybridoma	NBMPR-labelled material remaining in solution (%) [*]	Cytochalasin B-labelled material remaining in solution (%) [†]
11C4	55	105
3E3	72	98
5E11	72	104
2B11	99	102
64C7	102	99

^{*} About 58% of the radioactivity present in [³H]NBMPR-labelled membranes was associated with a single peak (M_r 64000) after SDS/polyacrylamide-gel electrophoresis.

[†] About 45% of the radioactivity present in [³H]cytochalasin B-labelled membranes was associated with a broad band (M_r 45000–66000) after SDS/polyacrylamide-gel electrophoresis.

suggested by the results presented in Table 2. These antibodies were capable of precipitating [³H]NBMPR-labelled material from Triton X-100 extracts of pig erythrocyte membranes. In contrast, antibody 2B11, which recognizes pig band 3, and antibody 64C7, which recognizes band 4.5 polypeptides in human erythrocytes (Jarvis *et al.*, 1985), did not bind to material photolabelled with [³H]NBMPR. The binding of antibodies 11C4, 3E3 and 5E11 to membrane material photolabelled with [³H]NBMPR was saturable, as is illustrated in Fig. 1 for antibody 11C4. The results obtained with antibody 5E11 suggest that this antibody (an IgM) has a lower affinity for NBMPR-binding polypeptides of pig erythrocytes than have antibodies 3E3 and 11C4, although other possibilities have not been excluded.

An objective of the present study was to obtain antibodies specific for band 4.5 polypeptides associated with nucleoside transport. Although erythrocytes from adult pigs lack glucose-transport activity, erythrocytes from neonatal pigs are able to transport glucose and their membranes contain band 4.5 polypeptides (M_r 45000–66000) that can be selectively photolabelled with [³H]cytochalasin B (J. D. Craik, R. Gottschalk, A. R. P. Paterson & C. E. Cass, unpublished work). The cytochalasin B-binding component of erythrocytes of neonatal pigs, like that of human erythrocytes, migrates in SDS/polyacrylamide gels as a broad band (M_r 45000–66000), whereas the NBMPR-binding component is larger (M_r 64000) and migrates as a sharper band. In the experiment of Table 3, membranes from erythrocytes of neonatal pigs were photolabelled with either [³H]NBMPR or [³H]cytochalasin B, and antibody reactivity was assessed by indirect immunoprecipitation of photolabelled material in Triton X-100 extracts. Antibodies 11C4, 3E3 and 5E11 precipitated NBMPR-, but not cytochalasin B-, labelled material, indicating that

Fig. 2. Reactivity of antibodies 11C4 and 3E3 with pig erythrocyte band 4.5 polypeptides on Western blots before and after treatment with endoglycosidase F

Protein-depleted pig erythrocyte ghosts, photolabelled with [³H]NBMPR as described in the Experimental section, were (a) solubilized in enzyme-digestion buffer immediately before electrophoresis, (b) solubilized in enzyme-digestion buffer and incubated at 37 °C for 14 h in the absence of endoglycosidase F, or (c) solubilized in enzyme-digestion buffer and incubated at 37 °C for 14 h with 5.5 units of endoglycosidase F/mg of protein. Samples were subjected to electrophoresis (10% acrylamide gel) and blotted on to nitrocellulose as described in the Experimental section. M_r values were determined by using protein standards run in adjacent lanes and blotted on to nitrocellulose. The stacking-gel/running-gel interfaces (i) and the tracking-dye positions (t) are indicated.

the antibodies were specific for the NBMPR-binding component and did not recognize the cytochalasin B-binding component of pig erythrocyte membranes.

Binding of monoclonal antibodies to band 4.5 polypeptides was confirmed in the experiments of Fig. 2, in which immunoreactivity of proteins from membranes of erythrocytes from adult pigs was determined after separation by SDS/polyacrylamide-gel electrophoresis and electrophoretic transfer to nitrocellulose. Antibodies 11C4 and 3E3 recognized band 4.5 polypeptides before and after treatment with endoglycosidase F, which cleaves the bond between *N*-acetylglucosaminyl(1-4)*N*-acetylglucosamine and asparagine of the core protein of high-mannose structures. Treatment with endoglycosidase F decreased the apparent M_r of [³H]NBMPR-labelled polypeptides of pig erythrocyte membranes from 64000 to 57000, as has been previously reported by Kwong *et al.* (1986). The downward shift in M_r , although not great, was reproducible and suggests that antibodies 11C4 and 3E3 are not directed against carbohydrate moieties of the NBMPR-binding protein. Similar results (not shown) were obtained with antibody 5E11.

The effects of proteolysis on immunoreactivity of the

Fig. 3. Tryptic cleavage of [^3H]NBMPR-photolabelled polypeptides of pig erythrocyte membranes

Protein-depleted ghosts, labelled with [^3H]NBMPR, as described in the Experimental section, were subjected to proteolysis as follows: (a) control (no trypsin); (b) 3.3 μg of trypsin/mg of sample protein, 4 $^{\circ}\text{C}$, 15 min; (c) 3.3 μg

Fig. 4. Binding of monoclonal antibody 11C4 to tryptic fragments of the NBMPR-binding protein

Western-blot analysis was conducted as described in the Experimental section on replicate samples (a–d) from the experiments described in Fig. 3. M_r standards were run simultaneously and were stained with Amido Black. The stacking-gel/running-gel interface (i) and the tracking-dye position (t) are indicated.

NBMPR-binding protein are illustrated in Fig. 3. Mild trypsin treatment of protein-depleted membranes photolabelled with [^3H]NBMPR generated a single radioactive fragment (M_r 43000), and extensive trypsin treatment generated a smaller fragment (M_r 26000). Both fragments migrated as relatively broad peaks, suggesting the presence of carbohydrate. After trypsin treatment, antibodies 11C4 and 3E3 bound to Western blots at positions exactly coincident with those of the two radiolabelled fragments (Fig. 4). Antibody 5E11, which apparently recognized a different determinant, bound only to the 43 kDa fragment on Western blots (results not shown). Janmohamed *et al.* (1985), who used similar trypsin treatment for proteolysis of membranes of human erythrocytes, reported the formation of two NBMPR-labelled fragments, a carbohydrate-containing fragment (M_r 38000) and a smaller carbohydrate-free fragment (M_r 23000).

The interaction of the antibodies with erythrocyte membranes from other species (mouse, rabbit, human)

of trypsin/mg of sample protein, 22 $^{\circ}\text{C}$, 60 min; or (d) 10 μg of trypsin/mg of sample protein, 22 $^{\circ}\text{C}$, 90 min. Polypeptides were separated by electrophoresis (12% acrylamide gel). The stacking-gel/running-gel interface (i), the tracking-dye position (t) and mobilities of polypeptide standards are indicated.

known to possess NBMPR-binding sites (Jarvis *et al.*, 1982) was examined by e.l.i.s.a. using glutaraldehyde-fixed erythrocyte ghosts (results not shown). Antibodies 11C4 and 3E3 did not bind to protein-depleted membranes of mouse, rabbit or human erythrocytes, and, although limited reactivity was seen with antibody 5E11 against protein-depleted membranes of human erythrocytes, the Western-blotting procedure showed that 5E11 did not recognize band 4.5 polypeptides of human erythrocyte membranes, but bound to a sharp band (M_r 31000) of unknown identity.

Agbanyo *et al.* (1986) have shown that the NBMPR-binding sites of pig erythrocytes are on the outward face of the membrane. Asymmetrical binding of NBMPR to sheep erythrocyte membranes has also been suggested from results of studies of the kinetics of inhibition of transport by NBMPR (Jarvis *et al.*, 1981). Since antibodies 11C4, 3E3 and 5E11 recognize determinants that are externally located, their effects on the reversible binding of [3 H]NBMPR to protein-depleted pig erythrocyte membranes were evaluated. None of the antibodies altered NBMPR binding to erythrocyte membranes under conditions that employed a molar excess of antibody over NBMPR-binding sites.

In conclusion, three antibodies with specificity for the NBMPR-binding component of pig erythrocytes membranes have been produced. The antibodies are directed against protein determinants present on the external surface of pig erythrocytes. The antibodies did not bind cytochalasin B-labelled proteins of erythrocytes of neonatal pigs or to NBMPR-labelled proteins of erythrocytes of humans, mice or rabbits. Because the antibodies interacted with NBMPR-labelled proteins from pig erythrocyte membranes in non-ionic-detergent solution, immunoaffinity chromatography would seem to be a means to purify the polypeptide further. Thus a detailed characterization of this polypeptide and an exploration of its role in nucleoside transport in pig erythrocytes are feasible.

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