Plasma-Membrane-Associated Immunoglobulins and other Polypeptides of Pig Mesenteric Node Lymphocytes

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Plasma membranes from pig mesenteric lymph-node lymphocytes contain a large number of polypeptide chains ranging in molecular weight from 20000 to greater than 470000, with a major component of 46000. There are approx. 12-15 glycoproteins. The membranes contain immunoglobulin G, which comprises 0.6% of the total protein. Immunoglobulin M is also detected, but has not been accurately quantified for technical reasons. Possible origins of the membrane-associated immunoglobulin are briefly discussed. It is concluded that the immunoglobulin is probably associated with the plasma membrane in the intact cell.

The role of the plasma membrane in immunological processes is the subject of a great deal of study and discussion (Marchalonis & Cone, 1973; Vitteta & Uhr, 1973). We have studied the membrane-associated immunoglobulin by means of which the cell is presumed to respond to immunogens with either immunological paralysis or antibody synthesis (Bretscher, 1972). Although the presence of immunoglobulin on the surface of some lymphocytes is clearly established, little is known about the structure of this immunoglobulin, the manner in which it is attached to the membrane, and the role which it plays in immune responses after binding the appropriate immunogen.

Methods

Preparation of membranes

Plasma membranes were prepared from whole pig mesenteric lymph nodes or thymus by the method of Allan & Crumpton (1970, 1972), except for modifications in the centrifugations. The homogenate was filtered through a plastic household strainer, mesh size 1 mm². The filtrate was centrifuged in an International model PR 6000 machine, in 50ml polycarbonate tubes at 3750g_{max} for 90 min at 4°C. The pellet, containing connective tissue, intact cells and nuclei, was discarded. The supernatant was centrifuged in Spinco SW 25.2 nitrocellulose tubes at 40000g_{av} for 90 min (4°C). The pellet ('crude membrane') was homogenized, re-centrifuged

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at 40000g for 90 min, and the clear supernatant was discarded. The pellet was dispersed with a glass rod and an equal volume of 80% (w/v) sucrose was added. The suspension was transferred to a Thomas Teflon homogenizer and was thoroughly dispersed in 40% (w/w) sucrose in 10mM-Tris-HCl [10mM-Tris adjusted to pH 7.4 (20°C) with 1M-HCl]. The refractive indices of all sucrose solutions were measured with a refractometer, and the concentrations and densities calculated from the International Critical Tables. The final volume was adjusted to 90 ml with 40% (w/w) sucrose, and three 30 ml portions of suspension were transferred to 60 ml tubes and overlaid with 25 ml of 27% (w/w) sucrose. The discontinuous gradients were centrifuged in a Spinco L2 65B centrifuge in an SW 25.2 rotor at 78000g_{av} for 18 h (4°C). Purified plasma membrane was collected as a white band at the interface of the two sucrose solutions, diluted with about 4 vol. of 10mM-Tris-HCl and re-centrifuged in the SW 25.2 rotor at 40000g_{av} for 60 min. The washed pellet was homogenized in 10mM-Tris-HCl-20% (w/w) sucrose and stored at -20°C. Membranes were also prepared from suspensions of washed lymph-node cells (Snary *et al.*, 1974).

Protein

Protein was measured by the Folin method of Lowry *et al.* (1951) with the following modifications. All the samples and standards were dissolved in 0.5% sodium dodecyl sulphate-0.1M-Na₂CO₃ and the absorbance was read in a Pye Unicam SP. 500 series 2 spectrophotometer at 750 nm. Bovine serum albumin (crystallized) [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.] was used as a standard. When necessary, standard protein curves were determined in the presence of bile salts.

Phospholipid analyses

Cholesterol and phosphorus were analysed by the methods of Huang *et al.* (1961) and McClare (1971) respectively. The finely divided membrane suspension in Tris-0.9% NaCl was extracted by a method adapted from Folch *et al.* (1956), in which 3 vol. of membrane in 0.9% NaCl was treated with 4 vol. of methanol, and then 8 vol. of chloroform was added. The original membrane and the lower phase of the Folch extract were analysed for cholesterol and phosphorus. The lower phase of the Folch extract was run on a one- or two-dimensional silica gel H t.l.c. plate, in chloroform-methanol-7M-NH₃ (46:18:3, by vol.) and chloroform-methanol-acetic acid-water-acetone (10:2:2:1:4, by vol.) as the first and second solvents respectively. After a preliminary qualitative analysis with phospholipid standards, the spots were either located with iodine, scraped off the plate and analysed for phosphorus, or the plates were sprayed with the Dittmer lipid phosphorus reagent (Dittmer & Lester, 1964), and the absorbance of the blue spots was measured *in situ* in a Vitatron densitometer.

Enzyme assays

Succinate dehydrogenase (EC 1.3.99.1) was assayed as succinate-2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase (Pennington, 1961; Porteous & Clark, 1965). The amount of protein assayed was between 0.2 mg and 1.2 mg. 5'-Nucleotidase activity (EC 3.1.3.5) was measured by the method of Michell & Hawthorne (1965), by using between 50 and 450 µg of protein. P_i was measured by the method of Ames (1966). Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Bergmeyer (1963) in the presence of 0.1% Triton X-100 (BDH Chemicals Ltd., Poole, Dorset, U.K.).

Polyacrylamide-slab-gel electrophoresis

Membrane samples containing 60–100 µg of protein were delipidated with chloroform-methanol (2:1, v/v), and the protein fraction was dissolved in 2% (w/v) sodium dodecyl sulphate-50 mM-Na₂CO₃ solution, reduced with 10% (v/v) 2-mercaptoethanol, and electrophoresed by the method of Neville (1971) and Neville & Glossmann (1971), in a slab gel apparatus (Raven Scientific Co., Haverhill, Suffolk, U.K.). The stacking gel contained 3.2% (w/v) (acrylamide + bisacrylamide), of which 6.25% (w/w) of the total acrylamide was methylenebisacrylamide. The composition of the separation gel was 11.1% (w/v) total acrylamide and 0.9% (w/w) bisacrylamide. All acrylamide stock solutions were stored in dark bottles at 4°C for no longer than 1 month; even during this short storage time, a progressive increase in electrophoretic mobility of the membrane poly-

peptides was noted. Electrophoresis was started at 30 mA (constant current) at room temperature (21°C) and terminated when the Bromophenol Blue tracking dye had migrated 10 cm into the separation gel; the dye front moved at about 6 cm/h. The gel slabs (1.5 mm thick) were stained for protein with Coomassie Brilliant Blue G 250 (Serva Biochemicals, Micro-bio Laboratories Ltd., Fulham, London SW6 3DX, U.K.) (Demus, 1973) and for carbohydrate (Kapitany & Zebrowski, 1971). When a gel was to be calibrated for estimation of molecular weights, a series of marker proteins were included, either individually or as a mixture, on the same gel as the membrane sample. The following proteins and molecular weights were used (Darnall & Klotz, 1972): catalase tetramer, 232 000; β-galactosidase monomer, 130 000; phosphorylase *a*, 92 500; bovine serum albumin, 68 000; catalase monomer, 57 500; ovalbumin, 43 000; chymotrypsinogen *a*, 30 000; carbonic anhydrase, 29 000; soya-bean trypsin inhibitor, 22 000; β-lactoglobulin, 17 500. The calibration curve of log molecular weight against mobility was approximately linear over the approximate molecular-weight range 100 000–30 000. Several low-molecular-weight proteins (including haemoglobin, lysozyme and pancreatic trypsin inhibitor) migrated at the dye front; the reason for this anomalous migration is not known, but it may be related to persulphate degradation (Chrumbach & Rodbard, 1971).

Antisera

Anti-(pig IgG*) was made by immunizing sheep with pig IgG; the antiserum reacted with light chains and γ chains, and was made specific for γ chain by passage through a Fab γ-chain-Sepharose column (Cuatrecasas *et al.*, 1968). Sheep anti-(pig IgM), prepared by immunizing with purified IgM, reacted mainly with μ chain and was made completely specific for μ chain by passage through a pig IgG-Sepharose column (Cuatrecasas *et al.*, 1968). Fluorescein derivatives of these antisera have previously been used in studies on intact pig lymphocytes and showed binding to 5–10% of mesenteric-node lymphocytes and to less than 0.1% of thymus lymphocytes (Binns *et al.*, 1972).

Immunodiffusion methods

Ouchterlony double immunodiffusion (Munoz, 1971) or Mancini radial immunodiffusion (Heremans, 1971) was carried out by standard methods, except that the agar gels contained 1% (v/v) sodium cholate (adjusted to pH 7.4 with 6M-NaOH). The appropriate antiserum was incorporated into the agar as indicated in the Results and Discussion section. Membrane samples were pelleted at 100 000g_{av.} for

* Abbreviations: IgG, immunoglobulin G; IgM, immunoglobulin M (macroglobulin).

60 min, and dissolved in 14% (w/v) sodium cholate (340 mM), at a final concentration of protein of 20–25 mg/ml. To ensure maximum solubilization, cholate was used at a concentration above its critical micelle concentration (about 45 mM) and at a detergent:protein ratio (w/w) of about 6–7:1. Control experiments indicated that the size of the precipitin ring, when 7S pig IgG was used, was essentially the same whether the sample was dissolved in 14% cholate or in phosphate-buffered saline (0.02 M- Na_2HPO_4 –0.15 M- NaCl , adjusted to pH 7.2 with 9 M- H_3PO_4). The plates were developed for 7–10 days, then washed successively in 1% sodium cholate, phosphate-buffered saline and water, 18–24 h in each solution. The concentrations of membrane immunoglobulin were estimated by projecting the dried and stained Mancini plates with a photographic enlarger, tracing the outline of the precipitin rings on paper, and weighing the circles. The weights of the circles are proportional to the concentration of the protein (Heremans, 1971). Standard immunoglobulins (purified pig serum IgG and IgM) were assayed on the same plates as the membranes to be estimated. The plates were photographed before or after staining, according to the intensity of the rings.

Electron microscopy

Material for electron microscopy was fixed as a suspension in 4% (v/v) glutaraldehyde (reagent grade; Koch-Light Laboratories, Colnbrook, Bucks., U.K.) in 0.1 M- Na_2HPO_4 buffer (adjusted to pH 7.2 with 9 M- H_3PO_4) for 30–60 min at room temperature, and then pelleted at 100 000 g_{av} for 30 min. The pellet was resuspended in the phosphate buffer without glutaraldehyde and re-centrifuged as described above. Post-fixation was with 1% (w/v) OsO_4 in 0.1 M-veronal-sodium acetate buffer, pH 7.2, for 60 min, followed by a water rinse. The pellets were stained in aq. 2% (w/v) uranyl acetate for 60 min, dehydrated in an alcohol series followed by a propylene oxide soak, and finally embedded in Araldite. Sections were stained as necessary with uranyl acetate (saturated in 50% ethanol) and lead citrate.

Results and Discussion

Preparation of plasma-membrane fractions

The yield of plasma-membrane protein was between 33 and 50 mg/100 g of wet lymph node. The highest yields of membrane were from pigs 8–12 weeks old.

The specific activity of 5'-nucleotidase was increased 6–10-fold, in the purified fraction, with a recovery of about 5% of the initial nucleotidase activity. These values must be interpreted with caution because the total nucleotidase activity

recovered in different preparations varied between 30 and 200% of the activity in the initial homogenate, and because of some doubt as to the use of 5'-nucleotidase and other enzymes as markers of the plasma membrane (Wallach & Lin, 1973; Warley & Cook, 1973). The purified membrane contained succinate dehydrogenase activity with a specific activity of similar value to that of the initial homogenate. This mitochondrial enzymic activity was not separable from the 5'-nucleotidase activity even after re-fractionation of the purified membrane on a 50–20% (w/w) continuous sucrose gradient, suggesting that the membrane fragments carrying the two activities had similar densities. Using the specific activity of the mitochondrial pellet, we have calculated that mitochondrial protein does not comprise more than 10% of the total protein in the gradient-purified plasma membrane. We assume that there are small amounts of membranes from other subcellular organelles.

Electron-microscopic appearance

The plasma-membrane fraction consisted of irregularly shaped vesicles, usually single layered, with an average diameter about 0.05–0.1 μm (500–1000 Å). The vesicles had a typical bilayer structure with occasional inclusions of smaller vesicles, vesicular fragments or amorphous material of unknown origin.

Phospholipid analyses

The major phospholipids of the membrane are listed in Table 1. The relative proportions of the two neutral phospholipids (phosphatidylcholine plus sphingomyelin) were rather variable, as indicated by their large standard deviations. The sum of phosphatidylcholine plus sphingomyelin was much more constant ($62.2 \pm 4.4\%$); a higher value for phosphatidylcholine was accompanied by a lower value for sphingomyelin, and vice versa. Phosphatidylinositol was present at around 1–4% of the total lipid phosphorus, but its proportion was too low to be measured accurately. Cardiolipin was not detected and therefore could account for no more than 1% of the lipid phosphorus.

Within the limits of experimental error, nearly all (99%) of the cholesterol in the membranes was found in the lower phase of the Folch extract, together with $80.6 \pm 1.5\%$ (three analyses) of the membrane phosphorus. The ratio cholesterol:membrane protein was 0.516 ± 0.023 (μmol):1 (mg) (three analyses), and the cholesterol:phospholipid molar ratio was 0.613 ± 0.017 :1.0 (four analyses). No protein was detected in the lower phase of the Folch extract.

The membranes had a mean density of 1.137. The membrane density and protein/cholesterol ratio

Table 1. *Phospholipids of pig mesenteric-node-lymphocyte plasma membrane*

The phospholipids were separated by chromatography as described in the text, and the spots were located with iodine, scraped off the plate and analysed for phosphorus. The values in the table are the means $\pm$ s.d. of six different membrane preparations.

Phospholipid	Percentage of total phospholipid
Phosphatidylcholine	47.3 $\pm$ 9.2
Phosphatidylethanolamine (including plasmalogen)	26.9 $\pm$ 3.7
Phosphatidylserine	10.9 $\pm$ 2.7
Sphingomyelin (+trace of phosphatidylinositol)	14.9 $\pm$ 6.9
Cardiolipin	Not detected

values are very similar to those obtained by Allan & Crumpton (1970). However, these workers found less phospholipid in the membranes, and therefore calculated a much higher cholesterol/phospholipid molar ratio of $1.01 \pm 0.03:1$. The different methods of extraction used by ourselves and by Allan & Crumpton (1970) may be responsible for the discrepancy in cholesterol/phospholipid ratios. After drying the membrane over P_2O_5 and extracting with chloroform-methanol (2:1, v/v), we found the phospholipids partially degraded to their lyso derivatives, and it is possible that some lipid phosphorus was lost as well. Taking the density of membrane protein as 1.3, the density of phospholipid plus cholesterol as 1.01 (Johnson & Buttress, 1973), and the molecular weight of phospholipid as 775, it can be calculated that our analysis values give a membrane density of 1.15, and those of Allan & Crumpton (1970) a density of 1.17. Minor membrane constituents were ignored in the calculation.

Plasma-membrane polypeptides

Polyacrylamide-gel electrophoresis showed a large number of polypeptide chains with mol.wts. from 20000 up to about 470000. The most prominent single band had mol.wt. 46000; different membrane preparations had virtually identical patterns (Plate 1). A comparable range of molecular weights, as well as a major polypeptide chain of about 46000 daltons, has been found in plasma membranes from kidney and liver cells (Neville & Glossmann, 1971), rat thymocytes (Zimmerman, 1974), human lymphocytes (Ragland *et al.*, 1973; Lopes *et al.*, 1973) and HeLa cells (Atkinson & Summers, 1971). The polyacrylamide-gel pattern was the same whether the membrane sample was fully reduced with 2-mercaptoethanol or not reduced, indicating that most of the membrane polypeptides are not cross-linked by intermolecular disulphide bonds.

The lack of effect of reduction was unexpected, because the membranes contain immunoglobulins, which are usually disulphide-linked multichain molecules (see below). In 100 μ g of membrane protein, there probably is a total of about 1 μ g of immunoglobulin [about 0.7 μ g and 0.3 μ g of heavy (H) and light (L) chains respectively]. Such small amounts will be difficult to identify in the complex electrophoretic pattern, especially since each stained band may contain more than one species of polypeptide chain.

We have tried to identify the immunoglobulin polypeptides as stained bands by adding pig IgG or IgM to the membrane sample before reduction and electrophoresis. One band in the light-chain region, mol.wt. about 20000, increased in staining intensity and probably contained the membrane-associated light chain. In the heavy-chain region, however, the effect of the added immunoglobulin was to diminish the resolution of several bands, rather than to increase the intensity of one band, although the heavy chain alone gave a distinct band; the reason for this effect is not known.

Plasma-membrane glycoproteins

Plate 2 shows that 12–15 periodic acid-Schiff-positive polypeptide chains were detected in pig plasma membrane, but this must be considered a minimum number. The staining procedure that we have used can detect as little as 2–3 μ g of carbohydrate (Kapitany & Zebrowski, 1971). Nevertheless, polypeptides in amounts of 10 μ g, even if they possessed as much as 20% carbohydrate, would not be seen, and many of the stainable polypeptides are present in amounts much less than 10 μ g. Although several of these glycopeptides had apparent mol.wts. greater than 300000, the remainder were distributed throughout the range of molecular weights. The very high molecular weight of plasma-membrane glycoproteins from tumour cells has been estimated by electron microscopy to vary from 55000 to more than 500000 (Slayter & Codington, 1973). We have not tried to determine their molecular weights, because many glycoproteins show anomalous mobilities in sodium dodecyl sulphate-polyacrylamide gels (Segrest *et al.*, 1971). (Ovalbumin did not show this anomalous behaviour, but had the appropriate R_f on a calibration curve derived from the series of carbohydrate-free standard proteins.)

It is sometimes difficult to identify a given polypeptide as a glycoprotein on the basis of similar electrophoretic mobilities, because of the apparent electrophoretic heterogeneity of several of the higher-molecular-weight glycoproteins, compared with the Coomassie Blue-positive bands in the same region of the gels. Band 1, with mol.wt. about 470000, appeared to be a glycoprotein, whereas

EXPLANATION OF PLATE I

Polypeptide chain composition of young pig mesenteric lymph-node plasma membranes, prepared from intact lymph node and analysed by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis

The molecular weights of the indicated membrane proteins have been calculated by comparing their mobilities with the mobilities of standard proteins in the same gel slabs. The membrane sample contains 100 μ g of protein and has been delipidated.

EXPLANATION OF PLATE 2

Polypeptide chain and glycoprotein patterns of young pig mesenteric lymph-node plasma membranes

The samples were electrophoresed on a single slab of polyacrylamide gel. After electrophoresis, the gel was sliced and one half stained for protein (samples 1-4) and the other half for carbohydrate (samples 5-11). The photographs of the two halves of the gel have been juxtaposed by aligning the ovalbumin bands. All membrane samples were delipidated. The samples and loadings were as follows: (1) and (2) ovalbumin, 50 μ g; (3) bovine serum albumin, 50 μ g; (4) plasma membrane, 100 μ g; (5), (6), (7) plasma membrane, 200 μ g, 100 μ g and 400 μ g respectively; (8) bovine serum albumin, 50 μ g; (9) plasma membrane, 600 μ g; (10), (11) ovalbumin, 50 μ g. The specificity of the glycoprotein stain for carbohydrate is indicated by the lack of detectable staining of bovine serum albumin. Proteins 1, 2 and 11 are identified in sample 4 by the corresponding black dots on the right side of the pattern.

EXPLANATION OF PLATE 3

Presence of membrane-associated IgG immunoglobulins in several different membrane preparations, demonstrated by Ouchterlony double immunodiffusion (a) and radial immunodiffusion (b)

(a) Antiserum in the centre wells is anti-(pig IgG), absorbed with insoluble Fab γ -chain, and specific for γ chains. The membranes have been dissolved in a final concentration of 7% (w/v) sodium cholate. Antigen wells contain the following samples and protein concentrations: (1) plasma membrane, whole mesenteric nodes of young pig, 18mg/ml; (2) pig IgG, 240 μ g/ml; (3) plasma membrane, whole mesenteric nodes of young pig, 20mg/ml; (4) pig IgG, 240 μ g/ml; (5) plasma membrane from washed cell suspension, 17mg/ml; (6) pig IgM, 1000 μ g/ml; (7) plasma membrane, whole mesenteric nodes of young pigs, 26mg/ml; (8) plasma membrane, whole thymus of young pig, 33mg/ml; (9) and (10) pig IgG, 100 μ g/ml; (11) plasma membrane, whole mesenteric lymph nodes of young pig, 50mg/ml; (12) pig IgG, 100 μ g/ml. Plates were photographed unstained after being developed for 14 days (left) and 2 days (right). The precipitin line from the washed cell suspension (well 5) is barely visible on this plate, but a sample of this same membrane preparation gives an unequivocal ring by radial immunodiffusion (Plate 3b, well 2C). The lack of reaction of the anti-(γ chain) with IgM demonstrates the absence of antibodies for light and μ chains. (b) Agar contains 0.14% (v/v) anti-(pig Ig) reacting with light and γ -chain determinants. Row 1 contains standard pig IgG at the following concentrations: (A) 200 μ g/ml; (B) 100 μ g/ml; (C) 50 μ g/ml. Row 2 contains membrane samples dissolved in 14% (w/v) cholate: (A) membrane from whole mesenteric lymph nodes of young pigs, 13.7mg/ml; (B) membranes from whole mesenteric lymph nodes of young pigs (different preparation) 14.3mg/ml; (C) membrane from washed suspension of young pig mesenteric lymph-node cells, 14.0mg/ml. Row 3: the left-hand well of each pair contains the same membrane samples as the corresponding well in row 2, and the right-hand well of each pair contains pig IgG, 200 μ g/ml. The plate was developed for 7 days before washing, and dried and stained after a total of 12 days, and photographed after staining. The rings of stained material immediately adjacent to antigen wells 3A, 3B and 3C are presumed to be non-immunological precipitates of membrane materials, after removal of detergent, because they are not usually visible in unstained gels and are also present in samples which do not contain immunoglobulin.

EXPLANATION OF PLATE 4

Presence of lymphocyte membrane-associated IgM demonstrated by Ouchterlony double diffusion (a) and by radial immunodiffusion (b)

(a) The membranes were dissolved in a final concentration of 14% (w/v) sodium cholate. The sample wells contain: (1) 7S pig serum IgM, partially reduced (unfractionated), 500 μ g/ml; (2) plasma membrane from young pig whole mesenteric lymph node, 25mg/ml; (3) 19S pig serum IgM, 700 μ g/ml; (4) specific sheep anti-(pig μ -chain antiserum), 1:4 dilution. Plate was photographed unstained after 48h incubation. Note the fusion of the precipitin line from the membrane with that of the adjacent serum 19S IgM and 7S IgM respectively, indicating identity of antigenic determinants recognized by this particular antiserum. Note also the curvature of the 19S IgM line towards the antigen well, which is due to the high molecular weight of the 19S IgM relative to that of the bulk of antibodies; the absence of this curvature from the membrane precipitin lines suggests that the membrane-associated IgM has a lower molecular weight than does 19S serum IgM. (b) The agar contains 0.44% (w/v) specific sheep anti-(pig $\mu\gamma$ -chain antiserum). The membrane was dissolved in sodium cholate (14%, w/v). The sample wells contain: (1) 19S pig IgM, 350 μ g/ml; (2) 7S pig IgM (partial reduction), 290 μ g/ml; (3) plasma membrane from young pig whole mesenteric lymph node, 26mg/ml. Plates were photographed unstained after 4 days. Note the two precipitin rings from the partially reduced IgM. The reason for the two rings is not known because only a single precipitin line is seen in Ouchterlony plates; only the sharper (inner) ring fuses with the unreduced 19S IgM.

band 3 stained only faintly for carbohydrate. Band 11, the most prominent polypeptide chain, is clearly not a glycoprotein, although there is a minor periodic acid-Schiff-positive band at its leading edge or immediately in front of it.

Membrane-associated immunoglobulins

The membranes contained immunoglobulin which gave a reaction of identity with pig IgG when a specific anti-(pig γ -chain antiserum) was used (Plate 3a). The presence of IgG in thymus membranes (Plate 3a, sample 8), which shows a reaction of identity with the IgG in lymph-node membranes (Chavin, 1974), should also be noted. In subsequent immunological analyses, radial immunodiffusion was used, because the latter method is semi-quantitative and more sensitive than double immunodiffusion. In samples which had larger amounts of immunoglobulin, a reaction of fusion could be shown between the rings from the membrane and from a standard sample in an adjacent well (Plate 3b). The slight spurring between the two rings does not necessarily indicate a lack of immunological identity, because identical samples of pig IgG placed in adjacent wells sometimes showed slight spurring over one another. In membrane samples containing small amounts of immunoglobulin, fusion was difficult to demonstrate; in such cases, the standard ring was usually partially interrupted and/or distorted by the membrane ring. Fusion and/or interruption were not caused by high concentrations of detergent, solutions of purified phospholipids or an antigenically unrelated precipitation ring. Membranes prepared from kidneys (presumably lacking immunoglobulin), or samples examined in gels containing normal

serum instead of anti-immunoglobulin, diffused only a short distance into the gel, without forming a distinct ring of precipitation; hence the rings were not the result of diffusional dilution of the detergent.

The membranes contained 0.6% (w/w) of their total protein as IgG (Table 2). The variation from one preparation of membrane to another may be the result of differences between animals or variable losses of membrane constituents in the course of purification. The immunoglobulin content of membranes prepared from washed cells was about the same as that from intact lymph nodes, whereas that of membranes from newborn-piglet lymphocytes (unsuckled and antigenically unstimulated) was much lower (see the next section, 'Origin of the membrane-associated immunoglobulins'). The class of immunoglobulin in these last two types of membranes was not determined.

A single precipitin line could be demonstrated with mesenteric-node membrane by Ouchterlony double immunodiffusion, by using a specific anti-(pig μ -chain antiserum); this line fused completely with both 19S and partially reduced 7S serum IgM (Plate 4a). The precipitin lines from the membrane are straight and do not show the curvature towards the antigen well which is characteristic of the high-molecular-weight 19S IgM; this suggests that the pig membrane IgM is not a pentameric 19S polymer. There is evidence in other species that membrane-associated IgM is present as monomeric subunits (Marchalonis & Cone, 1973; Vitteta & Uhr, 1973; Andersson *et al.*, 1974).

When Mancini radial immunodiffusion was used mesenteric node membranes gave a sharp precipitin ring which interrupted the ring corresponding to 19S serum IgM (Plate 4b). Preparations of 7S pig IgM (made by 'partial' reduction with 5 mM-dithiothreitol to cleave all interchain disulphide bridges) gave two precipitin rings, both of which were larger and less sharp than the single ring given by 19S IgM at a comparable concentration (Plate 4b); only the inner ring fused with the 19S ring. Although the partially reduced pig IgM, in aqueous solvents, sedimented as 7S subunits in the ultracentrifuge, under dissociating conditions in sodium dodecyl sulphate-polyacrylamide gels it migrated as a mixture of molecules smaller than H_2L_2 -chain complex (D. Beale, personal communication). The partially reduced IgM preparation was not used to estimate the amount of membrane IgM because of the difficulty of interpreting the double rings, and 19S IgM was not used because it is probably larger than the membrane IgM. For these reasons we have not tried to quantify the amount of IgM in pig membranes.

According to Allan & Crumpton (1970) and Snary *et al.* (1974), between 17 and 34 mg of membrane protein may be obtained from 5×10^{10} lymphocytes. If we take a value of about 1% of the total membrane

Table 2. *Immunoglobulin content of plasma membranes from pig mesenteric lymph-node lymphocytes*

The percentage was calculated from the concentration of immunoglobulin, in IgG equivalents, estimated by radial immunodiffusion, and the concentration of total protein, estimated by the Folin method (Lowry *et al.*, 1951). The antiserum was a sheep anti pig IgG, which reacted predominantly with γ determinants, and to a lesser extent with light chains.

Origin of membrane	Percentage of immunoglobulin (w/w)	Mean
Whole mesenteric node	0.9, 1.0	1.0
Whole mesenteric node	0.6, 0.9, 1.0	0.8
Whole mesenteric node	0.8, 0.6, 0.5, 0.5, 0.5	0.6
Whole mesenteric node	0.4, 0.3, 0.3	0.3
Whole mesenteric node	0.3, 0.2	0.3
Washed cell suspension	0.4, 0.5, 0.7	0.5
Neonatal mesenteric nodes, two pigs, pooled	0.2, 0.1, 0.1	0.1

protein for the immunoglobulin content (IgG and IgM) of pig mesenteric lymphocyte membranes, we may calculate that 10^6 cells contain 3–7 ng of immunoglobulin. If we assume an average molecular weight for the membrane-associated immunoglobulins of 160000, and if the immunoglobulins are evenly distributed among all of the lymphocytes, then each cell contains between 1.2×10^4 and 2.7×10^4 molecules; if the immunoglobulins are restricted to only the 10% of cells which have surface immunofluorescence, then each cell will contain between 1.2×10^5 and 2.7×10^5 molecules. In unfractionated mouse spleen cells, Grey *et al.* (1972) found 7.5 ng of kappa chain and 5.6 ng of μ -chain equivalents/ 10^6 cells. In purified unstimulated B lymphocytes from mouse spleen, Andersson *et al.* (1974) found between 3×10^4 and 15×10^4 molecules of membrane-associated 7–8S IgM/cell. In a subfraction of rat thoracic-duct lymphocytes, Jensenius & Williams (1974) detected between 2×10^4 and 15×10^4 molecules of surface immunoglobulin/cell. Thus the amounts of lymphocyte membrane-associated immunoglobulins in different species are approximately the same. Because these data are derived from different populations of cells, with the use of different methods of measurement, no further analysis seems warranted.

Origin of the membrane-associated immunoglobulin

There are several possible sites from which the membrane immunoglobulin might be derived, other than the plasma membrane itself.

(a) *Adsorption on to or trapping within the vesicles of immunoglobulin and other proteins from plasma or lymph.* Lymphocyte membranes prepared from extensively washed cells had essentially the same immunoglobulin content as membranes from intact lymph nodes. Pooled lymphocyte membranes from two unsuckled newborn piglets contained 0.1% immunoglobulin, compared with 0.6% for those from 3–4-month-old pigs; since the serum from the piglets contained about 88 and 113 μ g of immunoglobulin/ml respectively (Binns *et al.*, 1972; S. I. Chavin, unpublished work), i.e. less than about 1% of the amount in serum from 3–4-month-old pigs, it is unlikely that nearly the same amounts of serum immunoglobulin would be trapped in both cases. An analogous membrane fraction from whole kidneys of the same unsuckled piglets contained no detectable IgG; since the lower limit of detection was 12.5 μ g/ml the maximum immunoglobulin content can be calculated to be less than 0.07% of the total protein. We consider that these results make the trapping or adsorption of extracellular proteins (during the preparation of the membrane) unlikely possibilities, although the adsorption *in vivo* of cytophilic immunoglobulins cannot be excluded.

(b) *Contamination of the plasma-membrane fraction by cytoplasmic components, in particular, immunoglobulin-bearing polyribosomes.* The only direct evidence against this is the absence of morphologically identifiable polyribosomes from electron micrographs. The absence of soluble cytoplasmic proteins from the membrane fraction is indicated by the low lactate dehydrogenase activity (0.1% of the total enzymic activity in the crude lymph-node homogenate).

Concluding remarks

The cells from mesenteric lymph nodes are very heterogeneous. The predominant morphological type is a small lymphocyte (Allan & Crumpton, 1970), but there are also large lymphocytes, macrophages, plasma cells and erythrocytes. About 5–10% of the mesenteric lymph-node cells have surface IgG and/or IgM (Binns, 1973), 1–15% (mean 4.5%) have Fc receptors (Binns & Tonkin, 1974), and 15.6% have complement receptors (R. M. Binns & C. H. Tonkin, personal communication). These three are thought to be B-cell markers. The distribution of these markers on individual cells is not yet known. Another 30% of the cells have the properties attributed to T lymphocytes (Escadajillo & Binns, 1975). The membranes described in the present studies were derived from both B and T cells. If all of the immunoglobulin is derived from cells with detectable surface immunoglobulin, these cells must have 10–20 times the average amount of immunoglobulin. Alternatively, some of the cells without detectable surface immunoglobulin may have membrane-associated immunoglobulin. This possibility is suggested by our recent demonstration of immunoglobulin (0.3%, w/w, of the total membrane protein) in membranes derived from thymus cells (Chavin, 1974). Less than 0.1% of these cells possessed surface immunoglobulin by immunofluorescence or immunoperoxidase labelling (D. F. B. Symons & S. I. Chavin, unpublished work).

A final point, concerning the definition of a membrane protein, is particularly relevant to the present studies because lymphocyte plasma membranes may be in contact with immunoglobulins at both cytoplasmic and extracellular surfaces, as well as possessing a fraction of immunoglobulin which is truly a membrane component. Membrane-associated proteins may be firmly associated with the membrane (integral proteins) or weakly associated with the membrane surface (peripheral proteins) (Singer & Nicholson, 1972). The amount of a particular peripheral membrane protein may vary according to the manner of preparing the membranes (Coleman, 1974) and probably depends on other factors as well. In addition it has recently been shown that certain proteins can exist either as components of plasma

membranes or as soluble molecules; these proteins include the β_2 microglobulin (Tanigaki *et al.*, 1974), the apoproteins of human serum lipoproteins (Langdon, 1974), and a protein from fibroblast plasma membranes (Ruoslahti & Vaheri, 1974). Although membrane-associated immunoglobulins may be tentatively classified as peripheral membrane proteins, there are several important questions which remain to be answered. (1) Is there a minor subpopulation of membrane-associated immunoglobulins which is more or less confined to the membrane? (2) Is the membrane-associated population structurally and antigenically identical with the secreted soluble immunoglobulin? (3) Is the membrane immunoglobulin directly associated with the lipid bilayer regions of the membrane, or is it more loosely associated with another (unidentified) integral membrane protein (Ramasamy *et al.*, 1974)?

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