Difference in Carbohydrate Composition and a Possible Conformational Difference between Intracellular and Extracellular Immunoglobulin M†

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ABSTRACT: Intracellular 7S IgM_s subunits were purified from plasma cells of mouse plasmacytoma MOPC 104E by ammonium sulfate precipitation, agarose block electrophoresis, and sucrose gradient centrifugation. Intracellular IgM_s contains 23 glucosamine, 33 mannose, 0.5 galactose, and 2.5 fucose residues per 7S subunit, with no detectable amounts of neuraminic acid. Extracellular 19S IgM in two preparations either secreted from plasma cells or isolated from the serum of MOPC 104E tumor-bearing mice contained 26 and 26 glucosamine, 36 and 34 mannose, 14 and 10 galactose, and 16 and 8 fucose residues, with qualitatively detectable amounts of *N*-glycolylneuraminic acid in both preparations. All preparations also contained 2 to 3 residues of each glucose and galactosamine. IgM_s subunits (7S) obtained from extracellular 19S IgM by reduction in 5×10^{-4} M dithioerythritol could be reoxidized to 19S molecules. Intracellular 7S IgM_s subunits however, reduced at the same dithioerythritol concentration, could not be oxidized to 19S molecules. In mixtures with extracellular IgM intracellular subunits could be hybridized with extracellular subunits to 19S molecules. In equimolar mixtures, however, only 9% of the intracellular subunits appeared in hybrid 19S molecules, a 10^4 excess of extracellular subunits drawing 70% of the intracellular subunits into hybrid molecules. A conformational difference between intra- and extracellular IgM, possibly in the F_c portions of the molecules, is implied from these results.

Immunoglobulin M[1] is generally composed of five 7S subunits (IgM_s) which are linked by disulfide bonds in the F_c part of the molecule. Each IgM_s subunit consists of two heavy (μ) chains and two light chains bonded by disulfide bridges (for a review, see Metzger, 1970). IgM contains carbohydrate. While no information is available on the exact location and structure of these carbohydrate moieties in mouse IgM, such carbohydrate moieties have been located in human IgM at five sites in the constant sequence region of the μ chains. The human oligosaccharides are of two kinds, simple ones containing glucosamine and mannose residues, and complex ones containing glucosamine, mannose, galactose, fucose, and neuraminic acid residues (Shimizu *et al.*, 1971).

Biosynthesis of IgM has been studied mainly using the transplantable mouse plasma cell tumor MOPC 104E (McIntire *et al.*, 1965). In these tumor cells 7S IgM_s subunits formed from HL intermediates accumulate within plasma cells and are polymerized to 19S molecules very shortly before or simultaneously with secretion (Parkhouse and Askonas, 1969; Askonas and Parkhouse, 1971; Parkhouse, 1971). Incorporation of radioactive mannose, galactose and fucose into MOPC 104E mouse plasma tumor cell suspensions have suggested a stepwise addition of carbohydrate residues to IgM during the process of secretion. Mannose and glucosamines are added early on, while galactose and fucose are added just before, or at the time, IgM leaves the cell (Parkhouse and Melchers, 1971). These experiments suggested that addition of carbohydrate residues to IgM may somehow be correlated with the polymerization into secreted 19S IgM.

This paper reports the purification of intracellular IgM_s from MOPC 104E plasma cells and the analysis of its carbohydrate moieties. It has been found that 7S subunits obtained by partial reduction with dithioerythritol from 19S IgM could be reconstituted to 19S molecules by reoxidation. Intracellular 7S IgM_s subunits could be polymerized into 19S molecules in the presence of excess carrier 19S secreted IgM (Parkhouse *et al.*, 1970). Here we report experiments attempting to polymerize intracellular 7S IgM_s into 19S molecules by reduction and reoxidation in the absence of carrier extracellular 19S IgM and to polymerize intracellular 7S IgM_s with extracellular IgM in different molar ratios into hybrid 19S IgM molecules.

Materials and Methods

The MOPC 104E plasma cell tumor, synthesizing and secreting IgM and λ-type light chain (McIntire *et al.*, 1965), was kindly given to me by Dr. M. Potter, National Institutes of Health, Bethesda, Md., and maintained by subcutaneous transplantation in Balb/c mice. Tumors 2–4 weeks of age in transplant generations 25–40 were used.

Labeling *in vitro* of intracellular and extracellular protein by plasma cell tumor 104E with L-[4,5-^{3}H]leucine (The Radiochemical Centre, Amersham, batch 28, 22 Ci/mmole) was performed as described (Melchers, 1970). Immune coprecipitations were done with IgM- (μ,λ-) and μ-specific antisera as described (Parkhouse and Melchers, 1971). I thank Dr. R. M. E. Parkhouse, National Institute for Medical Research, Mill Hill, London, for a sample of μ-specific antiserum, reacting with μ-specific, but not with λ-specific determinants and Dr. A. Feinstein for a sample of sheep anti MOPC 104E 19S IgM serum. Sepharose–antiserum immunosorbents were prepared as described (Melchers and Sela, 1970).

Frozen solid MOPC 104E plasma cell tumor tissue (100 g) and 1×10^9 cells labeled for 5 hr with [^{3}H]leucine were lysed in 1000 ml of 0.3 M NaCl–0.01 M potassuim phosphate (pH 7.0, called buffer I) containing 1% Nonidet P-40 (NP-40, Shell Chem. Co., London) at 4° for 24 hr under vigorous

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[1] The nomenclature for human immunoglobulins ((1964), *Bull. WHO 30*, 447) will be followed.

stirring. The lysate was spun for 30 min at 40,000*g* in the SS-34 rotor of the Sorvall RC2-B centrifuge. The supernatant was subjected to stepwise precipitation with ammonium sulfate in the cold as described in the Results.

Preparative electrophoresis on agarose (Sea Kem) blocks in 0.05 M Veronal buffer (pH 8.6 for 72 hr at 10 V/cm, 2 mA/cm, and 4°) was done as described by Braun and Krause (1968). IgM was assayed for in ammonium sulfate (see Results) and agarose block fractions by Ouchterlony immunodiffusion tests using the IgM- and the μ-specific antiserum. The μ-containing fractions were pooled and dialyzed against 10 changes of 100 volumes excess of buffer I. They were concentrated by adjusting solutions with ammonium sulfate to 45% saturation in the cold.

Sucrose gradients were convex exponential varying from 5% to approximately 25% sucrose (Schwarz and Mann, Orangeburg, N. J., sucrose density grade, RNase free) in 0.3 M NaCl–0.02 M Tris-HCl (pH 8.0). They were spun with an SW-41 rotor for 14 hr at 40,000 rpm, 4°, in a Beckman L2-65B ultracentrifuge. The optical density profile of the sucrose gradients was recorded continuously with a flow cell system mounted in a Gilford Model 2400 spectrophotometer. The optical density scale recorded at 280 mμ in the figure is corrected for 1-cm light path. If radioactivity were analyzed, fractions of approximately 0.5 ml were collected directly into counting vials, 1.5 ml of H_2O was added, then mixed with 12 ml of Triton X-100 toluene scintillation liquid (Noll, 1969), and counted as described by Falvey and Staehelin (1970).

Protein peaks from sucrose gradients were dialyzed extensively against buffer I, concentrated by ammonium sulfate precipitation, and filtered over Bio-Gel-P10 columns in buffer I. Blank samples without protein were run through the purification procedure and analyzed for contaminating carbohydrate.

Polyacrylamide gel electrophoresis was done as described by Choules and Zimm (1965) and Maizel (1966).

Hydrolysis of the intracellular and extracellular IgM and the chemical analyses of the carbohydrate residues—neutral hexoses by gas chromatography as alditol hexaacetates (Kim *et al.*, 1967), hexosamines on the amino acid analyzer (Walborg *et al.*, 1963)—were done as described (Melchers and Knopf, 1967; Melchers, 1970).

Results

Purification of Intracellular IgM from MOPC 104E Plasma Cells. The detergent lysate of 100 g of solid MOPC 104E plasma tumor cells containing [^{3}H]leucine-labeled cells (see Materials and Methods) was subjected to stepwise ammonium sulfate precipitation by saturating first to 25%, then to 43%, then to 75% in the cold. The fraction precipitating between 25 and 43% saturation contained 97% of the myeloma protein serologically precipitable with μ-specific antiserum. Ribosomes in the redissolved 25–43% fraction were removed by centrifugation for 30 min at 40,000*g*. The stepwise ammonium sulfate precipitation was repeated on the supernatant and the resulting second 25–43% fraction used for subsequent purification by agarose block electrophoresis (see Materials and Methods). After electrophoresis for 72 hr myeloma protein serologically precipitable with μ-specific antiserum had migrated between 2 and 8 cm toward the anode. It was recovered from the agarose by repeated extraction with buffer I (see Materials and Methods) and concentrated by ammonium sulfate precipitation.

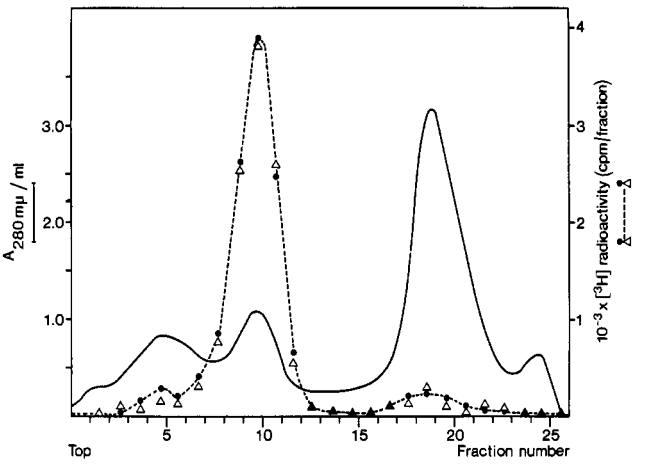

FIGURE 1: Sucrose gradient analysis of an aliquot of the IgM-containing fraction obtained from lysates of [^{3}H]leucine-labeled MOPC 104E tumor plasma cells by ammonium sulfate precipitation and agarose block electrophoresis (see Materials and Methods section). Gradient fractions were analyzed for ultraviolet-absorbing material ($A_{280\,m\mu}$) (—), for radioactivity precipitable by trichloroacetic acid (●), and by μ-specific antiserum (△).

The extensively dialyzed μ-containing agarose block fraction containing 5 mg/ml of protein was layered onto a 5–25% convex exponential sucrose gradient (see Materials and Methods). After centrifugation between 80 and 95% of the applied radioactivity was recovered in a peak sedimenting at a position on the gradient (fractions 8–11) coinciding with that of a 7S IgG_1 myeloma protein used as a marker (Figure 1). All of the radioactivity in the 7S peak was serologically precipitable by IgM- or μ-specific antiserum, while less than 5% of it was precipitated by an unspecific antiserum. Over 95% of the ultraviolet-absorbing material of the 7S peak was adsorbed to a Sepharose anti-μ antiserum immunosorbent column. Thus practically all the radioactive and ultraviolet-absorbing material in the 7S peak was serologically identifiable as IgM_s.

Fractions 18–22 of the gradient (Figure 1) contained 3–10% of the radioactivity and around 65–70% of the ultraviolet-absorbing material applied to the gradient. Migration on the gradient coincided with that of marker 19S IgM purified from serum of MOPC 104E tumor-bearing mice. Practically all of the radioactivity as well as the ultraviolet-absorbing material was serologically identifiable as IgM. When washed MOPC 104E tumor cells from single cell suspensions were used instead of solid tumor tissue for the preparation of intracellular IgM, the 19S peak decreased to approximately 10% of the total material applied onto the gradient. It appears, therefore, that most of the 19S material represents IgM already secreted from cells but trapped in intercellular spaces within the solid tumor tissue used in the preparation of intracellular IgM in Figure 1.

Upon reduction and alkylation of the 7S and 19S material (Figure 1) equimolar amounts of heavy and light chains were found in polyacrylamide gel electrophoresis, as judged by the distribution of [^{3}H]leucine radioactivity between the two chains.

Fractions 3–6 as well as 23 and 24 also contained serologically identifiable μ determinants.

From 100 g of tumor tissue 5 mg of intracellular 7S IgM_s and 22 mg of 19S IgM secreted into the intercellular spaces of the tumor were obtained.

TABLE I: Carbohydrate Composition of Intracellular 7S IgM_s and Extracellular 19S IgM from Mouse Plasma Cell Tumor MOPC 104E.

Residues	Moles of Monosaccharides/Mole of 7S IgM[a] Subunit		
	Intracellular 7S IgM_s	Extracellular 19S IgM from Cell Lysate	Extracellular 19S IgM from Serum
Glucosamine	23 ± 2	26 ± 3	26 ± 3
Galactosamine	2 ± 1	3 ± 1	2 ± 1
Mannose	33 ± 3	36 ± 3	34 ± 3
Galactose	0.5	14 ± 1	10 ± 1
Fucose	2.5 ± 1	16 ± 2	8 ± 1
Glucose	2.0 ± 1	2 ± 1	3 ± 2
N-Glycolylneuraminic acid[b]	Absent	Present	Present

[a] Purity of the protein was assumed to be 100%. 1 mg of protein was taken to correspond to 1.5 $A_{280\ m\mu}$ units. [b] Determined qualitatively as described (Melchers *et al.*, 1966).

TABLE II: Hybridization of Mixtures of [^{3}H]Leucine-Labeled Intracellular and Unlabeled Extracellular 7S IgM_s in Different Molar Ratios.

Molar Ratio of[a]		% of Total [^{3}H]Leucine Radioactivity Recovd in	
[^{3}H]-Leucine-Labeled Intracellular 7S IgM_s	Unlabeled Extracellular 7S IgM_s	7S Peak	19S Peak
1	0	>99	<1
1	0.1	96.5	3.5
1	0.5	93.5	6.5
1	1	91	9
1	2	86	14
1	10	75	25
1	100	58	42
1	1,000	42	58
1	10,000	30	70

[a] All mixtures were reduced and oxidized at 0.2 mg of protein in 0.3 M NaCl–0.05 M Tris-HCl (pH 8.0) with 10^{-3} M EDTA and 5×10^{-4} M dithioerythritol for 2 hr at room temperature. They contained a total of between 4×10^3 and 4×10^4 cpm of [^{3}H]leucine-labeled intracellular 7S IgM_s adjusted to the appropriate specific activity by unlabeled intracellular IgM_s.

Carbohydrate Composition of Intracellular and Extracellular IgM. IgM_s (7S) and IgM (19S) from the plasma cell lysate (Figure 1) as well as 19S IgM purified from serum of MOPC 104E tumor-bearing mice in the same way described above for protein from cell lysate were analyzed for their carbohydrate content. The results of the analyses, done in triplicate, are summarized in Table I.

The preparations of extracellular 19S IgM had only slightly more glucosamine and mannose residues and approximately the same amount of galactosamine than intracellular 7S IgM_s. Some glucose was found in all three preparations. Intracellular 7S IgM_s, in contrast to the extracellular 19S IgM preparations, contained very little fucose, barely detectable amounts of galactose and no detectable amounts of neuraminic acid. Extracellular 19S IgM, on the other hand, contained these monosaccharides, however in different amounts. The preparation of serum 19S IgM had less galactose and fucose residues than that of 19S IgM isolated from the cell lysate. Both 19S IgM preparations contained neuraminic acid, identified as *N*-glycolylneuraminic acid.

Reduction and Reoxidation of Mixtures of Intracellular 7S IgM_s and Extracellular 19S IgM. Conditions for reduction and oxidation of intracellular 7S IgM_s and extracellular 19S IgM were essentially those employed by Askonas and Parkhouse (1971). In pilot experiments extracellular 19S IgM was reduced at 0.2 mg/ml for 2 hr at room temperature with increasing concentrations of dithioerythritol. One aliquot of the reduced protein was analyzed for its size on sucrose gradients (see Materials and Methods) containing the same concentration of dithioerythritol that was used in the reduction. Another aliquot was oxidized after removal of dithioerythritol on a G-25 Sephadex column by standing in air at 4° overnight. The oxidized protein was analyzed for its size on sucrose gradients not containing dithioerythritol.

While 5×10^{-5} M dithioerythritol did not split the 19S molecules, a ten times higher concentration of it yielded 80–85% 7S IgM_s subunits and the rest in material sedimenting faster than 19S and partially to the bottom of the tube. Upon reoxidation 80–90% of the material reduced at 5×10^{-4} M dithioerythritol could be reconstituted to 19S IgM. Still higher concentrations of dithioerythritol, *e.g.*, 5×10^{-3} M, did not yield any appreciable amount of 7S IgM_s, since practically all the protein aggregated to a size bigger than 19S and partially sedimenting to the bottom of the tube, when analyzed in dithioerythritol-containing sucrose gradients. IgM reduced at this high dithioerythritol concentration could only partially be reoxidized to 19S molecules.

Different molar ratios of intracellular [^{3}H]leucine labeled 7S IgM_s and extracellular unlabeled 19S IgM (see Figure 1) were mixed and reduced at 5×10^{-4} M dithioerythritol. After removal of the dithioerythritol over a G-25 Sephadex column the protein mixture was oxidized by standing in air at 4° overnight. It was then analyzed for its size on sucrose gradients. Radioactivity was found only in the 7S or 19S regions, but not in other regions of the gradients. Table II summarizes these hybridization experiments. It shows that intracellular 7S IgM_s does not form any 19S molecules under the conditions used for reduction and oxidation. IgM_s (7S) forms more and more hybrid 19S molecules with subunits from extracellular 19S IgM as the excess in extracellular subunits in the mixtures increases. It can be hybridized to as high as 70% in 10^4-fold excess of extracellular over intracellular IgM subunits. The distribution of ultraviolet-absorbing material in the 19S and 7S peaks was approximately that expected from the radioactivity analyses in Table II. A minor amount (5–10%) was found sedimenting faster than 19 S and to the bottom of the tube after reduction and reoxidation. These results could be repeated when 19S IgM from serum was used instead of 19S IgM from the cell lysate.

Discussion

Different criteria can be employed in judging the purity of the intracellular 7S IgM_s fraction from sucrose gradients in Figure 1. Polyacrylamide gel electrophoresis does not detect any contamination. From the serological precipitations it can be concluded that the purity of the intracellular 7S IgM_s is as good as the one of the extracellular 19S IgM preparations used for the production of the antisera, which were employed in the serological tests. A minor contamination by any substance(s), X, cannot be excluded, appears however improbable, since X would have to exist in a 7S and a 19S form as does IgM. The methods for purifying intracellular MOPC 104E 7S IgM_s appear easy and may be applied in the isolation and purification of receptor IgM from lymphoid cells.

In accordance with results obtained by Parkhouse and Askonas (1969) most of the [^{3}H]leucine-labeled intracellular material was found to be 7 S, even though no sulfydryl blocking reagent was present when plasma cells were lysed (Askonas and Parkhouse, 1971). While most of the 19S IgM from cell lysates (see Figure 1) can be expected to be secreted IgM trapped in intercellular spaces of the tumors, it cannot be excluded that a part of the intracellular IgM may be polymerized to 19S IgM. The 10–15% of the total [^{3}H]leucine-labeled intracellular material found in the 19S region of the gradient in Figure 1 could either be secreted from, but sticking to cells, or truly intracellular in origin.

Incorporations of radioactive mannose, galactose and fucose into intra- and extracellular MOPC 104E IgM have suggested that glucosamine and mannose residues are added to IgM early on, while galactose and fucose residues are added just before, or at the time, IgM leaves the cell (Parkhouse and Melchers, 1971). The carbohydrate analyses in Table I justify these conclusions. Galactosamines (see Table I) occur in carbohydrate moieties of immunoglobulins linking these groups to the protein *via* hydroxyl groups of either serine or threonine (Smyth and Utsumi, 1967). Glucose however appears less likely to be an integral part of such carbohydrate groups of immunoglobulins. Here it may be of interest to note that a series of α-1,3-linked dextrans have been found to react as antigens with MOPC 104E IgM (Leon *et al.*, 1970). MOPC 104E 19S IgM isolated from the serum contains less fucose and galactose residues than 19S IgM isolated from the cell lysate (Table I). Since fucose and galactose residues can be expected to occur at terminal and semiterminal positions within carboyhdrate groups, this may mean that 19S IgM in the serum exists partially degraded in its carbohydrate groups.

The carbohydrate composition of the MOPC 104E IgM appears similar to that of three human myeloma IgM (Spragg and Clamp, 1969). This may indicate a similarity in the structure of the carbohydrate groups of mouse and human IgM.

Three forms of MOPC 104E IgM have been found. Two, intracellular 7S IgM_s and extracellular 19S IgM, occur naturally. The third, 7S extracellular IgM_s, is obtained experimentally from 19S IgM by reduction with dithioerythritol. The three forms may all have partially different conformations. The ability or inability to form 19S molecules (Table II) is taken as an indication for such differences in conformations.

The structural basis, resulting in a conformational difference between intracellular and extracellular IgM, could be the lack of galactose, fucose and neuraminic acid residues not yet attached to intracellular IgM_s (Table II) (Parkhouse and Melchers, 1971). It may indicate a role of carbohydrate residues in the determination of different conformations of a glycoprotein.

In a human 19S IgM carbohydrate groups located near the intersubunit disulfide bridges contain, besides glucosamine and mannose residues, galactose, fucose and neuraminic acid residues (Shimizu *et al.*, 1971). If we assume similar structures for the carbohydrate groups and the intersubunit disulfide bridges in mouse MOPC 104E IgM this may imply that the addition of galactose, fucose, and neuraminic acid residues at sites near the cysteines participating in the intersubunit disulfide bridges may induce conformational changes in the F_c portions of IgM leading to disulfide bridge formation and polymerization.

Intracellular subunits could be hybridized more and more into 19S molecules as the excess of extracellular subunits increased. This appears to be in agreement with the findings of Askonas and Parkhouse (1971) who used an excess of unlabeled extracellular IgM to polymerize labeled intracellular 7S IgM_s into 19S molecules. These results make it unlikely that a concentration of 5×10^{-4} M dithioerythritol did not remove the postulated block from the cysteines participating in the intersubunit disulfide bridges, thus preventing hybridization into 19S molecules. They may suggest that extracellular 7S IgM_s can form hybrid 19S molecules with intracellular 7S IgM_s by imposing, with its conformation, a conformational change in the intracellular IgM_s.

IgM has been found to represent the main cell surface receptor molecule in antigen-reactive cells, parts of the F_c portion of the molecules being buried in the surface membrane (for a review, see Greaves and Hogg, 1972). Combination of receptor with antigen results in a number of cellular reactions leading to proliferation and differentiation of clones of lymphoid cells. Conformational differences induced by antigen in the receptor molecules could mediate a signal to the cell (for a possible mechanism of such signal mediation, see Changeux *et al.*, 1967). So far conformational changes have been found only in the F_{ab} portions of a human myeloma 19S IgM isolated from serum (Ashman *et al.*, 1971; Ashman and Metzger, 1971). In view of its importance as receptor molecule it should be interesting to study the action of antigen on the conformation of the F_{ab} as well as F_c portions of intracellular 7S IgM_s.

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