Biosynthesis of the Carbohydrate Portion of Immunoglobulins

INCORPORATION OF RADIOACTIVE FUCOSE INTO IMMUNOGLOBULIN G1 SYNTHESIZED AND SECRETED BY MOUSE PLASMA-CELL TUMOUR MOPC 21

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(Received 1 June 1971)

Incorporation of radioactive fucose into the immunoglobulin G1 myeloma protein secreted by mouse plasma-cell tumour MOPC 21 is stereospecific for the L-isomer. Heavy chains of the secreted form of the myeloma protein carry 90% of the label in fucose residues of their carbohydrate moieties. A small but significant amount of the intracellular immunoglobulin G1 of the mouse plasma-cell tumour MOPC 21 appears to be labelled. Serum in the incubation medium supplies low-molecular-weight diffusible substances necessary to maintain continuous secretion of fucose-labelled myeloma protein beyond 2–3 h, and of leucine-labelled myeloma protein beyond 6–8 h. In medium containing extensively dialysed serum the secretion of leucine- and fucose-labelled myeloma protein can be restored by the addition of 250 μ M-D-mannose, 250 μ M-D-galactose and 250 μ M-glucosamine. Synthesis and secretion appear to be facilitated in the presence of these sugars, although secretion of myeloma protein devoid of terminal fucose residues is possible for a limited time-period.

The carbohydrate groups of immunoglobulins are assembled in a stepwise manner at different subcellular sites during transport of the protein through the plasma cell (Melchers, 1970, 1971a; Sherr & Uhr, 1970; Schenkein & Uhr, 1970; Uhr & Schenkein, 1970; Choi, Knopf & Lennox, 1971; Parkhouse & Melchers, 1971). Fucose is added just before, or at the time of, secretion. This has been demonstrated by chemical analysis of intracellular and extracellular myeloma protein (Melchers & Knopf, 1967; Melchers, 1971a) and by incorporation of label from radioactive mannose as precursor into predominantly the fucose positions of secreted myeloma proteins (Melchers, 1970; Parkhouse & Melchers, 1971). Incorporation of radioactive mannose and galactose into secreted myeloma protein commences without the lag time of 20–30 min (Helmreich, Kern & Eisen, 1961) observed for newly synthesized leucine-labelled protein (Melchers, 1970). This suggests that there is an intracellular pool of myeloma protein precursors, lacking the complete complement of carbohydrate, that accepts the fucose residues some time after the synthesis of its polypeptide chains and shortly before leaving the cell.

The present paper reports the stereospecific incorporation of L-fucose, but not D-fucose, into fucose residues within the carbohydrate portion of an IgG1* myeloma protein. Since fucose residues

appear to occupy terminal positions within the carbohydrate portion of IgG1 molecules (Clamp, Dawson & Franklin, 1968; Spragg & Clamp, 1969; Melchers, 1969), incorporation of radioactive fucose was used as a marker for the assembly of a complete carbohydrate complement. The supply of certain monosaccharides that occur in the carbohydrate portion of the IgG1 protein was varied in the medium used for the incorporation of radioactive fucose by plasma-cell-tumour suspensions. Its effect on the synthesis and secretion of [³H]fucose-labelled IgG1 (presumably complete in its carbohydrate and polypeptide portions) was studied. Comparison with the synthesis and secretion of [³H]leucine-labelled IgG1 (complete possibly only in its polypeptide portion) suggests that, under conditions of monosaccharide starvation, the plasma-cell tumour secretes myeloma protein incomplete in its carbohydrate portion for a limited period of time. The results are discussed in relation to the hypothesis (Melchers & Knopf, 1967; Swenson & Kern, 1967) that attachment of carbohydrate is requisite for the secretion of immunoglobulins.

MATERIALS AND METHODS

The plasma-cell tumour MOPC 21, transplantable in Balb/c mice, was given to me by Dr M. Potter, National Institutes of Health, Bethesda, Md., U.S.A. It was used in transplanation numbers 50–80 at 2–3 weeks after

* Abbreviation: IgG, immunoglobulin G.

transplantation. Cell suspensions were prepared and incubated with 50 mCi of D-[1-³H]fucose (840 mCi/mmol) as well as 50 mCi of L-[1-³H]fucose (920 mCi/mmol) (both batch 1; The Radiochemical Centre, Amersham, Bucks., U.K.)/ml as described for the incorporation of radioactive mannose and galactose (Melchers, 1970) with the following alterations: foetal calf serum (Ip-tested, batch R8050Q; Grand Island Biological Co., Grand Island, N.Y., U.S.A.) was dialysed against a balanced salt solution of Eagle's medium (Vogt & Dulbecco, 1963), as indicated in the Results section. In double-labelling experiments with L-[U-¹⁴C]leucine (270 mCi/mmol) (New England Nuclear Corp., Boston, Mass., U.S.A.) at 25 μ Ci/ml and L-[1-³H]fucose at 50 μ Ci/ml cell suspensions were incubated in glucose-free medium containing 40 μ M unlabelled leucine. Unlabelled D-mannose, D-galactose and D-glucosamine were from Mann Research Laboratories, New York, N.Y., U.S.A. They were analysed on Dowex 2 (X8) columns and on Amberlite CG-120 columns (see below for the analyses of radioactive hexoses and hexosamines, and Fig. 3) for contaminating hexoses and hexosamines and found to be better than 98% pure. Glucosamine solutions were neutralized with HCl before use. Determination of total trichloroacetic acid-precipitable radioactivity incorporated by plasma cells, separation of plasma cells from the medium, lysis of cell pellets with 0.5% (v/w) Nonidet P-40 (Shell Chemical Co., London S.E.1, U.K.) and determinations of serologically precipitable radioactivity with rabbit antisera specific (recogniz-

ing κ -type light chain as well as IgG Fc determinants) and unspecific (recognizing *Escherichia coli* β -galactosidase) for the IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 were carried out as described previously (Melchers, 1970). The pig anti-(rabbit Ig) antibody fraction used in the co-precipitations of rabbit antiserum-mouse myeloma protein complexes was kindly prepared by Dr F. Franek, Czechoslovakian Academy of Science, Prague, Czechoslovakia. Values obtained by immune precipitations with specific antiserum were corrected by subtracting from them the values obtained by precipitations with unspecific antiserum (Figs. 1, 4 and 5). Polyacrylamide-gel electrophoresis of immune precipitates and analysis of radioactivity incorporated into the carbohydrate portions of IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 were done as described previously (Melchers, 1970). Simultaneous counting of ¹⁴C and ³H radioactivity was done in the Beckman LS-250 scintillation counter, with the automatic quench control. For optimum separation of ³H from ¹⁴C radioactivity in channels 1 and 2 the ³H channel (channel 1) was narrowed from the upper end of the radioactivity spectrum to yield 48% of the ³H c.p.m. of the total spectrum (channel 3), whereas the ¹⁴C channel (channel 2) was narrowed from the lower end of the spectrum to yield 51% of the ¹⁴C c.p.m. of the total spectrum (channel 3). Under these conditions less than 0.5% of the ³H radioactivity in channel 1 was in channel 2, and 10–15% of the ¹⁴C radioactivity in channel 2 was in channel 1.

Fig. 1. Time-course of incorporation of (a) L-[1-³H]fucose and (b) D-[1-³H]fucose into total acid-precipitable material (□) and into intracellular (○) and secreted (Δ) IgG1 myeloma protein of mouse plasma-cell tumour MOPC 21 obtained by precipitation with specific antiserum. For details see the Materials and Methods section.

RESULTS

Incorporation of D-fucose and L-fucose

Incorporation of D-fucose and L-fucose into total acid-precipitable material and into immunologically characterized IgG1 myeloma protein of plasma-cell tumour MOPC 21 was performed with suspensions of plasma-cell tumour MOPC 21 in medium containing 3% (v/v) of non-dialysed foetal calf serum (with approx. 1g of glucose/l of serum). The incorporation rates into acid-precipitable material and into immunologically characterized IgG1 myeloma protein of plasma-cell tumour MOPC 21 were not constant, but decreased steadily. Only L-fucose, but not D-fucose, was incorporated into the immunologically characterized IgG1 myeloma protein of plasma-cell tumour MOPC 21 (Figs. 1a and 1b). L-Fucose-labelled IgG1 myeloma protein represented between 40 and 65% of the total acid-precipitable radioactivity. A small, but significant, amount of radioactive myeloma protein was found inside the cells (5–15% of the total acid-precipitable material).

Electrophoretic analysis of the radioactive IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 on sodium dodecyl sulphate-polyacrylamide gels indicated that the label was incorporated into heavy chains, but not into light chains (Fig. 2). Labelled myeloma protein was analysed for its radioactivity. Most of the radioactivity (90%) recovered in neutral hexoses from the Dowex 2 (X8) columns was in fucose; approx. 5% each was in mannose and galactose (Fig. 3). There was no detectable radioactivity in the hexosamine fraction.

Variation of monosaccharide content in the incubation medium

Effect on the synthesis and secretion of fucose-labelled IgG1 myeloma protein by plasma-cell tumour MOPC 21. Diffusible material of low molecular weight present in the foetal calf serum was diluted 1:10¹, 1:10², 1:10³ and 1:10⁴ by dialysis against Eagle's balanced salt solution (Vogt & Dulbecco, 1963). The glucose-free incubation medium was then supplied with these depleted foetal calf sera to a final concentration of 3% (v/v), and the incorporation of fucose by plasma-cell tumour MOPC 21 into immunologically characterized IgG1 myeloma protein was measured (Fig. 4). The total amount of intracellular fucose-labelled IgG1 myeloma protein did not change in the different media. Secretion of labelled IgG1 myeloma protein, however, ceased after 2–3 h. A 1:10¹ dilution of diffusible material in the foetal calf serum was sufficient to exert the effect; further dialysis of the

Fig. 2. Polyacrylamide-gel electrophoresis of (a) L-[4,5-³H₂]leucine-labelled and (b) L-[1-³H]fucose-labelled IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21. The labelled myeloma proteins were serologically precipitated with specific antiserum from culture media of 8 h incubations that had been 'cleaned' by an immune precipitation with unspecific antiserum. The precipitates were washed five times in iso-osmotic buffer, pH 7, reduced and alkylated (Schubert, 1970), subjected simultaneously in separate gels to electrophoresis on 7.5% cross-linked polyacrylamide gels containing 0.5M-urea, 0.1% sodium dodecyl sulphate, 0.2% 2-mercapto-ethanol and 0.01M-sodium phosphate buffer, pH 7.1, for 3.5 h at 5 mA/gel (Maizel, 1966) and assayed for radioactivity after dissolution of the gel fractions in Hyamine hydroxide (1% in methanol; The Radiochemical Centre)-pyridine-water (10:9:81, by vol.) (Choules & Zimm, 1965). Fractions are numbered from the positive to the negative electrode. The positions for leucine-labelled MOPC 21 heavy and light chains in the gel are indicated by the letters H and L respectively. For further details see the Materials and Methods section.

foetal calf serum did not decrease secretion of the fucose-labelled IgG1 myeloma protein much more. In all experiments the secreted IgG1 myeloma protein was assayed for its radioactivity as described above (see Figs 2 and 3) and 90% was found in the fucose residues of the heavy chains.

Diffusible material in the foetal calf serum therefore appears to enable plasma cells to secrete fucose-labelled IgG1 myeloma protein for periods of time longer than 2–3 h. An intracellular pool of myeloma protein capable of accepting fucose residues shortly before leaving the cell appears to

Fig. 3. Analysis of (a) radioactive standard hexoses and (b) the neutral hexose fraction on a 0.9 cm $\times$ 100 cm column of Dowex 2 (X8) (fraction volume 1.55 ml) (Walborg, Christensson & Gardell, 1965) obtained from IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 at 8 h after incorporation of L-[³H]fucose (see Fig. 1). For details see the Materials and Methods section.

sustain secretion of the fucose-labelled IgG1 myeloma protein for 2–3 h even when there is more extensive depletion of the foetal calf serum.

D-Galactose, D-glucosamine and D-mannose are present as residues within the carbohydrate moiety of the IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 (Melchers & Knopf, 1967), and appear to be used from the medium as precursors for incorporation into such residues (Melchers, 1970). Therefore glucose-free medium containing dialysed foetal calf serum was supplied with these sugars to study their possible efficacy in substituting for the diffusible material in foetal calf serum necessary for the maintenance of the synthesis and secretion of fucose-labelled IgG1 myeloma protein. With mannose, glucosamine and galactose in the medium the secretion of the fucose-labelled IgG1 myeloma protein beyond 3 h was not only restored, but increased to a constant rate of secretion for 13 h (Fig. 4).

Secretion of the fucose-labelled IgG1 myeloma protein could be reinitiated after starvation of the cells in glucose-free medium containing dialysed serum. This was achieved by the addition either of non-dialysed foetal calf serum to a final concentration of 3% (v/v) or, as shown in Fig. 5, of D-glucosamine, D-galactose and D-mannose to final concentrations of 250 μ M each. A lag period in the reinitiation of secretion was observed. The lag

period appeared to be longer the longer the cells had been starved. The rate of reinitiated secretion of the fucose-labelled IgG1 myeloma protein decreased with increasing length of starvation.

Effect on the synthesis and secretion of leucine-labelled IgG1 myeloma protein by plasma-cell tumour MOPC 21. If attachment of fucose residues to immunoglobulin is requisite for its transport and secretion from plasma cells, as has been suggested (Melchers & Knopf, 1967; Swenson & Kern, 1967), the secretion of the leucine-labelled IgG1 myeloma protein should cease at the same time that secretion of the fucose-labelled IgG1 myeloma protein does. Therefore the synthesis and secretion of double ([¹⁴C]leucine and [³H]fucose)-labelled IgG1 myeloma protein by plasma-cell tumour MOPC 21 was studied in glucose-free media containing 40 μ M unlabelled leucine supplied with dialysed foetal calf sera. Again the secretion of the fucose-labelled IgG1 myeloma protein stopped after 2–3 h. However, the secretion as well as the synthesis of the leucine-labelled IgG1 myeloma protein ceased only after 7–8 h (Fig. 6). Again a 1:10¹ dilution of diffusible material in the foetal calf serum was sufficient to halt secretion after 6–8 h; further dialysis of the serum appeared not to increase the inhibitory effect on secretion. It is evident that the leucine-labelled IgG1 myeloma protein continued to be secreted for approx. 4 h more after the fucose-

Fig. 4. Time-course of incorporation of L-[1-³H]fucose into IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 in medium containing 3% (v/v) of (a) undialysed (□), (b) dialysed (1:10⁴-diluted) (▽), (c) dialysed (1:10²-diluted) (▼), (d) dialysed (1:10³-diluted) (■), (e) dialysed (1:10⁴-diluted) serum (△) and (f) dialysed (1:10⁴-diluted) serum plus D-mannose, D-galactose and D-glucosamine (each 250 μM) (▲). Only the results for intracellularly labelled IgG1 myeloma protein from plasma-cell tumour incubated in dialysed (1:10⁴-diluted) serum (○) are given, since results obtained from incubations in the other media were indistinguishable from them. For details see the Materials and Methods section.

labelled IgG1 myeloma protein had ceased to be secreted. I conclude from these experiments that IgG1 myeloma protein incapable of accepting terminal fucose residues can be secreted from plasma cells.

Secretion of the double (leucine and fucose)-labelled IgG1 myeloma protein in media containing dialysed foetal calf serum could be not only restored, but increased to a constant rate by the addition of mannose, glucosamine and galactose (Fig. 6). These results (see also those for the fucose-labelled IgG1 myeloma protein in Fig. 4) indicate that these sugars facilitate secretion of the IgG1 myeloma protein in a glucose-free medium.

Synthesis of total intracellular leucine-labelled IgG1 myeloma protein was not changed in media containing either dialysed foetal calf serum or added sugars (Fig. 6). This appears to indicate that

Fig. 5. Time-course of incorporation of L-[1-³H]fucose into IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 in medium containing 1:10⁴-diluted dialysed foetal calf serum (○), to which D-glucosamine, D-galactose and D-mannose (each 250 μM) were added after 3 h (●) and 5 h (▲) of incubation. For comparison the secretion of IgG1 myeloma protein from cell suspensions in medium containing dialysed (1:10⁴-diluted) serum with these sugars added at zero time is given (---; taken from Fig. 4). For details see the Materials and Methods section.

continued synthesis is regulated by the capability of the plasma cells to secrete their IgG1 myeloma protein. Cell viability, as determined by uptake of fluorescein dibutyrate (Rotman & Papermaster, 1966; see Melchers, 1970) in the experiments, was approximately halved from initially above 80% to between 35 and 55%. There was no apparent correlation between cell viability and monosaccharide content in the medium.

DISCUSSION

Incorporation of L-fucose into the IgG1 myeloma protein secreted by plasma-cell tumour MOPC 21 confirms findings obtained by studying the incorporation of D-mannose (Melchers, 1970), namely that fucose residues are added some time after synthesis and shortly before secretion of the IgG1 myeloma protein from the plasma-cell tumour. Fucose can therefore be used as such or be formed from D-mannose for incorporation into immunoglobulin. It is not clear at present whether both pathways of incorporation are utilized simultaneously by the plasma-cell tumour.

Fig. 6. Time-course of simultaneous incorporation of L-[U-¹⁴C]leucine (○ and ●) and L-[³H]fucose (△ and ▲) into intracellular (● and ▲) and secreted (○ and △) IgG1 myeloma protein of plasma-cell tumour MOPC 21 in glucose-free medium containing 40 μM-leucine supplied with (a) dialysed (1:10⁴-diluted) foetal calf serum and (b) dialysed (1:10⁴-diluted) foetal calf serum plus D-mannose, D-galactose and D-glucosamine (each 250 μM). For details see the Materials and Methods section.

Fucose residues are found at non-reducing terminal positions within the carbohydrate portion (Clamp *et al.* 1968; Spragg & Clamp, 1969). Thus fucose-labelled immunoglobulin can be expected to

have other sugar residues attached e.g. *N*-acetylglucosamine, mannose and galactose residues, and therefore may be regarded as nearly or fully complete in its carbohydrate complement. The stereospecificity for the L-isomer can be used to compare the synthesis and location of nearly or fully completed immunoglobulin with the synthesis and location of another cell component incorporating D-fucose in double-labelling experiments within the same cells. It is noteworthy that surface-membrane-associated histocompatibility alloantigens (H-2), which appear to be genetically linked to or associated with immune responsiveness (McDevitt & Benacerraf, 1969), have been found to be associated with glycopeptide structures into which radioactive L-fucose could be incorporated (Muramatsu & Nathenson, 1970).

Dialysis of the serum added to the glucose-free medium results in loss of the capability for the secretion of the fucose-labelled IgG1 myeloma protein after 2–3 h, but of the leucine-labelled IgG1 myeloma protein only after 7–8 h, however. Leucine-labelled IgG1 myeloma protein represents all possible stages of synthesis and assembly of the protein, including the intermediates in the assembly of its carbohydrate portions (Melchers, 1970, 1971a). Secretion of the IgG1 myeloma protein therefore appears to continue for some time even though not all the sugar residues are added. The failure to accept fucose may be explained by a depletion of IgG1 myeloma protein molecules with a particular conformation in their carbohydrate moieties capable of accepting fucose residues.

The hypothesis that addition of all carbohydrate residues, including terminal fucose residues, is requisite for transport and secretion of the protein (Melchers & Knopf, 1967; Swenson & Kern, 1967) is therefore not tenable, at least not in this simple form. This can also be argued from evidence that immunoglobulin light chains can be secreted from plasma cells without the normal complement of carbohydrate present on the Fc portion of IgG (Schubert, 1970; Melchers, 1971b; Parkhouse & Melchers, 1971). All the evidence, including the results of the present paper, does not rule out the possibility that attachment to immunoglobulin of one or two sugar residues, e.g. *N*-acetylglucosamine or mannose, as sugars of the inner part of the carbohydrate moiety (Melchers, 1969) may bring about its selection for secretion from among all other intracellular proteins. It should also be remembered that plasma cells are end products of a differentiating cell clone secreting immunoglobulin probably without the triggering effect of antigen. It may well be that antigen-sensitive small lymphocytes regulate the synthesis and secretion of their immunoglobulin receptor by a much tighter control in the attachment of carbohydrate residues.

The observed lag period in the reinitiation of the secretion of the fucose-labelled IgG1 myeloma protein after replenishment of the incubation medium with either undialysed serum or with monosaccharides is reminiscent of the lag period observed in the secretion of newly synthesized leucine-labelled protein (Helmreich *et al.* 1961; Melchers, 1970), and is in contrast with the immediate secretion of the fucose-labelled IgG1 myeloma protein (Fig. 1). The lag period may be an experimental artifact due to the disturbance of cell suspensions caused by the sudden additions to the medium. It may, however, also be possible that incubation in glucose-free medium containing dialysed foetal calf serum depletes the plasma-cell tumour of intracellular structures involved in and capable of synthesizing, transferring and accepting fucose residues. In analogy with the normal secretion process such cells may need some time in the supplemented media for such structures to be resupplied.

Finally, heterogeneity within the carbohydrate moieties of immunoglobulins (Melchers, Lennox & Facon, 1966; Clamp *et al.* 1968; Spragg & Clamp, 1969) and more generally of glycoproteins (Gottschalk, 1969, and references cited therein), including those occurring in surface membranes of cells and viruses (Burge & Strauss, 1970), may be the result of incomplete synthesis of these carbohydrate groups as a result of monosaccharide starvation of glycoprotein-producing and -secreting cells.

Part of this work was done during a visit to the Department of Genetics, Stanford University Medical School, and was supported during this visit by U.S. Public Health Service Grant A1-08917-07 from the National Institutes of Health to Dr L. Herzenberg. I thank Dr Len Herzenberg and the members of his laboratory for their hospitality and their interest in this work. The able technical assistance of Miss Dorothee Jablonski is gratefully acknowledged.

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