Complement proteins C2, C4 and factor B

Effect of glycosylation on their secretion and catabolism

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Tunicamycin, an inhibitor of *N*-acetylglucosaminylpyrophosphopolyisoprenol-dependent glycosylation, was used to study the effect of glycosylation on the synthesis, post-translational modification, secretion and function of the complement proteins that are associated with the major histocompatibility complex in humans, mice and guinea pigs. Tunicamycin blocked glycosylation of pro-C4, C2 and factor B and inhibited secretion of the corresponding native complement proteins synthesized by guinea-pig peritoneal macrophages in tissue culture. In addition, underglycosylated pro-C4 was more rapidly catabolized intracellularly than the corresponding fully glycosylated pro-complement protein. C4 protein secreted by cells incubated with tunicamycin had approximately the same specific biological activity as the protein obtained from control culture media, suggesting that carbohydrate is not required for its activity in immune haemolysis. Direct studies of carbohydrate incorporation and the tunicamycin effect suggested an unequal distribution of sugar among the C4 subunits, with maximal incorporation of carbohydrate into α -, and less into the β -chain of the native protein.

The complement system consists of 20 plasma proteins that mediate many functions of the inflammatory response (Muller-Eberhard, 1975). Most of the complement proteins contain carbohydrate, the content of which varies considerably among individual complement components (Reid & Porter, 1980). The genes for three of the complement proteins, the second (C2) and fourth (C4) components and factor B, reside within the major histocompatibility complex in humans (Alper, 1981) and guinea pigs (Bitter-Suermann *et al.*, 1981). In mice the C4 gene is also linked to the H-2 histocompatibility complex (Shreffler, 1976) and evidence for H-2-linked control of C2 levels has been reported (Gorman *et al.*, 1980).

Several complement proteins, including the second (C2), third (C3), fourth (C4), and fifth (C5) components and factors B and D of the alternative complement pathway are synthesized by macrophages (Colten *et al.*, 1979). The fourth, as well as

the third and fifth components of complement are each known to be synthesized as single-chain precursor proteins that undergo proteolytic cleavage to generate the native multichain proteins (Fey & Colten, 1980). Among these, pro-C4, the precursor of C4, and its post-translational modification, have been most extensively studied. The precursor-product relationship between pro-C4 and C4 was established on the basis of kinetic studies of C4 production by macrophages in culture (Parker *et al.*, 1979), by studies showing partial structural identity between the two proteins (Parker *et al.*, 1979; Goldberger *et al.*, 1980; Parker *et al.*, 1980) and direct demonstration of conversion by plasmin of pro-C4 into C4 *in vitro* (Goldberger & Colten, 1980). Native C4 is a 200 000-dalton protein that consists of three polypeptides of approx. M_r 95 000 (α), 78 000 (β) and 31 000 (γ) (Schreiber *et al.*, 1974; Gigli *et al.*, 1977). C4 in human plasma contains mannose, galactose, glucosamine and sialic acid, and the total carbohydrate content accounts for approx. 7% of the total molecular weight (Gigli *et al.*, 1977). The α - and β -chains are glycosylated, but no accurate assessment of carbohydrate content in the γ -chain has been reported. C2 and factor B are each single-chain glycoproteins of M_r 102 000 and 95 000

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organisation (1968); SDS, sodium dodecyl sulphate; endo-H, endo- β -*N*-acetylglucosaminidase-H.

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respectively (Kerr & Porter, 1978). These two proteins are strikingly similar in structure and function.

Recently, Roos *et al.* (1980a) have shown that radiolabelled carbohydrate is incorporated into murine pro-C4 produced by macrophages in culture, that carbohydrate incorporation is blocked by tunicamycin (Roos *et al.*, 1980b) and that secretion of C4 is decreased in the presence of this inhibitor of dolichyl phosphate-dependent glycosylation. We confirm their findings and also report that tunicamycin inhibits glycosylation and secretion of the other major-histocompatibility-complex-linked complement proteins, C2 and factor B. In addition, in the case of C4, the underglycosylated pro-C4 protein is more rapidly catabolized than the corresponding fully glycosylated protein. Finally, underglycosylated native C4, produced in the presence of tunicamycin, had approximately the same specific biological activity as C4 secreted by cells in control media, suggesting that the carbohydrate moiety is not required for haemolytic activity.

Materials and methods

Materials

The following reagents were purchased as follows: tissue-culture media and sera from Microbiological Associates, Bethesda, MD, U.S.A., except that Dulbecco's modified Eagle's Medium lacking methionine was obtained from Gibco Laboratories, Grand Island, NY, U.S.A.; phenylmethanesulphonyl fluoride, Trizma base, dithiothreitol, leucine, Triton X-100, deoxycholate and calf thymus DNA from Sigma, St. Louis, MO, U.S.A.; trichloroacetic acid, NaCl, KCl and toluene from Mallinckrodt, St. Louis, MO, U.S.A.; acrylamide, *NNN'*-methylenebisacrylamide, *NNN'*-tetramethylethylenediamine and ammonium persulphate from Eastman Kodak, Rochester, NY, U.S.A.; 15 ³H-labelled amino acids mixture (NET 250), [³⁵S]methionine (1050 Ci/mmol), D-[1-¹⁴C]mannose (216.5 mCi/mmol), [U-¹⁴C]galactose (56.5 mCi/mmol) and *N*-acetyl[1-¹⁴C]glucosamine (58.2 mCi/mmol), D-[2-³H]mannose (15 μ Ci/mmol), En³Hance, PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] from New England Nuclear, Boston, MA, U.S.A.; mercaptoethanol from Bio-Rad, Richmond, CA, U.S.A.; Instagel from Packard Instruments, Downers Grove, IL, U.S.A.; tunicamycin (lot nos. 361-268-117 and 361-26E-239-A) from Lilly Research Laboratories, Indianapolis, IN, U.S.A.

Methods

Guinea-pig pseudoglobulin was prepared as de-

scribed by Hall & Colten (1977), and antiserum to guinea-pig C4 was raised by the method of Frank *et al.* (1971) in deficient guinea pigs. Antiserum to guinea-pig C2 was raised in a similar manner by immunizing C2-deficient guinea pigs (Bitter-Suermann *et al.*, 1981), and goat antiserum to human factor B, reactive with guinea-pig factor B, was purchased from Atlantic Antibodies (Scarborough, ME, U.S.A.). Rabbit antiserum to ovalbumin and the globulin fractions of each antiserum were prepared as described by Hall & Colten (1977).

Hartley-strain guinea pigs (400–500 g) were injected intraperitoneally with a 3% potato-starch suspension; peritoneal-exudate cells were harvested at 72 h. The cells were washed, resuspended in supplemented Medium 199, and plated at a concentration of 2×10^6 cells in 15 mm flat-bottom wells (Linbro tissue-culture plates; Linbro Scientifics, Hamden, CT, U.S.A.) or 1.5×10^7 cells in 60 mm tissue-culture dishes (Falcon Plastics, Oxnard, CA, U.S.A.). After incubation for 2–3 h at 37°C to permit adherence, the monolayers were washed with Hanks (1948) balanced salt solution and fed with one of the media described below according to the experimental design.

For most experiments, Medium 199, lacking 15 amino acids, contained 100 μ Ci of a mixture of 15 ³H-labelled amino acids, 8.3 μ Ci of [¹⁴C]galactose, 8.3 μ Ci of [¹⁴C]mannose and 8.3 μ Ci of [¹⁴C]*N*-acetylglucosamine/ml. In some, [³H]mannose (100 μ Ci/ml) was added to complete Medium 199. For experiments with [³⁵S]methionine (100 μ Ci/ml), Dulbecco's modified Eagle's medium without methionine was used. All media contained 10% (v/v) heat-inactivated dialysed foetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml).

The source and preparation of reagents for haemolytic assays of C2 and C4 and specificity and sensitivity of those assays were described previously (Rapp & Borsos, 1970). An estimate of complement production per cell was calculated by dividing the number of molecules/ml by the amount of DNA per plate. The DNA assay was performed exactly as previously described (Einstein *et al.*, 1976); 1 μ g of DNA is equivalent to $(1.0\text{--}1.5) \times 10^5$ cells. The limit of sensitivity of the assay is approx. 1 μ g of DNA.

Total protein synthesis was estimated by the method of Roberts & Paterson (1973) and specific protein synthesis by immunoprecipitation of radiolabelled protein as described by Hall & Colten (1977).

Polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970) or Weber & Osborn (1969), and the radiolabelled proteins were assessed by autoradiography or by scintillation spectrometry. A ¹⁴C-labelled protein mixture was used as an *M_r* standard (Amersham, Arlington Heights, IL, U.S.A.).

Results

Incorporation of mannose, galactose and N-acetylglucosamine into pro-C4 and C4

Cultures of guinea-pig peritoneal macrophages were incubated in media containing [^{14}C]mannose, [^{14}C]galactose and/or [^{14}C]N-acetylglucosamine and ^3H -labelled amino acids for 12 h. Pro-C4 from intracellular lysates and C4 secreted into the media were immunoprecipitated and electrophoresed on SDS/polyacrylamide gels under reducing conditions. The results (Figs. 1 and 2) show that radiolabelled carbohydrates were incorporated into pro-C4 and that galactose was incorporated predominantly into the α -chain of secreted C4. Mannose incorporation into α - and β -chains was approximately equal; little or no carbohydrate was found in the γ -chain of secreted C4. The pattern of incorporation of N-acetylglucosamine was similar to that of galactose (results not shown): i.e., predominantly into the α -chain.

Effect of tunicamycin on glycosylation and secretion of C2, C4 and factor B proteins

Analysis by SDS/polyacrylamide-gel electrophoresis of intracellular pro-C4, C2 and factor B and the corresponding native proteins secreted by macrophages cultured in the presence of tunica-

Fig. 2. SDS (1%)/5.6%-polyacrylamide-gel electrophoresis of C4 antigen (under reducing conditions) of tissue-culture media from cells cultured for 12 h in the presence of (a) [^{14}C]galactose, (b) [^{14}C]mannose and (c) ^3H -labelled amino acids

Control immunoprecipitates (ovalbumin-anti-ovalbumin) yielded no peaks of radioactivity.

Fig. 1. SDS (1%)/5.6% (w/v)-polyacrylamide-gel electrophoresis of C4 antigen under reducing conditions (50 mM-dithiothreitol) from lysates of cells cultured for 12 h in the presence of (a) [^{14}C]galactose, [^{14}C]mannose and [^{14}C]N-acetylglucosamine and (b) ^3H -labelled amino acids in a double-label experiment

Radioactivity (c.p.m.) in 2 mm gel slices is shown. Control immunoprecipitates (ovalbumin-anti-ovalbumin) yielded no peaks of radioactivity.

mycin is shown in Figs. 3 and 4. The α - and to a lesser extent the β -chain of secreted C4 from tunicamycin-treated cultures had apparent molecular sizes less than those from control cultures. An increase in mobility was also noted in pro-C4 derived from tunicamycin-treated cultures. There was a similar increase in mobilities of intracellular and secreted C2 and factor B proteins

Fig. 3. SDS (1%)/polyacrylamide-gel electrophoresis of intracellular (a) and extracellular (b) C4 antigen from peritoneal-macrophage cultures incubated in the presence of [^{35}S]methionine for 6 h with (+) and without (–) tunicamycin (TM) (lot 361-268-117; 5 $\mu\text{g}/\text{ml}$)

Intracellular material was electrophoresed under reducing conditions on 4.5% (w/v), and extracellular on 8%, gels. Other abbreviation used: Std, M_r standards.

Fig. 4. SDS (1%)/7%-polyacrylamide-gel electrophoresis of intracellular and secreted (a) C2 and (b) factor B (FB) proteins produced by peritoneal-macrophage cultures incubated for 6 h in medium containing [^{35}S]methionine with or without tunicamycin (5 $\mu\text{g}/\text{ml}$; lot 361-268-117)

Lane 1, intracellular; lane 2, extracellular; lane 3, intracellular with tunicamycin; lane 4, extracellular with tunicamycin.

produced by tunicamycin-treated cells (Fig. 4). In addition, a small but reproducible difference was noted in the mobility of intracellular C2 and factor B as compared with the respective secreted proteins in control cultures. The kinetics of C2 production (Fig. 5) revealed a rapid rate of secretion (30–60 min).

Fig. 5. Kinetics of C2 secretion in peritoneal macrophages pulsed for 30 min in medium containing [^{35}S]-methionine, after which monolayers were washed and fed with medium containing unlabelled amino acids.

At timed intervals, cell lysates (a, intracellular) and media (b, extracellular) were subjected to immunoprecipitation and analysis of each on 1%-SDS/7%-polyacrylamide-gel electrophoresis under reducing conditions as described in the Materials and methods section.

Fig. 6. Effect of tunicamycin on secretion and intracellular catabolism of C4 in peritoneal macrophages. Cells were preincubated in medium containing tunicamycin (lot no. 361-26E-239-A; $10\text{ }\mu\text{g/ml}$) or in medium alone. During the second hour [^{35}S]-methionine ($100\text{ }\mu\text{Ci/ml}$) was added. At zero time, each culture was washed and fed with medium containing unlabelled amino acids. Cell cultures previously exposed to tunicamycin were re-fed with medium containing tunicamycin at $10\text{ }\mu\text{g/ml}$. Open symbols show radiolabelled C4 antigen in control cultures, and solid symbols show it in tunicamycin-treated cultures. Triangular symbols (Δ, ▲) indicate extracellular, and circles (○, ●), intracellular, C4 antigen.

Similar kinetics were found for factor B (results not shown), but not for C4 (see Fig. 6).

Digestion of the carbohydrate moiety from intracellular pro-C4 with endo-H (Zilberstein *et al.*, 1980) generated a protein with a mobility similar to that of pro-C4 produced in tunicamycin-treated cultures; intracellular C2 protein was also susceptible to endo-H cleavage (results not shown). Native secreted C4 was not cleaved by endo-H. Incubation of pro-C4 or C2 in the cell lysates at 37°C in the absence of endo-H had no effect on their mobilities.

Tunicamycin in these experiments had little effect on incorporation of radiolabelled amino acids into total intracellular protein as assessed by trichloroacetic acid precipitation, but decreased the amount of extracellular total protein by 30% (Table 1). Net incorporation of ^3H -labelled amino acids into intracellular pro-C4 in tunicamycin-treated cultures was 96% of control, but extracellular C4 was decreased by 50%. There was no detectable mannose incorporation into pro-C4 under these conditions (results not shown). In the secreted C4 protein from cultures exposed to tunicamycin, incorporation of ^3H -labelled

Table 1. *Effect of tunicamycin (lot no. 361-268-117, preincubated 2 h) on the incorporation of ^3H -labelled amino acids into intracellular and extracellular total protein (trichloroacetic acid precipitate) and C4 antigen*
 Volumes refer to sample size

	$10^{-3} \times \text{Radioactivity (c.p.m.)}$			
	Tunicamycin (5 $\mu\text{g/ml}$)		Control	
	Total (5 μl)	C4 antigen (700 μl)	Total (5 μl)	C4 antigen (700 μl)
Extracellular	6.5	33.7	9.8	65.0
Intracellular	30.7	9.5	32.5	9.9

Table 2. *Effect of unlabelled C4 antigen on the recovery of radiolabelled C4 protein in cell lysate and culture medium*

Radiolabelled C4 antigen obtained from lysate and medium from peritoneal cells cultured for 6 h in medium containing 100 μCi of [^{35}S]methionine/ml was mixed with equal volumes of lysate buffer or with unlabelled lysate from C4-containing (lysate from normal cells incubated 6 h), or C4-lacking (lysate from C4-deficient cells incubated 6 h), samples. Portions (350 μl) of each sample (i.e. mixtures contained one half of the amount of radiolabelled protein present in the portion of the labelled lysate) was immunoprecipitated (with 2-fold antibody excess and carrier protein) as described in the Materials and methods section. The results show that the amount of radioactivity in the precipitates was independent of the amount of C4 antigen synthesized during a 6 h period.

Radiolabelled C4 antigen mixed with:	$10^{-3} \times \text{Radioactivity in C4 immunoprecipitates (c.p.m.)}$	
	Intracellular	Extracellular
None	24.6	78.9
Lysate from C4-deficient cells	11.6	36.4
Lysate from C4-sufficient cells	12.2	38.8
Buffer	11.7	38.1

amino acids and functional C4 activity were decreased by 50%, but mannose incorporation into C4 was decreased by more than 75%, indicating that the underglycosylated C4 was functional.

Effect of tunicamycin on intracellular catabolism and secretion of C4 protein

The above experiments suggested an effect of tunicamycin on the secretion of C4 protein. To further evaluate this finding, the following experiment was undertaken. Macrophage cultures were incubated for 2 h in media with or without tunicamycin (lot 361-26E-239-A) (10 $\mu\text{g/ml}$) and during the second hour pulsed with [^{35}S]methionine. The

Fig. 7. *Kinetics of secretion of functional C2 (a) and C4 (b) by peritoneal macrophages, preincubated in medium containing tunicamycin (5 $\mu\text{g/ml}$) for 2, 4 and 6 h*
 ●, Control. Preincubation: ○, 2 h; △, 4 h; □, 6 h.

cell monolayers were washed extensively, then fed with fresh medium containing unlabelled amino acids with or without tunicamycin (10 $\mu\text{g/ml}$). The amounts of radiolabelled pro-C4 protein in cell lysates and native C4 in culture media were determined at timed intervals. Under the conditions employed for the immunoprecipitation, new protein

synthesis after the 'pulse' period had no effect on the total recovery of labelled pro-C4 (Table 2), hence measurement of radiolabelled antigen during the 'chase' provided a measure of secretion and catabolism. This experiment (Fig. 6) showed that, in control cultures, intracellular pro-C4 progressively decreased in the first 6 h, during which time there was a corresponding increase in extracellular native C4. During the same time period, in tunicamycin-treated cultures, the decline in intracellular pro-C4 was markedly decreased, and less native C4 appeared in the extracellular medium. Within the next 3 h period, intracellular pro-C4 in tunicamycin-treated cultures rapidly decreased to levels similar to those of controls, but no secretion of labelled native C4 was detected.

Effect of tunicamycin on secretion of biologically active C2 and C4 proteins

Peritoneal-exudate cells were preincubated in medium with tunicamycin, the cell monolayers were washed and fed with fresh medium and the secretion of functional C4 and C2 was monitored. In these experiments, preincubation with 5 µg of tunicamycin (lot 361-268-117)/ml for 2, 4 and 6 h led to progressive inhibition of secretion of functional C2 and C4 (Fig. 7).

Discussion

Experimental evidence for the importance of post-translational processing of complement was obtained after the recognition that several of these components are synthesized as precursors of the native plasma proteins (Fey & Colten, 1980). Recent studies demonstrated cleavage by plasmin of the precursor proteins pro-C4 (Goldberger & Colten, 1980) and pro-C3 (Goldberger *et al.*, 1981), generating proteins with the size and subunit structure of native C4 and C3 respectively. Post-synthetic processing also determines in part the amount of biologically active C5 secreted by activated and resting macrophages (Ooi *et al.*, 1980). Furthermore, Morris & Paz (1980) have shown that postsynthetic hydroxylation of proline residues of C1q, a complement protein similar in structure to collagen (Reid & Porter, 1980), is ascorbate-dependent.

The present data indicate that, as with several other eukaryotic proteins (Hickman *et al.*, 1977; Fujisawa *et al.*, 1978; Hickman & Kornfeld, 1978; Rothman *et al.*, 1978) and viral proteins (Takatsuki & Tamura, 1971; Toneguzzo & Ghosh, 1977; Leavitt *et al.*, 1977; Schwarz *et al.*, 1976), post-translational glycosylation affects the size and secretion of the major-histocompatibility-complex-associated complement components C4, C2 and factor B.

Kinetic studies indicated a requirement for pre-treatment with tunicamycin to effect inhibition. It appears that the intracellular pool of C4 protein is relatively small by comparison with the amount synthesized during the time course of these experiments. An even smaller pool of intracellular C2 and factor B proteins is indicated by pulse-chase experiments that show secretion of C2 and factor B within approx. ½ h. Thus the requirement for pre-treatment with tunicamycin to achieve maximal inhibition of secretion suggested that transport of tunicamycin to its active site within the cell or the action of the agent at that site is the time-limiting factor. However, the presence of a small intracellular pool of glycosylated complement protein could not be ruled out.

Decreased secretion of C4 protein was accompanied by an increased rate of intracellular catabolism of pro-C4 in cells treated with tunicamycin. These data are in agreement with studies suggesting that the rate of catabolism of several other glycoproteins is a function of their carbohydrate composition (Olden *et al.*, 1979; Hortin & Boime, 1980).

Native C4, C2 and factor B secreted by tunicamycin-treated cells were underglycosylated as assessed by increased mobility of subunits of secreted C4 protein and of the single C2 and factor B bands on SDS/polyacrylamide-gel analysis. Nevertheless the corresponding underglycosylated C4 protein had about the same specific biological activity as native C4, i.e., protein secretion and haemolytic activity in media from tunicamycin-treated cultures were inhibited to the same extent. These findings suggest that the intact carbohydrate moiety of the native protein is not essential for the haemolytic activity of C4. In a similar manner, although tunicamycin blocks glycosylation of H-2 and HLA (histocompatibility antigens in mice and humans respectively) heavy chains these underglycosylated polypeptides are apparently capable of interaction with the β_2 -microglobulin subunit of the major-histocompatibility-complex antigens and are expressed at the cell surface (Ploegh *et al.*, 1981).

Incorporation of carbohydrate occurs before conversion of pro-C4 into C4, but glycosylation is probably not completed in the precursor protein. This conclusion is based on the observation that glycosylation of pro-C4 is tunicamycin-sensitive and that endo-H is capable of removing carbohydrate from the precursor, but not the native protein. The latter finding suggests that modification of the carbohydrate moiety occurs during maturation to native C4, since complex oligosaccharides are resistant to endo-H (Tarentino & Maley, 1974; Trimble *et al.*, 1978). A similar shift from endo-H-sensitivity to endo-H-resistance has been noted during maturation of the HLA heavy chain (Kran-

gel *et al.*, 1979). The apparent difference in size between intracellular and extracellular C2 and factor B proteins, even in the presence of tunicamycin, suggests the possibility that a second, tunicamycin-insensitive glycosylation step precedes secretion of these two proteins as well.

Taken together, the present data indicate that postsynthetic processing of the major-histocompatibility-complex-associated complement proteins includes dolichyl phosphate-dependent glycosylation and perhaps a second independent glycosylation step, and that, for C4, the intact carbohydrate moiety is not required for biological activity.

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