1979). The intracellular activities of these enzymes were also inhibited (by approx. 50%) during culture in the presence of tunicamycin.

Mycelia of A. niger were incubated for 24 h in the presence of D-(2-3H)mannose. The radiolabeled proteins secreted into the culture medium were separated by polyacylamide-gel electrophoresis. The results indicated that, in the presence of tunicamycin, the glycosylation of proteins that have the same electrophoretic mobility as the three glycosidases is inhibited (Speake et al., 1979).

Antiserum specific for β-N-acetylglucosaminidase was obtained after injection of the purified enzyme into rabbits. Immunization of the enzyme activity in the culture medium indicated that the decreased enzyme activity observed in the presence of tunicamycin was due to a decrease in the amount of immunodetectable enzyme (B. K. Speake, D. J. Malley & F. W. Hemming, unpublished work).

There are a number of possible explanations for the decreased amount of enzyme in the culture medium during growth of A. niger in the presence of tunicamycin. Inhibition of glycosylation of the newly synthesized enzyme may lead to impairment of its secretion. No intracellular accumulation of β-N-acetylglucosaminidase was, however, observed. Secondly, it is possible that the non-glycosylated enzyme may be especially susceptible to proteolytic degradation, either before or after secretion from the cell. Finally, inhibition of the synthesis of the protein portion of the specific enzyme has not been ruled out, although tunicamycin was found to have little effect on total protein synthesis. Obviously more work is needed to distinguish between these alternative explanations. It is however evident that glycosylation is of considerable functional importance in the production of secreted enzyme by this organism.

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The value of tunicamycin for studies on immunoglobulin biosynthesis

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All immunoglobulins are glycoproteins. Therefore tunicamycin affords the opportunity to block the addition of carbohydrate to immunoglobulins, allowing one to ask whether this impairs production or function of the molecule. Synthesis of nonglycosylated immunoglobulin polypeptide chains also allows one to ask whether this impairs production or function of the molecule. Synthesis of non-glycosylated enzyme may be especially susceptible to proteolytic degradation, either before or after secretion from the cell. Finally, inhibition of the synthesis of the protein portion of the specific enzyme has not been ruled out, although tunicamycin was found to have little effect on total protein synthesis. Obviously more work is needed to distinguish between these alternative explanations. It is however evident that glycosylation is of considerable functional importance in the production of secreted enzyme by this organism.

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lymphoblastoid cell lines active secretion and surface deposition apparently occur simultaneously. We have used lactoperoxidase-catalysed iodination of cell-surface proteins to identify membrane immunoglobulin and have followed these and all other biosynthetic experiments by techniques of specific antibody precipitation and polyacrylamide-gel-electrophoretic analysis.

Demonstration of immunoglobulin-heavy-chain signal peptide

The signal-peptide hypothesis (Blobel & Dobberstein, 1975) states that an N-terminal hydrophobic peptide initiates the synthesis of polypeptide chains destined for secretion, and this signal peptide functions by affecting the vectorial transfer of the nascent polypeptide chain across the membrane of the endoplasmic reticulum. The precursor form of the polypeptide chain is generally short-lived in vitro and the hydrophobic sequence is removed leaving the mature polypeptide chain. When a secretory polypeptide is synthesized in a cell-free translation system lacking the processing enzymes, the presence of the signal peptide on the product can be inferred by comparing the size of the cell-free product with the size of the mature polypeptide chain (Milstein et al., 1972). This method demonstrates clearly the precursor form of immunoglobulin light chain (Milstein et al., 1972; Burstein & Schecter, 1978). However, a simple size comparison is not informative for immunoglobulin heavy chains, which are glycosylated in their mature form. In the presence of tunicamycin, cells will produce a non-glycosylated form of the mature heavy chain, and comparison of this product with heavy chains synthesized in a wheat-germ-derived cell-free system reveals that the cell-free product has an apparent molecular weight higher than that of the non-glycosylated mature heavy chain. The apparent difference in molecular weight of approx. 2000 correlates well with the length of 1980
the precursor peptide determined by partial amino acid-sequence analysis (H. H. Singer et al., 1979). This type of experiment constitutes a general method for ascertaining whether an N-glycosylated polypeptide is initiated with a signal peptide.

**Enumerating N-glycosylation sites: evidence for a non-glycosylated polypeptide**

In using tunicamycin to ask questions about the role of glycosylation it is desirable to achieve conditions in which tunicamycin fully blocks the glycosylation of a particular polypeptide chain. How can one be sure that this has been achieved? The comparison described above between the cell-free product and the putative non-glycosylated polypeptide is one good piece of evidence, but that approach may not always be feasible. We have adopted the strategy that by varying the concentration of tunicamycin and the time of exposure of the cells to tunicamycin before biosynthetic labelling limit conditions can be achieved. In the approach to limit conditions a variety of partially glycosylated polypeptide chains can be identified. In our studies on the biosynthesis of \( \mu \) heavy chains, six different glycosylation states of \( \mu \)-chain have been identified under non-limit conditions of treatment with tunicamycin. From the complete structural analysis of these chains we know that there are five sites of N-glycosylation (Spragg & Clamp, 1969; Shimizu et al., 1971), so we would expect six forms of \( \mu \)-chain with a range of zero to five carbohydrate side chains attached. Under limit conditions only the lowest molecular form of \( \mu \)-chain, designated \( \mu_0 \), is biosynthetically labelled. Thus we feel confident that \( \mu_0 \) is the non-glycosylated form of \( \mu \)-chain. For a system where the number of sites of N-glycosylation are unknown this same approach could be used to count the number of sites.

**Non-glycosylated immunoglobulin polypeptide chains are correctly assembled and secreted**

Earlier studies in which the drugs 2-deoxyglucose (Melchers, 1973) or tunicamycin (Hickman & Kornfeld, 1978) were used have led to support for the idea that glycosylation is generally a necessary event for active secretion of immunoglobulin to occur. Our own studies challenge this suggestion. The extent of glycosylation of different classes of immunoglobulin is in the order: IgM* > IgA > IgG. We therefore examined the synthesis and secretion of each of these classes of immunoglobulin under limit conditions of treatment with tunicamycin. Murine myeloma cell lines synthesizing IgM, IgA or IgG were biosynthetically labelled under limit conditions, and the non-glycosylated forms of \( \mu \)-, \( \alpha \)- and \( \gamma \)-chains were identified intracellularly. The rate of secretion of assembled non-glycosylated IgG and IgA was similar to the rate of secretion of the glycosylated immunoglobulins. In these experiments there is some variability in the effect of tunicamycin on total protein synthesis, but no differential affect on the proportion of immunoglobulin production. Deriving limit conditions for the IgM-producing cell line required the use of higher concentrations of tunicamycin, and in this instance the proportion of \( \mu \)-chains synthesized relative to other cellular proteins is decreased, but only by about 25%. On the other hand, secretion of IgM under these limit conditions is reduced 5-fold. However, analysis of the IgM that is secreted shows that it is composed entirely of non-glycosylated \( \mu_0 \) chains apparently correctly assembled with light chains. Recently we have also identified J-chain present in this secreted non-glycosylated IgM. J-chain is a polypeptide of mol wt. approx. 15000 (normally heavily glycosylated to give an apparent mol wt. of 25000) that is found in pentameric 19S IgM (or polymeric IgA) at a ratio of one chain/polymeric molecule (Koshland, 1975). The presence of J-chain in the non-glycosylated IgM suggests that J-chain functions correctly in its non-glycosylated form.

*Abbreviations: IgM, IgA and IgG, immunoglobulins M, A, and G respectively.

**Membrane and secretory IgM have \( \mu \)-chains with different primary structures**

Many previous lines of evidence have pointed to a structural difference between membrane and secretory immunoglobulin without clearly indicating the nature of that difference (Melcher & Uhr, 1976, 1977). Using tunicamycin to synthesize non-glycosylated forms of membrane \( \mu \)-chain and secreted \( \mu \)-chain, we have shown that they differ in apparent molecular weights with the membrane form being the larger by approx. 3000 daltons (P. A. Singer et al., 1979a,b). We have also been able to separate the non-glycosylated \( \mu \)-chains preparatively and demonstrate that the difference resides at the C-terminus of the \( \mu \)-chain. We have postulated that the membrane form has an extra hydrophobic peptide at its C-terminus and that this peptide is involved in insertion of the \( \mu \)-chain into the surface membrane.

**Transfer of non-glycosylated \( \mu \)-chain to the plasma membrane has not been observed**

Human lymphoid cells synthesizing predominantly the membrane \( \mu \)-chain form have been held under limit conditions with tunicamycin and attempts have been made to chase the non-glycosylated \( \mu \)-chain from the cytoplasm to the plasma membrane. So far we have not obtained conditions under which we can detect non-glycosylated \( \mu \)-chain on the cell surface. As yet we have no explanation for this finding.

**Discussion**

The difference between our own data and that of Hickman & Kornfeld (1978) lies mainly in our demonstration that the non-glycosylated forms of IgM, IgA and IgG are able to be secreted. We agree entirely that the secretion of non-glycosylated IgM is inhibited relative to that of glycosylated IgM. We do not, however, see any inhibition of the synthesis of non-glycosylated IgA relative to glycosylated IgA, and in this case we studied a cell line (315.40) derived from the tumour studied by Hickman & Kornfeld (1978).

Our interpretation of the data is that glycosylation is not a necessary event for secretion of immunoglobulin, not even for IgM. Glycosylation commencing at the nascent polypeptide chain is able to influence the folding of the polypeptide chain. Hence in the absence of glycosylation the final tertiary, and possibly quaternary, structure of a protein may be different from the glycosylated form of the protein. The interpretation that the tertiary (quaternary) structure of non-glycosylated IgM reduces the efficiency of its secretion is in line with the demonstration of structural mutants of immunoglobulin light chain leading to blocked secretion (Mosmann et al., 1979). Two different structural mutations of immunoglobulin light chain were shown to affect some process apparently involved in the transfer of the light chains from the lumen of the endoplasmic reticulum via the Golgi apparatus to the outside of the cell. The light chains studied do not contain carbohydrate, so differences in glycosylation are not involved in the secretion defect. It would appear that, for proteins destined for secretion, there is a step in intracellular transport at which active selection of proteins with the correct three-dimensional structure takes place. An analogous selective step seems to be involved in the surface localization of viral glycoprotein. Knipe et al. (1977) reported two temperature-sensitive mutants of the G-protein of VS virus defective in the transfer of G-protein from the endoplasmic reticulum to the Golgi apparatus, thereby blocking insertion into the plasma membrane of the virus-infected cell. The failure to observe the transfer of non-glycosylated \( \mu \)-chain to the plasma membrane may be explained by structural differences in non-glycosylated IgM being actively selected against during intracellular transport. The greater sensitivity of membrane deposition relative to active secretion could be related to the fact that four-chain IgM is transferred to the plasma.  

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membrane, whereas pentamers of the four-chain structure are actively secreted.

Conclusions

Use of the drug tunicamycin has greatly facilitated study of many different aspects of immunoglobulin biosynthesis. The most striking conclusion is that blocking glycosylation does not prevent synthesis of immunoglobulin heavy chains either directly or by feedback; non-glycosylated heavy chains are able to assemble into immunoglobulin molecules capable of being secreted. Since glycosylation affects overall protein three-dimensional structure, differences in the structure of non-glycosylated immunoglobulins might be responsible for the failure of non-glycosylated IgM to be transferred to the cell surface, alterations in the details of assembly and secretion of the non-glycosylated immunoglobulins. Such changes in structure in the non-glycosylated molecules might also affect function, and studies on the function of non-glycosylated antibodies should prove rewarding.

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Effects of tunicamycin on insect cells

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Insects are intermediate hosts in the infectious processes of several human pathogens, for example arthropod-borne viruses and certain medically important protozoa. It has been known for some time that such enveloped RNA viruses acquire their glycosylated membrane components by virtue of infected-cell biosynthetic pathways (for a review, see Patzer et al., 1979). Membrane glycoproteins of viruses grown in insect cells appear similar in structure, apart from some important quantitative differences, to those established in mammalian tissues, indicating that analogous mechanisms exist for the assembly of protein-bound glycan chains. However, little is known directly about the mechanisms of glycoprotein biosynthesis in insect cells, although a detailed scheme is emerging for glycoprotein processing and assembly in other cells. It is of interest to know to what extent these processes are universal throughout phylogeny and to expose differences in insect cells that may serve as points of attack in pathogen control.

Polyprenoid intermediates

One critical step in the biosynthesis of glycoproteins in vertebrate cells is the formation of dolichol derivatives of sugars to act as intermediates in glycosylation reactions. Insect cells share with mammalian cells a common pathway for the assimilation of isoprenoids from acetyl-CoA. A significant difference, however, is that insect cells are unable to synthesize cholesterol from a common intermediate, mevalonic acid (Clayton, 1964).

Small isoprenoid polymers are known to function as hormones, pheromones and various attractants in insects (Karlson, 1970), but relatively little information is available on the occurrence of higher polymers, e.g. dolichol, in invertebrates and the regulation of their synthesis, presumably by mechanisms different from the inter-relationship between cholesterol biosynthesis and protein glycosylation in mammalian tissues (Mills & Adamany, 1978).

There appears to be some requirement for the participation of polyprenoid intermediates in invertebrate cell-glycosylation reactions, since dolichol (Beedle et al., 1975), dolichol phosphosphate (Quesada et al., 1975) and dolichol phosphomannose (Quesada et al., 1976) have been identified unequivocally in larval extracts. Additional evidence for dolichol pyrophosphate-linked oligosaccharides has also been obtained for the fruit-fly Ceratitis capitata larvae (Quesada & Belocopiow, 1978), although a precursor-product relationship between lipid intermediates and cellular glycoproteins has yet to be shown.

We have examined the occurrence of dolichol derivatives of sugars in a cultured cell line established from the larvae of the mosquito Aedes aegypti. Direct evidence for the synthesis of phosphorylated dolichol derivatives of sugars by mosquito cells has been obtained. Precursors of mosquito cell-membrane oligosaccharides are detected in microsomal fractions of cells catalysing the transfer of mannose and N-acetylglucosamine from GDP-[14C]mannose and UDP-N-acetyl-[14C]glucosamine. Substantial amounts of radioactivity are incorporated into chloroform/methanol (2:1, v/v) extracts, and the formation of these is stimulated by the addition of dolichol phosphate. The labelled intermediates formed from endogenous and exogenous dolichol acceptors show similar chromatographic characteristics upon t.l.c. in different solvent systems and are consistent with literature values for dolichol phosphomannose and pyrophospho-N-acetylglucosamine respectively. No effect upon the incorporation of radioactivity into dolichol phosphomannose is observed in the presence of tunicamycin, whereas the formation of dolichol pyrophosphate-N-acetylglucosamine is inhibited, as expected from the known specificity of this drug. In both cases, incorporation into higher oligosaccharides and protein fractions is significantly reduced upon addition of tunicamycin. These data indicate that lipid-bound oligosaccharides contain structurally similar units to those established to be present in mammalian cells. We present evidence now that such intermediates are involved in the glycosylation of mosquito cell proteins. Effects of tunicamycin on the incorporation of sugars into mosquito cell glycoproteins

We have found that cultured mosquito cells are sensitive to the effects of tunicamycin in vivo. The incorporation of radioactive mannose into acid-precipitable fractions is inhibited by more than 90% at 0.5 μg of tunicamycin/ml after 16 h at