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MODIFICATION OF BLOOD-BORNE ARREST PROPERTIES OF LYMPHOMA CELLS BY INHIBITORS OF PROTEIN GLYCOSYLATION SUGGESTS THE EXISTENCE OF ENDOGENOUS LECTINS*†

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ABSTRACT

The requirement for intact carbohydrates of glycoproteins at the cell surface was investigated after treatment of lymphoma cells with compounds which interfere at different steps in *N*-linked glycosylation: swainsonine and 1-deoxynojirimycin act at different levels during the processing, so that complex oligosaccharides cannot be formed; 2-deoxyglucose, β-hydroxynorvaline, and tunicamycin completely prevent the formation of *N*-linked (high-mannose as well as complex) oligosaccharides. The role of sialic acid was investigated by treating the cells with neuraminidase. These treatments resulted in altered patterns of surface-labelled glycoproteins after SDS–polyacrylamide gel electrophoresis. Blood-borne arrest of lymphoma cells in the spleen was sensitive to neuraminidase and to treatments interfering with the processing of complex *N*-linked oligosaccharides. It is suggested that carbohydrates are signals for cellular interactions involved in the recirculation and homing behaviour of lymphoid cells and probably interact with endogenous lectins at their site of homing.

INTRODUCTION

Cell-surface proteins play critical roles in cellular functions[1,2]. Although all cell-surface proteins seem to be glycosylated, the biological functions of their oligosaccharide moieties have not been elucidated. Since a particular glycoprotein may be glycosylated differently at various stages of differentiation, with a tendency towards increased complexity of the carbohydrate with increasing cellular differentiation, it was suggested[3] that different carbohydrate chains have different specific

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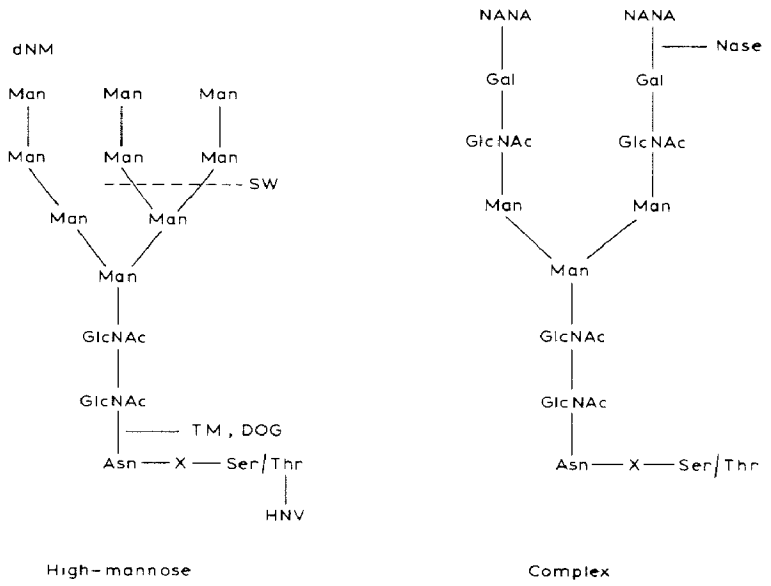

Fig. 1. Examples of high-mannose and complex asparagine-linked (*N*-linked) oligosaccharides found on membrane and secretory glycoproteins, and the sites of action of the various compounds used in this study. Replacement of threonine (Thr) in the polypeptide chain by β-hydroxynorvaline (HNV) prevents glycosylation of the asparagine (Asn) residue[20]. 2-Deoxyglucose[19] (DOG) and tunicamycin[20] (TM) prevent the transfer of lipid-linked oligosaccharide to asparagine, deoxynojirimycin[17] (dNM) prevents the action of glucosidase on high-mannose, glucose-containing precursors, swainsonine[12] (SW) is an inhibitor of mannosidase II which removes Man residues, and neuraminidase (Nase) hydrolyses the terminal sialic acid (NANA) residues.

functions. However, the idea that changes in carbohydrate structure are accompanied by changes in function is so far supported by experimental evidence only for lymphocyte homing. For instance, there is a correlation between the presence of sialic acid on cell surfaces and the ability to home into lymphoid tissues[4]. Carbohydrates have also been implicated in the interactions of tumour and normal cells (invasion, metastasis)[5–8].

In an attempt to further correlate differentiation-related changes in carbohydrate structure with cell function, we have checked the effects of compounds which interfere with different steps in the synthesis of *N*-linked carbohydrates of cell-surface glycoproteins (see Fig. 1). Inhibitors of processing strongly interfered with the arrest of lymphoma cells injected intravenously.

EXPERIMENTAL

Adult mice from the inbred strains C57BL/cnb and BALB/c were obtained from the breeding center of SCK/CEN.

The following cell lines were used: BL/VL_3, BL/RL12-NP (thymic lymphomas from C57BL/Ka mice), and BALB/nu_1 (a T-cell lymphoma induced in a BALB/c nu/nu mouse). They were cultivated as described[9,10].

Neuraminidase (Nase) was obtained from Behringwerke, lentil lectin–Sepharose and concanavalin A–Sepharose were from Pharmacia, and formalin-fixed *Staphylococcus aureus*, strain Cowan I was from BRL (Bethesda, MD); rabbit antilymphoma P1798 serum[11] was a gift from D. Hoessli (University of Geneva).

Swainsonine[12–16] (SW) was a gift from P. Dorling (School of Veterinary Studies, Murdoch, W. Australia) and 1-deoxynojirimycin[15–17] (dNM) was a gift from E. Truscheit and D. D. Schmidt (Bayer, Wuppertal, F.R.G.). Tunicamycin[13,15,16,18–20] (TM), β-hydroxynorvaline[20] (HNV), and 2-deoxy-D-*arabino*-hexose (2-deoxyglucose[19], DOG) were commercial products, SW and dNM were added to the cultures 48 h prior to harvest and all other drugs 24 h prior to harvest.

For neuraminidase treatment, 25×10^6 cells were resuspended in Hanks medium (3 mL), neuraminidase (0.05 U) from *Vibrio cholerae* was added, and the cells were kept for 45 min at 37°. Enzymic treatment was terminated by the addition of complete, cold culture medium followed by extensive washings.

Blood-borne arrest of lymphoma cells. — Cells were labelled by incubation for 30 min at 37° with 100 μCi ^{51}Cr (as $Na_2{}^{51}CrO_4$) per 3×10^7 cells in phosphate-buffered saline (PBS, 3 mL). The various drug treatments did not interfere with the uptake of chromate, nor with spontaneous release. Cells were injected i.v. into 3 or 4 syngeneic recipient mice (6×10^6 cells, *i.e.*, usually 50,000–150,000 c.p.m. per mouse). Radioactivity in the following organs was determined after 24 h: liver, spleen, kidney, lungs, thymus, and lymph nodes (axillary, inguinal, peri-aortic). The results are expressed as the percent of injected c.p.m. retained by an organ. The total recovered radioactivity was also calculated[21].

Cell-surface glycoproteins. — Lactoperoxidase-catalysed surface iodination, lysis with 1% Nonidet P40 (NP40) in PBS, immunoprecipitation or separation on lectin–Sepharose, SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography were done as described[11,22].

RESULTS

Biochemical controls. — The migration of surface proteins in SDS-PAGE was modified after treatment with various inhibitors of *N*-linked glycosylation or processing (Fig. 2). In most instances, the treatment resulted in an increased apparent mobility of the different labelled species, various drugs yielding different patterns. Treatment with inhibitors of glycosylation, however, did not prevent lectin binding, indicating that the surface molecules were still glycosylated.

Blood-borne arrest. — Following the intravenous injection of labelled lymphoma cells, radioactivity was recovered after 24 h mainly from the liver and the spleen (Table I). Despite some fluctuation from one experiment to another, the fraction of radioactivity recovered from the different experimental groups was constant within a given experiment. Arrest in the spleen was reduced in a significant manner after treatment with dNM or SW, but there was no increased trapping in the liver. However, HNV and TM had no clear effect. Treatment of the cells with

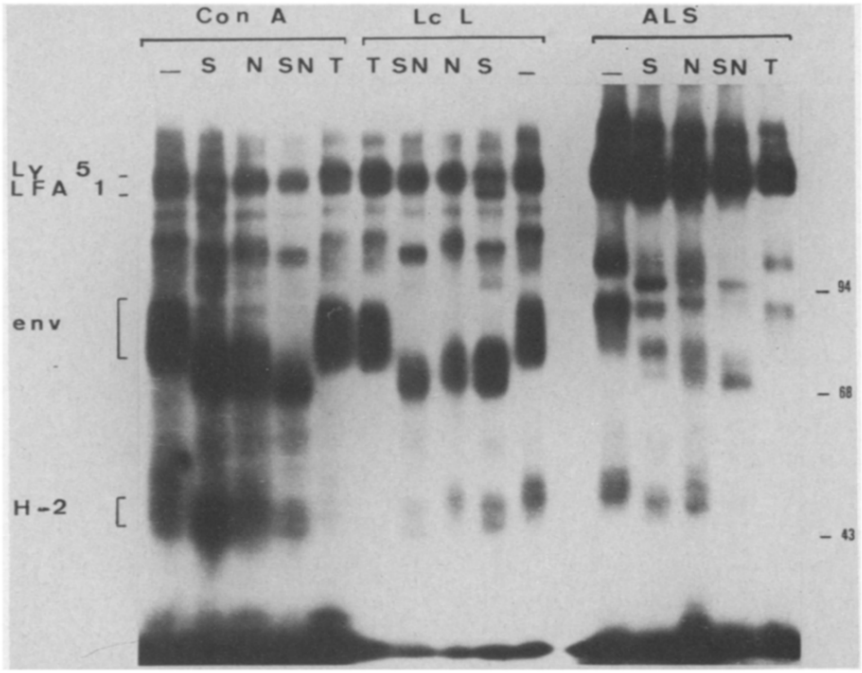

Fig. 2. Effect of inhibitors of glycosylation on membrane proteins. Autoradiograph of cell-surface iodinated peptides of BL/RL12 NP cells cultivated in the presence of SW (S, 0.2 μg/mL), dNM (N, 10mM), SW + dNM (SN) or TM (T, 50 μg/mL) or in regular medium (—). Concanavalin A–Sepharose (Con A), lentil lectin–Sepharose (LcL), or antiserum against lymphoma P 1798 followed by Staphylococci Cowan I (ALS) were used for selection. The eluates were analysed on 8% acrylamide gels in the presence of 2-mercaptoethanol. The position of molecular weight markers is indicated on the right. On the left, surface antigens are identified as in ref. 22. The antilymphoma serum poorly precipitates env-related peptides. Also, lectins and antilymphoma serum recognise different molecules between 100 and 170 kD.

Nase immediately before i.v. injection reduced arrest in the spleen. The effects of SW and Nase were additive, since radioactivity in the spleen was lower after combined treatment than after single treatments. Also, the effect of dNM was amplified by SW. Preliminary data with other inhibitors (given for 24 h) showed that mM 2-deoxy-2-fluoro-D-glucose or 0.3mM 2-deoxy-2-fluoro-D-mannose had no effect. In contrast, 1.5μM monensin very strongly decreased arrest in the spleen, whereas 60nM marcellomycin increased arrest.

DISCUSSION

In this study, the hypothesis that *N*-linked carbohydrates of surface glycoproteins play a role in membrane-mediated functions, with special concern for tumour cell localisation, was tested. Surface proteins were modified by culturing cells in the presence of inhibitors of glycosylation. Alternatively, surface proteins

were enzymically modified just before use. The effectiveness of the various procedures was determined by biochemical analysis and their functional consequences were assessed by measuring blood-borne arrest of lymphoma cells after i.v. injection.

The mode of action of the various compounds used in this study has been described (see Fig. 1). Surface proteins from lymphoma cells were clearly modified by the various treatments but no gross abnormalities in the patterns could be detected. Rather, there was a generalised increase in migration, which was different for each drug. Binding to lentil lectin and to concanavalin A indicated that the molecules were still, at least partially, glycosylated. This finding is not surprising after treatment with SW or dNM, which results in the synthesis of hybrid or high-mannose sugars that bind concanavalin A even better than complex *N*-linked carbohydrates[12,23,24]. After treatment with TM or with HNV, which prevent lipid-mediated *N*-linked glycosylation, binding could be explained by the slow turn-over of some glycoproteins. Alternatively, at the concentrations used, the drugs may have prevented *N*-linked glycosylation at certain sites only. An answer to this question will be provided by the structural analysis of the carbohydrates from control and treated cells, which is in progress. The pattern obtained with dNM plus SW was different from that obtained with either drug given alone, in accord with the data of Peyrieras *et al.*[16].

In several systems, cell–cell or cell–substrate interactions are prevented by the presence of simple sugars or glycolipids[8,13,25–27], thus suggesting the existence of endogenous lectins. To our knowledge, however, there has been no thorough investigation of the constraints imposed on carbohydrate structure for proper homing. Such a study was initiated by first following the effects of various treatments on the blood-borne arrest properties of several lymphoma lines.

Nase, SW, and dNM clearly reduced arrest in the spleen. Nase and SW had additive effects, as did dNM and SW. Each of these treatments results in the formation of abnormal *N*-linked carbohydrates. In contrast, TM and HNV completely prevent the occurrence of *N*-linked glycosylation and their lack of effect on blood-borne arrest suggests that the routing and/or arrest of injected cells is more adversely affected by the presence of abnormal carbohydrates (generated by SW and/or dNM) than by the absence of at least some *N*-linked carbohydrates (after treatment with TM or HNV). More precise biochemical data on the effect of these compounds are required. It is clear, however, from the SDS-PAGE (Fig. 2) and from other biological tests[28,29] that the treatments have biochemical and biological effects. Our data suggest that cell-surface carbohydrates play a role in the homing of lymphoma cells. Several groups have suggested that one or several glycoproteins at the lymphocyte surface are involved in the homing process but they have not attributed special functions to the sugar moieties of these proteins[30,31].

Clearly, inhibitors of glycosylation with very low toxicity (SW, dNM) are most useful for studying the molecular basis of cellular interactions such as homing. Bar-Shavit *et al.*[13] have recently reported impaired binding to bone after treatment

TABLE I

DISTRIBUTION OF ^{51}Cr-LABELLED LYMPHOMA CELLS 24 h AFTER I V INJECTION[a]

Inocula			*Spleen*		*Liver*		*Total recovered*		*Total/spleen*
Cells		*(c p m)*	*(A)*	%	*(B)*	%	*(C)*	%	*(C/A)*
BL/RL12 NP cells									
Expt 1	Control	(40,641)	1336 (906–1601)	3.3	9761 (8221–10643)	24 0	13417(11052–14570)	33 0	10.3 (9.1–12.2)
	SW	(56,954)	1068 (936–1399)	1.9	16260(15142–16846)	28 5	19889(18431–21552)	34.9	19.0(15.4–21.1)
	dNM	(65,974)	1269 (882–1664)	1 9	19316(17920–20870)	29 2	23451(21573–26340)	35.5	19.3(15.8–27.5)
	Nase	(28,422)	627 (587–662)	2.2	5712 (6363–5405)	20 1	8576 (8233–9090)	30.2	13.7(13.0–14.4)
Expt. 2	Control	(131,477)	7631(6787–8324)	5.8	45897(43745–47907)	34.9	57717(55660–59235)	43.9	7.6 (7.1–8.6)
	SW	(142,684)	4203(3181–4722)	2 9	49648(43518–54105)	34.8	59161(49511–66990)	41.5	14.2(12.2–15.6)
	dNM	(84,710)	2016(1804–2414)	2 4	27574(22856–33900)	32.6	32420(26790–38886)	38.3	17.2(14 1–21.9)
	dNM + SW	(71,536)	1400(1188–1646)	2 0	27700(24080–36880)	38.7	32420(28360–41874)	45.3	23.4(22 7–29.3)
	TM	(125,572)	5108(3532–7698)	4.1	40941(28200–52542)	32 6	49647(34288–61944)	39 5	10 3 (6 8–14.0)
Balb/Nu$_1$ cells									
Expt 3	Control	(190,025)	8818(7905–10867)	4.6	56869(54460–61719)	29 9	70117(67106–74751)	36.9	8.1 (6.4–9.0)
	SW	(62,941)	2136(2024–2301)	3.4	17626(16412–18642)	28.0	21628(20729–22456)	34.4	10.2 (9.1–10.8)
	SW + Nase	(109,795)	2476(1756–2940)	2.3	24241(16363–28167)	22.1	30142 (2744–35677)	27.5	12.2(11.1–13.5)
	Nase	(173,367)	5905(5395–6365)	3 4	52692(48074–63192)	30.4	62661(57673–75067)	36.1	10.6 (9.6–11.8)
BL/VL$_3$ cells									
Expt 4	Control	(33,910)	1264(1207–1407)	3.7	12739(12139–13175)	37 6	15349(14607–15672)	45 3	12 2(11.1–13 0)
	dNM	(13,210)	173 (142–200)	1 3	4411 (4143–4547)	33.4	5435 (5067–5625)	41.1	31.8(27.8–35.6)
Expt 5	Control	(57,397)	1311(1283–1347)	2.3	18899(17430–90294)	32 9	21373(20014–22399)	37.2	16.3(15.5–17.0)
	SW	(74,215)	614 (563–723)	0.8	20629(18841–21580)	27 8	23207(21185–24297)	31.3	38.2(32.3–43.2)
	dNM	(46,805)	488 (477–511)	1.0	15655(14449–16486)	33.4	17321(16952–18297)	37.0	35 5(32.7–37.5)
	HNV	(18,812)	475 (408–540)	2.5	6428 (6166–6907)	34.2	7551 (7195–8053)	40 1	14.8 (9.6–17.6)
Expt 6	Control	(51,537)	2095(1941–2207)	4.1	17952(17227–18728)	34.8	21364(20749–21984)	41 5	10.2 (9 8–11.3)
	SW	(54,257)	1233 (955–1453)	2 3	18089(17580–18800)	33.3	20877(20421–21299)	38 5	17.3(14.5–22.3)
	dNM	(53,071)	1491(1427–1586)	2.8	21919(20919–22744)	41.3	24585(23671–25355)	46.3	16.5(14.9–17.4)
	HNV	(35,507)	1687(1649–1792)	4 8	15046(13363–16814)	42.4	17753(16068–19521)	50.0	

Expt. 7	Control	(84,016)	4142(3330–4950)	4.9	25045(21877–26514)	29.8	31203(28775–32300)	37.1	7.8 (5.8–9.5)
	SW	(82,283)	2327(1532–2942)	2.8	23300(18483–27464)	28.3	27673(21650–33053)	33.6	12.2(11.2–14.1)
	Nase	(48,433)	1123 (994–1234)	2.3	13351(11977–14837)	27.6	16102(14820–17667)	33.2	14.4(13.0–15.9)
	SW + Nase	(47,774)	1426(1250–1644)	3.0	11449(10907–12221)	24.0	14268(13783–14777)	29.9	10.2 (8.4–11.9)
	TM	(75,112)	4218(3642–5070)	5.6	30150(28114–31314)	40.1	36380(33282–38901)	48.4	8.7 (7.5–9.6)
Expt. 8	Control	(100,613)	6094(5420–7299)	6.1	37187(34903–39620)	37.0	47808(43440–51685)	47.5	8.0 (6.8–9.5)
	dNM	(123,226)	3183(2742–3471)	2.6	48257(46603–50626)	39.2	55481(53713–57945)	45.0	17.5(15.6–19.6)
	HNV	(157,606)	6853(4470–9966)	4.3	48949(33569–67421)	31.1	62138(41069–90301)	39.4	9.1 (9.0–9.2)
	TM	(46,714)	2790(2631–2890)	6.0	16518(15004–18468)	35.4	20916(19284–22518)	44.8	7.5 (6.8–8.6)

[a]Batches of cells, grown for 48 h in the presence of SW (0.2 μg/mL) or dNM (10mM), or both, or for 24 h in the presence of HNV (10mM) or TM (50 μg/mL) treated (or not) with Nase, were labelled separately with ^{51}Cr. The same number of cells (6×10^6) was injected into 4 mice (3 in a few cases) even when the labelling efficiency had not been the same for all groups of cells. After 24 h, organs were removed and counted[21]. Liver and spleen accounted for at least 70% of the recovered activity. In a given experiment, both the activity recovered in the liver (B), and the total recovered activity (C) were fairly constant. Some treatments strongly decreased arrest in the spleen, as reflected by percent counts recovered in the spleen (A) or by the ratio C/A. Cells from the same cultures were used for Expt. 2 and for the experiment illustrated in Fig. 2. The C/A ratios were calculated for each mouse, and not from the average for C and A given here.

of macrophages with SW or TM. In their system, however, neuraminidase had no inhibitory effect.

Thus, inhibitors of glycosylation are useful tools for defining structures that are required for membrane-mediated functions. Our data add new evidence supporting the idea that complex sugars are important for blood-borne arrest of tumour cells, in agreement with recent reports[6,8]. Inhibitors of glycosylation have potential applications in the management of cancer. They may alter the metastatic potential of (residual) tumour cells, without reducing immune response against the tumour[14,32,33].

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