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REDUCTION IN CELL SURFACE CONCANAVALIN A BINDING AND MANNOSE INCORPORATION INTO GLYCOPROTEINS OF SARCOMA 180 BY 6-THIOGUANINE

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Abstract—Treatment of mice bearing Sarcoma 180 ascites cells with 20 mg/kg of 6-thioguanine for 1 hr caused a 50 per cent decrease in [2-^{3}H]mannose incorporation into glycoproteins; this inhibition continued for at least 6 hr after drug exposure. The decrease in labeling of mannose-containing glycoproteins produced by the 6-thiopurine was followed by a decrease in the specific binding of Concanavalin A to Sarcoma 180; this effect was observed as early as 12 hr after 6-thioguanine treatment. The decrease in specific binding of Concanavalin A to Sarcoma 180 24 hr after 6-thioguanine was due to a 20 per cent reduction in the number of Concanavalin A receptors available on the plasma membrane of these neoplastic cells. The binding of lectins specific for sugars not activated by GTP, such as Wheat Germ Agglutinin and *Ricinus communis*, was not decreased by treatment of cells with the purine antimetabolite. This disruption in the biochemical composition of the cell surface produced by 6-thioguanine was accompanied by morphological alterations in the microvillar content of Sarcoma 180 cells which were observed by scanning electron microscopy. The alterations in membrane composition and structure caused by this antineoplastic agent are hypothesized to be of importance in the delayed cytotoxicity of 6-thioguanine.

Differences in the structure of the plasma membrane of normal and malignant cells appear to contribute to altered properties of the neoplastic cells in characteristics such as cellular adhesiveness, contact inhibition of movement, and antigenicity [1, 2]. A number of studies have demonstrated that the glycoprotein composition of the plasma membrane is changed when cells assume a malignant phenotype [3]. The function of the carbohydrate moieties of membrane proteins and the significance of transformation associated changes in glycoproteins have not been precisely defined, although they are clearly of importance in the interaction of cells with their external environment [4]. Moreover, it is not certain whether differential sensitivities of normal and malignant cells to antineoplastic agents are due at least in part to differences at the plasma membrane level or whether cancer chemotherapeutic drugs have the capacity to rectify such membrane abnormalities. In this report, the effects of a clinically active antileukemic agent, 6-thioguanine (6-TG), on membrane glycoprotein composition and on cellular morphology have been studied.

The anticancer activity of 6-TG requires its conversion to the nucleotide form, 6-ThioGMP [5]. Such anabolic metabolism results in the incorporation of 6-TG into DNA and RNA [6, 7], inhibition of protein synthesis [8], inhibition of purine ribonucleotide interconversion [9], inhibition of fucose activation and incorporation into glycoproteins [10], and inhibition of purine nucleotide biosynthesis *de novo* [11]. A delayed cellular cytotoxicity is produced by 6-TG which has been considered supportive of the hypothesis that this agent exerts its action via incorporation into DNA [12]. Although the incorporation of the nucleotide form of this drug into cellular DNA may be critical for the expression of cytotoxicity [13], the mechanism by which the incorporated analog produces its ultimate cytotoxic action has not been delineated [14]. Moreover, Bieber *et al.* [15] have demonstrated that (a) 6-mercaptopurine is incorporated into DNA as 6-TG and (b) incorporation is greater in a 6-thiopurine-resistant tumor than in the corresponding susceptible parent subline [16]. In addition, Parks *et al.* [17] have reported that 6-selenoguanine, a structural analog of 6-TG, can cause delayed cytotoxicity similar to that produced by 6-TG, but does not appear to be incorporated into DNA. It is possible, therefore, that at least part of the cytotoxicity of 6-TG may be due to biochemical alterations other than incorporation into DNA.

6-TG produces a decrease in intracellular levels of GTP within 2 hr after exposure of cells in culture [13]. Since GTP is necessary for the addition of mannose and fucose units onto glycoproteins and glycolipids, 6-TG may alter membranes of sensitive cells by depletion of GTP pools. The observation that 6-TG inhibits the formation of GDP-fucose and the incorporation of fucose into glycoproteins *in vitro* and *in vivo* [10] is consistent with this hypothesis. However, since the addition of exogenous fucose onto glycoproteins may be a minor metabolic pathway [18], we have examined the effects of 6-TG on the incorporation of mannose into glycoproteins of Sarcoma 180 (S180) ascites cells *in vivo* to determine whether, in addition to fucose, other biosynthetic pathways which require the formation of guanine sugar nucleotides are inhibited. In addition, to ascertain whether plasma membrane changes are produced by this agent, we have also examined cell surface topography and the binding of various plant lectins to the surface of Sarcoma 180 cells collected from mice treated with 6-TG.

MATERIALS AND METHODS

Materials. 6-TG was generously provided by Dr. George H. Hitchings of the Burroughs–Wellcome Research Laboratories (Research Triangle Park, NC). D-[2-^{3}H]mannose was purchased from the Amersham Corp. (Arlington Heights, IL). [^{3}H]Concanavalin A ([^{3}H]Con A), [^{3}H]Wheat Germ Agglutinin ([^{3}H]WGA) and [^{3}H]*Ricinus communis*-120 ([^{3}H]Ricin) were obtained from the New England Nuclear Corp. (Boston, MA). Con A and *R. communis*-120 were purchased from Miles Laboratories Incorporated (Elkhart, IN) and WGA from Pharmacia Fine Chemicals Incorporated (Piscataway, NJ). α-Methyl mannoside, galactose and *N*-acetyl glucosamine were obtained from Sigma Corp. (St. Louis, MO).

Mannose incorporation into glycoprotein. CD-1 mice (Charles River Breeding Laboratories, North Wilmington, MA) were inoculated i.p. with 6×10^6 S180 cells and 6 days later were injected i.p. with 0.9% NaCl with or without 20 mg/kg of 6-TG. After various periods of time, the animals were injected i.p. with 5 μCi (0.5 μg) of D-[2-^{3}H]mannose and the cells were collected 10 min later. Glycoproteins were isolated by trichloroacetic acid (TCA) precipitation as described previously [10]. The protein content of the acid-precipitable material was measured by the method of Lowry *et al.* [19]. The rate of incorporation of [2-^{3}H]mannose into glycoprotein of S180 *in vivo* was found to be constant between 2.5 and 15 min. Most of the [^{3}H]mannose appeared to be incorporated into glycoproteins as mannose. Thus, 10 min after an injection of D-[2-^{3}H]mannose, over 80 per cent of the radioactivity found in the TCA precipitate of untreated control cells or cells from mice exposed to 6-TG for 2 hr co-migrated on descending paper chromatography (Whatman 3 MM; pyridine–ethylacetate–H_2O; 10:36:11.5 (v/v); 20 hr) with mannose after hydrolysis in 1 N H_2SO_4 for 4 hr at 100° [20]. Over 50 per cent of the acid-soluble radioactivity from control cells co-chromatographed with mannose and 25 per cent co-migrated with fucose.

Lectin binding. The binding of lectins to S180 cells was measured as described by Hwang *et al.* [21]. Various concentrations of radioactive Con A, WGA, or Ricin were incubated in Ca^{2+}, Mg^{2+}-free phosphate NaCl buffer (8.0 g NaCl, 0.2 g KCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$ and 0.2 g KH_2PO_4/liter of H_2O, pH 7.4) that contained 0.5% bovine serum albumin (BSA) for 10 min with or without 50 mM α-methyl Man, GlcNAc, or Gal, respectively. Cells (1.7×10^6) were added and the solution was shaken gently for 30 min at room temperature. The reaction was terminated by the addition of 20 vol. of ice-cold phosphate buffer containing 0.1% BSA. The cell suspension was filtered immediately with a GF/A filter (Whatman Ltd, England) which had been presoaked in 1% BSA, and washed three times with 5 ml of ice-cold phosphate buffer containing 0.1% BSA and monosaccharides when appropriate. The radioactivity on filters was determined in Biofluor (New England Nuclear Corp.) and the specific binding was calculated from the amount of radioactivity bound in the absence of monosaccharide minus the amount of radioactivity bound in its presence.

Scanning electron microscopy. Cells from untreated and 6-TG-treated mice bearing 6-day implants of S180 were collected 24 hr after 20 mg 6-TG/kg and were washed twice with phosphate buffered 0.9% NaCl. Cells were then fixed with 0.1 M phosphate buffered 0.9% NaCl, 0.05 M cacodylate, and 2.5% glutaraldehyde (pH 7.2) and allowed to settle for 30 min at room temperature. After two washes with 0.2 M cacodylate buffer, cells were exposed to 1% OsO_4 in 0.1 M cacodylate buffer for 4 min. Cells were then washed with 0.2 M cacodylate buffer and dehydrated on polycarbonate membranes (Nucleopore Corp., Pleasanton, CA) with increasing concentrations of ethanol until cells were in 100% ethanol; they were then subjected to critical point drying [22]. Cells were coated with 200 Å of gold–palladium with a Polaron diode sputter and examined with an Autoscan model U-1 scanning electron microscope (Etec Corp., Hayward, CA). Electron micrographs were coded randomly and two independent investigators who did not know the code scored over 350 cells for topographical features.

RESULTS

S180 cells from tumor-bearing mice, treated i.p. with a single dose of 20 mg of 6-TG/kg 1 hr before injection of [^{3}H]mannose, incorporated 48 per cent less radioactive mannose into glycoprotein than did cells from

Table 1. Effect of 6-thioguanine on the incorporation of [^{3}H]mannose into glycoproteins of S180 cells*

Hr after 6-TG	Counts/min/10^4 cells ± S.E.		Counts/min/mg protein ± S.E.	
	Control	6-TG	Control	6-TG
1	2.78 ± 0.47	1.44 ± 0.18†	1060 ± 180	520 ± 78†
2	2.33 ± 0.32	1.45 ± 0.17†	940 ± 130	580 ± 67†
6	2.48 ± 0.24	1.21 ± 0.04‡	ND§	ND§
24	3.24 ± 0.70	3.52 ± 0.65	1340 ± 290	1300 ± 240

* S180-bearing mice were injected i.p. with either 0.9% NaCl (control) or 0.9% NaCl containing 20 mg/kg of 6-TG. At various times thereafter, 5 μCi of [^{3}H]mannose was injected i.p. into each mouse. Cells were collected 10 min later, the cell number was determined, and radioactivity and protein in the TCA precipitable material of 1.7×10^7 cells were measured. Each value is the mean ± S.E. of separate determinations of cells from four to six mice. Control cells had an average of 251 pg of TCA precipitable protein/cell.

† $P < 0.05$.

‡ $P < 0.01$.

§ ND = not determined.

untreated control animals (Table 1). The decrease in mannose incorporation into these macromolecules produced by 6-TG continued for at least 6 hr after exposure to the purine antimetabolite; 24 hr after exposure to 6-TG, the amount of radiolabeled mannose utilized for the formation of glycoprotein was identical in control and treated cells. In non-drug-treated cells, approximately twice as much radioactivity per cell was acid-precipitable than was acid-soluble (Table 2). Furthermore, in contrast to the acid-precipitable material, the acid-soluble radioactivity found in cells collected from mice treated with 6-TG at 1, 2, 6, or 24 hr after drug exposure was not significantly different from that found in cells isolated from saline-treated animals. The finding that 6-TG did not cause a significant decrease in acid-soluble radioactivity suggests that this agent does not inhibit the transport of mannose across cellular membranes.

To determine whether inhibition of mannose incorporation into glycoprotein by 6-TG led to a change in mannose content on the cell surface, the specific binding of [^{3}H]Con A to S180 cells treated with this agent was measured. The binding of Con A, a mannose-specific plant lectin, to S180 cells 24 hr after exposure of cells *in vivo* to a single dose of 20 mg/kg of 6-TG was decreased significantly (Fig. 1A). When the binding was plotted by the method of Scatchard [23], straight lines were obtained, suggesting the presence of a single type of Con A receptor in both control and drug-treated cells (Fig. 1B). Thus, the decrease in Con A binding that occurs in 6-TG-treated cells appears to be due to a 20 per cent decrease in the number of Con A binding sites. It can be calculated that $2.55 \pm 0.11 \times 10^7$ Con A receptors were present on each untreated S180 cell, whereas tumor cell populations exposed to 6-TG *in vivo* possessed $2.01 \pm 0.11 \times 10^7$ receptors/cell. No significant difference was observed between untreated and analog-treated cells in the apparent affinity of the lectin for Con A receptors. In other experiments (data not shown), no decrease in the degree of [^{3}H]Con A binding was obtained with cells collected from mice 24 hr after treatment with 5 mg/kg of 6-TG and incubated with either 40 or 200 μg/ml of labeled Con A. The decrease in the number of [^{3}H]Con A molecules bound to S180 cells exposed *in vivo* to 20 mg 6-TG/kg was detected as early as 12 hr after exposure to the purine antimetabolite, but was not apparent at 2 hr after drug treatment (Fig. 2). Thus, the decrease in Con A binding occurs subsequent to the inhibition of [^{3}H]mannose incorporation into glycoprotein produced by 6-TG.

Table 2. Effect of 6-thioguanine on the acid-soluble [^{3}H]mannose content of S180 cells*

	Counts/min/10^4 cells ± S.E.	
Hr after 6-TG	Control	6-TG
1	1.23 ± 0.10	1.32 ± 0.18
2	1.17 ± 0.12	1.16 ± 0.11
6	1.05 ± 0.04	0.98 ± 0.12
24	1.26 ± 0.21	1.62 ± 0.27

* Mice were treated and cells were collected as described in Table 1. The radioactivity from 1.7×10^7 cells which was soluble in 10% TCA was measured. Each value is the mean ± S.E. of separate determinations of cells from four to six mice.

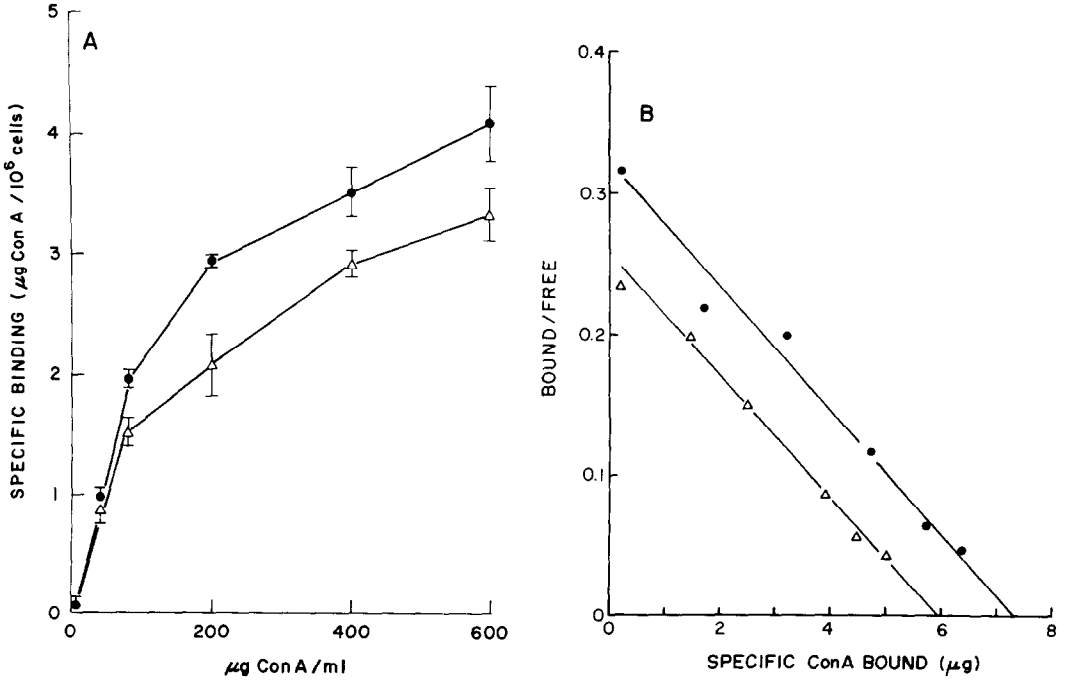

Fig 1. Binding of Con A to S180 cells 24 hr after exposure to 6-TG. Mice bearing 6-day implants of S180 were injected i.p. with 0.9% NaCl (control) or 0.9% NaCl containing 20 mg 6-TG/kg. Cells were collected 24 hr later, washed and 1.7×10^6 cells were added to preincubated solutions of [^{3}H]Con A in the absence and presence of 50 mM α-methyl mannoside. After a 30-min incubation period, cells were collected by filtration and the radioactivity therein was measured. Panel A: mean [^{3}H]Con A binding ± S.E. (n = 4). Panel B: Scatchard plot of data. Key: Untreated control (●); 6-TG treated (△).

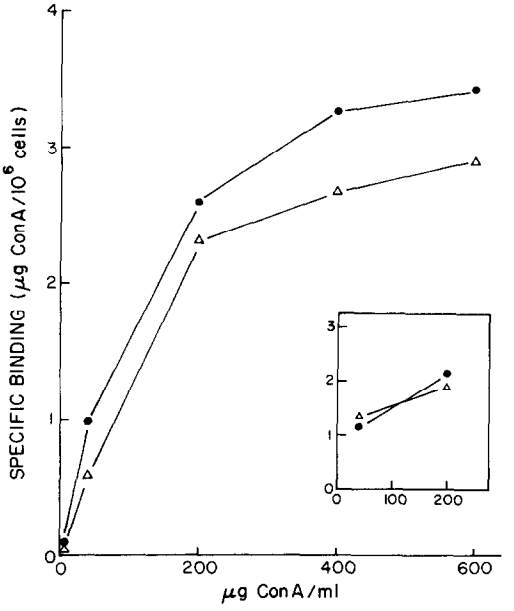

Fig. 2. Binding of [^{3}H]Con A to S180 cells 12 hr after exposure to 6-TG. Treatment and Con A binding were conducted as described in Fig. 1. Each point is the average of duplicate experiments. Inset: binding of Con A to S180 cells 2 hr after exposure to 6-TG. Key: untreated control (●); 6-TG treated (△).

The binding of lectins that have high affinity for carbohydrates activated by UTP (i.e. WGA and Ricin) was measured to ensure that the effects observed with Con A were specifically monitoring changes in the mannose content of the cell surface. In contrast to Con A binding, the specific binding of [^{3}H]WGA, a lectin which recognizes GlcNAc, and [^{3}H]Ricin, which rec-

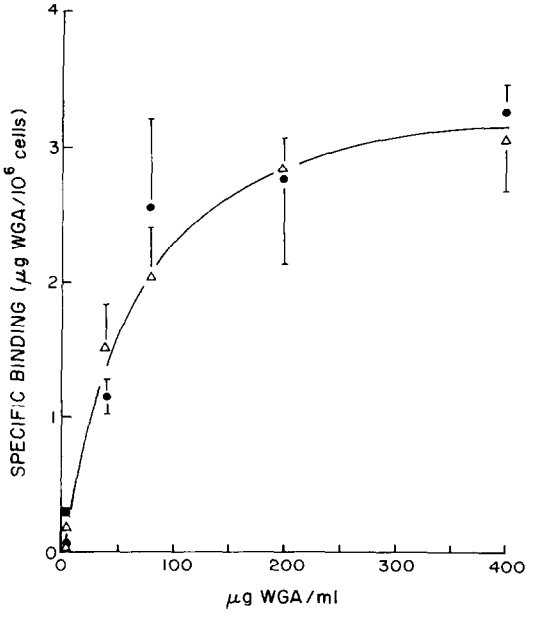

Fig. 3. Binding of [^{3}H]WGA to S180 cells 24 hr after exposure to 6-TG. WGA binding was determined in the presence or absence of 50 mM GlcNAc as described in Fig. 1. Each point is the result of three experiments ± S.E. Key: untreated control (●); 6-TG treated (△).

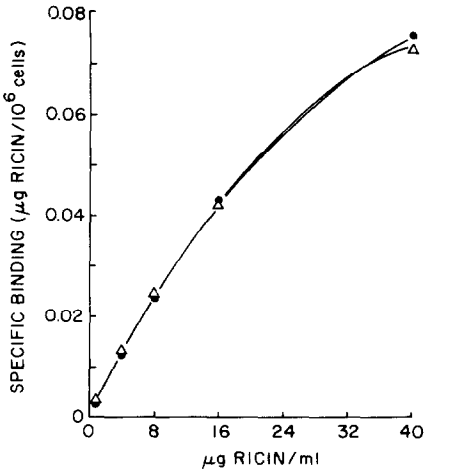

Fig. 4. Binding of [^{3}H]Ricin to S180 cells 24 hr after exposure to 6-TG. Cells were collected and 1.7 × 10^6 cells were added to preincubated solutions of [^{3}H]Ricin in the presence or absence of 50 mM Gal. After 30 min of incubation, cells were collected by filtration, and the radioactivity contained therein was measured. The results presented are representative data from one of five experiments. Key: untreated control (●); 6-TG treated (△).

ognizes Gal, to S180 cells 24 hr after *in vivo* exposure of cells to 20 mg/kg of 6-TG was not altered (Figs. 3 and 4, respectively). These findings are consistent with the observation that the incorporation of a UTP activated sugar, GlcN, into glycoprotein was not altered by 6-TG [10]. Thus, the reduction in the concentration of mannose moieties available for Con A binding on the surface membrane of S180 cells appears to be a specific phenomenon, since the binding of WGA and Ricin was not reduced.

The treatment of S180 cells *in vivo* with 20 mg/kg of 6-TG for 24 hr resulted in an increase in the percentage of cells in the population with thick microvilli and blebs. The percentage of cells with thick microvilli and blebs was scored independently by two investigators using a total of fifty coded micrographs from three separate experiments. An abnormal cell surface which included thick microvilli or numerous blebs was observed in 59.5 ± 8.7 per cent (mean ± S.E. of three experiments) of the cells from 6-TG-treated mice (Fig. 5). These topographical features were seen in only 19.9 ± 6.6 per cent of the cells from control mice.

DISCUSSION

Treatment of Sarcoma 180 cells *in vivo* with a cytotoxic dose of 6-TG [24] results in a decrease in the incorporation of [^{3}H]mannose into cellular glycoproteins (Fig. 1) which occurs prior to the reported reduction in cell viability produced by this agent [24]. Mannose has a particularly important role in the synthesis of complex glycoproteins, since it comprises the internal core region of many oligosaccharide chains. It has been suggested [25] that the formation of a mannose-rich precursor occurs, followed by cleavage of several terminal mannose units and the addition of other sugar residues. Thus, interference with the addition of mannose units to glycoproteins might be expected to result in major alterations in the primary structure of complex cellular glycoproteins. In a previous study [10], we

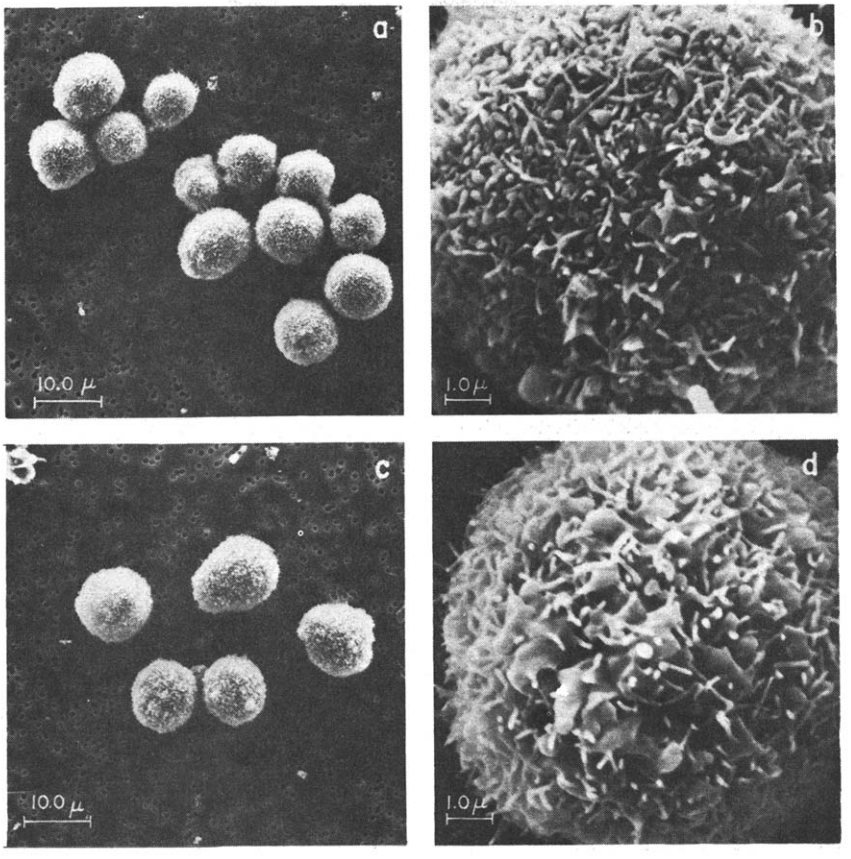

Fig. 5. Topographical changes in S180 cells 24 hr after exposure to 20 mg/kg of 6-TG *in vivo*. Panels a and b: untreated control cells; panels c and d: 6-TG-treated cells. Magnification 1800 diameters (bars equal 10 μM) in panels a and c; magnification 7500 diameters (bars equal 1 μM) in panels b and d.

reported that 6-TG produced a dose-related decrease in the synthesis of [^{3}H]fucose-containing glycoproteins both *in vivo* and in culture. This decrease appeared to be due to a reduction in the rate of formation of GDP-fucose, which resulted in a decrease in the intracellular content of this sugar nucleotide. Since the majority of GDP-fucose in mammalian cells is believed to originate from GDP-mannose [18], we suggested [10] that the activation of mannose and its subsequent incorporation into membrane macromolecules might also be reduced significantly in 6-TG-treated cells. The data presented in this report (Table 1) support this contention. Since [^{3}H]glucosamine incorporation into glycoprotein of S180 was not reduced after a similar dose of 6-TG [10], it is unlikely that the inhibition of [^{3}H]mannose incorporation into glycoprotein by 6-TG was due to inhibition of protein synthesis. Furthermore, the observation of Kwan *et al.* [8], which showed no significant decrease in protein synthesis of S180 ascites cells 1 hr after their exposure to a 30 mg/kg dose of 6-TG, also supports the concept that inhibition of protein synthesis is not the cause of the reduction in fucose and mannose incorporation into glycoprotein after treatment with the purine antimetabolite.

The decrease in [^{3}H]fucose and [^{3}H]mannose incorporation into glycoprotein produced by 6-TG appears at a time when significant amounts of intracellular 6-thioGMP are present [26], implying that generation of this metabolite is essential for the observed effects. Since (a) intracellular GTP pools are reduced by 6-TG during this period (see Ref. 13; J. S. Lazo and A. C. Sartorelli, unpublished observations), and (b) GTP is essential for the synthesis of mannosyl- and fucosyl-containing glycoproteins and glycolipids, it is possible that guanosine sugar nucleotide activation may be acutely sensitive to the intracellular content of GTP relative to other biochemical reactions which require guanine nucleotides.

A decrease in the binding of Con A to plasma membranes and an alteration in cell topography occurred in drug-treated cells subsequent to the decrease in the specific activity of mannose-containing glycoproteins (Figs. 1 and 5). Although the relationship between cell topography and mannose content is uncertain, the decrease in the amount of Con A bound to 6-TG-treated S180 cells appears to be due to 20 per cent fewer Con A receptors available for binding. Although the specific activity of [^{3}H]mannose-labeled glycoprotein was reduced markedly by 2 hr after exposure of cells to 6-TG (Table 1), no decrease in the extent of Con A binding was detected at this time (Fig. 2). Moreover, 24 hr after treatment of S180 with 6-thiopurine, when the incorporation of [^{3}H]mannose into glycoproteins of treated and control cells was similar, a decrease occurred in the number of Con A molecules bound by 6-TG-treated cells. These findings suggest that the receptors for Con A on S180 cells that are being measured may be only a small subset of the mannose-containing

glycoproteins and that they have a relatively slow turnover rate. These results are similar to those of Duksin and Bornstein [27] who reported that a dose of the antibiotic tunicamycin, which reduced [^{3}H]mannose incorporation into glycoprotein by 99 per cent, caused only a 20–30 per cent decrease in Con A binding.

In contrast to the effects of the 6-thiopurine on the specific binding of Con A, the specific binding of WGA and Ricin was not altered by treatment with this agent. WGA and Ricin are lectins specific for GlcNAc and Gal, respectively, sugars which are activated by UTP rather than GTP [3]. GlcNAc is usually found proximal to the mannose core in complex oligosaccharides. Consequently, a decrease in WGA binding might not be expected in 6-TG-treated cells if the mannose-deficient oligosaccharides appeared on the plasma membrane. Ricin, however, binds to Gal, which is usually the penultimate sugar in complex oligosaccharides. Thus, on S180 cells, the molecules of Ricin might be bound either to oligosaccharides which lack core mannose units or to a subset of complex oligosaccharides which have a much slower turnover than those glycoproteins involved in Con A binding. Duksin and Bornstein [27] have reported similar results with tunicamycin in that a level of tunicamycin which reduced [^{3}H]mannose incorporation by 99 per cent and Con A binding and cell agglutination in transformed cells did not affect agglutination by WGA or soybean agglutinin. Thus, while mannose may be a core element of many glycoproteins, inhibition of its incorporation into these macromolecules does not necessarily result in a reduction in the degree of binding of lectins specific for other sugars. Duksin and Bornstein [27] have speculated that the cytotoxicity of tunicamycin is due to its ability to inhibit glycoprotein synthesis. Although the mechanism by which interference with the formation of glycoproteins can result in cell death is not certain, there are a number of examples which demonstrate that altered glycosylation can lead to changes in the disposition of glycoproteins and, possibly, the characteristics of cells. Thus, Melchers [28] has shown that glycosylation is necessary for the movement of some peptides from the ribosomes through the cisternae of the rough endoplasmic reticulum to the cisternae of the smooth endoplasmic reticulum. Inhibition of glycosylation leads to a decrease in the secretion of some glycoproteins, such as the LETS protein [27] and IgA and IgE [29]. Furthermore, the inability of cells to synthesize normal oligosaccharides appears to result in altered cellular morphology and adhesion [27, 30]. Treatment of malignant cells with neuraminidase has been reported to lead to an increase in their immunogenicity [31].

Whether inhibition of glycosylation can cause cell death is unresolved; however, when glycoprotein biosynthesis is altered by a chemical agent in concert with other actions, such as the incorporation of 6-thioGMP into DNA, the multiple lesions created may lessen the probability of cellular repair and ultimately lead to cell death. The disruption of glycoprotein synthesis by 6-TG occurs shortly after drug exposure and occurs to a greater degree in 6-thiopurine-sensitive cells than in a drug-resistant variant [10]. These findings are consistent with the concept that modification of surface properties by 6-TG may be an obligatory step for cell death.

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