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STUDIES ON THE POSSIBLE CHEMICAL, IMMUNOCHEMICAL AND MORPHOLOGICAL DIFFERENCES AT THE CELL SURFACES OF IMMUNOSENSITIVE AND IMMUNORESISTANT, MOLONEY VIRUS-INDUCED, LYMPHOMA CELL-LINES*,†

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ABSTRACT

Comparison was made of several cell-surface parameters in the immunosensitive, Moloney virus-induced, mouse lymphoma, YAC, and its immunoresistant variant, YACIR. The characteristics of the two cell lines appeared to be similar by most of the criteria employed. The poly(acrylamide)-gel electrophoresis (with sodium dodecyl sulfate) patterns, after staining with Coomassie Brilliant Blue, of detergent-solubilized materials, appeared to be identical. After elution from a gel-filtration column, no major differences were observed in the protein profiles of material cleaved from viable cells by proteolysis. Scanning and transmission electron microscopy revealed no major differences between the YAC and YACIR cells. The concentration of the lectins, *Ricinus communis* agglutinin, concanavalin A, wheat-germ agglutinin, and *Solanum tuberosum* (potato) agglutinin, required to agglutinate viable cells of the two lines were not significantly different. Neither cell was agglutinated by the lectins from *Dolichos biflorus* or *Vicia graminea*. Significant differences were, however, observed in the concentrations of lectin from

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Arachis hypogaea (peanut) needed to agglutinate the two cells. Although similar amounts (184–188 $\mu g/10^9$ cells) of sialic acid were released from viable cells by neuraminidase (*V. cholerae*), striking differences were observed in the composition of this material: 48% of *N*-glycolylneuraminic acid for YAC and 15% for YACIR. The remainder was *N*-acetylneuraminic acid for each cell line.

INTRODUCTION

Immunoresistant tumor-cells, which are able to escape from or resist the immune defenses of the host, may owe their properties to one or more escape mechanisms. In immunoresistant, TA3 mammary-carcinoma ascites cells, antigen masking by high-molecular-weight, cell-surface glycoprotein molecules[1-3], and blocking of immune lymphocytes by antigen–antibody complexes[4,5], have been suggested. In the same system, immunoresistance also appears to be associated with tumor-cell pleomorphism, with high concentrations of microvilli[6]. Additional escape-mechanisms have been suggested for other tumors[7-10]. Very little is, however, known of the actions or interactions of macromolecules, at the cell surfaces of tumor cells, that might modify antigenic activity or that might alter the cytotoxic action of host lymphocytes, macrophages, or antibodies.

This study is an extension of previous work on an allogenic system with TA3 tumors of the strain A mouse, in which the major histocompatibility antigens (H-2^a) of the tumor cell are the targets of the immune factors of the foreign-strain mice[1-3,11-13]. Possible differences in cell-surface glycoproteins between an immunosensitive, Moloney virus-induced, lymphoma (YAC) and an immunoresistant line (YACIR) of the same tumor[14] have been studied. In this syngeneic system, the major target of the immune defense is the Moloney virus-induced, cell-surface antigen (MCSA), which is the tumor-rejection antigen[15].

EXPERIMENTAL

Ascites cells. — The Moloney virus-induced, murine lymphoma cells YAC and YACIR were developed and maintained at the Karolinska Institutet, as previously described[14]. The cells were shipped *in vivo* to Massachusetts General Hospital during passage 131 of YAC and 26 of YACIR. They were maintained in the ascites form by serial passages in syngeneic A/WySn mice (purchased from Jackson Laboratory, Bar Harbor, ME 04609).

Treatment of cells with neuraminidase. — Sialic acid was released from intact cells by incubation of cells ($1–2 \times 10^8$) with neuraminidase (0.5 U, EC 3.2.1.18, *V. cholerae*, 1.0 U/mL; Behring Diagnostics, Somerville, NJ 08876) in phosphate-buffered saline solution[16] (4 mL) for 80 min at 37°. Cells were washed twice with buffer[16], and the pooled, supernatant solutions centrifuged to remove all insoluble material.

Composition of sialic acid. — The sialic acid released by neuraminidase was

determined by the thiobarbituric acid method[17]. To determine the ratios of *N*-acetyl to *N*-glycolyl to total neuraminic acid, the solution was made salt-free by ion-exchange chromatography[18,19], and the sialic acid present in the residue was converted into the per-*O*-(trimethylsilyl)ated methyl glycoside methyl esters as described previously[19]. The sialic acid derivatives were fractionated, and identified, by gas–liquid chromatography (g.l.c.)[19,20].

Proteolysis. — Cells free from erythrocytes were incubated with L-1-tosylamido-2-phenylethyl chloromethyl ketone–trypsin (EC 3.4.21.4, Worthington Biochemical Corp., Freehold, NJ 07728) at 4° in three sequential incubations of 30 min each, as previously described[12]. After removal of the cells and other debris by centrifugation, the pooled trypsinates were centrifuged at 20 000 *g* for 30 min at 0–4°. Aliquots used in the determination of sialic acid[17] and protein[21] and for analysis by g.l.c. of sialic acid composition[19], total carbohydrate[20], and amino acids[22] were taken. The remainder was lyophilized and later used for fractionation, as described next.

Fractionation of glycopeptides by gel filtration. — The lyophilized proteolyzate aforementioned was fractionated at 4° on a column (3.5 × 42 cm) of Bio-Gel P-2 (200–400 mesh) with 50mM pyridine acetate (pH 5.3) as eluant. The fraction of highest molecular weight (excluded from the column) was subsequently eluted at 4° from a column (2.6 × 96 cm) of Bio-Gel P-100 (100–200 mesh) with 0.10mM pyridine acetate (pH 5.3). Fractions (6 mL) were collected and analyzed[20,22].

Lectins. — *Ricinus communis* agglutinin (RCA), concanavalin A (con A), wheat-germ agglutinin (WGA), and *Solanum tuberosum* (potato) agglutinin (STA) were prepared as described previously[23]. *Dolichos biflorus* lectin and *Arachis hypogaea* (peanut) lectin were purchased from Sigma Chemical Co. (St. Louis, MO 14508). *Vicia graminea* seeds, obtained from Dr. H. C. D. DeWit (Wageningen, Holland), were extracted as previously described[5].

Agglutination of tumor cells. — Agglutination experiments were performed at 20–23°, in a series of small tubes, with 50 μL of cells (4×10^6 cells/mL) added to buffer (50 μL) containing lectin at appropriate, 2-fold serial dilutions. After 60 min with periodic mixing, the proportion of agglutinated cells was determined with a hemocytometer. The concentration of lectin (μg/mL) required to effect the agglutination of 50% of the cells was determined from the graph.

Adsorption of Vicia graminea *lectin by intact cells.* — The adsorption of *Vicia graminea* lectin by intact cells was performed as previously described[11]. Determination of the amount of lectin adsorbed by YAC and YACIR ascites cells was expressed as a proportion of that adsorbed by TA3-Ha ascites cells. Data were plotted as the percent of type N-specific, human erythrocytes agglutinated *versus* the number of tumor cells used in that adsorption of lectin. The number of cells needed in order to adsorb an amount of lectin such that the remaining lectin was sufficient to cause the agglutination of 50% of the erythrocytes was compared with a value similarly obtained for TA3-Ha cells. In these experiments, the relative numbers of erythrocytes were measured by lysing with Triton X-100, and then recording the

liberated hemoglobin by the absorption at 550 nm in a spectrophotometer, as described previously[5,11].

Electron microscopy. — For transmission electron-microscopy, cells were fixed in glutaraldehyde and osmium tetraoxide, as previously described[24], and were embedded in Spurr's low-viscosity resin[25]. For scanning electron-microscopy, cells were fixed, as previously reported[24], and were critical-point dried. Two electron microscopes, a JEOL 100S and a JEOL JSM U-3 scanning electron-microscope, were used.

RESULTS

Quantity and composition of neuraminidase-released sialic acid. — No significant difference could be found between the amount of sialic acid released from viable YAC and YACIR ascites cells by neuraminidase. Average values from more than three independent experiments with each cell line were 188 and 184 $\mu g/10^9$ cells for YAC and YACIR cells, respectively (see Table I). Compositions of the sialic acid enzymically released, however, showed marked differences. The proportion of *N*-glycolylneuraminic acid in neuraminidase-released sialic acid was 48% for the YAC and 15% for the YACIR cell-line. The remainder of the sialic acid from each line was found to be *N*-acetylneuraminic acid.

Amount and composition of bound sialic acid released from viable YAC and YACIR cells by proteolysis. — The proportion of sialic acid released by neuraminidase treatment of glycoproteins cleaved from viable YAC and YACIR cells by proteolysis was 34 and 27%, respectively, of that released by neuraminidase treatment of whole cells. Neuraminidase treatment of the cell residues remaining after proteolysis released only an additional 19 and 18%, respectively, of the total cleaved by neuraminidase, probably because of extensive

TABLE I

AMOUNT AND COMPOSITION OF SIALIC ACIDS IN YAC AND YACIR CELLS

Treatment of cells	*Cells*			
	YAC		*YACIR*	
	Amount ($\mu g/10^9$ cells)	*NeuGc[a] (%)*	*Amount ($\mu g/10^9$ cells)*	*NeuGc (%)*
Neuraminidase	188 ±20	48 ±2	184 ±20	15 ±1
Trypsin[b]				
Supernatant solution	64 ±5	29 ±2	50 ±4	18 ±1
Residue	36 ±3	59 ±2	34 ±3	9 ±1

[a]NeuGc, *N*-glycolylneuraminic acid. The rest is *N*-acetylneuraminic acid. [b]A modified trypsin, 1-1-tosylamido-2-phenylethyl chloromethyl ketone–trypsin, was used

agglutination of the cells. The proportion of *N*-glycolyl to total neuraminic acid of the supernatant and the residue from the YAC cell was found to be 59% in each case, but the proportion of this component in comparable fractions from the YACIR cell-line was only 18 and 9%, respectively.

Material released by proteolysis. — The composition and total yield of the carbohydrate material released from the two cell lines during three successive incubations with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin at 4° appeared to be approximately the same. The major carbohydrate component present in the cleaved material from each cell line was 2-acetamido-2-deoxy-D-glucose (GlcNAc). Present in smaller proportions were mannose, galactose, 2-acetamido-2-deoxy-D-galactose (GalNAc), and sialic acid. Glucose was detected in amounts approximately equal to those of galactose and mannose, but the source of the glucose was probably not the released glycopeptides. The small differences in the total carbohydrate content detected in the supernatant solutions, as determined by g.l.c. (0.11 mg from 10^9 YAC and 0.10 mg from 10^9 YACIR cells), cannot be considered significant.

In order to determine whether differences in either carbohydrate or amino acid components existed in the individual glycopeptide fractions cleaved by proteolysis from the two cells, the trypsinates from the two cells were fractionated by gel filtration on the same Bio-Gel P-100 column. The protein content of each fraction was determined by the method of Lowry *et al.*[21]. Small differences in the elution profiles of material from the two cells were observed (not shown), but no major difference was found. Neither cell line exhibited major peaks at or near the excluded volume, and, for each cell, 90% of the protein was eluted as a single, large, broad peak of average estimated M_r <20 000.

The effluent fractions from each cell treatment were divided, and pooled into eight portions, and carbohydrate and amino acid compositions determined. Owing to the extremely small quantities of material available for the high-molecular-weight fraction, accurate measurements were difficult to obtain. The relative amounts of some carbohydrate components, such as galactose and sialic acid, did indeed exhibit marked differences in the fractions early eluted, but failure to obtain the same results consistently with a few μg of material from separate, chromatographic separations suggests that the differences may be artifactual. It is noteworthy, however, that, in material eluted in effluent volumes of 120 to 240 mL, representing the first three fractions analyzed, the proportion of GalNAc in material from each cell line was equal to that of GlcNAc. This result suggests the presence, in both cell lines, of protease-resistant glycoproteins containing a high proportion of *O*-glycosyl-linked carbohydrate chains. These same fractions gave consistently higher values of serine or threonine than the later fractions, a result that is consistent with the presence of *O*-linked chains[26].

Agglutination by lectins. — In experiments with several lectins, only minor differences in the agglutinability of the YAC and YACIR cells were observed. Agglutination curves for two lectins, con A (a) and RCA (b), are given in Fig. 1.

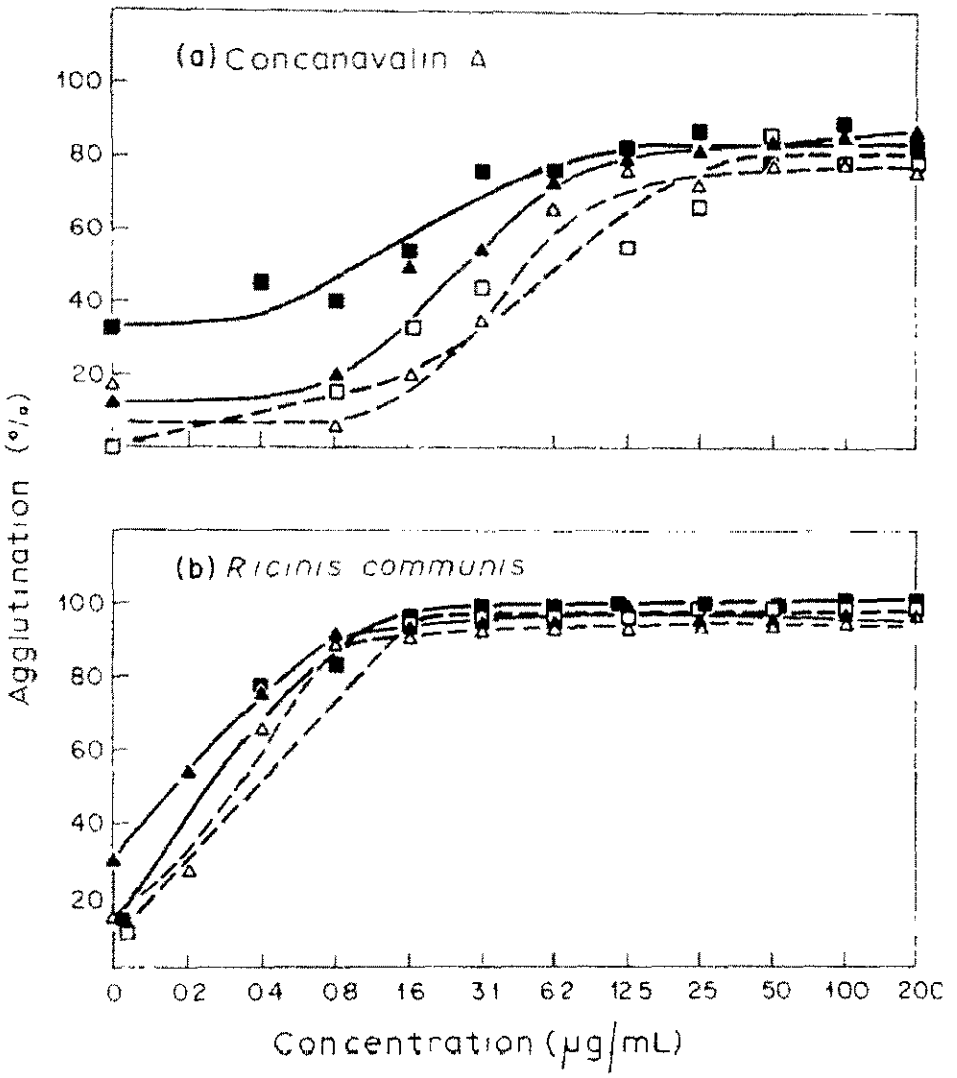

Fig. 1. Agglutination of ascites cells (untreated YAC, □---□; neuraminidase-treated YAC, ■---■, untreated YACIR, △---△, neuraminidase-treated YACIR, ▲---▲), in phosphate-buffered saline at 20–23°. Lectins: (a) concanavalin A, (b) *Ricinus communis* agglutinin

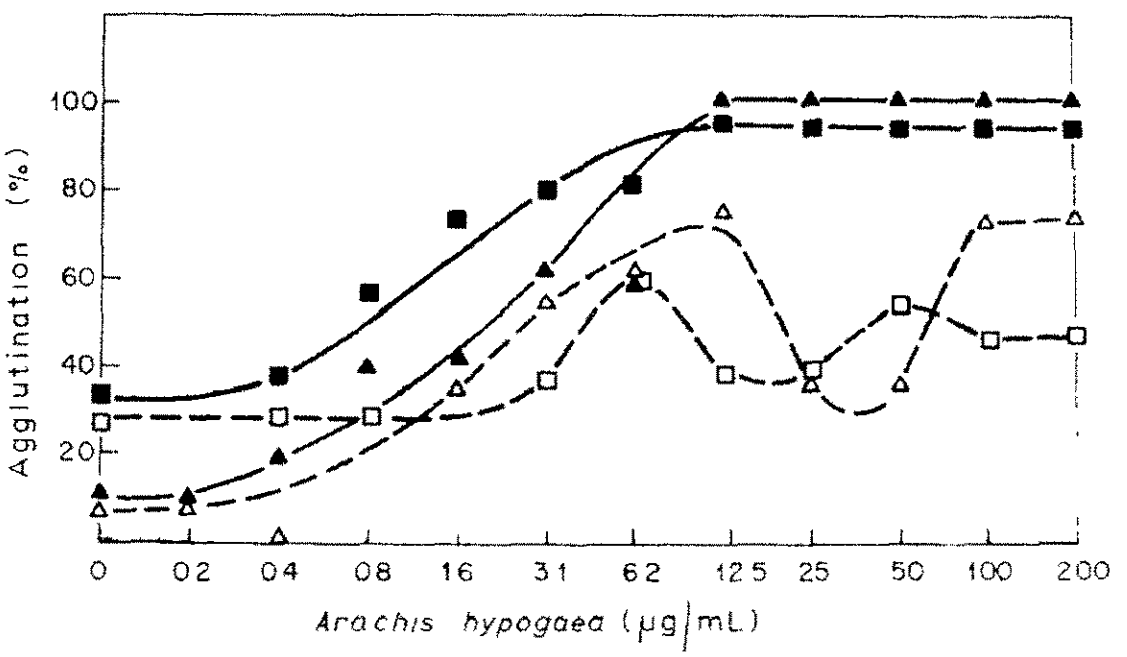

Fig. 2. Agglutination of ascites cells, as described for Fig. 1. Lectin *Arachis hypogaea*, peanut lectin.

TABLE II

AGGLUTINATION OF CELLS BY LECTINS

Lectin	*Cell line*	*Lectin concentration required for agglutination (μg/mL)*[a]	
		Untreated cells	*Neuraminidase-treated cells*
Concanavalin A	YAC	<2	2 ±1
	YACIR	3 ±1	2 ±1
Ricinus communis agglutinin	YAC	<1	<1
	YACIR	<1	<1
Wheat-germ agglutinin	YAC	<2	
	YACIR	1 ±0.5	6 ±2
Peanut lectin	YAC	(4 ±1)[b]; 40 ±20	1 ±0.5
	YACIR	(2 ±1)[b]; 70 ±10	2 ±1
Dolichos biflorus agglutinin	YAC	>200	
	YACIR	>200	>200
Potato lectin	YAC	4 ±2	
	YACIR	5 ±2	

[a]Values represent the concentrations of lectin (μg/mL) needed to agglutinate 50% of cells (2×10^6 cells/mL) at 20–25°. [b]Values in parentheses represent the first of two separate agglutinations observed (see Fig. 2).

The results of an experiment with *A. hypogaea* (peanut) lectin are presented in Fig. 2. The results obtained with these and other lectins are tabulated in Table II. As shown in Fig. 2, the agglutinations of YACIR and YAC cells by peanut lectin followed similarly unusual, but repeatable, courses. The curves for the two cells were not, however, identical. Agglutination reached 50% at 2–4 μg of lectin per mL, but at concentrations of ~20–30 μg per mL, 50% of the cells agglutinated. The cells again agglutinated at higher concentrations (40–70 μg) of lectin.

YACIR and YAC cells were agglutinated equally well by either con A or RCA. After neuraminidase treatment, however, each cell line was agglutinated at a low concentration of lectin. No major differences between these lines were observed with the other lectins studied. YAC and YACIR cells were agglutinated at approximately the same concentration (4–5 μg/mL) of potato lectin. Each line was agglutinated by a low concentration (1–2 μg/mL) of wheat-germ agglutinin. More lectin (6 μg/mL) was required after neuraminidase treatment. In these experiments with wheat-germ agglutinin, 95–100% of the neuraminidase-susceptible sialic acid was removed from each cell line. No agglutination of YAC or YACIR cells was observed with *Dolichos biflorus* or *Vicia graminea* lectins, either before or after removal of sialic acid.

Adsorption of Vicia graminea *lectin by untreated and neuraminidase-treated cells.* — Hemagglutinations by solutions of the lectin after adsorption by intact YAC or YACIR cells indicated the presence of relatively few receptors on either of the two cell lines, as compared to the TA3-Ha cell, a cell that contains receptors

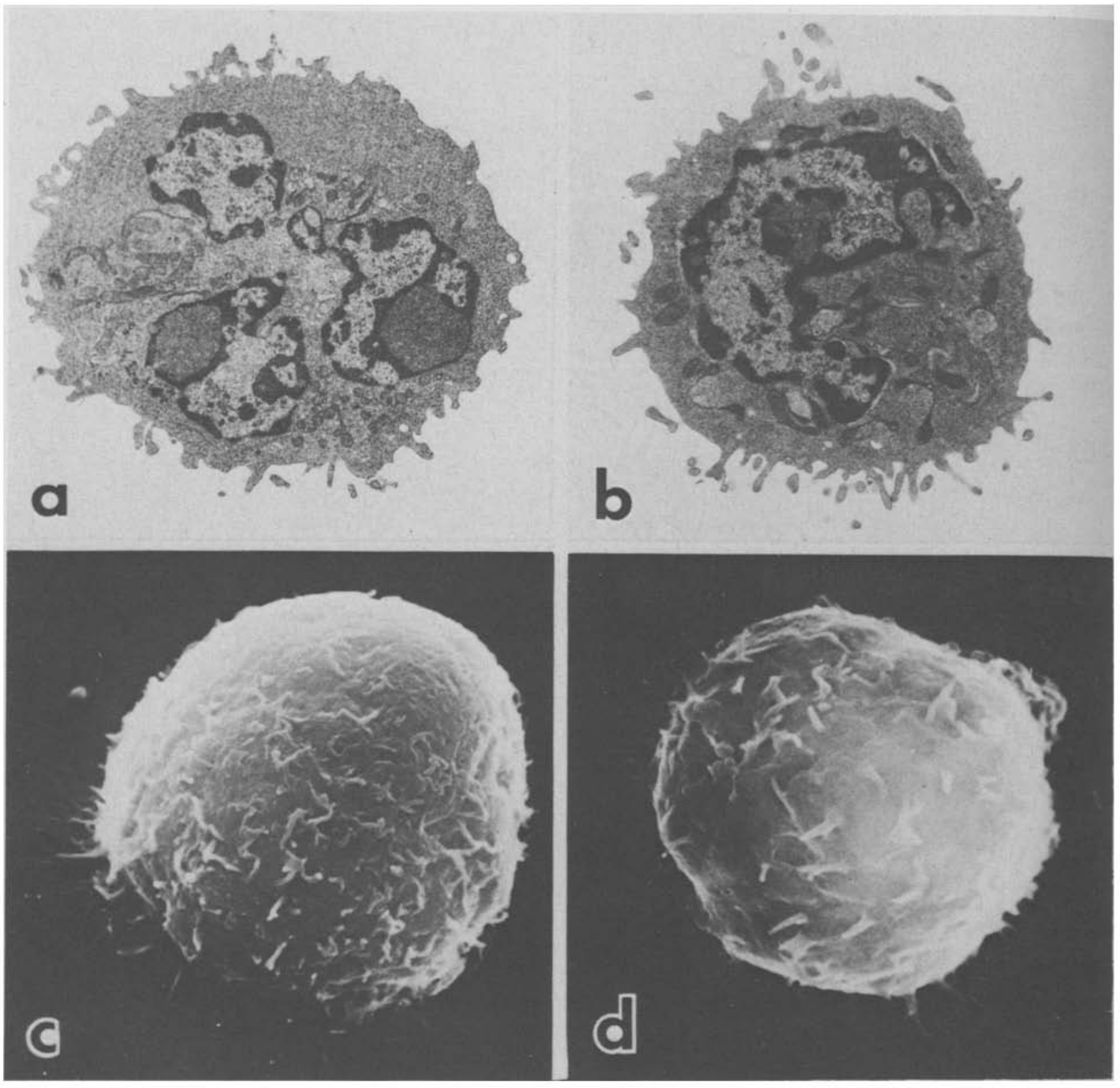

Fig. 3 (a) Transmission electron-micrograph of a YAC cell (× 5 000) (b) Transmission electron micrograph of a YACIR cell (× 7 500) (c) Scanning electron-micrograph of a YAC cell adherent to a glass coverslip (× 4 500) (d) Scanning electron-micrograph of a YACIR cell adherent to a glass coverslip (× 6 500)

located in epiglycanin molecules[2]. The level of lectin adsorption either by neuraminidase-treated or untreated YACIR, or YAC, cells, suggests the presence of <0.3% as many receptors per cell on these cells as on the TA3-Ha cell.

Cell morphology. — When examined by transmission electron-microscopy, YAC (see Fig. 3a) and YACIR (see Fig. 3b) cells were found to have pleomorphic nuclei containing large nucleoli. The nuclei are frequently lobulated. The cytoplasm contains mitochondria, a paranuclear Golgi apparatus, and abundant free ribosomes. Virus particles were also evident in the cytoplasm. The surfaces of these cells, as observed by transmission electron-microscopy and scanning electron-microscopy (see Figs. 3c and 3d) have numerous small, irregular microvilli and

lamellopodia. YAC (see Fig. 3c) and YACIR (see Fig. 3d) cells are adherent to the glass substrate by filopodia and lamellopodia. It was concluded that, at the ultrastructural level, the YAC and YACIR cells possess quite similar features.

DISCUSSION

No general mechanism for the immunoresistance developed by tumor cells has been proposed. Indeed, the mechanism employed by a cell selected on the basis of resistance to anti-MCSA antiserum *in vitro*, as well as to the immune defenses of the immunized host *in vivo*[14,15], such as the YACIR lymphoma, may not be expected to be similar to that of a mammary carcinoma which became immunoresistant "spontaneously" during routine passage in the syngeneic host, such as the TA3-Ha (ref. 27) or TA3-MM (refs. 11 and 12) ascites cells. Similarly, little is known regarding possible differences that may exist in escape processes involving histocompatibility antigens, as compared to those involving such tumor-specific antigens as MCSA. In a recent report, however, Dalianis *et al.*[28] found that the rejection of the same Moloney lymphoma (YAC) as used in the present study, in syngeneic, preimmunized host-cells, is strongly influenced by the expression of the H-2 antigen on the cell surface.

The present investigation shows no evidence for the presence in the variant line YACIR of a major glycoprotein having physicochemical properties resembling those of epiglycanin[12,29–31], which has been implicated in antigen masking[1–3]. Receptors for *Vicia graminea* lectin, which are present in epiglycanin[32], appear to be totally absent from the surface of the YACIR, as well as the YAC, cell. Furthermore, no fraction having carbohydrate or amino acid compositions similar to those of epiglycanin[12] was isolated from the trypsinates of YAC and YACIR cells, and no qualitative difference between the detergent-solubilized proteins, as stained by Coomassie Blue, was observed for the two cell lines (gels not shown), in agreement with the findings of Troy *et al.*[33]. The major cell-surface glycoproteins of the YAC and YACIR cells are very similar, as shown by carbohydrate and amino acid compositions of proteolyzates (data not given). Some difference may be found in the fractions containing high-molecular-weight material, but only amounts of material too small for the present study were available. The similarity of the cell-surface glycoproteins of both cells was confirmed by their positive agglutination with six different plant-agglutinins (see Table II), and negative agglutination with the lectin from *V. graminea*. Of five of the lectins, the concentrations required for agglutination (see Table II) were almost identical for the two cells. However, in addition to the presence of specific carbohydrate or glycopeptide structures, various factors are required at the cell surface for the agglutination of eukaryotic cells by lectins[34]. These include the arrangement and lateral mobility of receptors[34].

In order to examine this requirement, the agglutinability of each cell line was determined by microscope examination over a wide range of lectin concentrations (see Figs. 1 and 2). No significant differences were observed with the lectins from

wheat germ, *Dolichos biflorus*, and potato (not shown), and the two lectins, con A and *Ricinus communis* agglutinin (see Fig. 1). For *Arachis hypogaea* (peanut) lectin, however, unusual, but repeatable, differences were found (see Fig. 2). Two separate, optimal values of agglutination were observed for each cell, but these values were significantly different for each cell. The meaning of these two maxima is not clear, the results probably reflecting small differences in the concentration and/or exposure at the cell surfaces of the disaccharide *O*-β-D-galactopyranosyl-(1→3)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl) residue, which is linked to serine or threonine and is the receptor for the peanut lectin[35]. A high concentration of this oligosaccharide chain is present at the surface of the immunoresistant TA3-Ha ascites cell, but absent at the surface of the immunosensitive TA3-St ascites cell[32].

Unlike the findings in the TA3 mammary-carcinoma ascites cells, where immunoresistance was accompanied by marked changes in cell-surface morphology[6,24], no significant differences were observed in the two cell lines by either transmission or scanning electron-microscopy (see Fig. 3).

The most striking difference between the YAC and YACIR ascites cells is the far lower proportion of *N*-glycolylneuraminic acid (15%) released from the immunoresistant YACIR line (see Table I), as compared to that from the immunosensitive YAC line (48%). These findings are consistent with the previous observation that the immunoresistant, mammary-carcinoma line, TA3-Ha, possesses a lower proportion (7%) of *N*-glycolylneuraminic acid than the immunosensitive cell, TA3-St (20%). This relationship cannot, however, be generalized. An immunoselected variant of the TA3-St ascites cell, TA3-St/ticol (ref. 28), which is weakly immunoresistant, did indeed possess a lower proportion of *N*-glycolylneuraminic acid (9%) than the immunosensitive, control cell, TA3-St (ref. 36) (20%), but a variant spontaneously developed *in vivo*, and having strong immunoresistance, namely, TA3-St/ticol/-A, possesses 93% of *N*-glycolylneuraminic acid[37].

A relationship between the development of immunoresistance in the YAC cell and the change in sialic acid composition would be plausible, as sialic acid has been implicated in masking crypt antigens, resulting in enhanced transplantability[38], and its concentration has been correlated with immunoresistance in a mouse L cell line[39]. Little has been reported, however, regarding a specific role for terminal *N*-glycolylneuraminic acid groups, as contrasted to that of *N*-acetylneuraminic acid groups, at cell surfaces. Bhavanandan and Katlic[40] found that sialoglycoproteins having terminal *N*-acetylneuraminic acid residues bind to wheat-germ agglutinin, but those with *N*-glycolylneuraminic acid residues do not, which suggests that the composition of sialic acid at cell surfaces may affect both the conformation and the arrangement of cell-surface glycoproteins, as well as receptor activity. Possible relationships between the differences in physicochemical properties of cell-surface glycoproteins of the immunoresistant YACIR cell and the immunosensitive YAC

cell and the differences in the apparent molecular weights of macromolecules containing Moloney-virus induced, cell-surface antigenic activity from the two lines, as reported by Troy and co-workers[41], are not yet evident.

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