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In Vivo Release of Glycoprotein I from the Ha Subline of TA_3 Murine Tumor into Ascites Fluid and Serum

(tumor antigen/lectin/quantitative hemagglutination-inhibition)

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ABSTRACT **Previous studies have demonstrated that a unique glycoprotein can be cleaved by trypsin from the plasma membrane of the Ha, but not the St, subline of the TA_3 murine mammary adenocarcinoma. Using an automated quantitative method for measurement of trypsin-cleaved fragments (glycoprotein fraction I) by inhibition of *Vicia graminea* lectin hemagglutination, we find evidence that glycoprotein fraction I-like molecules appear in the ascites fluid and serum of the Ha-bearing, but not the St-bearing, syngeneic mice. These molecules were shown by gel filtration to be larger than the trypsin-cleaved glycoprotein fraction I but have a carbohydrate composition very similar to glycoprotein fraction I. It is likely that these ascites and serum glycoproteins have been released *in vivo* from the membranes of the viable Ha tumor cells. In view of the ability of the Ha, but not the St, cells to grow in allogeneic recipients, it is possible that these circulating membrane-derived molecules may be playing a blocking role in the immune response to the tumor.**

Two sublines, Ha and St, of the murine mammary adenocarcinoma TA_3 have been extensively studied (1–9). The Ha subline is of special interest because it has lost strain specificity and is able to grow in H-2 incompatible allogeneic hosts (1–4). There are several theories to explain this biologic behavior. Although the Ha subline grows marginally faster than the St in syngeneic recipients, this increment is not thought sufficient to explain the difference in malignancy (2). There is evidence that the Ha cells are less immunogenic than St cells (4) and are less susceptible to antibody-mediated and cellular immune destruction *in vitro* (3).

The biologic behavior of the Ha subline may be related to a unique glycoprotein fragment (GPF-I) that has been removed from the surface membrane of the Ha, but not the St, subline by trypsin cleavage (6, 7). This glycoprotein is present in large amounts on the membrane and has been extensively characterized chemically (6, 7) and physically (10). It has been suggested that these glycoprotein molecules may sterically block access to H-2 antigens, since the ability of the Ha cells to absorb anti-H-2 sera is considerably increased after the cells have been disrupted by lyophilization (4, 5). An alternative possibility, suggested by the recent work of the Hellströms and others (11–14), is that the GPF-I molecules are released from the surface membranes of Ha cells *in vivo*, enter the circulation, and participate in blocking an immune mechanism that would otherwise be capable of eliminating the cells. Such a finding would be especially attractive because of the extensive characterization that has already been accomplished for the plasma membrane-derived GPF-I. In this study, we have taken advantage of the recent demonstration (15) that GPF-I molecules have receptor sites for the lectin *Vicia graminea* and have developed an automated hemagglutination-inhibition assay for GPF-I molecules. Using this assay, we have demonstrated that GPF-I-like molecules are released from Ha cells into the ascites fluid and enter the circulation.

Abbreviations: GPF-I, glycoprotein fraction I; PBS, phosphate-buffered saline (pH 7.2); PCA, perchloric acid.

MATERIALS AND METHODS

Tumors. The TA_3 mammary adenocarcinoma arose spontaneously in 1949 in an A/HeHa female mouse (1). Two independent conversions from the solid to the ascites form of the tumor resulted in the sublines TA_3-St and TA_3-Ha. Both sublines were originally strain specific, but the Ha subline subsequently lost strain specificity during passage in syngeneic A/HeHa mice (1). The TA_3-Ha subline was obtained from Dr. T. S. Hauschka, Roswell Park Memorial Institute, in 1965, and the TA_3-St subline was obtained from Dr. G. Klein, Karolinska Institutet, in 1970. Both sublines have been maintained by weekly intraperitoneal transfers in syngeneic A/HeHa mice with inocula of 8×10^4 Ha cells or 1.3×10^5 St cells. There was never any gross evidence of tumor metastasis outside the peritoneal cavity.

Serum and Ascites Fluid. Serum was obtained by cardiac puncture from ether-anesthetized TA_3-Ha or TA_3-St tumor-bearing A/HeHa mice, before the abdominal cavity was opened to remove tumor cells, and from control normal A/HeHa mice. In some instances, blood was obtained from the tails of the Ha-bearing mice at intervals from the time of tumor inoculation until the mice were killed. All blood samples were allowed to clot for 1 hr, and the serum was freed of cells by successive centrifugations at 1,000 and 10,000 $\times g$. Examination of buffy coats of whole blood was performed in several instances and no circulating tumor cells were seen.

The ascites fluid was obtained from the Ha and St tumor-bearing mice at the time of weekly transfer by sterile aspiration through the exposed peritoneal membrane. The volume of ascites fluid was on an average 2–2.5 ml and was usually straw-colored. The fluid was placed in a measured volume of sterile Dulbecco balanced salt solution, and the tumor cells were enumerated with a Spencer hemocytometer. The total cell count was on an average 2×10^8 (range 8×10^7 to 3×10^8), with greater than 95% viability as measured by trypan

blue exclusion. Variable numbers of erythrocytes were seen microscopically. The diluted ascites fluid was freed of cells by successive centrifugations at 100 × *g* and 10,000 × *g* followed by passage through a Millipore filter (0.45 μm).

Glycoprotein Fraction I (GPF-I). The sample used as a standard in the *Vicia graminea* inhibitory tests (see below) was obtained from viable TA_3-Ha cells by successive incubations with TPCK-trypsin (18 μg/ml) at 4° and was purified by gel filtration on columns of Bio-Gel P-4 and P-100, as described (6). Peak material from the P-100 column was further fractionated by Sepharose 4B gel filtration, as reported (10), and Fraction C, after lyophilization, was used as a standard in the *Vicia graminea* assay.

Perchloric Acid Extraction of Serum and Ascites Fluid. Aliquots of serum and ascites fluid to be tested for *Vicia graminea* inhibition (see below) were made 0.6 M with perchloric acid (PCA) for 30 min at room temperature. After centrifugation, the glycoprotein-rich supernatant solutions were neutralized and dialyzed against phosphate-buffered saline, pH 7.2 (PBS). The PCA caused precipitation of proteins, including any possible contaminating cell-membrane fragments of tumor cells and any hemagglutinating antibodies that might interfere with the hemagglutination-inhibition assay. It had been shown in preliminary tests that such PCA extraction did not precipitate GPF-I or interfere with its *Vicia*-inhibitory activity.

Hemagglutination-Inhibition Assay for GPF-I. Seeds of the legume, *Vicia graminea*, were extracted by grinding 20 seeds in 2 ml of PBS and incubating the mixture for 30 min at 37°. The lectin-containing extract was cleared at 40,000 × *g* by centrifugation and was filtered and stored in small aliquots at −20°. Hemagglutination by the lectin was quantitatively measured with a continuous flow automated system (Technicon Hemagglutinating System) (16). Washed human O+, MN erythrocytes, adjusted to an hematocrit of 16% in a solution of PBS containing 0.5% Ficoll, were sampled continuously at a rate of 6 ml/hr. These cells were joined by an equal stream containing alternating aliquots of the lectin, diluted 1/500 with PBS (each aliquot was sampled for 2 min), and aliquots of plain PBS (sampled for 4 min) used to wash out and segregate each lectin aliquot. The erythrocytes and lectin (or PBS) were segmented by small air bubbles, introduced to facilitate mixing during passage through coiled glass tubing, and agglutination was promoted by the addition to the stream of 1.5% polyvinylpyrrolidone. Agglutinates formed in proportion to the amount of lectin in each aliquot. The agglutinates then settled to the bottom of the stream and were removed from the system. The remaining unagglutinated erythrocytes were lysed with Triton X-100 and the amount of hemoglobin, measured from the absorption at 550 nm, was recorded continuously. The recording showed a baseline, corresponding to periods in which PBS without lectin had been sampled, and intermittent peaks (with decreased A_{550}) corresponding to the sampling of aliquots of lectin. The more lectin present, the greater the peak height (baseline A_{550} − peak A_{550}).

For the quantitative hemagglutination-inhibition assay of GPF-I and the unknown ascites and serum samples, a standard amount of lectin extract (1 ml with a 1/500 final dilution) was preincubated for 1 hr at 4° with various amounts of the GPF-I or dilutions of ascites fluid or serum. Control tubes contained only the standard amount of lectin in PBS. These tubes were then tested for hemagglutination in the continuous flow system. Inhibition by the GPF-I or ascites or serum extracts resulted in diminution of the height of the peak of lectin agglutination. This measured diminution could be expressed as percent inhibition of the lectin by simultaneously determining the peak heights given by serial dilutions of this standard amount of lectin.

Inhibition of hemagglutination by the unknown ascites and serum samples was related to the amount of standard GPF-I that gave the same degree of inhibition. The ascites and serum levels of inhibitory substances were expressed as μg equivalents of GPF-I.

Controls for the specificity of the lectin inhibition included normal A/HeHa serum and human A, B, and H blood-group glycoproteins (generous gift of Prof. W. Watkins).

Sepharose 4B Gel Filtration. Aliquots of pooled serum (2.5 ml) and pooled ascites fluid (2.0 ml) from TA_3-Ha tumor-bearing mice were filtered (Millipore), dialyzed against distilled water, and then against 0.05 M pyridine acetate, pH 5.3. Any insoluble material was removed by centrifugation and discarded. The supernatant solutions were applied in turn to the identical column of Sepharose 4B (1.5 × 85 cm), after extensive washing with the eluent, and eluted at 4° with the pyridine acetate buffer. An aliquot (0.2-ml) of each fraction (3.0-ml) was used for a protein determination (17), and the remaining portion was lyophilized and tested for *Vicia*-inhibitory activity. A sample of GPF-I (870-μg) was then applied to the same column for comparison with the serum and ascites fluid activities.

Carbohydrate Analysis. A larger aliquot of pooled Ha ascites fluid was fractionated through a Bio-Gel P-4 column (2.7 × 77 cm) and eluted with 0.05 M pyridine acetate, pH 5.3. The excluded material was lyophilized and applied to a Sepharose 4B column (2.6 × 70 cm) and eluted with the same buffer. The peak of *Vicia*-inhibiting material (which was eluted well ahead of the bulk of the protein) was lyophilized. Dry samples were heated in a closed vessel at 80° with freshly prepared methanolic HCl (1.0 M) for 20 hr. Per(trimethylsilyl)ated methyl glycosides were prepared, as previously described (6), by the method of Reinhold (18). Gas-liquid chromatography was performed on a Perkin-Elmer Gas Chromatograph, model 900, with a column of OV 17 (Supelco, Inc., Bellefonte, Pa.).

RESULTS

Automated Hemagglutination by Vicia graminea and Inhibition by GPF-I. Fig. 1 shows the progressive inhibition of the lectin by increasing amounts of GPF-I. Duplicate determinations, expressed as percent inhibition of the lectin, varied by less than 5%. Less than 10 ng of GPF-I in 1 ml of the diluted lectin still gave measurable inhibition. No inhibition was caused by 1 μg of blood-group A, B, or H glycoproteins.

Inhibition of Vicia Extract by Ascites Fluid and Serum. Very high inhibitory activity was consistently found in the ascites fluid of TA_3Ha-bearing syngeneic mice. This activity was not dialyzable, indicating a macromolecular structure. The results of measuring the *Vicia*-inhibiting activity of PCA supernatant solutions of a number of Ha and St ascites fluids are shown in Fig. 2. The results are expressed in terms of GPF-I equivalents. It was necessary to greatly dilute the Ha ascites fluid in order to measure the exact levels of GPF-I

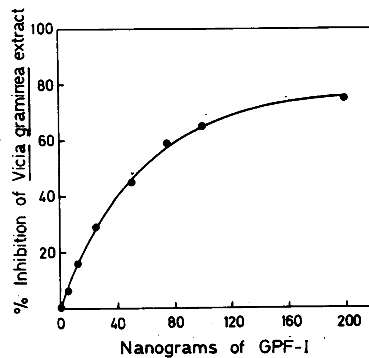

FIG. 1. Standard inhibition curve of *Vicia graminea* hemagglutination by glycoprotein I (GPF-I). Serial amounts of the GPF-I were added to a constant amount of the lectin (1/500 final dilution), and the lectin was tested for hemagglutination in the AutoAnalyzer system. The amount of inhibition was proportional to the amount of GPF-I. As little as 5 ng of GPF-I gave measurable inhibition.

equivalent activity, which varied from 44 to 510 μg/ml (median value 150 μg) of ascites fluid. There was no strict correlation between the ascites fluid activity and the number of tumor cells that was present per ml of fluid. The levels of activity in the ascites fluid of St mice were less than 0.5 μg/ml, even though the St ascites cell counts were in the same range as the Ha counts.

In order to rule out the possibility that the St ascites levels appeared erroneously low due to the presence of an inhibitor, such as antibody, which could prevent binding by the *Vicia* lectin, exogenous GPF-I and Ha ascites fluid were each added to aliquots of St ascites fluid. The anticipated level of lectin-inhibiting activity was found in each instance whether or not the final mixture was extracted with PCA before the assay.

Serum from Ha- and St-bearing mice, obtained after 1 week of tumor growth, along with serum from normal mice, were tested for *Vicia*-inhibiting activity. The results of quantitating the activities of the PCA supernatant solutions of Ha,

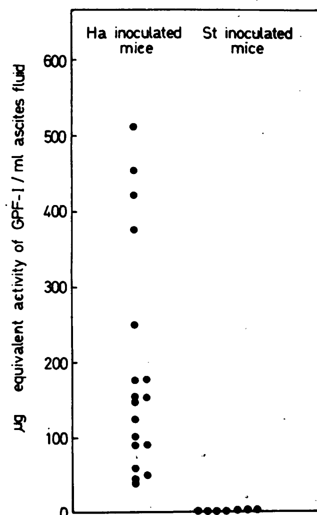

FIG. 2. *Vicia*-inhibiting activity in the ascites fluid of Ha and St tumor-bearing mice expressed as the equivalent amount of GPF-I giving the same amount of inhibition. The median value for the Ha mice was 150 μg/ml of fluid. Each of the St mice had less than 0.5 μg/ml of fluid, even though the tumor cell counts were comparable in each case.

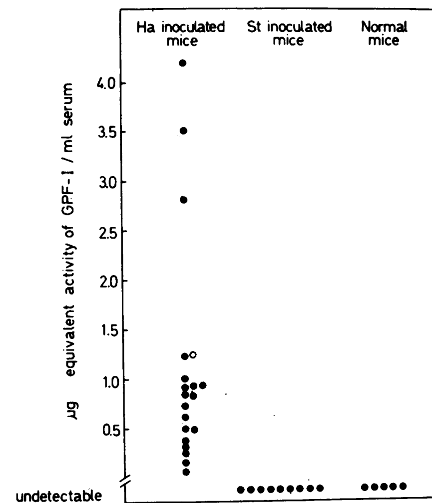

FIG. 3. *Vicia*-inhibiting activity in the serum of Ha and St tumor-bearing mice and normal control A/HeHa mice. The Ha values ranged from 0.5 μg to 4.2 μg/ml (median about 1.0 μg). None of the St-bearing or normal control mice had detectable levels. One Ha point (*open circle*) is a pool of nine Ha-bearing sera.

St, and normal sera are shown in Fig. 3. Again, there was a considerable range of values for the Ha sera (0.05–4.2 μg/ml, median 1 μg/ml). In most instances, ascites fluid and serum were obtained from the same mouse. The ascites fluid concentrations were always much higher than the serum, but the ratios of ascites to serum activities varied considerably. No activity was detectable in the sera from the St-bearing or the normal control mice, and, from the amount of serum tested, their levels must have been less than 0.1 μg/ml, which was the lower limit of detectability because of limited amounts of St serum.

Serial Measurements of Activity in the Serum of Ha-Bearing Mice. In order to study the time course of appearance of the serum *Vicia*-inhibiting activity in Ha-bearing mice, several

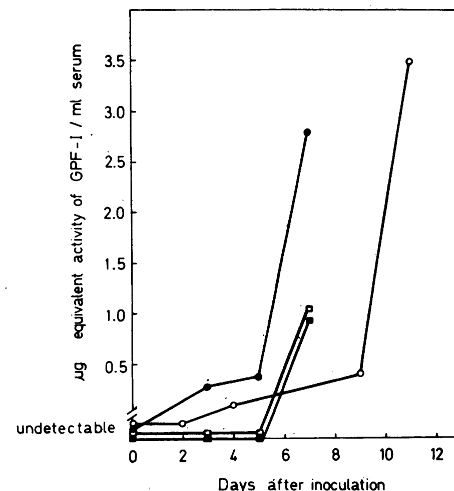

FIG. 4. Measurement of serial levels of *Vicia*-inhibiting activity in the serum of four Ha-bearing mice from time of inoculation with tumor cells until they were killed. The mouse represented by *open circles* was inoculated with 10^4 Ha cells and was killed on day 11. The other three mice received 8×10^4 Ha cells and were killed on day 7.

mice were bled from the tail at intervals from the time of intraperitoneal inoculation with Ha cells until the day they were killed, when they were bled from the heart and the Ha cells and ascites fluid were harvested. Fig. 4 shows the *Vicia*-inhibitory activity of serial serum samples from four mice, three of which were inoculated with 8×10^4 Ha cells and killed on day 7 and one of which was given only 10^4 Ha cells, to prolong the growth period, and was killed on day 11. In each case, there was a latent period, in which no activity could be detected, followed by a rapid rise in activity at the end of the study.

Sepharose 4B Fractionation of Ascites Fluid, Serum, and GPF-I. In order to compare the molecular size of the-*Vicia*-inhibiting material, which appeared in Ha ascites fluid and serum, with that of GPF-I, which had been trypsin-cleaved from Ha cells, pooled samples of ascites fluid and serum and a sample of GPF-I were prepared and fractionated sequentially on Sepharose 4B. Fig. 5A shows the elution profile for the GPF-I. The peak of activity emerged at 60–90 ml. Fig. *5B* and *5C* show the elution curves for the Ha ascites fluid and serum, respectively. The curve of the *Vicia*-inhibiting activity in each case showed a peak at 45–57 ml (clearly ahead of GPF-I) with a descending shoulder at 60–78 ml in the case of the serum, and in each case, the peak of *Vicia* activity was eluted well ahead of the bulk of the noninhibiting proteins.

Carbohydrate Composition of Ha Ascites Inhibitory Material. Since the Ha ascites fluid contained a large amount of the *Vicia*-inhibiting activity and since this activity was eluted well ahead of the detectable protein, it was reasoned that the material in this region was composed predominantly of the inhibitory molecules. A pool of Ha ascites was fractionated through columns of BioGel P-4 and then Sepharose 4B, and the inhibitory material was lyophilized and analyzed by gas-liquid chromatography, as described above. Due to the small quantity of material, an accurate weight of the sample was not obtained. Table 1 compares the relative proportions of the carbohydrate components of this fraction with those of GPF-I (6). Assigning *N*-acetylglucosamine the value of 1.0, it can be seen that the relative values for the other sugars are very similar for the ascites material and the GPF-I.

TABLE 1. *Relative molar proportions of the carbohydrate components of the Vicia inhibition material from the ascites fluid of TA3-Ha-bearing mice and of GPF-I*

Carbohydrate component	Relative moles (GNAc = 1.0)* Ascites fluid fraction	Glycoprotein fraction 1†
Galactose	4.3	3.8
Mannose	0.1	0.1
N-Acetylgalactosamine	2.4	2.1
N-Acetylglucosamine	1.0	1.0
N-Acetylneuraminic acid	0.8	0.9

* All values were determined by gas-liquid chromatography. *GNAc*, *N*-acetylglucosamine.

† Data from ref. 6.

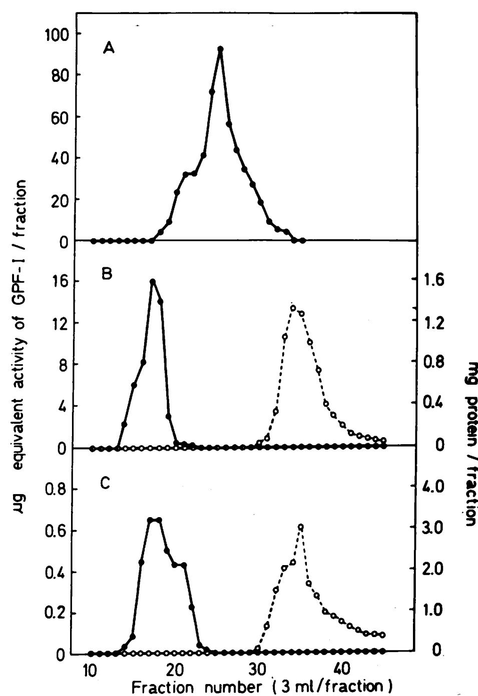

FIG. 5. Sepharose 4B column (1.5 × 85 cm) fractionation of GPF-I (*A*), pooled Ha-bearing mouse ascites fluid (*B*), and pooled Ha-bearing serum (*C*). Three-milliliter fractions were taken, and aliquots were used for *Vicia*-inhibiting activity (*closed circles*) and for total protein (*open circles*). The ascites and serum activities emerged well ahead of the trypsin-derived GPF-I activity and also well ahead of the bulk of the other proteins present.

DISCUSSION

In this study we have found that molecules with the properties of the trypsin-cleaved fragments (GPF-I) of a unique surface-membrane glycoprotein of TA_3-Ha ascites cells (6) appear in the ascites fluid and serum of syngeneic mice bearing this tumor. Our detection method was based on the recent demonstration (15) that GPF-I strongly inhibits the agglutination of human erythrocytes of NN or MN specificities by the lectin of *Vicia graminea*. The receptor for this lectin is believed to require the sequence, Gal → GalNAc → Serine (or threonine) (19, 20), and we have evidence (Codington, J. F. and Jeanloz, R. W., in preparation) that this carbohydrate chain is present in GPF-I.

The evidence that we are measuring a GPF-I-like substance in the ascites fluid and serum of Ha-bearing mice is as follows: (*1*) The sera and ascites fluids of St-bearing mice were essentially negative for the *Vicia*-inhibiting activity. It has been shown (7) that trypsin cleavage of the St subline does not yield a detectable amount of GPF-I, and we have shown (Codington, J. F., Cooper, A. G., Brown, M. C., and Jeanloz, R. W., in preparation) by absorption of the *Vicia* lectin with serial dilutions of Ha and St cells that there are over 100 times more *Vicia* receptors on the Ha than the St cell. Furthermore, this *Vicia*-inhibiting activity is removed from the Ha cells by trypsin in parallel with GPF-I removal. (*2*) When serial dilutions of the serum or ascites fluid were measured, the inhibition curves were superimposable on that of the standard GPF-I. (*3*) In several instances, we obtained serial serum samples from Ha-bearing mice by bleeding from the tail and showed that the *Vicia*-inhibiting activity was initially absent and rose toward the end of the *in vivo* Ha cell

growing period. (*4*) The *Vicia*-inhibiting material in the serum and ascites fluid was, like GPF-I, composed of nondialyzable, PCA-soluble, macromolecules. (*5*) Lastly, carbohydrate analysis of the Sepharose 4B peak of the ascites fluid *Vicia*-inhibiting activity showed the same carbohydrate components in very similar relative composition to that of purified GPF-I.

The most likely explanation for our findings is that the Ha tumor cells are releasing *in vivo* the surface-membrane glycoprotein that includes the GPF-I portion. The release of some components of the plasma membrane has been well documented for cells *in vitro*, and is believed to be a mechanism of metabolic turnover of membrane constituents (21-25). The amount of GPF-I-like activity found in the ascites fluid represents a large percentage of the total *Vicia*-absorbing activity on the surface of the Ha cells present in the fluid. Since very few dead tumor cells are found, it is unlikely that this activity was released from dead or dying cells. Furthermore, in unpublished studies, we showed that dead Ha cells incubated *in vitro* did not release GPF-I activity into the medium.

An important consideration is the structural relationship of the released glycoprotein molecules to the trypsin-cleaved fragments, GPF-I, on the one hand, and to the intact plasma-membrane molecules, on the other hand. In view of the earlier elution from Sepharose 4B of the *in vivo* released molecules compared with the trypsin-released molecules, it would appear, unless aggregation occurs, that the former are of significantly larger molecular weight (26), since the substances in the body fluids are probably of similar rod-like conformation. It is likely that the enzymatically released GPF-I fragments have been cleaved from the external end of the molecule, and that the *in vivo* released molecules include the GPF-I portion and an additional more proximal segment. Recent reports by Marchesi and coworkers (27, 28) suggest that such a situation obtains in the case of the major glycoprotein of the erythrocyte membrane. More complete characterization of the glycoprotein isolated from the ascites fluid should help to resolve this question for the TA_3Ha cell.

It is not known whether the *in vivo*-released glycoprotein molecules have any biologic significance. One possibility would be that of a blocking role (11-14). Ha, but not St, tumor cells can survive in an allogeneic host even though, in each case, the host responds with lymphocytes capable of destroying the tumor cells (8). The Ha, but not the St cells, have been shown here to release surface-membrane glycoproteins into the ascites fluid and serum and these molecules could exert an inhibitory or blocking effect on the immune cells. Circulating blocking factors are thought to consist of either free membrane-derived antigen or antigen complexed with antibody (11-14). Because of the ability to characterize the released glycoprotein molecules and to measure their levels in minute amounts of biologic fluids, the TA_3-Ha cell should provide a good model for understanding the mechanism of release and the biologic significance of circulating tumor-membrane molecules. Lastly, it is possible that the methodology used here, with the advantages of being quantitative, rapid, automated, and nonradioactive, may find application in other areas of tumor antigen detection.

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