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Production of Monoclonal Antibodies Against a Cell Surface Concanavalin A Binding Glycoprotein

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Concanavalin A-binding (Con A)-binding cell surface glycoproteins were isolated, via Con A-affinity chromatography, from Triton X-100-solubilized Chinese hamster ovary (CHO) cell plasma membranes. The Con A binding glycoproteins isolated in this manner displayed a significantly different profile on sodium dodecyl sulfate–polyacrylamide gels than did the Triton-soluble surface components, which were not retarded by the Con A-Sepharose column. [^{125}I]-Con A overlays of the pooled column fractions displayed on sodium dodecyl sulfate–polyacrylamide gel electro-phoresis (SDS-PAGE) demonstrated that there were virtually no Con A receptors associated with the unretarded peak released by the Con A-Sepharose column, whereas the material which was bound and specifically eluted from the Con A-Sepharose column with the sugar hapten α-methyl-D-mannopyranoside contained at least 15 prominent bands which bound [^{125}I]-Con A.

In order to produce monoclonal antibodies against various cell surface Con A receptors, Balb/c mice were immunized with the pooled Con A receptor fraction. Following immunization spleens were excised from the animals and single spleen cell suspensions were fused with mouse myeloma P3/X63-Ag8 cells. Numerous hybridoma clones were subsequently picked on the basis of their ability to secrete antibody which could bind to both live and glutaraldehyde-fixed CHO cells as well as to the Triton-soluble fraction isolated from the CHO plasma membrane fraction. Antibody from two of these clones was able to precipitate a single [^{125}I]-labeled CHO surface component of ∿265,000 daltons.

Key words: **plasma membrane, lectin receptors, affinity chromatography, membrane proteins, hybridoma, monoclonal antibody**

A great deal of work over the last decade has dealt with the structure and function of the plasma membrane. Many investigators have used lectins as probes to study cell surface architecture [1–3]. While much useful information concerning membrane structure

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and function has been obtained with use of lectins, a problem remains in that although the binding of lectins to cell surface components occurs through specific carbohydrate-lectin interactions, a given lectin may bind to a variety of different molecules at the cell surface, all of which possess the appropriate carbohydrate determinant. This problem in specificity is particularly vexing if one is interested in studying a single molecule on the cell surface as opposed to analyzing the characteristics of a class of cell surface components. This problem can be circumvented by using a more specific probe to investigate cell surface structure.

The probe most frequently used to answer questions regarding individual surface components is an antibody prepared against a specific membrane component. Unfortunately, owing in part to the extreme complexity of membrane structure [4] relatively few membrane proteins have been purified to homogeneity and therefore the production of monoclonal antibodies against surface proteins has been achieved in only a very few instances. Fortunately, the pioneering work of Kohler and Milstein [5,6] has recently provided a technology which offers the potential of preparing monoclonal antibody against a variety of membrane proteins and glycoproteins. In Kohler and Milstein's approach, monoclonal antibody production is achieved by cloning cell hybrids produced between membrane protein activated spleen cells and a myeloma cell line maintained in vitro. This technology has an extreme advantage over more traditional approaches to antibody production in that a single membrane protein does not have to be isolated and purified to homogeneity in order to produce a homogeneous antibody. Furthermore once a hybridoma clone producing a desired antibody has been established, the actively secreting clone can serve as a source of large amounts of the particular antibody. In this manuscript we report the application of the hybridoma technology of Kohler and Milstein [5,6] to the production of monoclonal antibodies against a specific Con A receptor localized to the cell surface of Chinese hamster ovary (CHO) cells.

MATERIALS AND METHODS

Cell Lines

The H-7_W subclone of Chinese hamster ovary cells was maintained in McCoy's 5A medium + 10% (v/v) fetal calf serum as previously described [7].

The myeloma cell line P3/X63-Ag8 [8] was maintained in Dulbecco's modified Eagle's medium containing 7.5% (v/v) heat-inactivated fetal calf serum, 7.5% (v/v) gamma-globulin-free horse serum, and 1% penicillin-streptomycin (DME-P). The cells were routinely passaged 1:20 upon reaching a cell density of 5×10^6 to 1×10^7 per milliliter.

Hybridomas derived from the fusion of spleen cells and myeloma cells were maintained in medium consisting of 7 parts DEM-P and 3 parts DME-P conditioned by the myeloma cells (designated DME-PC). The hybridoma nomenclature used throughout this manuscript follows the nomenclature recently described by Springer et al [9] except that the hybridomas derived from mice immunized with the Con A receptors (peak B, Fig 2) carry the prefix BI.

Whole-Cell Labeling Procedures

H-7_W cells were removed from the substratum using CMF-PBS-EDTA containing 1 gm/liter glucose as previously described [7]. Following removal from the substratum, the cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended in PBS to a final density of 2×10^7 cells per milliliter. Cell surface sialoglyco-

proteins were labeled using the $NaIO_4/NaB^3H_4$ technique described by Gahmberg and Anderson [10], while membrane proteins containing accessible tyrosine residues were labeled with [^{125}I] using the lactoperoxidase technique described by Phillips and Morrison [11]. Immediately after labeling with either reagent the cells were washed extensively with PBS.

Plasma Membrane Isolation and Solubilization

Subcellular fractions enriched in plasma membrane were isolated according to the aqueous two-phase polymer techniques of Brunette and Till [12]. Following isolation the membrane fraction was resuspended in 1 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and incubated for 10 min at room temperature in order to remove excess Zn^{2+}. The membrane fraction was then pelleted at 11,000g for 10 min in a Sorvall HB-4 rotor. The membranes were then resuspended (at a concentration of 1–2 mg protein per milliliter buffer) in 50 mM sodium borate (pH 7.4) containing 0.5% Triton X-100 and were incubated for 30 min at room temperature. Finally the insoluble material was pelleted at 11,000g (10 min) in a Sorvall HB-4 rotor and the Triton-soluble (T_S) fraction was collected.

Electron Microscopy

Membranes were prepared for electron microscopy as previously described [7].

Con A Affinity Chromatography

Sepharose 4B (Pharmacia, Piscataway, New Jersey) was activated with cyanogen bromide according to the procedure of Cuatrecasas [13]. A 30-ml portion of a solution containing 100 mg affinity-purified Con A, 1 mM α-methyl-D-mannopyranoside (α-MM), 1 M NaCl, and 0.13 M $NaHCO_3$ was added to 30 ml activated Sepharose 4B and gently agitated over night at 4°C. Following the overnight incubation, 0.5 ml ethanolamine was added to the reaction mixture and the agitation was continued for 2 h at 4°C. The Con A-Sepharose resin was then washed sequentially with 1 liter of each of the following: 0.1 M $NaHCO_3$; 1 M NaCl; Tris-Triton buffer (0.1 M NaCl, 0.1% Triton X-100, 0.7 mM $CaCl_2$, 0.01% NaN_3, 10 mM Tris-HCl, pH 7.4 [14]); Tris-Triton buffer containing 100 mM α-MM; and finally Tris-Triton buffer. This procedure produced a Con A-to-Sepharose coupling efficiency of at least 90%.

Following coupling the Con A-Sepharose was poured into a column and washed over night with 50 mM sodium acetate containing 4.5 M urea, pH 6.5 [15]. The column was subsequently re-equilibrated in Tris-Triton buffer prior to application of the sample. The radiolabeled T_S fraction derived from the isolated plasma membrane was allowed to percolate onto the column matrix and then the column flow was stopped. The sample was incubated with the Con A-Sepharose for 1–3 h prior to resumption of flow. After the nonretarded fractions were eluted from the affinity matrix, Tris-Triton buffer containing 100 mM α-MM was added and the Con A-receptor glycoproteins were eluted. The hapten-released material was pooled and dialyzed against Tris-Triton buffer at 4°C.

SDS Polyacrylamide Gel Electrophoresis

Membrane proteins and glycoproteins were separated on slab gels according to the discontinuous technique of Laemmli [16]. The stacking gel consisted of 5.6% acrylamide (w/w), while the separating gel was a linear gradient of 7.5–12.5% acrylamide. Following electrophoresis the gels were fixed in trichloroacetic (TCA) acid, stained with Coomassie blue, and destained as previously described [7].

[^{125}I]-Con A Overlays

Con A was iodinated by the lactoperoxidase/[^{125}I] technique of Martinozzi and Moscona [17] and subsequently affinity-purified on a Sephadex G-100 column [18]. [^{125}I]-Con A overlays of the slab gels were performed according to the method of Burridge [19]. After extensive washing of the overlaid gels to remove unbound [^{125}I]-Con A, the gels were dried and an autoradiogram was produced.

Immunization and Cell Fusion

T_S membrane proteins (peak A or peak B, Fig. 2) were concentrated (10–20-fold) using polyethylene glycol (mol wt 6,000) and mixed with an equal volume of complete Freund's adjuvant. Balb/c mice were initially immunized with 20 μg of protein via subcutaneous and intramuscular injections. The mice were boosted at 7 and 14 days with a single injection of 20 μg of the concentrated T_S membrane proteins (minus the Freund's adjuvant) via intraperitoneal and intravenous injections, respectively. Three days after the last injection the spleens were excised aseptically and a spleen cell suspension was prepared as described by Simrell and Klein [20]. The spleen cells were then fused with the mouse myeloma P3/X63-Ag8 cells according to procedures previously described [21,22]. The hybrids were initially seeded (in 0.1-ml aliquots) into 96 well microtiter plates in hypoxanthine/aminopterin/thymidine (HAT) selective medium. The hybridoma cultures were maintained in HAT medium for ∿3 weeks and then gradually transferred to DME-P.

Poly-L-lysine Attachment of Cells to Microtiter Plates and Radioimmunoassay

Hybridoma colonies were screened for the production of antibodies specific for the CHO cell surface by assay of the capacity of components from the medium in which the hybridomas were growing to bind to glutaraldehyde-fixed H-7_W cells using the radioimmunoassay (RIA) described by Simrell and Klein [20]. Briefly, 25 μl of poly-L-lysine (PLL) in PBS was added to each well of a U-bottom flex vinyl microtiter plate and incubated for 45 min at room temperature (RT). The PLL was then aspirated from the wells and the plates were washed twice with PBS. A CHO cell suspension (50 μl) containing 3×10^6 cells per milliliter PBS was added to each well and the cells were allowed to attach to the PLL-coated microtiter plates for 60 min (RT). The attached cells were subsequently washed three times with PBS. Then 25 μl of PBS containing 10% gamma-globulin-free horse serum and 0.1% NaN_3 was added to each well and the incubation was continued for 10 min. Each well was then washed twice with PBS and the cells were fixed with 25 μl of 0.1% glutaraldehyde in PBS (5 min, RT). Following fixation the cells were washed three more times with PBS, and 25 μl of 100 mM glycine in PBS was added to each well (5 min, RT). Finally the cells were washed twice with PBS and stored (4°C) in 25 μl of PBS containing 10% gamma-globulin-free horse serum and 0.1% NaN_3. Live (ie, nonfixed) cells were prepared for the RIA as above except both the glutaraldehyde fixation and the subsequent wash with glycine were omitted. In our "live cell" RIA the cells were maintained at 0°C throughout the binding assay. The ability of hybridoma supernates to detect soluble antigen was determined by using as the target membrane proteins or glycoproteins (peak A or B, Fig. 2) dried (37°C) over night onto individual microtiter wells and then fixed with absolute methanol (5 min at RT) prior to use in the RIA.

In order to quantitate antibody production, 25 μl of the test solution (hybridoma supernatant, whole serum, ascites fluid, etc) was added to the attached cells and allowed to incubate for 90 min at either room temperature (for fixed cells) or 0°C (for "live" cells). Following incubation with the test solution, the cells were washed three times with

PBS containing 1% gamma-globulin-free horse serum and 0.1% NaN_3. Then 25 μl of affinity-purified [23] [^{125}I]-labeled [24] rabbit anti-mouse IgG serum was added to each well (40,000 cpm/well) and the incubation was continued for 90 min at either room temperature or 0°C. Subsequently the cells were washed three more times with PBS containing 1% gamma-globulin-free horse serum and 0.1% NaN_3. Finally the individual wells were cut out of the microtiter plate and counted in a gamma counter.

Cloning

Hybridoma colonies were cloned in soft agar [25] using a stock solution of 2% (w/v) agar without a cellular feeder layer. The hard agar underlayer was composed of Dulbecco modified Eagle's medium containing 25% DME-PC, 7% heat-inactivated fetal calf serum, 7% gamma-globulin-free horse serum, and 1% penicillin-streptomycin. The soft agar overlay in which the cells were suspended was composed of 0.33% (w/v) agar containing the same nutrients as the hard agar.

Immunoprecipitation

Immunoprecipitations of [^{125}I]-labeled, $T_S$membrane components were performed with whole antisera and hybridoma-secreted monoclonal antibody.

In our immunoprecipitation protocol four 150-μl aliquots of inactivated Staphylococcus aureus (Pansorbin, Calbiochem, La Jolla, California) were centrifuged for 3 min in 1.5-ml Eppendorf cups in an Eppendorf microfuge. Aliquots (475 μl) of [^{125}I]-labeled T_S membrane proteins ($\sim$2.2 $\times$ 10^6aliquot) were added to each of the Pansorbin pellets and mixed well. After incubation at 4°C for 30 min the material was pelleted and the supernates were placed into new Eppendorf cups. This step "cleared" $\sim$1% of the T_S counts, suggesting that a minor component(s) of the $T_S$membrane fraction bound to the Pansorbin. A ten-fold concentrated DME-PC solution (100 μl) was then added to the Pansorbin-cleared, [^{125}I]-labeled membrane components and incubated 6 h at 4°C. Then 10 μl rabbit anti-mouse IgG serum (IgG fraction, Miles Yeda, 1.6 mg antibody per milliliter) or another appropriate antiserum was added, and the samples were mixed well and incubated over night at 4°C. The next day the samples were transferred to Eppendorf cups containing the pellet from 150 μl of Pansorbin and were incubated for another 1.5 h (4°C). Following this incubation the material was again pelleted in a microfuge and the supernates were collected. Finally 100 μl of the antibody to be used in the immunoprecipitation was added to the Triton-soluble [^{125}I]-labeled membrane proteins and incubated 6 h (4°C). After 6 h, 10 μl of rabbit anti-mouse IgG (or another appropriate second antibody) was added to the solution and the samples were again incubated over night at 4°C. The following day the supernates were added to a pellet from 150 μl Pansorbin and incubated 1.5 h at 4°C, and the immunotitrated material that adsorbed to the Pansorbin was pelleted. The supernates were removed, the pellets were washed twice with 500 μl Tris-Triton buffer containing 5 mg/ml ovalbumin, and then the adsorbed material was removed from the Pansorbin by boiling for 5 min in Laemmli sample buffer [16].

RESULTS

A plasma membrane-enriched fraction was isolated from the H-7_Wsubclone of CHO cells according to a modification [7] of the two phase aqueous polymer membrane isolation technique of Brunette and Till [12]. This membrane fraction has been extensively character-

ized in a previous publication [7], where it was shown to be highly enriched in plasma membrane. An electron micrograph of the isolated material is displayed in Figure 1.

In order to solubilize the majority of the membrane fraction, the membranes were incubated in 1 mM Tris-HCl (pH 7.4) containing 10 mM EDTA (to remove excess Zn^{2+}), pelleted, and then resuspended for 30 min at RT in 50 mM sodium borate (pH 7.4) containing 0.5% Triton X-100. Finally the insoluble material was pelleted at 12,000g for 15 min and both the Triton-soluble (T_s) and Triton-insoluble (T_i) fractions were collected. Using this solubilization protocol ∿ 80% of the membrane fraction is solubilized. Figure 3A displays a Coomasie blue-stained SDS-PAGE in which it can be seen that the plasma membrane-enriched fraction as well as the T_s and T_i fractions are easily distinguishable from each other.

The T_s fraction was concentrated approximately ten-fold and then applied to a urea-stripped Con A-Sepharose affinity column (see Materials and Methods). Figure 2 presents the elution profile from the affinity column. Three peaks can be collected from the column. Peak A is the nonretarded fraction which is eluted in the Tris-Triton buffer (see Materials and Methods) without α-MM. Peak B is eluted with Tris-Triton buffer containing 100 mM α-MM, while peak C can only be eluted with Tris-Triton buffer containing 4.5 M urea. Peak C contains both non-Con A-binding proteins and Con A receptors as well as a large amount of Con A (data not shown). Peak A accounts for ∿65% of the radioactivity applied to the column, peak B accounts for ∿25% of the applied material, and peak C accounts for another 5% of the applied material. Approximately 5% of the T_s fraction remains bound to the column even after the 4.5 M urea wash.

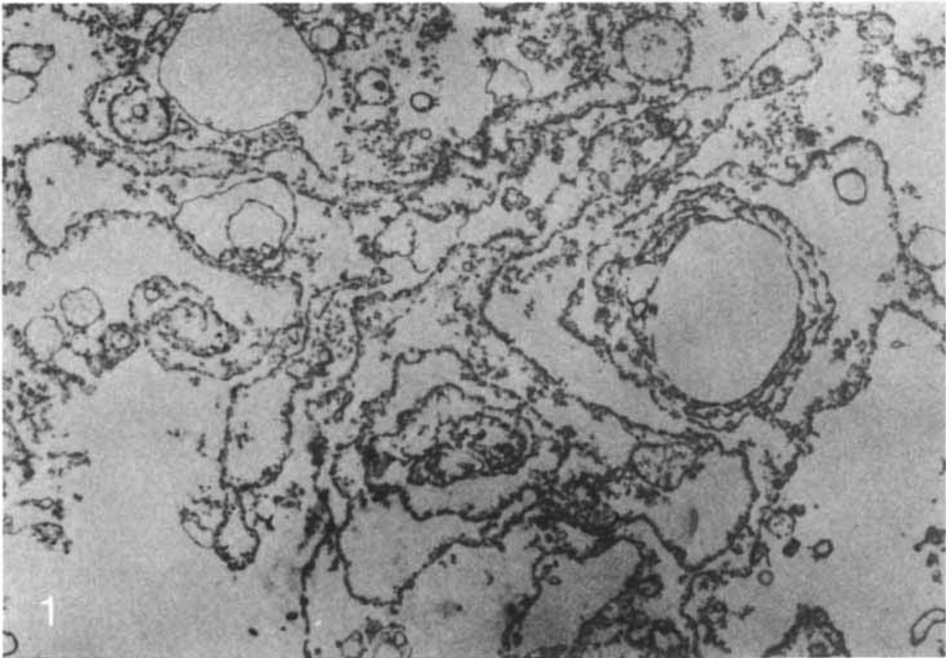

Fig. 1. Electron micrograph of H-7_w membrane fraction (× 5,000). The "particles" seen attached to the membranes do not have the dimensions of ribosomes. The "particles" probably represent aggregates of actin associated with the inner face of the surface membrane [26].

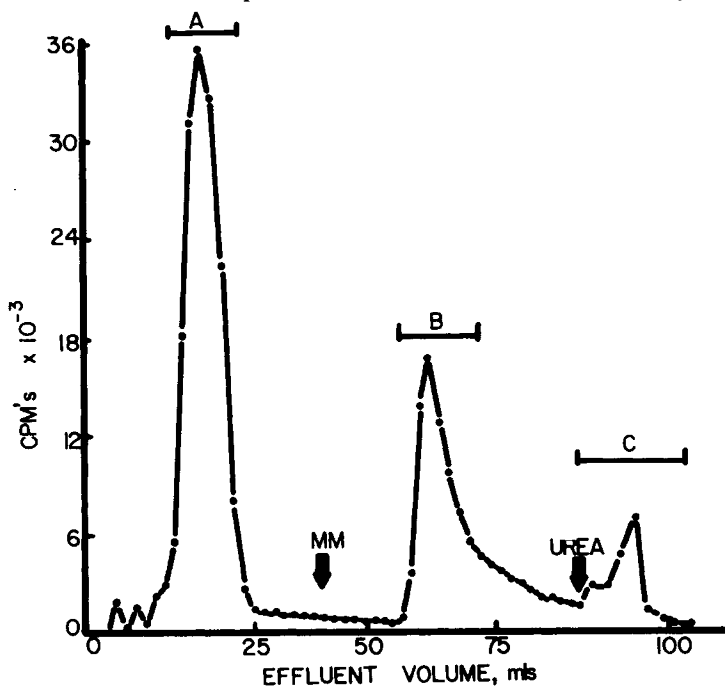

Fig. 2. Elution profile of Con A-Sepharose Column. The column was prepared and run as described in Materials and Methods. In this experiment 2 × 10^7 H-7_W cells were labeled with [^{125}I]/lactoperoxidase [26] prior to membrane isolation and then the radiolabeled membrane was used as a "tracer" with membrane isolated from 1.6 × 10^8 H-7_W cells. In the particular experiment shown, 5.4 mg of protein was applied to the column and 1.8-ml fractions were collected. Peak A represents the nonretarded membrane components, peak B the Con A-binding membrane components, and peak C the nonspecifically adsorbed membrane components which could be eluted with 4.5 M urea.

Figure 3A contains the Coomasie blue staining profile on an SDS-PAGE of the material recovered from the affinity column. As can be seen, peaks A and B are clearly distinguishable from each other as well as from the T_S fraction and the isolated membrane. The profile presented in Figure 3Ae resolves approximately 15 major Con A receptors. Two-dimensional analysis of this fraction according to the technique of O'Farrell [27] resolves peak B into at least 50 distinct spots, all capable of binding [^{125}I]-Con A (data not shown). Figure 3B is an [^{125}I]-Con A overlay [19] of the SDS-PAGE displayed in Figure 3A. Figure 3B demonstrates that the majority of the Con A receptors present in the membrane-enriched fraction are soluble in Triton (lane b) and that virtually all of them can be recovered in peak B of the Con A affinity column (lane e, Fig. 3B).

Following isolation, the Con A-binding glycoproteins (peak B, Fig. 2) were concentrated and used as a complex antigen for the production of antiserum and hybridomas against the Con A-binding glycoproteins.

Production of Antiserum

Balb/c mice were injected with concentrated peak B material as described in Materials and Methods. Following the completion of the immunization protocol the animals' sera were collected and the spleens excised for subsequent fusion with the myeloma cells.

The serum derived from clotted whole blood was tested for its ability to bind to glutaral-

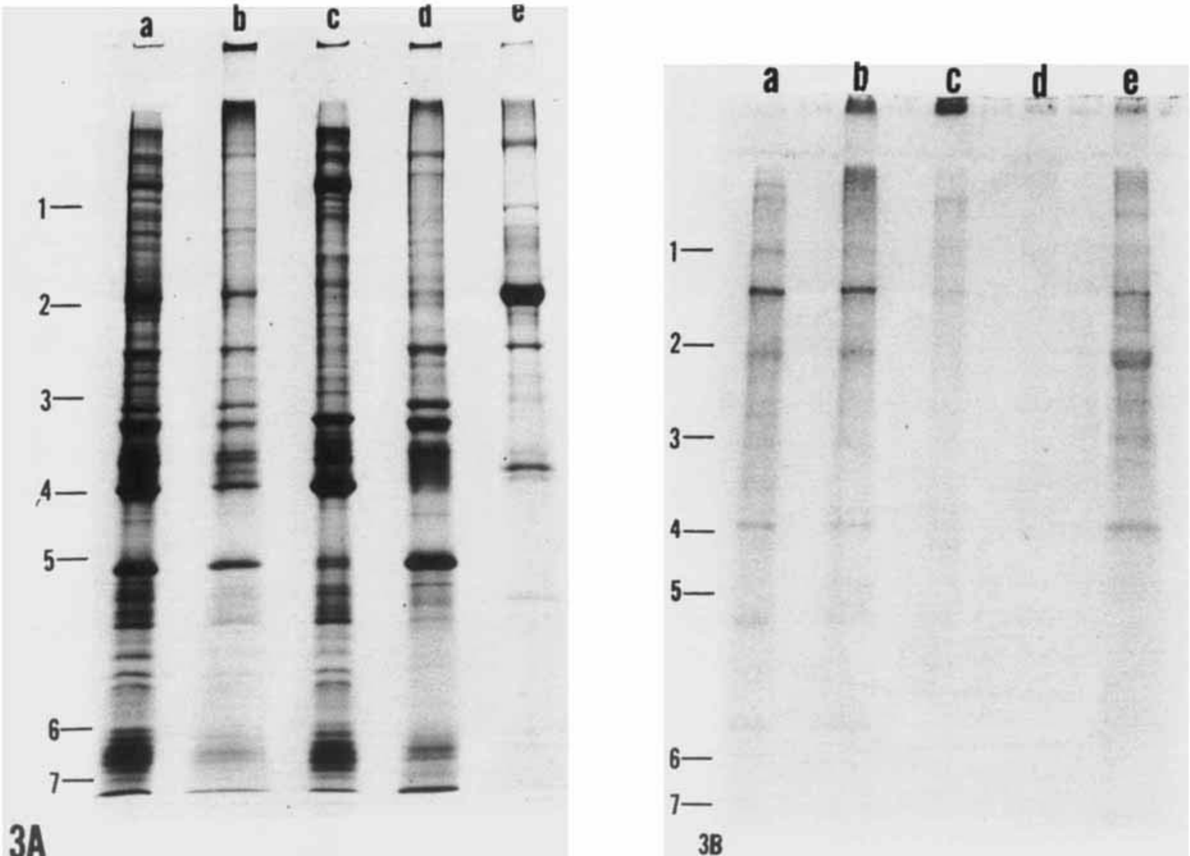

Fig. 3. A: Coomasie blue staining profile of subfractions: a) plasma membrane-enriched fraction; b) T_s fraction from plasma membrane; c) T_i fraction from plasma membrane; d) nonretarded peak off Con A-Sepharose column (peak A); e) α-MM-eluted peak off Con A-Sepharose column (peak B). The components were separated on a discontinuous 7.5–12.5% SDS-PAGE with a 5.6% stacking gel; 50 μg protein was added to each track. Molecular weight markers: 1) myosin, 200,000; 2) phosphorylase A, 100,000; 3) bovine serum albumin, 69,000; 4) ovalbumin, 43,000; 5) DNase I, 35,000; 6) soybean trypsin inhibitor, 23,000; 7) cytochrome c, 13,500. B: [^{125}I]-Con A overlay of Coomasie blue-stained gel presented in A. Overlay was performed as described by Burridge [19]. Molecular weight markers are the same as in A.

Table I. Antiserum Binding to CHO cells

Antiserum	cpm [^{125}I]-anti-IgG bound to glutaraldehyde-fixed cells
Preimmune	1,500
Immune serum (peak B)	12,000
Immune serum (10 × Tris-Triton)	1,700
Immune serum (nuclear envelope)	1,600

Preimmune serum was drawn from a nonimmunized mouse. Antisera against peak B (Fig. 2) and Tris-Triton were prepared as described in Materials and Methods. Immune serum was also prepared against a Triton-solubilized fraction of CHO cell nuclear envelopes [28].

dehyde-fixed CHO cells and the soluble antigens in peaks A and B (Fig. 2) as described in Materials and Methods. Table I demonstrates that antiserum derived from peak B-injected animals binds to glutaraldehyde-fixed CHO cells significantly more efficiently than do pre-immune sera, immune sera produced against ten-fold concentrated Tris-Triton (the carrier of peak B), or antisera produced against a T_S fraction derived from the nuclear envelope of CHO cells [28]. Furthermore, antisera derived from animals injected with peak B material preferentially recognize peak B material relative to peak A in the soluble antigen assay described in Materials and Methods (Fig. 4). Conversely antisera derived from animals injected with concentrated peak A material preferentially recognize soluble peak A antigen (Fig. 4). Immunoprecipitation of iodinated T_S surface components with antisera to peak A or peak B results in the recovery of different radio-labeled components in the precipitate. These components can be matched to specific peak A or peak B components displayed in Figure 3 (data not shown).

Production of Hybridomas

The spleen cell suspension derived from the peak B-immunized Balb/c mice was fused with mouse myeloma cells P3/X63-Ag8 as previously described [21,22] and distributed into microtiter test plate wells. Approximately 2 weeks later the supernates from those wells that showed good growth were tested for the presence of antibody which could cross-react with glutaraldehyde-fixed CHO cells. In the particular fusion being described in this manuscript only 9 of 101 growing hybridoma cultures produced an antibody capable of cross-reacting with glutaraldehyde-fixed CHO cells (Fig. 5). The average fusion we now do in the laboratory routinely results in a fusion in which ∼30% of the growing colonies secrete antibody which cross-reacts with glutaraldehyde-fixed CHO cells (data not shown).

Figure 6 displays a titration of the antibody secreted by one of the hybridoma colonies (BI41) against soluble peak A and peak B antigen. As can be seen, the antibody secreted by this colony shows virtually no binding to peak A even at a 1:1 dilution, while the midpoint in the titration curve against soluble peak B occurs between a 1:64 and a 1:128 dilution. A similar titration curve has been derived for the other eight colonies which were scored as positive in their ability to bind to glutaraldehyde-fixed CHO cells.

Selection and Characterization of Clones

Colonies BI41 and BI69 proved to be most efficient in producing clones in soft agar, and therefore the data to be discussed in this manuscript will be limited to the results obtained with these two clones. As can be seen in Table II, colony BI41 produced

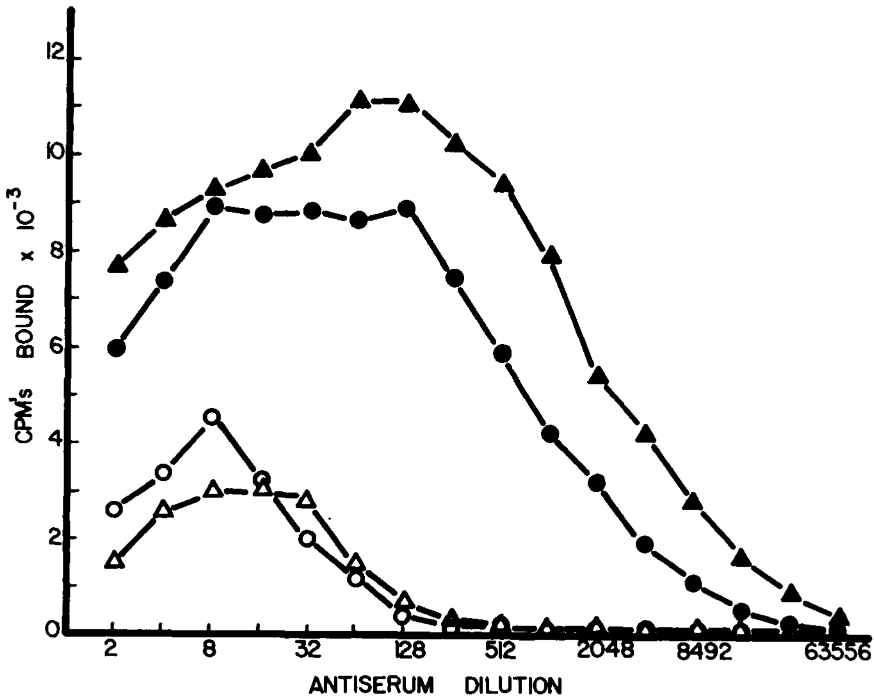

Fig. 4. Antiserum binding to soluble peaks A and B. Peaks A and B were isolated as described in Materials and Methods and Figure 2. The soluble proteins and glycoproteins from each fraction were dried down and fixed to microtiter wells as described in Materials and Methods. Finally an RIA on the soluble antigens was performed as described in Materials and Methods. ▲, peak B antiserum binding to peak B; △, peak B antiserum binding to peak A; ●, peak A antiserum binding to peak A; ○, peak A antiserum binding to peak B.

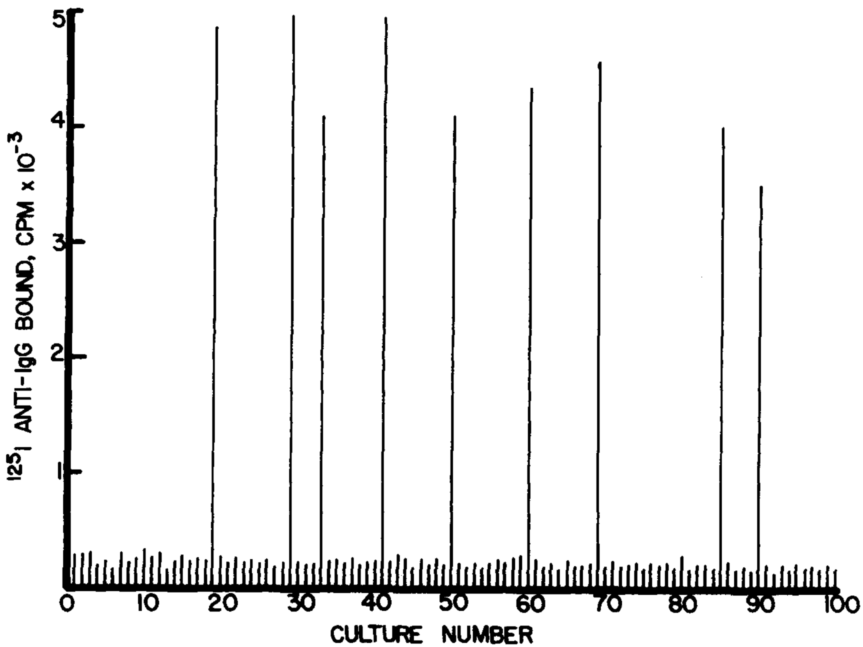

Fig. 5. Hybridoma supernate binding to glutaraldehyde-fixed CHO cells. Culture fluid (25 μl) was aseptically removed from the growing hybridoma cultures approximately 2 weeks after the initial fusion and tested for its ability to bind to glutaraldehyde-fixed CHO cells in the RIA described in detail in Materials and Methods.

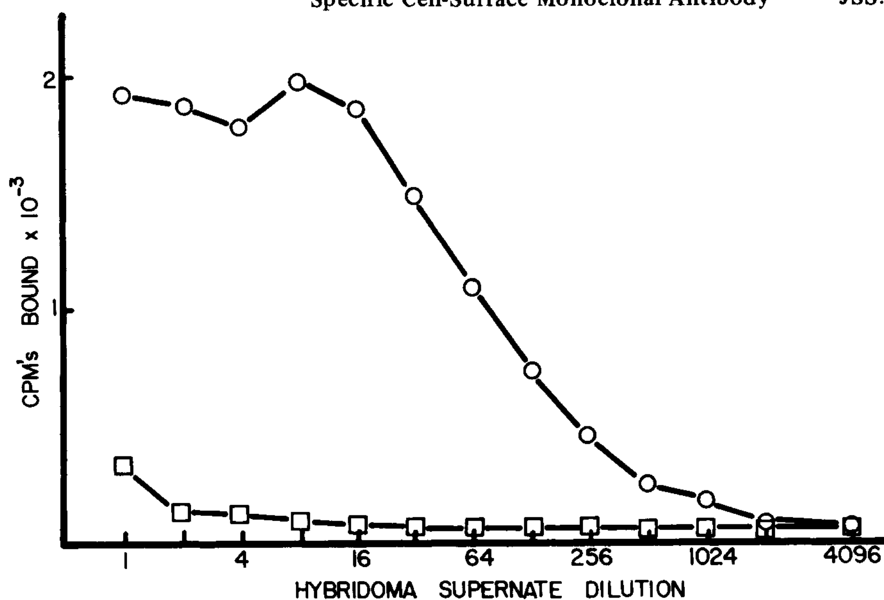

Fig. 6 Hybridoma supernate binding to peaks A and B. Peaks A and B were isolated as described in Materials and Methods and Figure 2. The soluble proteins and glycoproteins were dried down and fixed to microtiter wells as described in Materials and Methods. Finally an RIA on the soluble antigens was performed as described in Materials and Methods. ○, Supernate binding to peak B; □, supernate binding to peak A.

TABLE II. Binding of Clonal Supernates

	Glutaraldehyde-fixed CHO cells	$[^{125}I]$-anti-IgG bound			
		Live cells (0°C)	Peak A	Peak B	Fetuin
BI41.1	240	160	150	150	100
BI41.2	270	300	100	100	100
BI41.3.	270	250	100	100	100
BI41.4	4,500	2,500	1,700	10,600	100
BI69.1	4,000	3,000	1,800	7,800	100
BI69.2	280	300	100	100	100
BI69.3	250	200	100	100	100
BI69.4	4,600	2,910	1,600	10,120	100

The RIAs were performed as described in Materials and Methods. Among the other 21 clones selected from the BI41 colony, 18 showed positive binding to glutaraldehyde-fixed CHO cells.

23 clones that were subsequently picked and grown to high cell density in vitro. Clone BI69 produced two actively growing clones that were also picked (Table II). Both clones secreted antibodies of the gamma-globulin class 1 variety.

It is worth noting that in those cases where it has been studied the clonal supernates show an apparently higher efficiency of binding to glutaraldehyde-fixed CHO cells than to live cells maintained at 0°C (Table II). This may result from a glutaraldehyde-mediated preservation of the antigenic structures recognized by the particular antibody or to down-modulation of surface antigens on the live cells subsequent to antibody binding. It should also be

TABLE III. Antibody Binding to CHO, 3T3 (Balb/c A-31), and NIL 8 M-2 Cells

Clonal supernate	Cell line	cpm [^{125}I]-anti-IgG bound
BI41.4	CHO (H-7_w)	5,000
	NIL 8 M-2	3,790
	Balb/c A-31	147
BI69.1	CHO (H-7_w)	4,000
	NIL 8 M-2	2,800
	Balb/c A-31	125

Antibody binding to glutaraldehyde-fixed cells was determined as described in Materials and Methods.

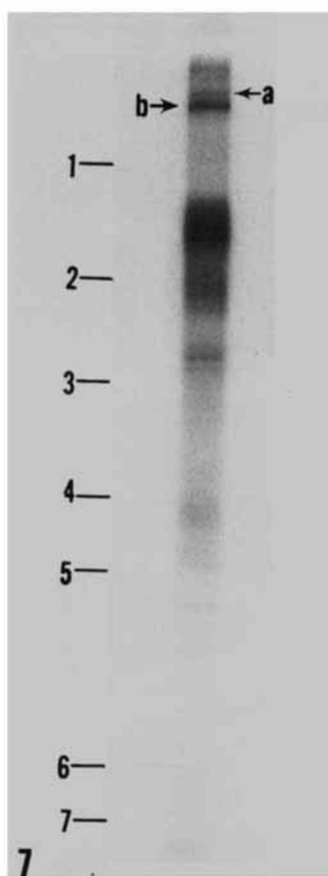

Fig. 7. Iodination of surface proteins and glycoproteins. H-7_w cells were iodinated according to the technique of Phillips and Morrison [11]. A plasma membrane-enriched fraction was then isolated [12], the membranes were solubilized in SDS, and the proteins and glycoproteins were separated on a 7.5–12.5% Laemmli discontinuous SDS-PAGE [16]. Approximately 100,000 cpm was applied to the gel. After separation the gel was dried and an autoradiograph was prepared. Molecular weight markers [1–7] as in Figure 3. Arrow (a) identifies the 265,000-dalton component, arrow (b) the 250,000-dalton component.

noted that where it has been tested the clonal supernates that have proved active in their ability to bind to glutaraldehyde-fixed CHO cells were capable of efficiently distinguishing between peak A and peak B material in the soluble antigen assay (Table II). None of the active supernates tested showed any binding to fetuin (an excellent Con A receptor, [29]), which suggests but by no means proves that the antigenic determinant recognized by the monoclonal antibody is the peptide rather than the oligosaccharide portion of the Con A-binding glycoprotein.

Table III demonstrates that the antibody secreted by BI41.4 and BI69.1 binds efficiently to CHO cells and NIL 8 M-2 cells (another hamster-S derived cell line provided to us by Dr. Richard Hynes). Interestingly the antibody secreted by these two clones does not bind to a mouse-derived permanent cell line, Balb/c A-31 (a 3T3 clone, Table III). These antibodies also show no binding to a wide variety of chicken, mouse, and human cell lines (data not shown). These data clearly suggest that the antibodies secreted by BI41.4 and BI69.1 are "hamster-specific."

Figure 7 is an autoradiograph of iodinated surface components of the H-7_W CHO cell clone. As can be seen, the pattern of surface iodination is complex [7]; however, one band does clearly stand out. This band electrophoreses with a molecular weight of $\sim$250,000 daltons and can be labeled with [^{3}H]-glucosamine or with $NaIO_4/NaB^3H_4$ as previously described [7]. Above this major band is a more minor band which displays an apparent molecular weight of $\sim$265,000. Figure 8 is an autoradiograph of an immunoprecipitate of iodinated $T_S$components (see Fig. 3A) that had been incubated with antibodies derived from clone BI41.4, as described in Materials and Methods. As can be seen, the antibodies secreted by clone BI41.4 precipitate an iodinated species which runs at $\sim$265,000 daltons. This band represents 2–3% of the T_S iodinated surface components and almost certainly corresponds to the more "minor" high-molecular-weight band identified in Figure 7 that has an apparent molecular weight of $\sim$265,000. The same iodinated species is precipitated by the supernate derived from clone BI69.4 (data not shown) as well as the other clones derived from BI41 (Table II).

DISCUSSION

The data presented in this manuscript clearly demonstrate that one can use Con A affinity chromatography to isolate cell surface Con A-binding glycoproteins from a Triton X-100-soluble fraction of a plasma membrane-enriched organelle isolate. Furthermore the data clearly demonstrate that immunization of Balb/c mice with *all* of the T_S Con A receptors followed by fusion of the spleen cells with mouse myeloma cells will produce a series of hybridoma clones which secrete antibodies capable of recognizing Con A receptors associated with the CHO cell surface as well as T_S Con A receptors. Furthermore the antibodies derived from the clonal hybridoma supernates can be used, in conjunction with S aureus protein A, to immunoprecipitate specific Con A receptors from the T_S fraction of the iodinated surface membranes.

The work described in this manuscript demonstrates that we have been able to select a series of hybridoma clones which secrete a monoclonal antibody against a surface component of $\sim$265,000 mol wt. Extensive work in our laboratory has demonstrated that this high-molecular-weight glycoprotein is not fibronectin. Among the evidence we have gathered to support this conclusion are the following points: 1) anti-fibronectin antibody does not precipitate the 265,000-dalton CHO cell surface component; 2) the monoclonal antibodies described in this manuscript precipitate a 265,000-dalton NIL cell surface component but not

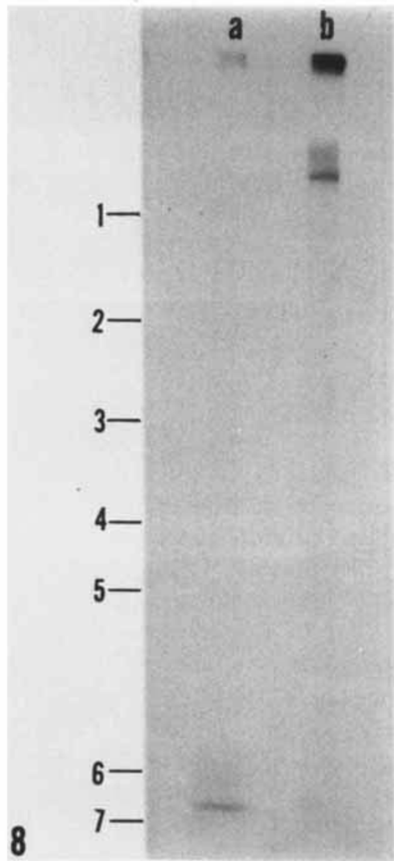

Fig. 8. Immunoprecipitation with clone BI41.4. H-7_W cells were labeled with [^{125}I], the surface membrane was isolated, and the T_S fraction prepared. The immunoprecipitation was performed as described in Materials and Methods. a: T_S fraction was taken through the immunoprecipitation protocol as outlined in Materials and Methods using supernate from myeloma cells that had not been fused with spleen cells as the source of antibody ($\sim$7,000 cpm applied). Supernate from spleen-myeloma fusions which showed no binding to the CHO cells also failed to precipitate any surface component. b: Immunoprecipitation with BI41.4 supernate ($\sim$7,000 cpm applied). None of the "clearings" which occurred prior to the addition of the antibody precipitated any components that could be detected on SDS-PAGE.

NIL cell fibronectin which can be precipitated by an antibody prepared against NIL cell fibronectin; and 3) the monoclonal antibodies do not precipitate purified NIL cell fibronectin. All of these data as well as an extensive characterization of the synthesis, turnover, and topologic localization of this 265,000-dalton CHO cell surface component are the subject of a manuscript about to be submitted for publication (Starling and Noonan, manuscript in preparation).

Finally we would like to point out that we also have derived monoclonal antibodies against other Con A-binding surface glycoproteins as well as some non-Con A-binding surface components. It is our intention to utilize these monoclonal antibodies to study the lateral mobility of individual membrane proteins and glycoproteins. We also intend to use these antibodies in efforts to isolate CHO cell variants lacking specific surface receptors.

It is our hope that such cell variants might provide us with information relating to the physiologic role of some membrane components in such biologically important phenomena as cell-cell and cell-substrate adhesion.

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REFERENCES

1. Nicolson GL: Int Rev Cytol 39:89, 1974.
2. Goldstein IJ, Hayes CE: Adv Carbohydr Chem Biochem 35:127, 1978.
3. Noonan KD: In Nicolau C (ed): "Virus-transformed Cell Membranes." London: Academic, 1978, pp 281–371.
4. Singer SJ: Ann Rev Biochem 43:805, 1974.
5. Kohler G, Milstein C: Nature 256:495, 1974.
6. Kohler G, Milstein C: Eur J Immunol 6:511, 1976.
7. Noonan KD: Biochim Biophys Acta 551:22, 1979.
8. Kohler G, Milstein C: Nature 244:42, 1973.
9. Springer T, Galfne G, Secher DS, Milstein C: Eur J Immunol 8:539, 1978.
10. Gahmberg CG, Anderson LC: J Biol 252:5888, 1977.
11. Phillips, DE, Morrison M: Biochem Biophys Res Commun 40:284, 1970.
12. Brunette DM, Till JE: J Membr Biol 5:215, 1971.
13. Cuatrecasas P: J Biol Chem 245:3059, 1977.
14. Findley JB: J Biol Chem 249:4398, 1974.
15. Jacobs S, Schechter Y, Bissel K, Cuatrecasas P: Biochem Biophys Res Commun 77:981, 1977.
16. Laemmli UK:Nature 227:680, 1970.
17. Martinozzi M, Moscona A: Exp Cell Res 94:253, 1975.
18. Agrawal BBS, Goldstein IJ: Biochem J 96:23c, 1965.
19. Burridge K: Proc Natl Acad Sci USA 73:4457, 1976.
20. Simrell CR, Klein PA: J Immunol. In press.
21. Koprowski H, Gerhard W, Croce CM: Proc Natl Acad Sci USA 74:2985, 1977.
22. Gerhard W, Croce CM, Lopes D, Koprowski H: Proc Natl Acad Sci USA 75:1510, 1978.
23. Guimezanes A, Fridman WH, Gisler RH, Korrilsky FM: Eur J Immunol 6:69, 1976.
24. Hunter R: Proc Soc Exp Biol Med 133:989, 1970.
25. Coffins P, Scharff MD: Proc Natl Acad Sci USA 68:2A, 1971.
26. Moore PB, Ownby CL, Carraway KL: Exp Cell Res 115:331, 1978.
27. O'Farrell PH: J Biol Chem 250:4007, 1975.
28. Noonan NE, Noonan KD: In preparation.
29. Sela BA, Wang JL, Edelman GM: J Biol Chem 250:7535, 1976.