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Antigenic distinctions of glycoproteins in plasma and mitochondrial membranes of lymphoid cells neoplastically transformed by simian virus 40

(membrane proteins/bidimensional immune electrophoresis/bidimensional isoelectric focusing-immune electrophoresis)

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ABSTRACT **Highly purified plasma membranes from hamster lymphocytes transformed by simian virus 40 (GD 248) were compared with the membranes of normal cells by crossed immune electrophoresis, crossed-line immune electrophoresis, and bidimensional isoelectric focusing-immune electrophoresis. Antiserum raised by inoculation of guinea pigs with GD 248 membranes was used as serologic reagent, either directly or after absorption with membranes from normal cells.**

Bidimensional immune electrophoresis reveals the presence in the plasma membranes of GD 248 cells of at least three antigens not detectable in the membranes from the normal cell population. At least two of these are also present in the mitochondrial membranes of GD 248 cells, but none could be detected in membranes of embryonic fibroblasts. Bidimensional isoelectric focusing-immune electrophoresis indicates that the distinctive antigens of the GD 248 membranes are glycoproteins.

The plasma membranes of neoplastic cells differ biochemically from those of normal cells (1, 2). Furthermore, indirect experimental approaches indicate that cultured fibroblasts, neoplastically converted by oncogenic viruses, are at least quantitatively deficient in some membrane or membrane-associated proteins (2–10), while acquiring new protein entities identified as transplantation antigens (11).

Our own study, comparing, by isoelectric focusing (12), highly purified plasma membranes (13) from normal hamster lymphocytes and from hamster lymphocytes transformed by simian virus 40 (GD 248), revealed two generalized differences: (*a*) overall depression on the isoelectric points of the glycosylated proteins in GD 248 membranes and (*b*) nonspecific elevation of the isoelectric points of nonglycosylated proteins due to increased side-chain amidation. This study also demonstrated the appearance in the membranes of transformed cells of a putative "new" protein with a pI of 4.5. Subsequent experimentation (14), utilizing bidimensional immune electrophoresis for membrane protein analysis, revealed that the membranes of transformed cells contain at least two antigenic components not detected in the membranes of the normal lymphocyte population.

We now extend these observations in two directions. (*i*) By bidimensional isoelectric focusing-immune electrophoresis we identify the antigenic distinction of the GD 248 membranes with specific, modified glycoproteins. (*ii*) We demonstrate that distinctive glycoprotein antigens in GD 248 plasma membranes occur also in the mitochondria of GD 248 cells.

MATERIALS AND METHODS

Chemicals. Unless stated otherwise, all chemicals used were of highest purity grade available. Triton X-100, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and bovine serum albumin were obtained from Sigma (St. Louis, Mo.); agarose (lot 102D) from Litex (Denmark); acrylamide, *N,N'*-methylene-bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine (Temed), ammonium persulfate, and Coomassie brilliant blue from Bio-Rad Laboratories (Richmond, Calif.), complete Freund's adjuvant from Difco Laboratories (Detroit, Mich.), and ampholytes (Ampholine pH 3.5–10.0) from LKB (Upsala, Sweden). Embryonal hamster fibroblasts were purchased from Flow Laboratories (Rockville, Md.).

Membranes. Isolation of normal and GD 248 lymphocytes from outbred golden Syrian hamsters, as well as the purification of these cells' plasma membranes and mitochondria, were as described (13). For immune electrophoresis and isoelectric focusing, the plasma membrane proteins were extracted as in ref. (12) using 1 mM Hepes, pH 8.5, 1% in Triton X-100. The mitochondria, devoid of plasma membrane material according to multiple marker criteria, were collected from the dextran step gradient described in ref. 13. They were then washed once in 65 mM NaCl, 10 mM Hepes, 2 mM $MgCl_2$, 75 mM KCl, and 2 mM EDTA, pH 7.4, collected by centrifugation at 4×10^5 *g*·min, and then freed of soluble proteins by successive hypotonic shocks in 10 and 1 mM Hepes, pH 7.5, as described for microsomal membrane vesicles (13, 15), and collected by centrifugation at 8×10^6 *g*·min. Mitochondrial membrane proteins were extracted as described for plasma membranes (14). The results presented are characteristic of at least five membrane preparations.

Antiserum against Membrane Proteins. We used antiserum prepared by injecting guinea pigs with plasma-membrane-enriched, mitochondria-free membranes from GD 248 cells (13) in complete Freund's adjuvant, using the immunization scheme described (14), but collecting antiserum 10 days after the third booster.

Absorption of Antiserum. To identify antigenic differences between the membranes of normal and GD 248 lymphocytes, we absorbed the antiserum with a mixed particle fraction from disrupted normal lymphocytes. For this, the subcellular particles of the lysed lymphocytes were collected by centrifugation at 8×10^6 *g*·min after earlier removal of nuclei by low-speed centrifugation (13). Particles (200 μg of protein) were then dispersed in 100 μl of antiserum and incubated first at 4° and then at 37° (25 min at each temperature). Up to four absorptions were used; the particles were removed at 8×10^6 *g*·min each time and replaced with fresh particles. The dilution introduced during the absorption procedure was corrected for by diluting unabsorbed serum correspondingly in comparative studies.

Instruments. Bidimensional immune electrophoresis was performed with the model 2117 Multiphor (LKB, Sweden). Isoelectric focusing in polyacrylamide was carried out with an

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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apparatus described (16), built by Medical Research Apparatus (Boston, Mass.) and a Buchler (Fort Lee, N.J.) power supply (model 3-1155) with both current and voltage regulation. Coomassie-stained gels were scanned in a Gilford 2400-S spectrophotometer. Centrifugations were in a Spinco L2 65B ultracentrifuge and an air-driven ultracentrifuge (Airfuge, Beckman Instruments).

Isoelectric Focusing. Analytical focusing was performed, following the principles described before (12), using slab gels formed with 4% acrylamide, crosslinked with 2.5% bisacrylamide, containing 2% ampholytes, 8 M urea, 1% Triton X-100, and 10% sucrose. For one 50 × 90 × 2 mm slab gel we mixed the following solutions: 1.2 ml of acrylamide/bisacrylamide (40%/2.5%), 0.6 ml of Ampholine pH 3.5–10.0 (40% wt/vol), 0.6 ml of 10 M urea 12% in sucrose and 1.2% in Triton X-100, 0.4 ml of deionized water, 0.24 ml of 2% ammonium persulfate, and 0.03 ml of 10% Temed. The mixture was agitated, degassed thoroughly, and then cast immediately, introducing three equally spaced, 4 × 10 × 1.5 mm sample wells near the cathodal ends of the gels. The gels were used 3–4 hr after casting. Prior to sample introduction into the wells, contact with the buffer compartments was established via two 1-cm wide, double-layered filter paper wicks. The one for the cathodal end was first soaked in 1 M NaOH and the one for the anodal end in 1 M H_3PO_4. The gels were then cooled to 6° in the electrophoresis apparatus and pre-electrophoresed for 10 min at 2.5 mA per slab. The sample (maximally 350 μg of protein per well) was then applied in three aliquots, introduced at 20-min intervals. Focusing was initiated at a constant current of 2.5 mA per gel until the voltage had reached 250 V. Thereafter, focusing was continued at a constant voltage of 250 V for 16 hr. During this time the current dropped below 0.5 mA. The temperature was maintained at 6° throughout.

Bidimensional Immune Electrophoresis. This was carried out as described in our earlier report (14). Because of the wide range in the staining intensities of immune precipitates, immunoplates were photographed at several exposures. The exposures presented are those which most faithfully demonstrate the immune precipitates under discussion.

Bidimensional Focusing-Immune Electrophoresis. We have showed that components C3 and C4 of guinea pig complement, which adsorb to erythrocyte surfaces, can be characterized by sequential electrofocusing and bidimensional immune eletrophoresis (17). In this experimentation, *adsorbed water-soluble serum proteins* were desorbed by saline extraction of erythrocyte membranes produced by complement lysis, concentrated, and electrofocused. More recently, Bhakdi *et al.* (18) have extended this sequential approach in their studies on the terminal complement complex on erythrocyte membranes by directly polymerizing the focusing gel into the immune electrophoresis plate. They solubilized membrane proteins and proteins adsorbed to the membrane in 1% Triton X-100 and focused in polyacrylamide containing 1% Triton X-100. However, because a satisfactory fractionation of membrane proteins can only be achieved in 1% Triton, 8 M urea (19), Bhakdi's technique cannot be generally applied to the analysis of membrane proteins.

To our knowledge, this approach has not heretofore been successful in the analysis of individual *integral* membrane proteins nor of the full protein complement of a given membrane. This is in part due to the fact that such proteins are not water-soluble. We developed a nonsequential procedure, in which isoelectric focusing replaces the standard first dimension step of conventional bidimensional immune electrophoresis, but which retains the high resolution achieved by our focusing procedure (12). Because this resolution requires the presence of 8 M urea, which can interfere with immune precipitation, special steps must be taken to avoid this interference.

In our technique we electrofocus as described above. Then we cut out the 10 × 90 mm gel strips containing the focused proteins and slice them longitudinally into two 5-mm halves. One of these is fixed and stained to localize the focused bands. We use a variation of Coomassie blue staining ref. (12) to avoid ampholyte interference. The other is washed 3 times in 20 ml of Tris/glycine (0.038 M/0.1 M), pH 8.7, 1% in Triton X-100, for 10 min each time. These washes eliminate the pH gradient, which would interfere with the uniform immobilization of antibody at the pH, 8.7, used for immune electrophoresis. The washes also remove the urea. The washing conditions were established by pilot experiments on standard antigen–antibody systems.

For immune electrophoresis we cast 80 × 80 mm immunoplates in two sections: (*a*) a cathodal 30 × 80 ×1.5 mm agarose strip without antibody and (*b*) a 50 × 80 × 1.5 mm area containing 0.275 ml of antiserum. Buffers and other conditions were as in ref. 14. A rinsed focusing strip was then placed atop the serum-free agarose with the focusing axis perpendicular to the direction of immune electrophoresis, and the nearest edge of the focusing strip 4 mm away from the interface between the two agarose domains. The 4-mm interval was found sufficient to provide uniform migration of proteins from the focusing gel prior to contact with precipitating antibody. The focused proteins were then electrophoresed out of the polyacrylamide strip, through the antibody-free zone, into the antibody—containing agarose at pH 8.7, 10 V per slab for 20 hr at 6° (14). Staining was as described (14).

Other Determinations. Protein was assayed by the ninhydrin method (12). pH gradients were determined as described (12).

RESULTS

Solubilization of Membrane Proteins. Using nonsedimentability at 8×10^6 *g*·min as a criterion for solubility, we find as before (12, 14) that more than 90% of the plasma membrane proteins of both normal and GD 248 cells can be solubilized by two extractions with 1 mM Hepes, 1% Triton X-100, pH 7.5. The identical procedure solubilized 70–80% of the mitochondrial protein of both cell types.

Absorption of Antiserum Against GD 248 Membranes. As shown before (14) and below, antisera produced by immunization with GD 248 membranes form multiple precipitation arcs upon crossed immune electrophoresis against membranes from both normal and GD 248 cells, as well as several precipitates apparently unique to the latter. We have assessed absorption in terms of reduction in the height of arcs common to normal and GD 248 cells (see ref. 14) at given antigen/antibody ratios. We find that four absorptions of antiserum (100 μl) with particulate material (200 μg of protein) caused maximal absorption, leaving only three persistent precipitation arcs.

Plasma Membranes. As before (14), our antiserum indicates the presence in the membranes of GD 248 cells of at least three antigens lacking in the membranes of the control cell population. However, our new antiserum against GD 248 membranes does not produce a precipitation pattern in bidimensional immune electrophoresis exactly identical to that reported before (14).

The "new" antigens stand out when one compares the bidimensional immune electrophoresis pattern of GD 248 membranes (Fig. 1A) with the pattern obtained by crossed-line immune electrophoresis (Fig. 1B). In the latter technique a strip

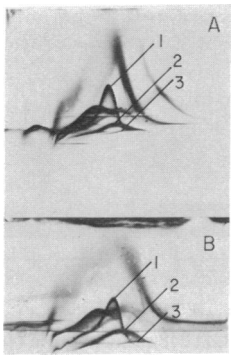

FIG. 1. Bidimensional immune electrophoresis of solubilized plasma membranes. Membranes solubilized in Triton X-100 were first separated in the first dimension (horizontal) and then electrophoresed in the second dimension (vertical) into agarose containing 40 μl/ml of agarose of a guinea pig antiserum raised against membranes of GD 248 cells. Coomassie blue staining of the 50 × 50 mm immunoplates. (A) Crossed immune electrophoresis of GD 248 plasma membranes (300 μg) against antiserum to GD 248 plasma membrane. Components 1–3 are not found when membranes of normal cells are used (14). (B) Crossed-line immune electrophoresis. Conditions are as in panel A, except for inclusion of an intermediate strip containing 400 μg of protein from normal lymphocytes. Note that components 1–3 are not displaced upward (anodally), indicating nonidentity with normal membrane material.

of agarose containing 400 μg of membrane protein from normal cells was interposed between the electrophoretically separated GD 248 material and the antibody- containing agarose prior to electrophoresis in the second dimension. This procedure indicates crossreactivity by upward (anodal) displacement and horizontal deformation of immune precipitates (20). As shown in Fig. 1, three components do not meet these criteria of crossreactivity and must therefore be absent in the membranes of normal cells. Membranes from embryonal hamster fibroblasts, when used in crossed-line immune electrophoresis, gave no evidence of crossreactivity with the "new" components of GD 248 cells.

That the membranes of the control cell population do indeed lack the antigens in question is documented by the experiments shown in Fig. 2. The results after partial (two) absorption (Fig. 2A) demonstrates that absorption does not simply displace the immune precipitates. Thus, although the heights of the precipitation arcs near the well and common to both membrane categories are increased, indicating a decrease in antibody (20), the precipitates still originate from the well. However, four absorptions (Fig. 2B) eliminate all antigenic components detected by bidimensional immune electrophoresis, except the three GD 248—derived entities already defined (Fig 1). Of these, component 2 appears antigenically heterogeneous, since it consists of at least two separate rockets (Fig. 2A and B).

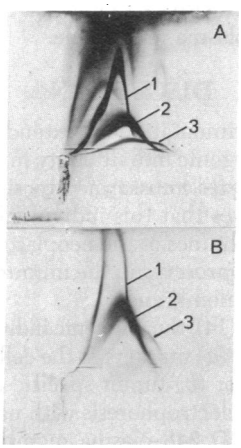

FIG. 2. Bidimensional immune electrophoresis of solubilized plasma membranes. GD 248 membrane proteins (300 μg) solubilized in Triton X-100 were first electrophoresed horizontally and then in the second dimension (vertically) into agarose containing antiserum (against membranes from GD 248 cells) absorbed with particulate material from normal cells (200 μg of protein per 100 μl of antiserum). (A) Two absorptions. (B) Four absorptions. The amount of antiserum was 30 or 160 μl/ml of agarose, respectively, corresponding to 40 μl of unabsorbed serum per ml of agarose.

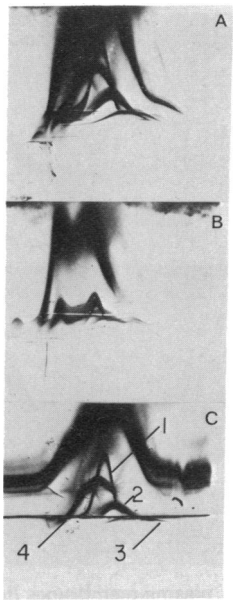

FIG. 3. Immunoelectrophoretic comparison of plasma membranes and mitochondria from GD 248 cells. (A) Crossed immune electrophoresis of GD 248 plasma membranes and (B) of GD 248 mitochondria. (C) Crossed-line immune electrophoresis of GD 248 plasma membranes compared with GD 248 mitochondria. In A–C, 350 μg of membrane protein was separated in the first dimension (horizontal). Crossed-line immune electrophoresis (C) was as in panel A, but 450 μg of mitochondrial membrane protein was polymerized into the intermediate strip. Note that components 1 and 2 arise from the mitochondrial precipitation line, a reaction of identity (20). For details see text.

The increased heights of the precipitates remaining after absorption is attributed in part to the possibility that we undercorrect for the dilution produced by adsorption. Another problem could be nonspecific adsorption during the repeated absorption steps. This appears likely since the relative heights of the persistent precipitates do not change during absorption.

Mitochondria. Comparison of the precipitation patterns obtained by crossed immune electrophoresis of GD 248 plasma membranes (Fig. 3A) and mitochondria (Fig. 3B) suggests that the two membrane categories share multiple antigens, although plasma membranes contain these in larger proportion (identical amounts of membrane protein were applied to plates A and B). Importantly, however, results of crossed-line immune electrophoresis of plasma membranes from GD 248 cells through an intermediate strip containing 400 μg of protein from GD 248 mitochondria (Fig. 3C) into antiserum against GD 248 plasma membranes suggest that GD 248 mitochondria contain at least two of the "neoantigens" already found in GD 248 plasma membranes.

The reasons for this conclusion are as follows: the antiserum against GD 248 plasma membrane reacts with mitochondrial components over the entire range of electrophoretic mobilities.

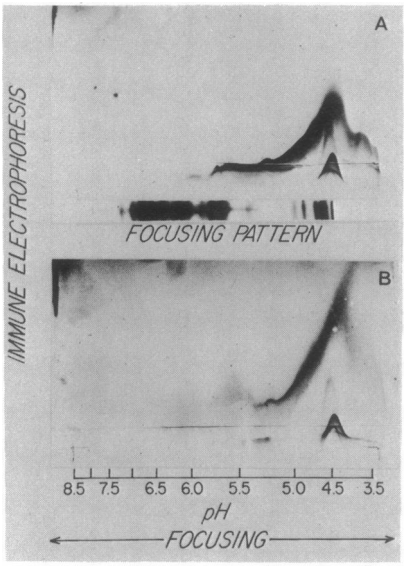

FIG. 4. Focusing of plasma membranes linked to immune electrophoresis. Four hundred micrograms of Triton-solubilized plasma membrane protein from GD 248 cells were fractionated by isoelectric focusing in polyacrylamide gel slabs in the first dimension (horizontal). The focused material was then electrophoresed in the second dimension (vertical) into antiserum-containing agarose (see *Materials and Methods*). (A) Unabsorbed serum (44 μl/ml of agarose). (B) Serum absorbed four times (140 μl/ml of agarose). The focusing pattern is shown in plate A and the pH gradient in the focusing gel is given in plate B.

Therefore, electrophoresis of the horizontal strip of mitochondrial protein, with its low antigen content, against the high-titer antiserum produces rapid immune deposition, yielding the horizontal precipitation line (Fig. 3C). Crossed-line immune electrophoresis using separated, antigen-rich plasma membrane material produces (*a*) an anodally displaced, broad, and complex front due to multiple crossreacting components and (*b*) two precipitation arcs (components 1 and 2) *arising from the mitochondrial precipitation line*. The latter pattern is a reaction of identity (20). We cannot be certain about component 3, since the precipitation arc expected in this position, although present (Fig. 3C), does not arise from the mitochondrial precipitation line. Component 4, a precipitate originating below the mitochondrial precipitation line, represents plasma membrane antigens entirely lacking in the mitochondria.

Bidimensional Isoelectric Focusing-Immune Electrophoresis of Plasma Membranes. Our previous focusing experiments (12) have shown that the plasma membranes of GD 248 cells differ from those of normal cells in (*a*) a generalized shift to low pH of the pI values of glycosylated proteins, (*b*) a possibly new glycoprotein with a pI of 4.5, and (*c*) a generalized shift to alkaline pH of nonglycosylated proteins. In order to relate these properties to the antigenic singularities of GD 248 membranes, we have combined isoelectric focusing and immune electrophoresis in a two-dimensional approach (Fig. 4).

Using unabsorbed serum (Fig. 4A) we find a sharp immune rocket at pH 3.5, which can be ascribed to acid glycolipids (12). The complex immune precipitate between pH 3.5 and 6.0 encompasses the chemically determined glycoprotein distribution of GD 248 membranes (12). We also observe two distinct rockets at pH 4.5, one at pH 4.7, a small arc at pH 5.8, and a coherent precipitation front between pH 5.0 and 5.8.

With absorbed serum, however (Fig. 4B), immune precipitation becomes restricted to the rockets at pH 4.5, the rocket at pH 4.7, and the broad precipitate between pH 4.2 and 5.3. The two rockets at pH 4.5 plus that at pH 4.7 correspond to three precipitates that distinguish the plasma membranes of GD 248 cells from those of normal lymphocytes upon crossed immune electrophoresis.

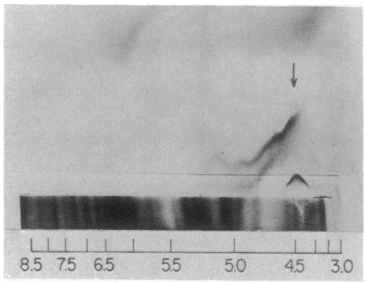

FIG. 5. Focusing of mitochondria linked to immune electrophoresis. Membranes from GD 248 mitochondria (400 μg of protein) were fractionated by isoelectric focusing in the first dimension (horizontal) and identified by immune electrophoresis in the second dimension (vertical). The focused material was electrophoresed into antibody-containing agarose (44 μl/ml of agarose). The patterns of focusing and immunoprecipitation are shown together with the pH gradient. The arrow indicates the position and height of the rocket at pH 4.7.

Bidimensional Isoelectric Focusing-Immune Electrophoresis of Mitochondria. The immune precipitates produced by electrophoresing focused mitochondria into agarose containing antiserum against GD 248 plasma membranes is shown in Fig. 5. The following similarities to the patterns obtained with plasma membranes are important: (*a*) the complex precipitation front between pH about 4.0 and pH about 5.2, (*b*) the presence of a precipitation arc at pH 4.5, and (*c*) the occurrence of a fainter rocket at pH 4.7 (arrow).

The mitochondrial pattern differs from that obtained with plasma membranes in the following respects: (*a*) it lacks the rocket at pH 3.5, the front above pH 5.2, and the rocket at pH 5.8; and (*b*) the height ratio of the rocket at pH 4.5 relative to that at pH 4.7 is 0.14 compared with 0.5 for plasma membranes. Items (*a*) and (*b*) indicate that the immune precipitates obtained with GD 248 mitochondria are not due to contamination of the immunizing material with mitochondria nor of the analyzed mitochondria with plasma membrane.

DISCUSSION

Our present experimentation (*a*) extends prior studies (14) documenting an antigenic individuality in plasma membrane proteins of lymphocytes transformed by simian virus 40 (GD 248), (*b*) demonstrates that this individuality extends to the *mitochondrial* membranes of the neoplastic cells, and (*c*) implicates certain *glycoproteins* in the immunological individuality of the GD 248 membranes.

As already treated (14), the antigenic individuality of GD 248 membranes could be (*a*) markers of the cell clone from which GD 248 originated or (*b*) tumor-specific components. Bidimensional immune electrophoresis with native and absorbed antiserum against GD 248 plasma membrane reveals three antigenic components in the plasma membranes of GD 248 cells that are not detected in the membranes of normal cells; one of these (component 2) contains at least two subcomponents. The lack of fusion between the three precipitation arcs indicates that these represent different antigens rather than an electrophoretic anomaly. The fact that the precipitates persist after extensive absorption speaks against, but does not fully exclude, the possibility that the antigens represent clonal markers of minor

segments in the reference population. The lack of detectable crossreactivity with embryonic material tends to minimize the possibility that the "new" antigens represent carcinoembryonic antigens [Forssman antigen, a carcinoembryonic antigen, is easily detected by our focusing-immune electrophoresis method (19)]. The data on mitochondria fit those on plasma membranes although they are inconclusive with regard to component 3. Also, the proportion of "new" antigens in mitochondria is generally less than in plasma membranes.

Bidimensional isoelectric focusing-immune electrophoresis sensitively and precisely defines membrane antigens according to their isoelectric points. This approach has allowed us to document that the "new" antigens of GD 248 membranes focus in the pH region encompassing the pI values of these membranes' glycoproteins. Two types of precipitate occur: (*a*) two sets of rockets occurring at specific pH values and (*b*) a high front, extending over a wide range of acid pH, due to nontumor-specific immune precipitation and imperfect separation of antigens.

Of major importance are the defined rockets occurring at pH 4.5 and 4.7 with both plasma membranes and mitochondria. The precipitate at pH 4.5 clearly contains two components in the case of GD 248 plasma membranes, appears heterogeneous also in the case of GD 248 mitochondria, and corresponds in pI to a glycoprotein component, B, *not* observed in the membranes of normal cells (12). It represents an entity that appears to be novel, both physicochemically and immunologically. However, the single rocket at pH 4.7 corresponds in pI to a glycoprotein component, 8, observed in both normal and transformed cells (12). Our techniques do not yet allow us to unambiguously relate the three "new" components revealed by crossed immune electrophoresis to those identified by the separation by isoelectric focusing. However, we suspect that the precipitate at pH 4.7 represents component 1 and that components 2 and 3 focus at pH 4.5. This question, as well as the relationship of the new components in GD 248 membranes detected by dodecyl sulfate-polyacrylamide gel electrophoresis (14) to those under discussion here, can be resolved by linking the latter technique bidimensionally to focusing (19) and immune electrophoresis.

We cannot now concretely eliminate the possibility that the "individuality" of GD 248 membranes represents clonal markers rather than products of the simian virus 40 genome (13, 14). However, it is fruitful to view the antigenic complexity we observe in terms of the concept (21) that transformed cells bear "hybrid" antigens, containing elements coded by the host genome and elements coded by the virus. It is also possible that we are detecting both membrane-associated simian virus 40 tumor (T)-antigen and transplantation antigen.

The fact that mitochondrial membranes share at least some of the antigenic distinctions found in plasma membranes serves to emphasize a too often ignored circumstance, that the mitochondria of tumor cells commonly exhibit numerous pleiotypic anomalies that can, in fact, account for many functional peculiarities of tumor cells (22, 23).

Note Added in Proof. We now find molecular weights of about 53,000 and 100,000, respectively, for the pI 4.5 and 4.7 components.

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