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Analytical Techniques for Cell Fractions

XXVIII. Dissection of Complex Antigenic Mixtures Using Monoclonal Antibodies and Two-Dimensional Gel Electrophoresis[1]

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Disposable microimmunoadsorbent columns containing *Staphylococcus* Protein A and monoclonal antibodies were used to bind antigenic proteins from a mixture. Eluates from these columns were directly analyzed by electrophoresis on two-dimensional (2-D) gels. In this way, biochemical and biophysical information on the bound antigen and on the specific antibody can be obtained simultaneously. The microimmunoadsorbents are easy to handle and in conjunction with multiple 2-D gel systems provide a means for screening large numbers of myeloma hybrids for specificity to antigens in complex mixtures.

Two-dimensional (2-D)[3] electrophoresis in polyacrylamide gels, as developed by O'Farrell (1) and others (2–4), gives the highest resolution of any analytical system at present available for the investigation of complex mixtures of proteins. Recent technical developments (5,6) have made it possible to perform large numbers of highly reproducible 2-D gel electrophoreses and to contemplate the complete analysis of human cells and fluids in terms of the protein gene products they contain (7,8). Concurrently it has also become possible, through the use of cell fusion techniques as developed by Köhler and Milstein (9,10), to produce cell lines which secrete chemically homogeneous monoclonal antibodies to desired antigens. Such antibodies show exquisite specificity and are thus excellent probes for antigenic molecules. Unlike antibodies in conventional antisera, monoclonal antibodies of extremely high titer can be made against those molecules in a mixture which are not immunodominant (11). There is also no need for purification of the immunizing antigens in the monoclonal antibody technique as the hybrids producing antibody specific for a given antigen are isolated by cloning of the cells. An animal may be immunized with a mixture of antigenic molecules and, after fusion, monoclonal antibodies to each antigenic component in the mixture can be separated by conventional tissue culture procedures. (For discussion of the advantages of monoclonal antibodies see Refs. (11,12).)

It is clear that even taken individually, multiple 2-D gel electrophoresis and monoclonal antibodies are powerful tools for analysis of molecular mixtures. In this paper, we describe an extremely versatile system for dissection of complex antigenic mixtures by combining these two techniques. We used monoclonal antibodies to "pull

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[3] Abbreviations used: 2-D, two-dimensional; Ig, immunoglobulin; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate.

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out" antigenic proteins from a mixture and analyzed the antigens and their specific antibodies simultaneously by electrophoresis on 2-D gels. To do this we prepared microimmunoadsorbent columns using Protein A–Sepharose and antibody from the ascites fluid of mice bearing cloned myeloma hybrids. The columns bind the appropriate antigen and can be eluted with extremely small volumes (20 μl) suitable for direct analysis on 2-D gels. The microimmunoadsorbent columns are disposable and easy to handle in large numbers; therefore they provide a means for screening extensive sets of fusion clones for specificity to "spots" (proteins) in the 2-D pattern of the immunizing mixture. Using this system, it is theoretically possible to derive monoclonal antibodies to all antigenic proteins in a mixture, to analyze the antigens individually, and simultaneously to obtain information on the specific monoclonal antibody—all using extremely small amounts of material.

MATERIALS AND METHODS

Monoclonal antibodies. Cell fusion techniques (9,10) were used to produce myeloma hybrids which secrete immunoglobulins specific for antigenic sites on trypanosome variable antigens. A doubly cloned myeloma hybrid (V3/16.15.1) was used in the present study. This hybrid was produced by fusing the parental tumor line X63-Ag8 (9) with spleen cells from BALB/c mice which had been hyperimmunized with trypanosome variable surface glycoprotein antigen 11E3 (see below). The myloma hybrid secretes IgG monoclonal antibodies which bind to the purified variable antigen in a radioimmunoassay and do not bind any of eight other variable antigens tested. A detailed description of the derivation of this and other hybrids and the specificities of their secreted immunoglobulins will be published elsewhere (Pearson *et al.*, in preparation). A control antibody used in the present study is from hybrid B5/4.1.4 which produces an IgG antibody specific for bovine IgM molecules. Its derivation and specificity will also be published elsewhere (Pinder *et al.*, in preparation).

Preparation of ascites fluid. Female BALB/c mice were injected intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co. Inc., Milwaukee, Wis.) and 14 days later with 10^7 viable X63-Ag8 parental tumor cells or with V3/16.15.1 or B5/4.1.4 myeloma hybrid cells. After 10 days, peritoneal swelling had occurred in most mice, indicating that ascitic tumors had grown. The ascites fluid was removed from these mice and was depleted of cells by centrifugation at 750*g* for 5 min. The clear fluid was heat inactivated (56°C, 30 min), sterilized by filtration through a Millipore filter (0.45 μm), and stored at −20°C in 0.5-ml aliquots. Just prior to use the ascites fluid was thawed at 37°C and ultracentrifuged in a Beckman microfuge to remove aggregated material.

Trypanosome variable antigens. Trypanosome surface glycoprotein antigens were purified from cloned populations of *Trypanosoma brucei* (11E3, 19E1, and 19E2) by the method of Cross (13). Iodination of 1.0 μg of each variable antigen was performed by the chloramine-T method (14).

Trypanosome extracts. T. brucei clone 11E3 was prepared by injecting a single trypanosome from strain 427 (13) into the peritoneum of a lethally irradiated mouse. A "stabilate" was prepared after further passage in irradiated mice. This "stabilate" of parasitemic mouse blood was stored in liquid nitrogen and used to infect lethally irradiated rats (13). Rats were exsanguinated on the third day of infection and trypanosomes were isolated by passage of a buffy coat layer through DEAE-cellulose by the method of Lanham and Godfrey (15).

Nonidet P-40 (NP-40) extracts were prepared by resuspending 10^9 washed trypanosomes in 1.0 ml of 0.25% NP-40. After 5 min at 20°C, the nuclei were removed by centrifugation at 3000*g* and the NP-40 solubilized material was stored in liquid nitrogen. Im-

mediately prior to use, the frozen lysate was thawed at 37°C and ultracentrifuged for 5 min in a Beckman microfuge in order to remove aggregated material.

Preparation of microimmunoadsorbent columns. Disposable microimmunoadsorbent columns were made using Eppendorf pipet tips supported in 2-ml plastic test tubes as follows: Four millimeters was cut off the small end of yellow Eppendorf pipet tips (1–100 μl, Brinkmann Instruments, Inc., Westbury, N. Y., Catalog No. 2235 130-3) using a razor blade. The bottom 2 mm was then plugged with a wisp of surgical cotton using a 25-μl glass disposable microsampling pipet (Corning Glass Works, Corning, N. Y., Catalog No. 7099-S) to press the cotton lightly into place. These plugged pipet tips (microcolumns) were then inserted into blue Eppendorf pipet tips (101–1000 μl, Brinkmann Instruments, Inc., Catalog No. 2235 090-1) which had been cut off 1 cm from the large end, and the assembly was placed into a plastic test tube (Luckham LP3, Sussex, England) as shown in Fig. 1. The use of these particular Eppendorf tips and LP3 tubes is important as the size of the components and the flanges on the upper ends of the Eppendorf tips allow air to escape during sample application, washing, and elution procedures.

Columns were packed at room temperature by pipetting 400 μl of a 10% suspension of Protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden) in phosphate-buffered saline (PBS) into each pipet tip and allowing the PBS to run through. Because of the small size of the columns, there is no danger of their running dry, so they need little attention during the loading or running procedures. After packing, the columns can be stored at 4°C after topping up with PBS (+0.1% sodium azide to prevent bacterial growth).

Using the microimmunoadsorbent columns. The columns were washed once with PBS at room temperature and the excess was allowed to run through to the surface

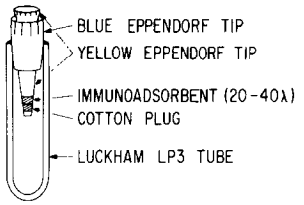

FIG. 1. Diagram of microimmunoadsorbent column. A section of the large end of a blue Eppendorf tip serves as an adaptor holding the yellow tip column. A firm cotton plug holds the immunoadsorbent in place. This arrangement can be centrifuged to dry the immunoadsorbent and, after rehydration with one column volume of a dissociating elution buffer, to collect the eluate. Almost all of the bound protein can be eluted in one column volume (20–40 μl).

of the Protein A bed. Fifty microliters of neat ascites fluid (containing the specific or control monoclonal antibodies) was applied to the column and gently blown into the Protein A. After 15 min incubation at room temperature, the column was filled with PBS and washed by gentle blowing until the PBS reached the level of the Protein A bed. Fifty microliters of antigen (or mixture containing the antigen) was then added, blown into the adsorbent, and incubated for 15 min at room temperature. After filling with PBS and washing as before, the column assembly was placed into a fresh LP3 tube and again topped up with PBS. The tubes were then centrifuged at 800*g* for 5 min, effectively drying the immunoadsorbent. Finally, the column assembly was transferred to another LP3 tube and 40 μl of a 1/2 dilution of sample preparation buffer (2% $NaDodSO_4$/5% 2-mercaptoethanol/10% glycerol) was added. This rehydrated the dried immunoadsorbent. After incubation at room temperature for 30 min, the columns were eluted by centrifuging at 2000*g* for 10 min. The eluate (then in 40 μl of sample preparation buffer) was heated to 95°C for 5 min and was either stored at −20°C or directly applied to isoelectric focusing gels for 2-D analysis (see below). Denaturation in $NaDodSO_4$ and 2-mercaptoethanol was used in order to separate tightly bound complexes. It has

been shown (7,16) that the $NaDodSO_4$ is removed from proteins during isoelectric focusing in urea/NP-40.

The above procedure describes the use of the micro-Protein A columns with monoclonal antibodies to trypanosome variable antigens but should be applicable to any antibody–antigen combination as long as the antibody binds to Protein A. Alternatively, an immunoadsorbent made by coupling an antiimmunoglobulin reagent to Sepharose CL-4B as a first step gives similar results. Direct coupling of monoclonal antibody to Sepharose beads is also possible.

Two-dimensional electrophoresis. The method used involves several modifications of the procedure originally described by O'Farrell (1). These include the use of $NaDodSO_4$ denaturation (16) and the use of apparatus for simultaneously casting and running 20 isoelectric focusing gels (the ISO apparatus) or 10 gradient $NaDodSO_4$ slab gels (the DALT apparatus). The 2-D method is called the ISO–DALT system since the separation of proteins in the first dimension is by ISOelectric focusing and in the second dimension by molecular weight as expressed in DALTons. Detailed descriptions of the apparatus and operating procedures are described elsewhere (5,6).

Isoelectric focusing gels were prefocused for 1 h at 200 V prior to sample loading and were then run at 600 V for 15 h. LKB Ampholines (pH 3.5–10) were used throughout. Slab gels were 10–15% gradients of acrylamide crosslinked with 0.26–0.4% bisacrylamide and were run at 400 mA constant current. To stain proteins, the slab gels were immersed and rocked gently for 12 h in 50% ethanol/10% acetic acid/0.2% Coomassie brilliant blue R250, then destained in three changes (2-h intervals) of 10% ethanol/2% acetic acid. Photographs were taken with an Olympus OM-1 camera fitted with a macrolens and 35-mm Kodak Panatomic-X film. An optical filter (Y2) was used to increase the contrast of the blue "spots." All gels are shown with the acid end to the left and increasing acrylamide concentration from top to bottom (i.e., sample origin is at upper right).

RESULTS

Two-Dimensional Analysis of Trypanosome Variable Antigens

Purified variable antigens were simultaneously run on 2-D gels either separately or as a mixture (Fig. 2). Antigens 19E1 and 19E2 both showed a single major spot with a fainter spot to their left (Figs. 2A and B). The minor spot is probably due to deamidation of the glycoproteins since charged sugars have not been found on variable antigens (17). No heterogeneity in apparent molecular weight was seen with antigens 19E1 and 19E2. Antigen 11E3, on the other hand, showed marked heterogeneity in apparent molecular weight (Fig. 2C). A uniform charge difference was seen between the native and presumably deamidated form for each of the spots differing in molecular weight. For purposes of comparison, a mixture of the three variable antigens was run together on a single gel (Fig. 2D).

Two-Dimensional Analysis of NP-40 Lysate from Trypanosome Clone 11E3

A proportion of the gel of whole NP-40 extract of trypanosomes (clone 11E3) is shown in Fig. 3. A number of protein spots can be seen. Predominant among these are a series which correspond in charge and molecular weight to those obtained with purified 11E3 variable antigen (Fig. 2C).

Two-Dimensional Analysis of Ascites Fluid Containing Monoclonal Antibodies

Figure 4 shows the 2-D patterns obtained with whole ascites fluid from mice bearing the X63-Ag8 parental myeloma cells (Fig. 4A) or the myeloma hybrid V3/16.15.1 (Fig. 4B). Although both ascites fluids contain a fair amount of host immunoglobulin and other serum proteins, the heavy- and

light-chain spots of the monoclonal antibodies are easily discernible as more predominant "spots." In Fig. 4B, heavy- and light-chain spots corresponding to the γ_1 heavy chain and κ light chain of the X63-Ag8 myeloma parent can also be clearly seen. This is not surprising as the myeloma hybrid V3/16.15.1 secretes the γ_1 and κ chains of the X63-Ag8 parental tumor cell in hybrid molecules with the specific heavy and light chains of the spleen cell parent. By superimposing the gels, it is possible to identify the heavy and light chains of the specific antibody. (For discussion of heavy- and light-chain nomenclature, see Refs. (10,11).)

Ascites fluids from both X63-Ag8 cells and V3/16.15.1 myeloma hybrids were passed through the Protein A columns and bound material was eluted and analyzed on 2-D gels. Protein A-bound material from X63-Ag8 ascites fluid appears as a series of spots running near the 50,000 molecular weight region (IgG heavy chains) and a series of spots corresponding to the light-chain region running at about 23,000 molecular weight (Fig. 5A). The faint spots were presumably due to host antibodies in the ascites fluid. The major heavy-chain and light-chain spots seen in 2-D patterns of the whole X63-Ag8 ascites are also evident. This is rather unexpected as the X63-Ag8 cells secrete an IgG_1 molecule (9) and since murine IgG_1 does not usually bind to *Staphylococcus* Protein A (18). It has recently been shown, however, that IgG_1 molecules bind weakly to Protein A at physiological pH (19). When material eluted from the V3/16.15.1–Protein A column was analyzed, predominant heavy-chain and light-chain spots were seen in addition to the faint spots due to host immunoglobulin in the ascites fluids (Fig. 5B). The trypanosome-specific antibody is therefore probably of the IgG_2 or IgG_3 class since it binds to Protein A, although IgG_1 molecules produced in some parasitic infections may also bind strongly to Protein A (18).

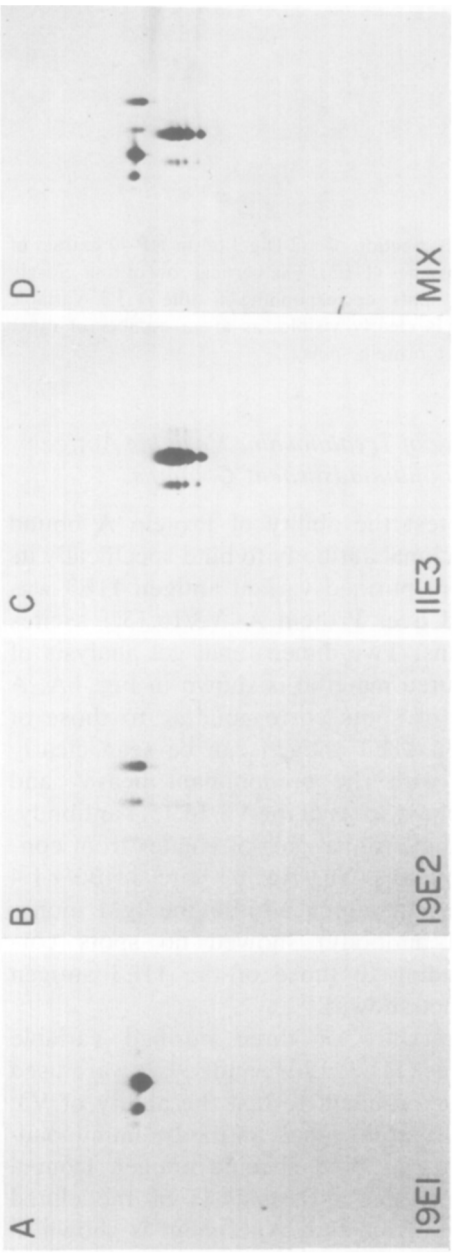

FIG. 2. Sections of 2-D gels of purified trypanosome variable surface antigens. Panel D shows the relationship of the three proteins, obtained by running a mixture of them on a single gel.

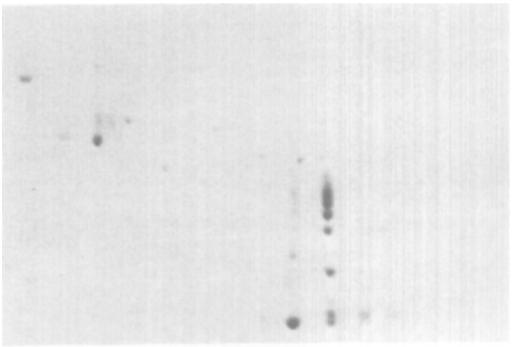

FIG. 3. Section of a 2-D gel of an NP-40 extract of trypanosomes (11E3). The vertical row of four closely spaced spots corresponding to the 11E3 variable antigen is clearly visible, as are a number of other unknown protein spots.

Binding of Trypanosome Variable Antigens to Immunoadsorbent Columns

To test the ability of Protein A bound monoclonal antibody to bind specifically its antigen, purified variant antigen 11E3 was passed over Protein A–V3/16.15.1 ascites columns. Two-dimensional gel analysis of the eluted material is shown in Fig. 6A. A series of spots corresponding to those of purified 11E3 antigen can be seen clearly along with the predominant heavy- and light-chain spots of the V3/16.15.1 antibody. Two-dimensional gels of eluates from control columns (X63-AgG8 ascites or B5/4.1.4 ascites containing anti-bovine IgM monoclonal antibody) showed no spots corresponding to those of the 11E3 antigen (gels not shown).

A mixture of three purified variable antigens (11E3, 19E1, and 19E2) were used in an experiment to test the ability of V3/16.15.1 monoclonal antibody immunoadsorbents to bind specific antigen from a mixture. The 2-D analysis of the eluted material from this experiment is shown in Fig. 6B. Only the spots corresponding to variable antigen 11E3 are seen. The antigens 19E1 and 19E2 did not bind to the column at all. A similar experiment was performed using NP-40 lysates of cloned

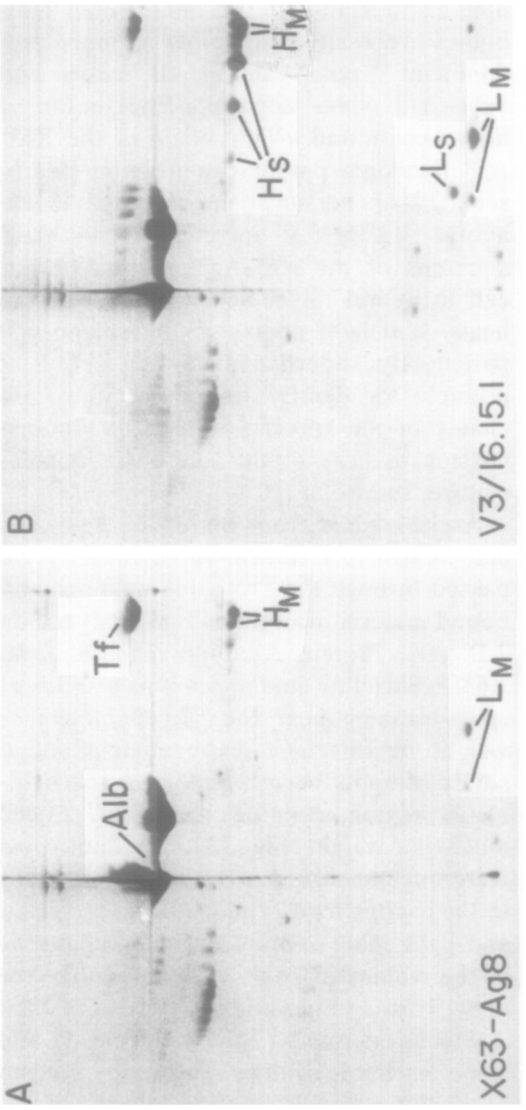

FIG. 4. Two-dimensional gels of ascites fluid from mice bearing the X63-Ag8 parental myeloma and V3/16.15.1 myeloma hybrid. In panel A, the horizontal row of spots halfway down the right-hand edge of the pattern corresponds to the X63-Ag8 IgG_1 heavy chain (H_M), while the spot near the bottom and one-third the way from right to left is the X63-Ag8 κ light chain (L_M). The remaining predominant spots are mouse serum proteins (Alb; albumin, Tf; transferrin). The V3/16.15.1 cells (panel B) produce X63-Ag8 heavy chains plus extra spots (variable antigen-specific heavy chain, H_S) just to the left of them, and X63-Ag8 light chains plus a variable antigen-specific light chain (L_S, above and to the left).

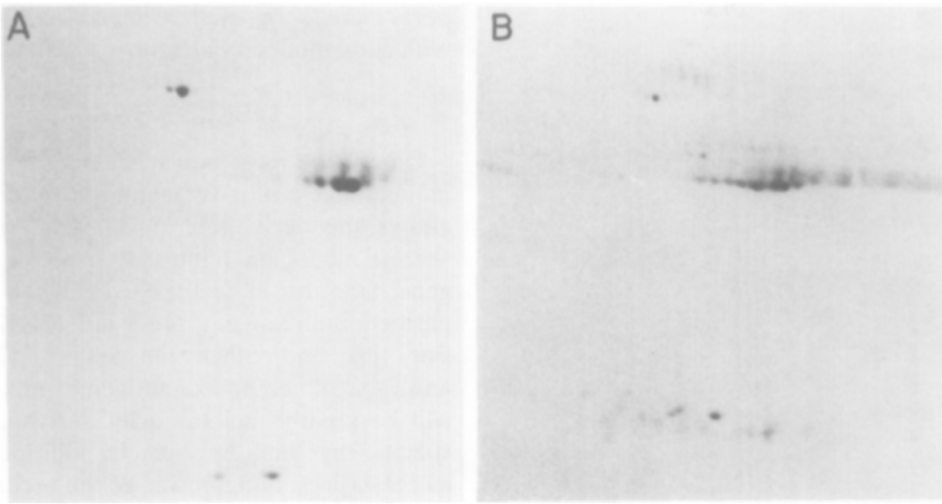

FIG. 5. Two-dimensional gels of X63-Ag8 (panel A) and V3/16.15.1 (panel B) ascites fluid proteins which bound to Protein A–Sepharose. The antibody heavy and light chains appear as in Fig. 4, but serum contamination has been practically eliminated; only traces of albumin (upper left) remain.

trypanosomes (11E3). Variable antigen 11E3 was specifically removed from the mixture of trypanosome proteins (Fig. 6C). Control immunoadsorbents (X63-Ag8 ascites–Protein A and B5/4.1.4 ascites–Protein A) did not bind any proteins in the above two experiments; nor was there any nonspecific sticking of trypanosome components to Protein A columns alone (gels not shown). Binding of ^{125}I-labeled variable antigen 11E3 was used to quantitate the degree of binding to and elution of antigen from the microcolumns. The results of one experiment are shown in Table 1. A clear difference in binding of ^{125}I-11E3 antigen can be seen in comparing the specific (column 1) and control (column 2) monoclonal immunoadsorbents. The specific immunoadsorbent bound 80% of the TCA-precipitable radioactivity added to the column. It is likely that the 20% of counts which did not bind to the adsorbent were on molecules which were not antigenically intact as monoclonal antibody was present in saturating amounts. More than 6% of the total added radioactivity bound nonspecifically to the Protein A–Sepharose column alone and was difficult to elute with $NaDodSO_4$/mercaptoethanol sample buffer. This nonspecific binding could be reduced but not eliminated by including protein (bovine serum albumin or fetal calf serum) in the PBS used for packing, running, and washing the columns (data not shown).

In preliminary experiments with certain ascites fluids we have also found a fairly large amount of nonspecific binding of iodinated antigens. By diluting out the ascites containing specific monoclonal antibodies and those containing control antibodies, the majority of this nonspecific binding could be eliminated without any effect on specific binding. It is possible that the high nonspecific binding occurs because of antigen "trapping" in columns saturated with immunoglobulins binding to the Protein A, thus explaining why only some ascites fluids present this problem. To avoid nonspecific binding of antigen, we have successfully used cell culture supernatants containing monoclonal antibodies. Especially promising for this purpose is the use of Iscove's serum-free medium (20) which contains only two different proteins and sup-

ports excellent growth of myeloma hybrids with good monoclonal antibody production.

DISCUSSION

One of the most powerful aspects of the monoclonal antibody technique is that it allows the derivation of cell lines which secrete monoclonal antibody to single antigenic sites on molecules even when nonpurified immunogens are used (11,12,21). For this and other reasons (11,12,21), analysis of complex antigenic mixtures will be greatly facilitated by derivation of specific myeloma hybrids. In addition, the increased resolving power of the technique will allow analysis of antigens which are not immunodominant, including many previously undiscovered. However, as a result of this increased resolving power, a problem arises. Because it is possible to derive many different myeloma hybrids from a single fusion, analyzing the specificities and biochemistry of the monoclonal antibodies themselves becomes labor intensive. In this paper, we have described a system which allows the screening of large numbers of myeloma hybrids for specificity to antigens in the immunizing mixture and which, at the same time, gives biochemical information on the specific monoclonal antibody. Using the multiple 2-D gel electrophoresis system (ISO–DALT), it is possible for three people to run 80 or more 2-D gels per day (7). In conjunction with the microimmunoadsorbents described in this paper, it is therefore possible to screen 80 or more monoclonal antibodies per day for their specificity and biochemical char-

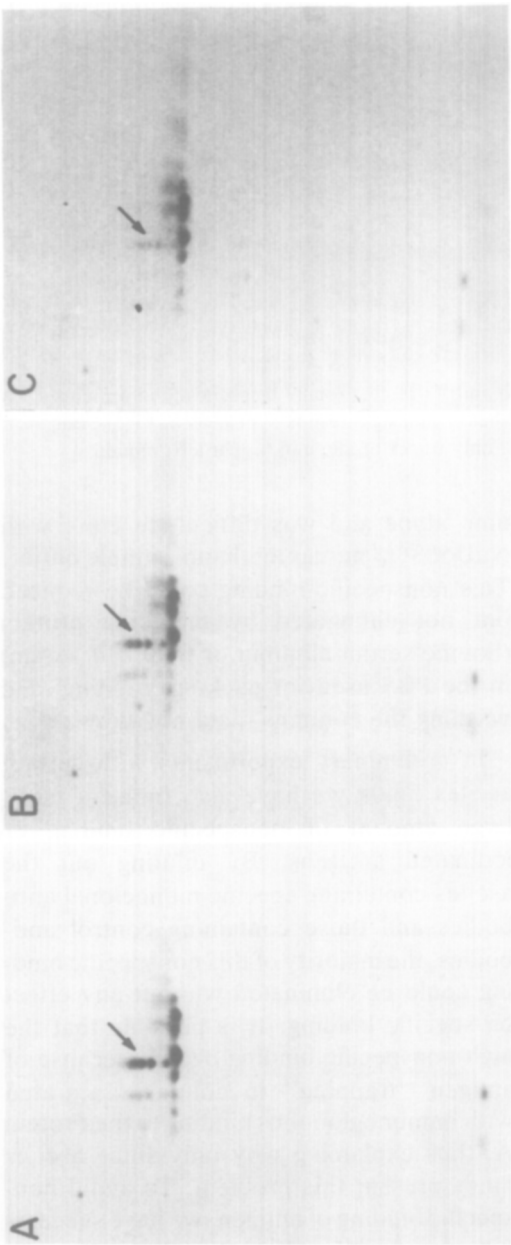

FIG. 6. Two-dimensional gels of proteins bound to V3/16.15.1–Protein A–microcolumns loaded with: (A) pure 11E3 variable antigen (the antigen against which V3/16.15.1 was made); (B) a mixture of three variable antigens (11E3, 19E1, and 19E2); and (C) NP-40 extract of 11E3 trypanosomes. In each case, the 11E3 variable antigen (indicated by arrows) bound to the column, but other variable antigens (B) or trypanosome proteins (C) did not.

TABLE 1

QUANTITATION OF BINDING OF ^{125}I-LABELED VARIABLE ANTIGEN 11E3 TO MICROIMMUNOADSORBENT COLUMNS

Column	Immunoadsorbent[a]	TCA-precipitable ^{125}I-11E3 added[b] (cpm)	cpm bound[c] (%)	cpm eluted[d] (%)
1	V3/16.15.1–Protein A–Sepharose	19,000	15,207 (80.0)	14,219 (93.5)
2	B5/4.1.4–Protein A–Sepharose	19,000	1,471 (7.7)	835 (56.7)
3	PBS–Protein A–Sepharose	19,000	1,291 (6.7)	918 (71.1)

[a] Fifty microliters each of neat ascites fluid was added to columns 1 and 2 and 50 μl of PBS to column 3. The entire procedure was carried out as described under Materials and Methods.

[b] 23,000 cpm of ^{125}I-11E3 was added to each column in 50 μl total volume in PBS. Cold 10% TCA precipitated 19,000 cpm from this. Specific activity of the ^{125}I-11E3 antigen was 9.1 μCi/μg. The amount of ^{125}I-11E3 antigen added to each tube is therefore approximately 1 ng of protein.

[c] Counts per minute ^{125}I bound, expressed as a percentage of the total TCA-precipitable counts per minute added to the column.

[d] Counts per minute ^{125}I eluted with $NaDodSO_4$/mercaptoethanol buffer, expressed as a percentage of the counts per minute bound to the column.

acteristics. In addition, only microgram quantities of antigens and antibodies are required for a complete analysis.

We used African trypanosomes and their purified variable antigens as a model system to study the effectiveness of the combined methods for dissection of antigens from a mixture. By mixing three different purified variable antigens, we showed that a single antigen could be "pulled out" of a mixture by its specific monoclonal antibody. Similarly, the antigen could be isolated from NP-40 lysates of whole trypanosomes, demonstrating the usefulness of the technique for analysis of cell surface antigens. In this regard, the experiments using ^{125}I-labeled variable antigen indicate that radiolabeling of antigens presents no major problems for this technique although there is some nonspecific binding of ^{125}I-labeled material to Sepharose (21).

One of the most appealing aspects of the system described here is that along with information on the bound antigen, biochemical and biophysical data on the specific monoclonal antibody itself are obtained simultaneously. For example, since the gels are run under reducing conditions and in two dimensions, the heavy and light chains of the antibody are completely separated thus revealing the chain composition of the secreted immunoglobulin. The system is therefore excellent for screening of chain-secreting variants of the myeloma hybrids and at the same time tests the antigen-binding activity of the variants. Heavy- and light-chain analysis of myeloma hybrids has until now depended on separate isoelectric focusing and $NaDodSO_4$–polyacrylamide gel electrophoresis analyses (10) of ^{14}C-labeled secreted products.

Another powerful feature of combining monoclonal immunoadsorbents and 2-D gel electrophoresis is that antigenic sites can be assigned to distinct peptides within a mixture by peptide mapping on 2-D gels, an approach we are currently using to localize the variable antigenic sites on surface antigens of African trypanosomes.

The widespread use of monoclonal antibodies in biology and medicine requires production of large numbers of myeloma hybrids. The system outlined in this paper will facilitate the screening of such hybrids.

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