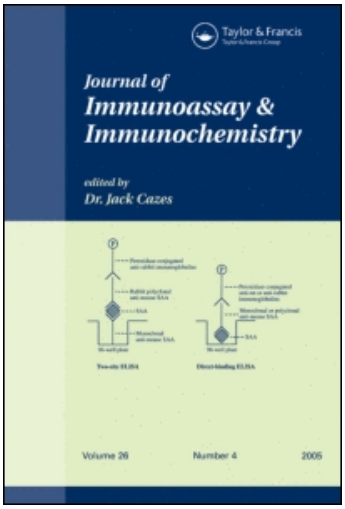

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Michael Brunner[ab]; Stephen Wachtel[ac]; Sing Chin[d]
[a] Center for Reproductive Biology, Collierville, TN [b] Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, NJ [c] Department of Obstetrics and Gynecology, University of Tennessee, Memphis, Tennessee [d] Department of Blood Derivatives,New York, The New York Blood Center, New York

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PURIFICATION AND ANALYSIS OF A SEX-SPECIFIC ANTIBODY

Michael Brunner[1,2], Stephen Wachtel[1,3] and Sing Chin[4]

[1]Center for Reproductive Biology, Collierville, TN 38017
[2]UMDNJ - New Jersey Medical School,
Department of Microbiology and Molecular Genetics,
185 South Orange Avenue, Newark, NJ 07103;
[3]University of Tennessee, Memphis, Tennessee 38163;
Department of Obstetrics and Gynecology,
[4]The New York Blood Center
Department of Blood Derivatives
New York, New York 10021

ABSTRACT

Analysis of the male-specific H-Y antigen is difficult, in part, because of the tendency of "male-specific" antisera to bind cell surface components found in male and in female cells. Some insight as to the nature of that difficulty is provided by biochemical evaluation of H-Y antibody. In this study, purification of a monoclonal H-Y antibody with ammonium sulfate or Protein-A Sepharose, revealed the possibility of microheterogeneity. Despite evidence of multiple subtypes, Protein-A elution profiles suggested that the male-specific activity of the antibody resided in an IgG2a moiety. This was borne out by decreased activity after absorption of the IgG2a subtype with male cells, and by reaction of the monoclonal antibody with mouse subtype-specific antisera in an ELISA. Combined analysis using biological (absorption), biochemical (EF and PAGE) and immunological (ELISA) methods could find applicability in other complex systems.

INTRODUCTION

Histocompatibility-Y (H-Y) antigen is the name given to a male-specific cell surface component that is identified by

antibodies originating in male-sensitized female mice. Although discovered on the cell surface, H-Y has recently been detected in solution -- notably in the supernatant fluid of Daudi cell cultures (see discussion) and in preparation of mouse testis supernatant (TS). Thus reaction of a monoclonal H-Y antibody with mouse TS is detected in the enzyme-linked immunosorbent assay (ELISA), for example, and that reaction is obviated when the antibody is first absorbed with male cells, but not female cells (1). A difficulty with that system is that mouse TS is a crude antigen source; it contains mouse globulin, with which secondary antibody (antimouse Ig) can react. It is therefore useful to conjugate the primary, monoclonal, antibody with enzyme or biotin, directly, to avoid nonspecific binding in the ELISA.

We have described an antibody secreted by a hybridoma clone gw-16, that exhibits male-specific, or more precisely, heterogametic-sex-specific reactivity in the ELISA (2). When purifying antibody gw-16 from ascites fluid, in preparation for attachment of enzyme or biotin, we had the opportunity to evaluate a number of questions concerning the biochemistry of the antibody: (a) whether it consisted of a (single) unique class of immunoglobulin; (b) whether the various fractions contained single components; and (c) whether the various procedures involved in the purification could bring about loss of sex-specific activity.

The techniques employed in purification and the analyses are presented in the following paragraphs.

MATERIALS AND METHODS

Analytical Electrofocusing in Polyacrylamide Gels

Analytical electrofocusing was performed with a LKB Multiphor horizontal electrophoresis apparatus using an Ampholine PAG Plate (LKB) with a pH range of 3.5 - 9.5. Quadruplicate samples were applied and run at a power setting of 30 watts for 1.5 hours and stained with Coomassie Brilliant Blue R-250.

Polyacrylamide Disc Gel Electrophoresis

Samples were denatured by heating in a buffer containing 1% sodium doedecyl sulfate (SDS), 50 mM Tris pH 6.8, 5% glycerol, bromphenol blue and 3% v/v β-mercaptoethanol to obtain reducing conditions, and subjected to electrophoresis on a 12% acrylamide vertical slab gel as previously described (3). Proteins were stained with Coomassie Brillant Blue R-250.

DEAE Affi-Gel Blue Chromatography

One ml of centrifuged ascites fluid was added to a 7 ml column of DEAE Affi-Gel Blue (Bio-Rad) according to Bruck et al. (4). The column was washed and the fractions were eluted with a linear 0-100 mM NaCl gradient. Peak fractions were tested for IgG by the ELISA; fractions 40-70 were concentrated by ultrafiltration (Amicon).

Direct Cell Binding Assay

Ninety-six well tissue culture plates (Falcon) were blocked with 1% gelatin in phosphate-buffered saline to which male or female C57BL mouse lymph node cells were added (2×10^5 cells per well). The cells were reacted with dilutions of ammonium-sulfate-precipitated (Protein-A column) "pass-through" fractions, along with dilutions of ascites fluid and/or purified antibody as positive controls. The reaction was analyzed by use of β-galactoside-conjugated $F(ab')_2$ sheep antimouse antibody (BRL) with 2 mM monothioglycerol in place of 2-mercaptoethanol (5) and p-nitrophenyl-β-D galactoside as substrate. Removal of reagents and cell washings was accomplished by centrifugation of the plates at 400 x g followed by manual discarding of supernatant. Results of the ELISA were read at 414 nm wavelength on a Titertek Multiscan MC reader.

Mouse Monoclonal Subtype Assay

The mouse Ig subtype identification kit (Boehringer Mannheim) depends on rabbit antimouse subclass specific sera to detect the primary reaction between the antibody (ascites fluid) and its antigen (mouse TS or Daudi supernatant) coated on a plate. A peroxidase-labeled goat antirabbit IgG is subsequently reacted and 2,2'Azino-di-(3-ethyl-benzthiazoline sulfate) substrate is used to generate a green color read at 414 nm on an ELISA plate reader.

Biotinylated ELISA

Ascites fluid was purified, biotinylated, and used in an ELISA as in our previous study (1). PA purified biotinylated antibody was absorbed with twelve million male or female C57BL mouse spleen cells on ice for 1 hour and used in the ELISA at 1/340 (2).

Ascites Fluid ELISA

Ascites fluid was absorbed with five million male or female C57BL mouse lymph node cells on ice for one hour and used in an ELISA. The plate was coated with Daudi supernatant (antigen source) and blocked with 0.5% gelatin. Primary antibody (absorbed and unabsorbed ascites fluid) was then reacted at 1/90 dilution. Peroxidase-labeled goat-antimouse (Tago) was then applied and orthophenylenediamine used as substrate. The plate was read on ELISA reader at 492 nm.

H-Y Antibody

The monoclonal antibody GW-16 (ATCC designation HB9487) was described previouslly (17). Briefly, spleen cells from male-sensitized B6 female mice were fused with P3/NS1/1-Ag4-1 myeloma cells and the hybridomas were selected, cloned, and recloned several times using a cytotoxicity test with supernatants from microtiter wells. The hybridomas were passaged as ascites tumors in (BALBXB6) F_1 female mice.

RESULTS AND DISCUSSION

Purification of Antibody

A number of purification techniques were applied. The DEAE Affi-Gel Blue method (4) gave an immunoglobulin peak distinct from the peaks for transferrin and albumin (Fig. 1). But the method was slow and tedious, involving salt gradient elution and concentration of the globulin, and thus inappropriate for general use.

Ammonium sulfate precipitation (43% saturation) gave a "semi-pure" product (Figs. 2, 3), but affinity chromatography with Protein-A (PA) Sepharose, with or without prior ammonium sulfate treatment, proved to be the quickest and most efficient method (2).

The general method calls for application of the sample to the PA column at pH 7-8 and elution at pH 3.5, in glycine-HCl buffer. To reveal separate subclass activity that might be present, we reduced the pH gradually from 6 to 4.5 (citrate buffer) and then to 3.5 (glycine-HCl buffer), during elution of the antibody according to the method of Ey et al. (6). Only one sharp peak was obtained at pH 4.5 (Fig. 4), representing the point at which the major portion of IgG_{2a} is expected to appear (the OD of the pooled fractions representing the peak was equal to the OD of the total protein applied to the column). It should be emphasized that occurrence of the single peak does not rule out

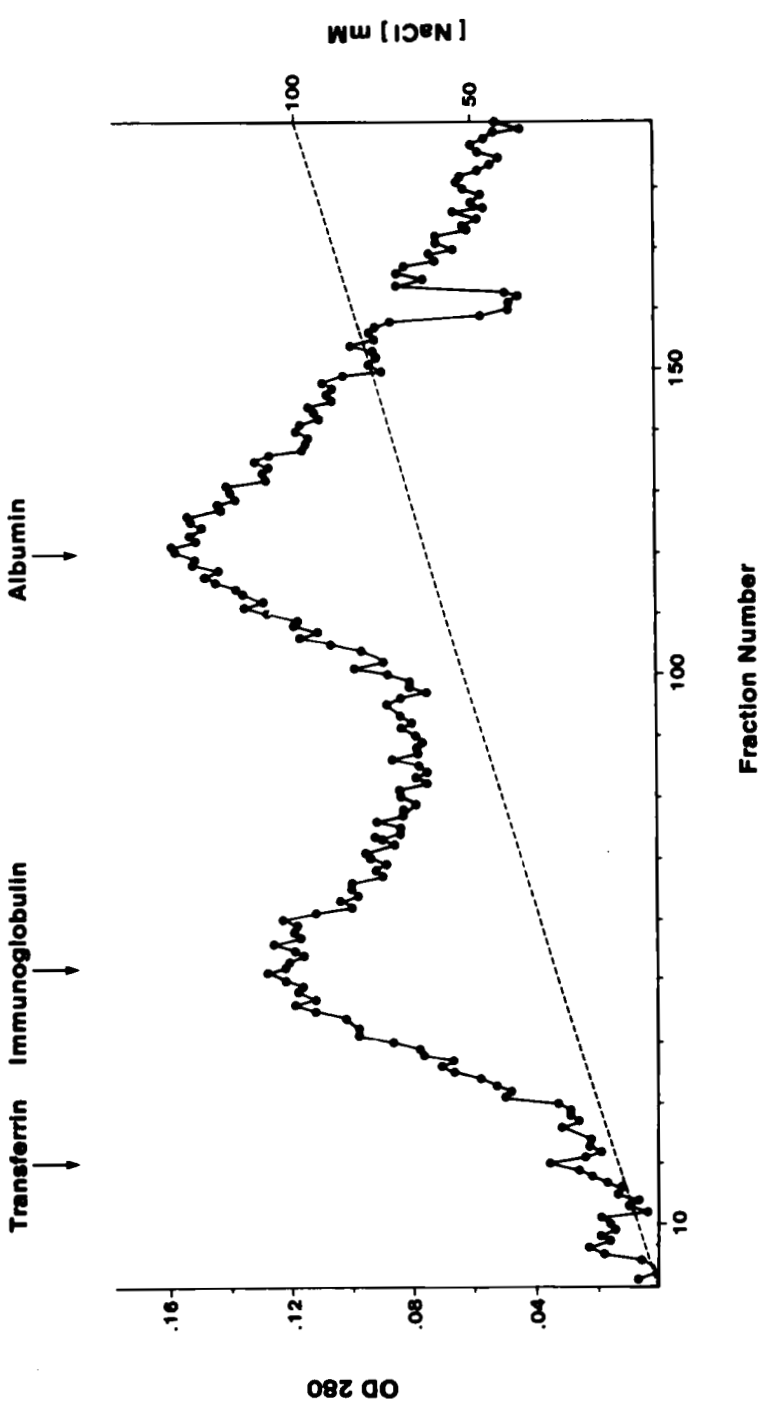

FIGURE 1. Elution of monoclonal H-Y antibody from a DEAE Affi-Gel Blue column. Ascites fluid was ultracentrifuged, dialyzed and layered on a 7 ml column in 20 mM Tris-HCl, pH 7.2. One ml fractions were eluted by a 0-100 mM NaCl gradient and peak fractions (arrows) were tested by FLISA for IgG.

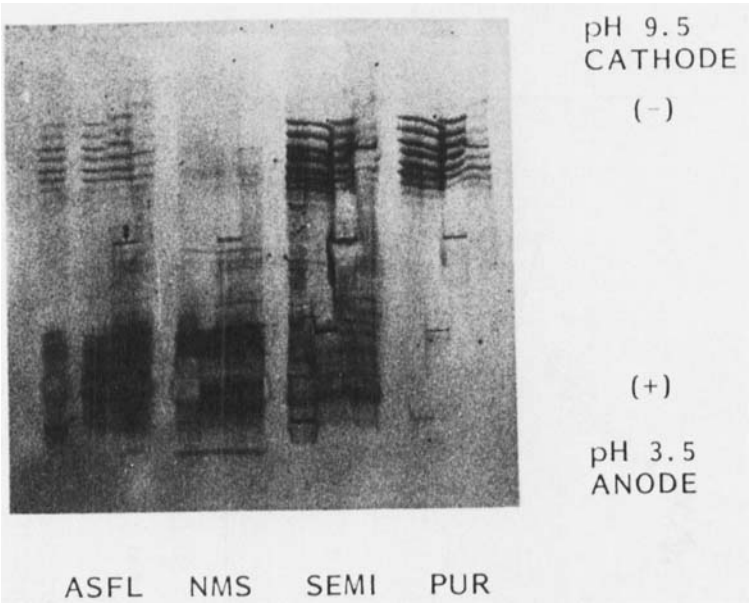

FIGURE 2. Analytical electrofocusing of gw-16. Quadruplicate samples were run on PAG plate with a pH range of 3.5 - 9.5. ASFL, ascites fluid (lanes 1-4); NMS, normal mouse serum (lanes 5-8); SEMI, semipurified antibody (lanes 9-12); PUR, purified antibody (lanes 13-16). Note heterogenous bands near the cathode (top) darker in SEMI and PUR and virtually absent in NMS. Other serum proteins (eg. albumin) migrated towards the anode.

the presence of IgG_1, the majority of which might be expected to elute at pH 6, because this column does have a capacity, albeit reduced, for IgG_1 (7). In fact the portion of IgG_1 that did bind (when present) eluted at pH 4.5 together with the IgG_{2a}, and was thereby obscured.

Column "pass through" fractions were precipitated with ammonium sulfate and tested for direct binding on male or female mouse lymph node cells, during similar tests with ascites fluid

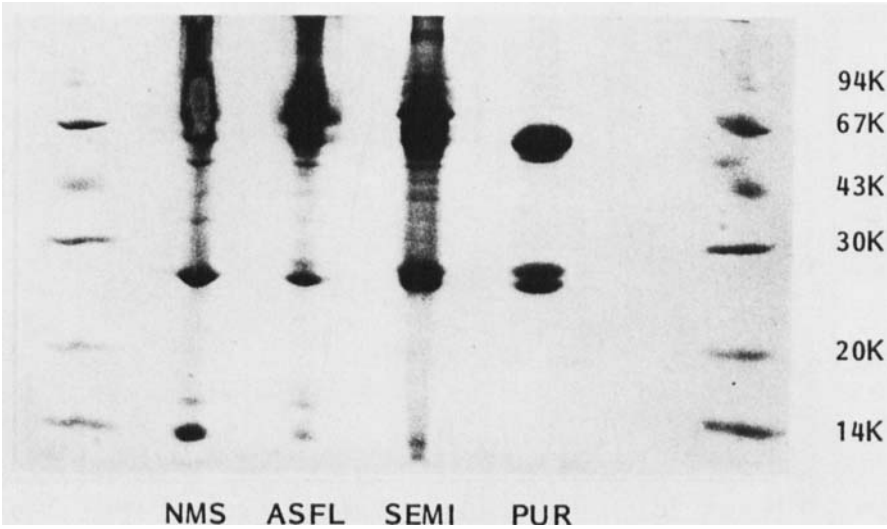

FIGURE 3. Polyacrylamide disc gel electrophoresis of monoclonal antibody. Reduced samples were analyzed by running on gels. NMS, normal mouse serum; ASFL, ascites fluid; SEMI, semipurified; PUR, purified antibody. A doublet at the light chain position is apparent in PUR; the doblet and the heavy chain are the only proteins apparent in the preparation.

and purified antibody. No activity above background was observed in the pass-through fractions.

Analytical Electrofocusing

Antibody obtained from ascites fluid by sequential purification, was analyzed by electrofocusing (EF) and reduced SDS-polyacrylamide disc gel electrophoresis. The EF patterns obtained upon sequential purification of ascites fluid (corrected for protein by diluting 1:10 to approximate the dilution of

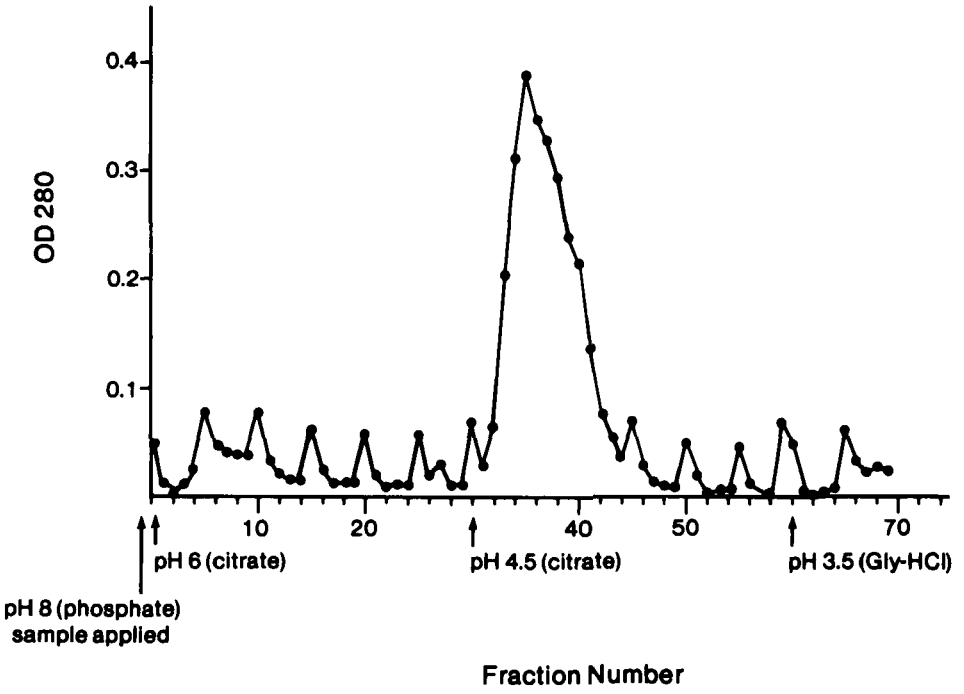

FIGURE 4. Sequential elution of antibody from PA Sepharose by stepwise decrease in pH. Eluted fractions were read at OD 280. Major peak fractions eluting at pH 4.5 were pooled for further use as purified antibody.

purified or partially purified antibody) is represented in lanes 1-4 (ASFL). Note bands of pI 8 near the cathode; these indicate microheterogeneity of the globulin (antibody). Bands representing other serum proteins (eg. albumin) were found near the anode. Lanes 5-8 (NMS) contain bands from normal mouse serum, a negative control exhibiting reduced or neglibible globulin content in comparison with that of the ascites fluid. Lanes 9-12 (SEMI), containing the partially-purified sample, corrected for protein,

exhibit dark globulin bands. This is due to a concentrating effect of precipitation with 43% ammonium sulfate. Note that other serum proteins are present. Lanes 13-16 (PUR) show dark bands of pure globulin, present after affinity chromatography with PA Sepharose. In this case other serum proteins are absent.

Seven closely-spaced bands were noted in our EF patterns of crude, partially-purified and purified antibody (Fig. 2). Similar patterns were noted previously upon electrofocusing of preparations of polyclonal and monoclonal antibody in other laboratories (8-13). The palisade pattern that we obtained has also been described previously with monoclonal antibodies (8, 9), and has been attributed to a variety of causes, including molecular "sieving" effects of the polyacrylamide gel (10), complexing of homogenous protein with various ampholine species (13), and even post-synthetic modifications of monoclonal antibody (resulting in alterations in electrical charge when the antibody comes into contact with certain serum components -- as in passaging the hybridoma, and purifying the antibody from serum and ascites fluid).

SDS Disc Polyacrylamide Gel Electrophoresis

To analyze the antibody heavy and light chains obtained after sequential purification of the monoclonal antibody, we performed SDS disc gel electrophoresis of serum proteins under reducing conditions. A representative gel is depiced in Fig. 3. Note that

albumin and other high and low molecular weight proteins that were still present in the partially purified sample disappear in the PA Sepharose purified sample. Relatively pure collections of heavy chains (mol. wt. 50,000) and light chains (mol. wt. 25,000) remained. The doublet at the light chain position could signify presence of κ and λ (light) chains. The faster migrating κ chain could be contamination from the NS-1 myeloma (a parent of the hybridoma) or alternatively an artifact of the procedure. In any event differential mobility of κ and λ chains has been reported previously (14) and has been attributed to differences in the binding capacity of SDS for κ and λ chains, differences in isoelectric points, differences in carbohydrate content, or molecular configuration.

Immunological Subclass of the Monoclonal

Analysis of the ascites fluid by Ouchterlony immunodiffusion in the presence of commercial anti-class and -subclass antibodies (Miles Laboratories) revealed the presence of class IgG, the absence of class IgM, and the presence of subclass IgG_1 molecules. Analysis with the Boehringer-Mannheim subclass ELISA test kit, with mouse TS as H-Y antigen source (1), revealed the presence of both IgG_1, and IgG_{2a} heavy chains, and κ light chains with lesser amounts of λ chains (Fig. 5).

Sex Specificity of the Antibody

Specific binding of the antibody to male cells could be

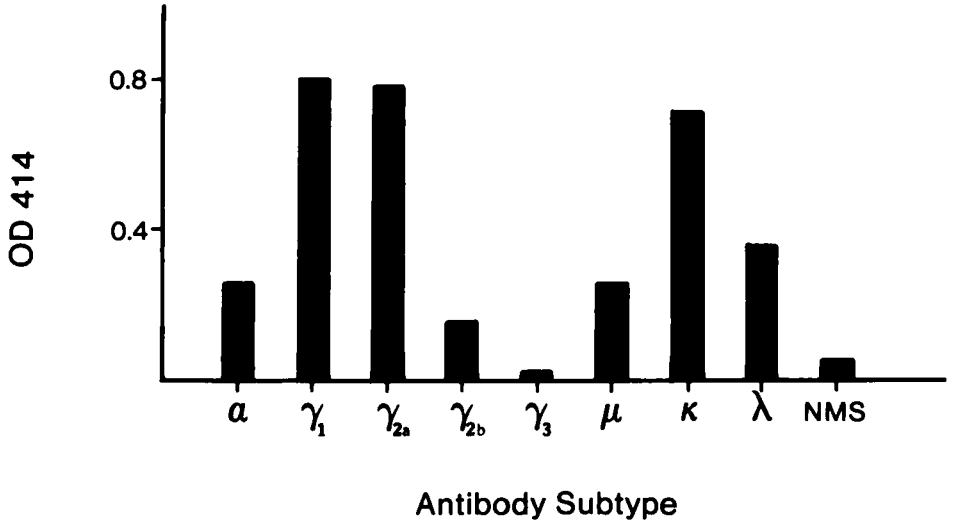

FIGURE 5. Subtype analysis of monoclonal H-Y antibody. The antibody reacted with testis supernatant bound to the plate. Then rabbit antimouse subtype serum was added; the resulting OD from peroxidase labeled antirabbit is represented as a bar graph.

demonstrated by serological absorption. Cells from highly inbred male or female mice were suspended in equal portions of the antibody; after 1 hour the antibody was tested for residual activity -- as in the ELISA. Loss of activity of male-absorbed antibody in comparison with the activity in female-absorbed or unabsorbed antibody provided a measure of the sex-specificity of the antibody. Fig. 6 shows two examples of the results obtained in this type of procedure. Two different preparations of biotinylated, purified antibody were employed in the ELISA. The antibody was divided into equal portions: one portion was absorbed with lymph node cells from C57BL female mice; another

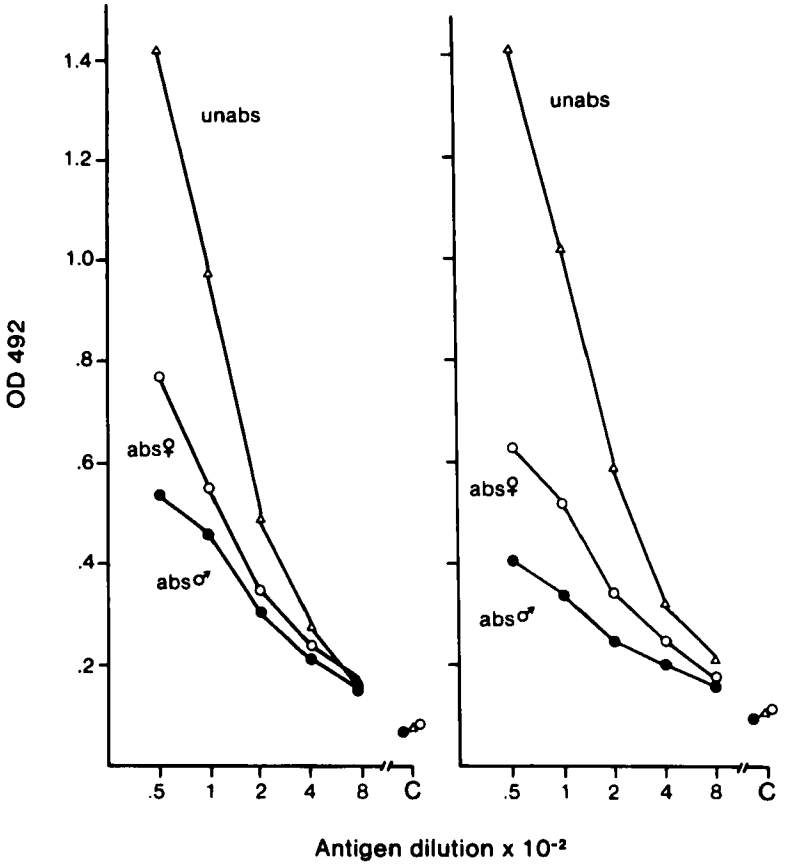

FIGURE 6. Absorption with biotinylated antibody demonstrates sex specificity in the ELISA. Two similar results underscore dependability and stability of the antibody preparations. One experiment was performed with newly biotinylated antibody, whereas, the other was performed with antibody maintained at 4°C for two months. Twelve million of each of male (●) and female (○) spleen cells were used for the absorption. Unabsorbed control (△) refers to antibody without cells. Mouse testis supernatant was used as H-Y antigen source.

portion was absorbed with corresponding cells from C57BL male mice; and the third was unabsorbed.

In similar experiments using ascites fluid as primary antibody source instead of biotinylated antibody, with peroxidase labeled goat-antimouse as second antibody in a conventional ELISA, three similarly-treated portions were tested for residual activity against Daudi cell supernatant fluid (H-Y antigen source). The results of two repeated experiments indicated male-specific binding (Fig. 7a, b). To determine whether that male-specific binding could be assigned to a particular IgG subclass, we reacted aliquots of the absorbed antibody in the Boehringer-Mannheim subclass ELISA, again using Daudi supernatant fluid as an antigen source. The results are depicted in Fig. 7c, d. The values for both IgG_1 and IgG_{2a} were reduced after absorption with male cells. Male specificity of the subclass reaction (Fig. 7a, b) could nevertheless be attributed to the IgG_2a moiety, since the values for the female-absorbed antibody approximated those for the unabsorbed aliquot in tests with IgG_{2a}, whereas this did not occur in tests with IgG_1.

CONCLUSION

The ascites fluid from which our antibody is collected contains IgG_1 and IgG_{2a} heavy chains, and κ and λ light chains. Indications are that antibody gw-16 is an IgG_{2a} monoclonal, but there are evidently alternative pairs of immunoglobulin heavy and

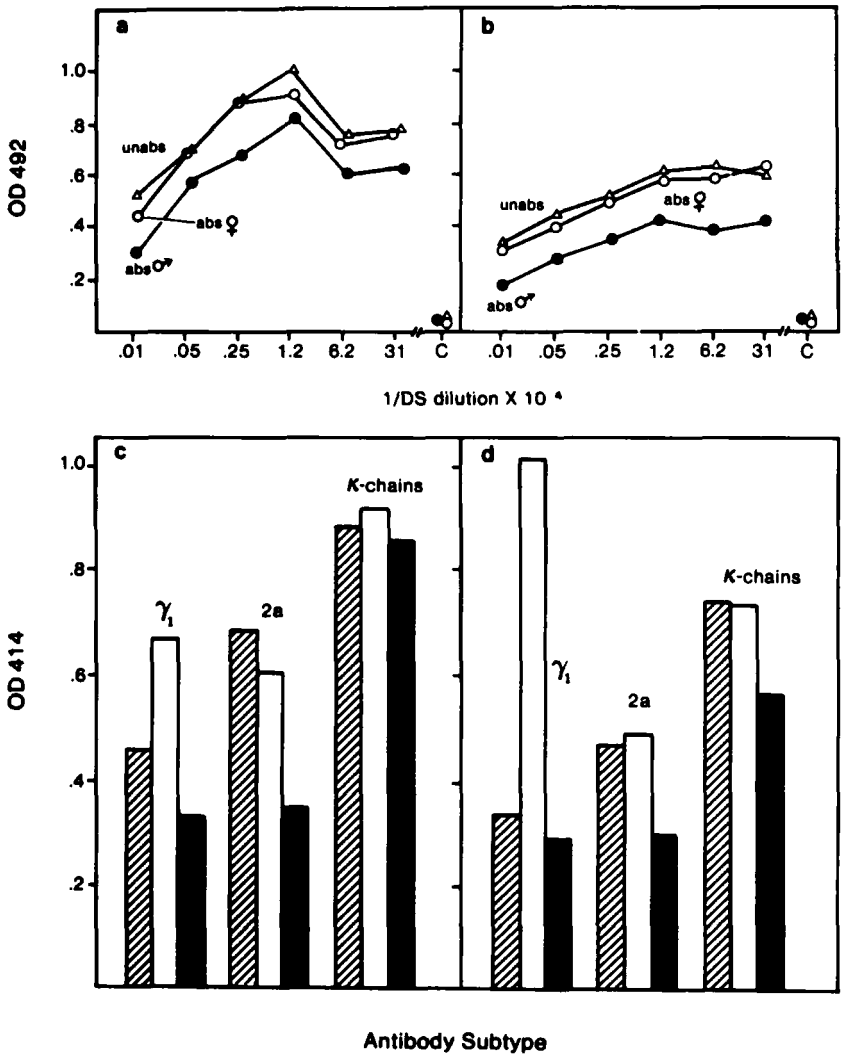

FIGURE 7. Sex specific absorption and corresponding analysis of antibody subtype activity. 7a, 7 b are two experiments showing ELISAs with ascites fluid (gw-16) used for absorption of male (●) or female (○) lymph node cells (5×10^6) or unabsorbed control (△) (i.e., antibody with no cells) followed by addition of peroxidase labeled antimouse antibody to assay for residual ascites fluid (antibody) activity. This consisted of that antibody which after absorption reacted with Daudi supernatant bound to microplate as H-Y antigen source. 7c, 7d represent experiments corresponding to figs 7a, 7b respectively, demonstrating subtype ELISAs of the absorbed ascites fluid. Daudi supernatant was used as H-Y antigen source. Cross-hatched bars represent activity of unabsorbed antibody; open bar and black bar represent activity of female-absorbed and male-absorbed antibody respectively.

light chains in the ascites fluid. Appearance of these alternative pairs could be artifactual, or due to the presence of a contaminant immunoglobulin lacking specificity for the H-Y antigen. As to the question which of those chains is responsible for the male-specific activity of the monoclonal antibody, some 95% or more of mouse immunoglobulins contain κ light chains (15). It could be argued that the presence of the κ species is due to secretion by the myeloma parent, or alternatively that it is a contaminant or artifact. But the fact that the antibody can be diluted 1/1000 in the ELISA and retain activity argues for its contribution to the sex-specificity of the monoclonal (and in fact there is a hint of male-specific reactivity of κ in Fig. 7c, d). Recently, Ware et al. (16) has described a rat antimouse kappa-chain-specific monoclonal antibody that we might be able to use to ascertain the specificity of the light chain in our antibody. This antimouse kappa-chain should identify only kappa-specific mouse antibody in an ELISA when used in lieu of goat antimouse.

Our gw-16 anti H-Y monoclonal appears to have specificity in the IgG_{2a} heavy chains because (i) the subclass ELISA shows strong IgG_{2a} reactivity, (ii) the elution product of the PA column (which has a limited capacity for IgG_1) is sex-specific, (iii) the column pass-through fraction (most likely IgG_1) is not sex-specific, (iv) the subclass ELISA using absorbed IgG_{2a} heavy chains resembles the ELISA with absorbed intact antibody, and (v) the major peak from the PA column has sex specific reactivity and elutes at pH 4.5 --

at which IgG_{2a} is known to elute. Furthermore, this monoclonal antibody was originally selected for complement-mediated cytotoxicity against mouse epididymal spermatozoa, and IgG_1 does not fix complement.

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Correspondence should be addressed to Dr. Michael Brunner, Department of Microbiology and Molecular Genetics, New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103 USA.

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