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MONOCLONAL ANTIBODIES AGAINST OVALBUMIN

Robert J. Bjercke, Peggy M. Hale,

Wayne D. Mercer, and William L. McGuire

University of Texas Health Science Center

Department of Medicine, San Antonio, Texas 78284

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SUMMARY: Fusion of cells of the NS-1 mouse myeloma line with spleen cells from BALB/c mice immunized against ovalbumin produced hybrid cells which continuously secrete antibodies specific for ovalbumin. One of these cells was used to establish a cloned line. Studies of its antibody obtained either from ascites fluid or from medium from hybridoma cultures showed high titer and specificity against ovalbumin using the double antibody technique with rabbit anti-mouse immunobeads; the antibody proved to be of the IgG_1 (kappa) subclass and type.

The application of cell hybridization technology to the fusion of spleen cells from immunized animals and myeloma cell lines has produced continuous cell lines that secrete monoclonal antibodies (1-3). The majority of the studies have involved the production of monoclonal antibodies specific for various cell surface antigens, such as histocompatibility antigen (3, 4), T lymphocyte antigen (5, 6, 7), human HLA and blood group antigens (8), and tumor cell antigens (6, 9, 10). More recent studies have produced monoclonal antibodies specific for purified soluble antigens (11-13). Hybridoma reagents make it possible to examine individually a complex set of determinants from a single antigen molecule, and hence may be used for determining the full fidelity of transcription and translation of gene sequences transferred to different cell types of various species. The cloning of the full length chicken ovalbumin gene (14) makes the ovalbumin molecule a candidate for such studies.

METHODS

Cell Lines and Reagents: BALB/c mice were purchased from Charles River, Boston, MA. The cell line NS-1 (P3-NS-1-Ag4-1) was generously supplied by Salk Distribution Center, La Jolla, CA. Ovalbumin was purchased from Cappel Labs. Cultures were maintained in RPMI-1640 medium with glutamine (GIBCO, Grand Island, N.Y.) supplemented

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with 15% New Born Calf serum (NBS; GIBCO), 10 units/ml penicillin, 100 µg/ml streptomycin.

Immunization: Ovalbumin in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 at 24°C) was emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously (s.c.) in four 8 week old female BALB/c mice. Each dose consisted of 500 µg ovalbumin and multiple doses were given s.c. in equal volumes of incomplete Freund's adjuvant at monthly periods. A positive serum titre was obtained after three injections. The final fourth dose prior to fusion was given intraperitoneally (i.p.) without adjuvant.

Spleen Cell Hybridization: Spleens were removed from immunized animals four days after the final injection and cell suspensions were made through a stainless steel screen followed by passages through 18, 22, and 25 gauge needles in serum-free RPMI-1640 medium. Red blood cells were lysed in 0.83% NH_4Cl at room temperature for 90 seconds. Fusion was performed by a modification of the procedure described by Milstein (2). Mouse myeloma cells (final 5×10^6 per ml) were mixed with spleen cells (final 2×10^7 per ml) and centrifuged at 400 xg for 5 minutes. The cells were suspended in 50% polyethylene glycol (PEG 1500, Koch-Light, Research Products International, Elk Grove, Ill.) and diluted to a final 5% PEG concentration over a 10 minute period in Ca^{+2} and serum-free RPMI-1640 medium. After centrifugation at 400 xg for 10 minutes the cells were plated at 10^6 cells/well in 96-well microtest dishes (Falcon 3040) in RPMI- 1640 containing 15% heat-inactivated NBS overnight. The medium was changed to medium containing hypoxanthine-aminopterin-thymidine (HAT) for hybrid selection. After daily feeding with HAT medium for three days, fresh medium was added at 3-4 day intervals for three weeks. Supernatants from wells were assayed for ovalbumin binding activity (see below) and switched to HT medium, excluding aminopterin, for the next several feedings. Cells from positive wells were cloned by limiting dilution in microtest wells. Clones were injected i.p. at $5\text{-}10 \times 10^6$ hybridoma cells into BALB/c mice injected i.p. two weeks previously with 0.5 ml pristane (Aldrich Chemicals, Milwaukee, WI). Ascites fluid began to accumulate 7-14 days later and was collected by insertion of an 18 gauge needle into the peritoneal cavity.

Radioiodination of Antigens: Antigens were radioiodinated using either the Chloramine T or lactoperoxidase methods. The entire procedures were carried out in the hood. Using the Chloramine T method, 10 µg of cytosol proteins isolated from chick oviducts or 1.25 µg of purified chicken ovalbumin (Cappel Laboratories, PA) were mixed with 200 µl of 0.2 M Na phosphate, pH 7.4, and 3 mCi of Na ^{125}I (Amersham/Searle, 13-17 mCi/µg). The reaction solution was kept in an ice bath. A solution of 1 mg/ml of Chloramine T in 0.2 M phosphate buffer, pH 7.2, was prepared fresh, kept on an ice bath, and diluted 1:8 with phosphate buffer just prior to use. Fifty microliters of diluted Chloramine T were added to the reaction solution, which was then shaken vigorously for 1 min. The reaction was allowed to continue for an additional 5 min. Precipitability with trichloracetic acid (TCA) was then checked by adding 5 µl of the reaction solution to 1 ml of phosphate buffer, followed by addition of 50 µl of this diluted reaction solution and 75 µl of 3% bovine serum albumin (BSA) to 0.5 ml of 6% TCA. The entire solution was vortexed and centrifuged. Supernatants and pellets were counted.

Using the lactoperoxidase method, 10 µg of purified ovalbumin were reacted with 1 mCi of Na ^{125}I and Enzymobead™ Reagent (Bio-Rad, Richmond, CA) as described in the pamphlet accompanying the reagent. Using either method of radioiodination, TCA-precipitabilities of 15-30% were obtained. Note that only the Chloramine T method of radioiodination was used with oviduct cytosol proteins.

Unreacted ^{125}I was removed using a Sephadex G10 column in the case of oviduct cytosol proteins and a Sephadex G75 column (both 0.6 x 20 cm, Pharmacia) in the case of ovalbumin. Columns were equilibrated with 0.15 M NaCl, 10 mM Na phosphate, pH 7.4, (PBS), plus 0.1% BSA using the lactoperoxidase method and minus BSA using the Chloramine T method. In the case of ovalbumin, unreacted ^{125}I was still associated with the protein peak from the column. Further removal of free ^{125}I was achieved by dialysis

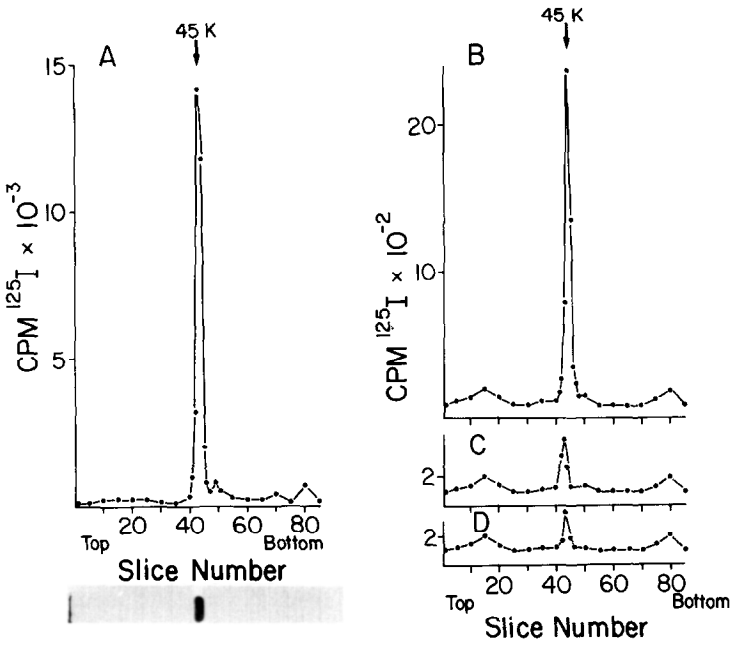

Figure 1: SDS polyacrylamide gel electrophoretic profiles. (A) ^{125}I-labeled ovalbumin used to screen hybridomas for production of antibody to ovalbumin. Below (A) is stained gel of the ovalbumin before labeling, injected as antigen. (B) Extract from immunobeads after incubation (see Methods) with ^{125}I- labeled ovalbumin and ascites fluid (1:20,000 dilution) from mouse injected with cloned hybridoma. (C) Same as B but also including 25 µg cold ovalbumin in the incubation. (D) same as B but omitting ascites fluid.

for 24 hrs against 100 volumes of 6 M urea, 10 mM Tris, pH 7.2, followed by 2 changes of 100 volumes each of PBS plus BSA. Radiolabeled antigens were stored at -20° C in PBS-50% glycerol.

Antibody Assay: Immunobeads (Biorad, Richmond, CA) were used as second antibody to bind mouse antibodies. (^{125}I)ovalbumin (40,000-80,000 cpm) was added to the monoclonal antibody to be assayed and was allowed to shake overnight at 4° C. (One µg of ovalbumin were added in cold competed assays.) Fifty µg rabbit anti-mouse immunobeads was added and allowed to shake 4 hours at 4° C. The beads were washed three times with PBS and counted in the gamma counter. For some experiments, beads were then extracted overnight at room temperature with SDS gel sample buffer consisting of 6 M urea, 1% SDS, 5% β-mercaptoethanol, and 0.125 M Tris, pH 6.8. The extract was then loaded onto SDS-polyacrylamide gels for electrophoresis.

Analysis of Antibody Class and Type: Ovalbumin adsorbed to acrylamide beads was used to bind mouse antibodies (15). The appropriate antibody or a blank with RIA buffer (0.01 M sodium phosphate, 0.15 M NaCl, 0.1% gelatin, 0.1% sodium azide, pH 6.9 at 4°C) were incubated with ovalbumin beads for 60 min at room temperature. The beads were washed three times with PBS buffer. Beads were incubated 60 min at RT with 1:100 dilutions in RIA buffer of rabbit antisera specific for mouse IgM, total IgG (H+L chains), IgG, IgG_{2A}, IgG_{2B}, IgG_3, IgA, and kappa or lambda light chains (Miles Research, Elkhart, IN.) After washing the beads the beads were incubated for 60 min at room temperature with (^{125}I)$F(ab')_2$ goat anti-rabbit antiserum. The beads were counted in a gamma counter after additional washes in PBS.

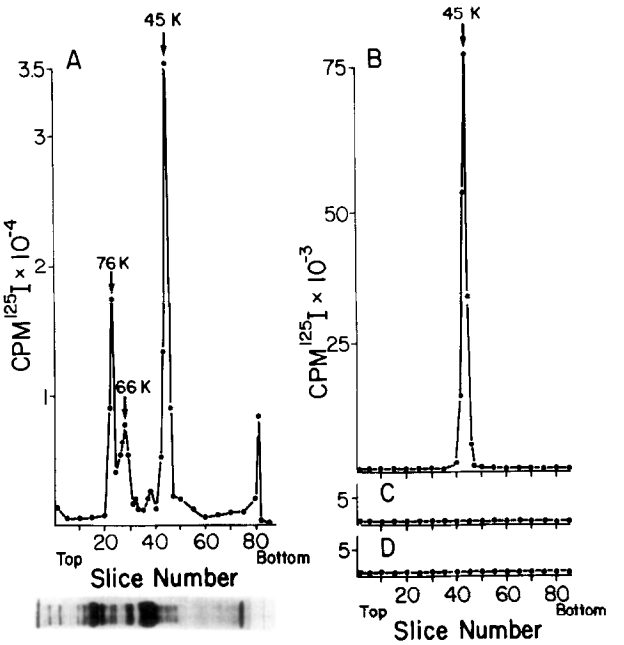

Figure 2: SDS polyacrylamide gel electrophoretic profiles. (A) ^{125}I-labeled oviduct cytosol proteins used to show specificity of monoclonal antibody from cloned hybridoma. Below (A) is stained gel of oviduct cytosol. (B) Extract from immunobeads after incubation (see Methods) with ^{125}I-labeled oviduct cytosol proteins and ascites fluid (1:20,000 dilution) from mouse injected with cloned hybridoma. (C) Same as B but also including 25 µg cold ovalbumin in the incubation. (D) Same as B but omitting ascites fluid.

Polyacrylamide Gel Electrophoresis: Polyacrylamide gel electrophoresis was performed in a slab apparatus (Bio-Rad Model 220) according to the method of Laemmli (16) as modified by Wyckoff et al. (17). Gels were sliced into 1 mm slices and counted in a gamma counter.

RESULTS AND DISCUSSION

Approximately 90% of the microtitre wells plated with fused cells grew hybridomas. To detect those hybrids producing antibodies specific for ovalbumin, supernatants from 9 microtitre dishes were pooled in 27 groups. These were tested for the presence of ovalbumin by the double-antibody technique with immunobeads. Eighty-one percent of the pooled supernatants proved positive, of which five pooled hybridoma fractions maintained ovalbumin binding after several cell divisions. Cloned cell lines were obtained by limiting dilution with the hybridoma with the highest binding activity being re-cloned. The ascites fluid obtained from pristane-primed mice injected with cloned hybridoma cells was titrated at 1:50,000 dilution for maximum ovalbumin binding using the immunobead assay.

Table 1

ANTIBODY TYPING

Binding by isotype specific antibodies, cpm

Sample	Kappa	Lambda	IgM	IgG (H+L)	IgG_1	IgG_{2A}	IgG_{2B}	IgG_3	IgA
(1)	2500	551	487	4279	2190	128	1408	611	384
(2)	2205	461	94	2780	1911	251	386	375	378
(3)	1610	40	35	2095	1320	30	192	75	146

(1) Serum from immunized mice
(2) Pooled ovalbumin hybridomas
(3) Ovalbumin hybridoma clone

Ovalbumin adsorbed beads (20% w/v in PBS) in 0.1 ml were incubated with 0.5 ml of 1:100 dilution in RIA buffer of pooled mouse serum from tail bleedings of immunized mice, or ascites from pristane primed mice injected either with the pooled hybridoma cells or with the cloned hybridoma cell. A second incubation was performed with the beads and 0.1 ml of 1:100 dilution in RIA buffer of rabbit antisera specific for mouse antibody subclasses and isotypes. ^{125}I-labeled $F(ab')_2$, 30,000 cpm, was incubated with the beads during a third incubation. All incubations were performed for one hour at room temperature (see Methods for other details). Blank values, obtained by substituting RIA buffer and omitting the serum and ascites during the first incubating, ranged from 400-800 cpm using the various typing reagent. Blank values have been subtracted from experimental values shown above.

The chromatographically purified ovalbumin used as antigen was shown to contain a single band on SDS gels (Fig. 1). The radioiodinated ovalbumin, using either the chloramine T or lactoperoxidase method for iodinating the ovalbumin, showed a single radioactive band with the same mobility as the stained protein band (Fig. 1, A). When (^{125}I) ovalbumin was immunoprecipitated with the cloned mouse hybridoma antibodies adsorbed to rabbit anti-mouse immunobeads, the extract of the immunobeads with urea-gel buffer (Fig. 1, B) show a single radioactive peak on SDS-gels identical to that obtained for (^{125}I) ovalbumin when radioactive ovalbumin was competed with cold ovalbumin (Fig. 1, C), radioactivity on the gels was reduced to the same similar background levels as obtained omitting the mouse anti-ovalbumin antibodies (Fig. 1, D).

To further confirm specificity, we used total ^{125}I-protein from oviduct cytosol. (It was necessary to use the chloramine T method to iodinate chick cytosol proteins as lactoperoxidase was ineffective.) Ovalbumin is the major protein obtained from chick oviduct (Fig. 2) and is the major radioactive peak of the iodinated cytosol proteins (Fig. 2, A). The mouse antibody selectively binds the native (^{125}I)ovalbumin from oviduct

cytosol, as shown by its elution as a single peak from the immunobeads (Fig. 2, B). Binding of the (^{125}I)ovalbumin by the monoclonal antibody was fully competed by cold ovalbumin (Fig. 2, C) and only background levels of radioactivity were obtained from immunobeads incubated with labeled cytosol but without mouse anti- ovalbumin before the elution with urea (Fig. 2, D).

The subclass and type of the new monoclonal antibody were determined with a series of specific antisera and found to be IgG1 (kappa) (Table 1). For comparison, the analyses of the original hyperimmune mouse serum and the pooled hybridoma supernatant are also shown. We are now cloning four other independently arising hybridomas with anti-ovalbumin specificities. Only one other report of a monoclonal antibody against ovalbumin has appeared (18), in that case an IgE intended for allergy studies. Monoclonal anti-ovalbumin antibodies can be used to examine the amount and fidelity of transcription, translation, and glycosylation of ovalbumin from cloned chicken genes incorporated into cells of other species.

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