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MONOCLONAL ANTIBODIES TO CONNECTIVE TISSUE MACROMOLECULES: TYPE II COLLAGEN*

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SUMMARY: A monoclonal antibody against chick type II collagen has been produced by lymphocyte-myeloma cell hybridization. The antibody, harvested either from spent medium of hybridoma cultures or from ascites fluid of hybridoma-containing mice, has an extremely high titer against type II collagen but shows no activity against type I. The antigenic site of the collagen seems to be located within the helical portion of native molecules. Using fluorescence histochemical procedures, the antibody can be used to localize type II collagen in sectioned material.

INTRODUCTION: To advance our biochemical and cytological investigations of the extracellular matrices involved in morphogenesis, we have undertaken the production of monoclonal antibodies to the various genetic types of collagen found in the embryonic chick. Monoclonal antibodies produced by lymphocyte-myeloma hybrids, hybridomas, have a number of highly desirable characteristics, including a single definable specificity, reproducibility, and large yields of immunoglobulin (1,2). We have recently reported the production of a monoclonal antibody to chick type I collagen (3), and now we report the production of a monoclonal antibody to chick type II collagen. This collagen, once thought to be found exclusively in cartilage (4), has recently been identified as a major constituent of several extracellular matrices in the developing chick eye (5-7), sometimes in association with other collagens. The early corneal epithelium synthesizes both types I and II

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ABBREVIATIONS: HAT = hypoxanthine-aminopterine-thymidine; RBC = red blood cells; PEG= polyethylene glycol; PBS = phosptate-buffered saline, pH 7.2 (17).

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(5), which are deposited in the primary corneal stroma (8). The neural retina produces about 90% type II collagen and 10% of a new genetic type (7). Thus, monoclonal atibodies to type II will be extremely useful for research on both the developing eye and skeletal system.

MATERIALS AND METHODS: Preparation of Collagens

Type II collagen was extracted by limited 4°C pepsinization of adult chick sternal cartilages. Neutral salt soluble type I collagen was extracted from lathyritic chicks with 0.4 ionic strength potassium phosphate buffer pH 7.6. All of the collagens were purified by multiple salt and ethanol precipitations from neutral solutions and salt precipitations from acid solutions. Type II collagen was separated from type I by repeated fractional salt precipitations.

Preparation of mouse lymphocytes and cell hybridization

Parental mouse myeloma cells (MPC 11 45.6 TG 1.7) (9) were cultured in complete medium composed of Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum plus antibodies (all obtained from GIBCO). Splenic lymphocytes were obtained from ASW/SN mice (Jackson Laboratories) which had previously been injected with approximately 0.2mg of chick type II collagen in complete Freund's adjuvant, and several months later had received a booster injection of collagen in incomplete Freund's. Three days after the booster, two mice were killed by cervical dislocation, the spleens removed and minced, and the cells liberated by agitation on a vortex mixer.

Four simultaneous hybridizations were performed using PEG according to Gefter et al (10). Myeloma cells (10^6) were washed twice in serum-free medium and then mixed with 1-3 x 10^7 spleen cells. The cells were centrifuged at 250 g for 5 min and 0.2 ml of 37.5% PEG in serum-free medium was added. The cells were resuspended and then centrifuged for 3 min at 350 g, followed by 2.5 min at 500 g, and were allowed to sit undisturbed for 2.5 min. The cells were washed with serum-free medium, dispersed in 20 ml of complete medium, and put into a 37°C humidified incubator for 48 hours to allow recovery.

For initial selection, the cells from each fusion were transferred into 50 ml of HAT medium (11) (complete medium supplemented with 10^{-4} M hypoxanthine; 4 x 10^{-7} M aminopterin; 1.6 x 10^{-5} M thymidine; and 3 x 10^{-6} M glycine) and dispersed into two 24-well plates. The hybridomas in wells positive for antibody (see below) were cloned by limiting dilution in 96-well flat bottom plates and then were recloned at least one additional time.

Antibody was obtained from spent culture medium or from the ascites fluid of athymic, nude mice (nu/nu) which had been injected 2-3 weeks previously with 1-5 x 10^6 cloned hybridoma cells.

Antibody assay

Antibody titers were routinely assayed by passive hemagglutination (12). Collagens (0.3% solutions in 0.1 M calcium acetate) were coupled to human type O-positive RBCs (10% solution) with glutaraldehyde. Then 100 µl aliquots of the derivatized RBCs (diluted 1/20) were added to 100 µl portions of antibody containing solution that had been serially diluted in round bottom microtiter plates, and the plates were allowed to develop overnight at 4°C.

Immunohistochemistry

Sections were stained with antibodies using a double-layered technique. Fourteen day embryonic chick tibias were fixed in 1% paraformaldehyde for 15 min, followed by quenching of free aldehyde groups in 0.15M tris HCl, pH 7.4. The tissue was quick-frozen to liquid N_2 temperature, and 8 μ sections were cut on a cryostat. Sections were air dried onto albumin-coated slides at room temperature for 1-2 hr and were then treated with testicular hyaluronidase (Sigma, Type I) in PBS (4000 U/ml) for 20 min at 37° (13). Subsequently, the sections were treated with the hybridoma antibody, diluted with PBS, for 1 hr at room temperature followed by washing in three changes of PBS. The secondary antibody, consisting of rhodamine conjugated IgG fraction of rabbit anti-mouse IgG (Cappel Laboratories, Lot 10169; diluted 4:1500 with PBS) was applied and allowed to react with the section for 1 hr in the dark. The sections were rinsed as above, and were then coated with glycerol: PBS (90:10) before attaching coverslips. Slides were viewed in a Zeiss photomicroscope III using the rhodamine filter set.

RESULTS AND DISCUSSION:

After hybridization and plating in HAT medium, 2 of 196 wells in 24-well plates were positive for anti-type II activity using passive hemagglutination. We do not yet know whether they represent two different antibodies. Cells from the two positive wells (II_6B_3 and II_1D_4) were cloned and 600 wells containing cells were tested for antibody, yielding 6 positives. Thus, from the number of positive wells in the two culture steps (2/196 for the 24-well plates multiplied by 6/600 for the microtiter plates), it was calculated that about 1 in 10,000 hybrid cells (those capable of growing in HAT) was a specific antibody producer. Positive wells were recloned and two of the subclones of II_1B_6 ($15A_4$ and $7E_8$) were recloned a third time. Each yielded $>$ 90% antibody-positive wells, thus confirming their monoclonality and stability.

Antibody titers were determined by passive hemagglutination (Table I) and since the titers were determined by 2-fold serial dilutions, the data is expressed as

TABLE I

Titers ($-\log_2$) of II_6B_3 subclone $15A_4$

Antibody Source	RBCs Coated	Titers
(1) culture medium	chick II	10.6 $\pm$0.69
(2) culture medium	chick I	0
(3) ascites fluid	chick II	21.2 $\pm$1.95
(4) ascites fuid	chick I	0

($-\log_2$). When tested against chick type II collagen, spend medium from 10 separate cultures of subclone $15A_4$ had an average titer of 10.6. Such medium can be obtained in virtually unlimited quantities and since it showed no crossreactivity against type I collagen (see below), it does not require affinity chromatographic purification . In comparison, an anti-type II collagen animal serum would have a similar titer (unpublished results); however, such sera are only available in relatively small quantities, and considerable antibody is lost during the affinity chromatographic purification necessary to eliminate crossreactivity. A much higher titer monoclonal antibody could be obtained by injecting $1\text{-}5 \times 10^6$ hybridoma cells into the peritoneal cavity of athymic, nude mice (nu/nu), and harvesting the ascites fluid 16 days later (Table 1). The ascites fluid from 7 hybridoma-containing nude mice (3-6 ml/mouse) had an average titer of 21 ± 1.95, which indicates a detectable average antibody response at an absolute dilution of greater than 2 million. The ascites fluid from the mouse with the highest titer antibody had detectable activity at a dilution of 32 million. Neither the culture medium nor the ascites fluid had any detectable activity when tested against chick type I collagen.

Since the type II collagen used for immunization and assay had been extracted by limited pepsin digestion, the molecules were probably devoid of nonhelical (telopeptide) extensions. Thus the antigenic site probably resides within the helical portion of the molecule. To test whether the antigenic site in addition is conformation dependent and present only when the collagen is in the native state, we performed inhibition of hemagglutination using native and denatured type II collagens (Figure 1). When native collagen was tested using 10-fold serial dilutions, a concentration dependence was observed. Collagen solutions of 10^{-1} mg/ml lowered the titer from a control value of 7 to an inhibited value of 2, whereas a concentration of 10^{-4} mg/ml caused negligible inhibition. Thermally denatured collagen caused no inhibition. Upon renaturation, inhibitory activity was regained. In addition, similar inhibitory activity was found for renatured molecules produced from α 1 (II) chains which had been further purified by carboxymethylcellulose chromatography (data not shown). That the antibody does not bind to denatured

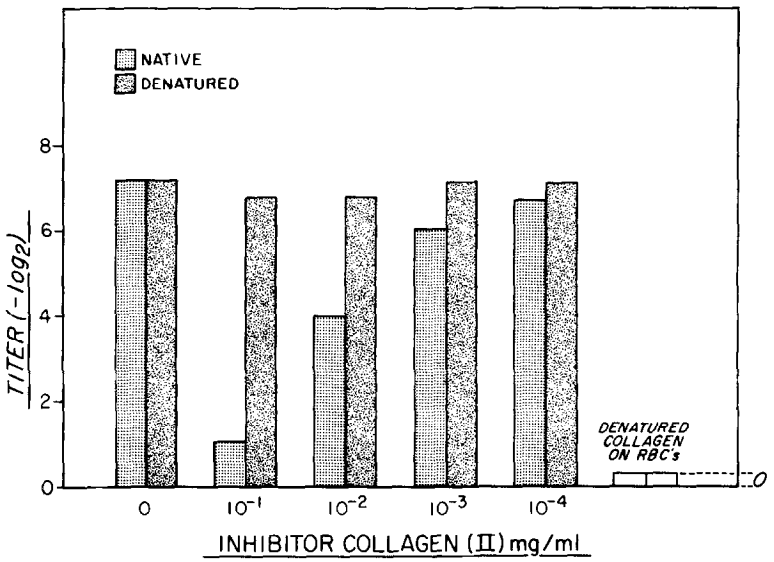

Figure 1: Native and thermally denatured (50° for 30 min) type II collagens were examined for their ability to inhibit the hemagglutination of type II-coated RBCs by II_6B_3 ($15A_4$) antibody. Inhibitor collagens at the concentrations shown in the figure were mixed with serial dilutions of antibody and allowed to incubate at 4° for 1 hour. The RBCs coated with native type II were added and allowed to react overnight at 4°. An additional experiment was to thermally denature the type II collagen on the RBCs in situ. The coated RBCs were heated to 50°C for 30 min., and after cooling were tested for agglutinability with antibody (see last two bars).

collagen was further demonstrated by using thermally denatured collagen on the RBC's surface. Such cells were not agglutinated by antibody (Figure 1, last two bars). These results are consistent with ones found for conventional anti-collagen antibodies produced in mice (14).

For assay purposes, antibody from ascites fluid was isolated by affinity chromatography on type II collagen-coupled Sepharose. Greater than 99% of the antibody activity bound to the resin, as tested by passive hemagglutination. Subsequently, this could be removed in active form by elution with 3M potassium thiocyanate. The eluted material, when analyzed by immunoelectrophoresis and double immunodiffusion against anti-mouse IgG, IgM or IgA, showed precipitant bands only against the anti-IgG sera (data not shown). The amount of active, affinity-purified IgG in ascites fluid, measured both by spectrophotometry ($O.D._{280}$) of solutions and by weight of lyophilized material, averaged > 90 mg per mouse.

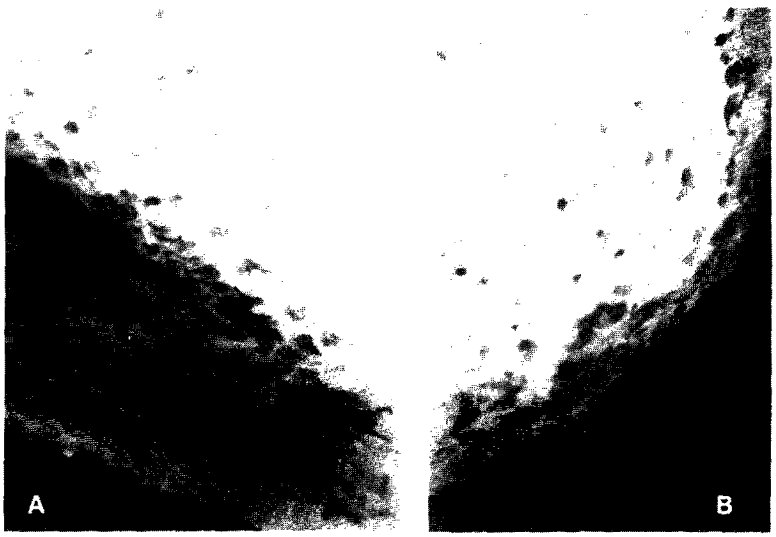

Figure 2: Sections of 14-day embryonic chick tibias stained with type II monoclonal antibody using a double-layered technique. A = ascites diluted 6000 x; B = ascites diluted 300,000 x. Photographic magnifications: A = 800 x; B = 640 x.

Lastly, the type II antibody was used in immunohistochemical procedures as we reported previously for the monoclonal antibody to type I collagen (3). Sections from 14-day cartilagenous chick tibia provided a good test object for cytological specificity of type I and II antibodies; cartilage contains type II collagen (15), whereas the surrounding perichondrium and loose connective tissue contain type I but not type II (13). Figure 2 shows the specificity for the type II antibody from ascites fluid at two different concentrations. Bright fluorescence was found over the cartilage matrix, with little if any on the perichondrium and surrounding connective tissue. Good fluorescence was still observed at a 300,000 fold dilution (Figure 2b). These results suggest, in addition, that the antibody does not react with type III collagen which also should be found in the unstained, non-cartilagenous tissue (16).

The monoclonal antibodies should be particularly useful probes for analyzing biosynthetic transitions in tissues producing more than one genetic type of collagen during development or remodelling. If directed against a single, unique determinant, they may also allow one to probe specific regions of molecular or fibrillar structure, or to identify molecular variants within a single collagen type.

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