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Production and Characterization of a Monoclonal Antibody Cross-Reactive with Most Group A Trichothecenes

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A monoclonal antibody cross-reactive with most group A trichothecenes was produced by fusion of P3/NS-1/1-AG4-1 myeloma cells with spleen cells isolated from a BALB/c mouse that had been immunized with 3-acetyl-neosolaniol-hemisuccinate conjugated to bovine serum albumin. One stable clone, H159B1D5, which produced monoclonal antibody that bound with both T-2 toxin and diacetoxyscirpenol (DAS) was obtained after subcloning. Enzyme-linked immunosorbent assay (ELISA) revealed that the antibody belongs to the immunoglobulin G1 (kappa chain) isotype and had binding constants of 2.81×10^9, 1.05×10^9, and 1.57×10^8 liters per mole for T-2 tetraol tetraacetate, T-2 toxin, and DAS, respectively. The relative cross-reactivities of the antibody with T-2 tetraol tetraacetate, T-2 toxin, and DAS were 200, 100, and 20, respectively, with tritiated T-2 toxin as the marker ligand. The relative cross-reactivities for the above toxins were 667, 100, and 73, respectively, with tritiated DAS as the marker ligand. No cross-reaction with HT-2 and deoxynivalenol triacetate was observed in either system. By using this monoclonal antibody, an indirect ELISA for analysis of T-2 toxin was also developed. The linear portion of the standard curve for analysis of T-2 toxin in each analysis by radioimmunoassay and ELISA was in the range of 0.1 to 2 ng and 0.05 to 1.0 ng, respectively.

Among a number of trichothecene mycotoxins produced by various *Fusarium*, *Myrothecium*, *Trichoderma*, and *Stachybotrys* species, T-2 toxin has attracted considerable attention (1, 5, 21). Interest in T-2 toxin and other related trichothecene mycotoxins has been stimulated by the potential hazard to humans and animals (5). Investigations in our laboratory was well as others have led to several sensitive and specific immunoassay systems, including radioimmunoassay (RIA), direct enzyme-linked immunosorbent assay (ELISA), and indirect ELISA, for the detection of T-2 toxin in foods, feeds, and biological fluids (2, 4, 7–10, 12, 15, 17, 19, 20). Such development has led to a demand for specific and well-characterized uniform antibody against T-2 toxin. Monoclonal antibody is suitable for this purpose. Unfortunately, the monoclonal antibodies obtained from hybridoma cell lines currently available have low affinities toward T-2 (11, 13, 14) compared with those of polyclonal antibodies. The binding constants of the monoclonal antibodies for T-2 toxin currently available were reported to be 10 to 100 times lower than those of polyclonal antibodies. Three of these monoclonal antibodies have strong cross-reactions with HT-2 toxin, a major T-2 toxin metabolite (13, 14). One monoclonal antibody had strong cross-reactivity with 3'-OH T-2 toxin (11). In the present study, attempts to produce high-affinity monoclonal antibody were made.

The approaches that have been generally used for the production of either polyclonal or monoclonal antibody against T-2 toxin involve the use of immunogens which were prepared by conjugation of T-2 toxin through the functional hydroxyl group at the C-3 position to a carrier protein (3, 4, 9, 11, 13, 14, 27). An alternative approach for conjugation of T-2 toxin to the carrier protein was made most recently in our laboratory (23). Instead of conjugation of T-2 toxin at the C-3 position, the toxin was coupled to the protein at the C-8 position and the hemisuccinate (HS) of 3-acetyl-neosolaniol (3-Ac-NEOS) was used for conjugation to bovine serum albumin (BSA). This conjugate, 3-Ac-NEOS–HS–BSA, has proved to be an effective immunogen that gave a quick immune response in rabbits (23). The antibody elicited by this new immunogen can recognize several group A trichothecenes including T-2 toxin and diacetoxyscirpenol (DAS). Consequently, this new immunogen was selected for the production of monoclonal antibody for T-2 toxin in the present study. Details of the production and characterization of this new monoclonal antibody are presented here.

MATERIALS AND METHODS

Materials. T-2 toxin was produced by *Fusarium sporotrichioides* (previously *F. tricinctum*) 696 (kindly supplied by E. B. Smalley of the University of Wisconsin) in cracked corn at 15°C under the conditions described by Cullen et al. (6). HT-2 toxin and T-2 tetraol were prepared as described previously (9, 23, 24). T-2 tetraol tetraacetate (T-2-4ol-4Ac) was prepared by hydrolysis of T-2 toxin to T-2 tetraol (23) and then conversion to the tetraacetate by acetylation under conditions described before (23). DAS was purchased from Calbiochem-Behring, La Jolla, Calif. Tritiated T-2 toxin and tritiated DAS with specific activities of 19 and 8.8 Ci/mM were prepared according to the procedure of Wallace et al. (22) as modified by Chu et al. (3). Bovine serum albumin (BSA, radioimmunoassay grade), goat anti-mouse immunoglobulin G, and goat anti-rabbit immunoglobulin G were purchased from Sigma Chemical Co., St. Louis, Mo. Water-soluble carbodiimide, i.e., 1-ethyl-3,3-dimethylamino-propyl-carbodiimide, was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Immunogen 3-Ac-NEOS–HS–BSA was prepared by the method of Wei and Chu (23). Complete Freund adjuvant containing *Mycobacterium tuberculosis* (H 37 Ra) was obtained from Difco Laboratories, Detroit, Mich. Polyethylene glycol 1000 was purchased from the J. T. Baker Chemical Co., Phillipsburg, N.J. Dulbecco modified Eagle medium, fetal calf serum, and penicillin-streptomycin were obtained from GIBCO Laboratories, Grand Island, N.Y. Hypoxanthine, thymidine, and aminopterin were purchased

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from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The myeloma cell line P3/NS-1/1-AG4-1 was obtained from the American Type Culture Collection, Rockville, Md. Polylysine-carboxymethyl-T-2 toxin (PLL-CMO-T-2 toxin) was prepared by the method of Zhang et al. (27). Virus-free BALB/c mice were obtained from Harlan Sprague Dawley, Inc., Madison, Wis. All chemicals and organic solvents were reagent grade or better.

Immunization procedures. The immunogen (3-Ac-NEOS–HS–BSA) was prepared by dissolving it in 0.01 M phosphate-buffered saline (pH 7.5)–0.85% NaCl (PBS), then emulsifying it with 2 volumes of complete Freund adjuvant. Six 8-week-old BALB/c mice were given 10 μg of the 3-Ac-NEOS–BSA conjugate subcutaneously at three sites on the back. The mice received booster injections intraperitoneally with the same amount of conjugate (but without adjuvant) every 3 weeks after the initial immunization (25). Blood samples were removed from the tails at intervals after each boost, and the plasma was diluted 1:10 with PBS and assayed by RIA for antibody titer. At 4 days before fusion (6 weeks after the initial immunization, with two booster injections during this period), the mouse exhibiting the highest titer was given an intravenous injection of 10 μg of immunogen, followed by intraperitoneal injections 3 and 2 days before fusion (25).

Detection of antibody titer in mouse serum-ascites fluids. The antibody titer of the mouse serum was determined by an RIA by using protocols essentially the same as those described before (3). Before the assay, the antiserum was precipitated with ammonium sulfate to a final saturation of 33.3%. The precipitate was dissolved in water and reprecipitated twice. Finally, the precipitates were reconstituted to half of the original volume with distilled water, dialyzed against distilled water for 0.5 to 1.0 h and then against PBS overnight at 4°C, and frozen or subsequently lyophilized. For antibody titer determination, a serial dilution was made with PBS containing 0.01% BSA. A 100-μl volume of the diluted antibody preparation was mixed with 0.1 ml of tritiated T-2 toxin in PBS (10,000 dpm; 19 Ci/mmol) and incubated at 4°C overnight. Separation of bound and free ligand was achieved by an ammonium sulfate precipitation method described earlier (3). Antibody titer was defined as the reciprocal of the amount of antiserum (in milliliters) required to give 50% binding of tritiated toxin under the conditions described. For antibody titer determination, [^{3}H]T-2 toxin and [^{3}H]DAS were used as the marker ligands.

Cell line and culture media. The myeloma cell line (P3/NS-1/1-AG4-1) used for fusion was grown in a medium which consisted of Dulbecco modified Eagle medium with 20% fetal calf serum, 1 mM sodium pyruvate, 60 μM hypoxanthine, 20 μM thymidine, and penicillin-streptomycin at a final concentration of 100 U/ml of each (HT medium). Cloning medium consisted of HT medium plus normal mouse erythrocytes at a concentration of 0.5%. Hybridomas were selected by growth in HAT medium, which consisted of HT medium plus 0.5 μM aminopterin (18, 25).

Cell fusion and cloning. The mouse with the highest polyclonal titer (i.e., 315) was sacrificed by cervical dislocation. The spleen was aseptically removed and then mashed with a glass pestle through a Cellector tissue sieve, producing a single-cell suspension which was combined with 10^7 myeloma cells. The cells were centrifuged, suspended in a very small amount of HT medium, and then fused by the addition of 2.0 ml of 40% polyethylene glycol 1000 in Dulbecco modified Eagle medium. After incubation for 1 to 2 h, the cells were pelleted, suspended in HAT medium plus 0.5% mouse erythrocytes, and plated in Costar 96-well tissue culture plates. The colonies were fed every 5 days with fresh medium. Hybridomas were screened for specific antibodies against T-2 toxin and DAS when the colonies reached at least one-half confluency in the well (approximately 8 to 12 days after fusion). Positive wells were cloned by the limiting dilution method (18) into 96-well tissue culture plates. Positive hybrid clone culture supernatant was collected for antibody characterization; cultures were grown to late stationary phase, and the spent medium was recovered and frozen.

Screening of culture supernatant for anti-T-2 toxin antibody. The RIA protocol used to screen the hybridoma cells was similar to the one described above for the polyclonal mouse sera, except that 0.1 ml of a 1:2 dilution of the culture supernatant solution (diluted with PBS) was incubated with either 0.1 ml (5,000 dpm) of tritiated T-2 toxin or with 0.1 ml of tritiated DAS (5,000 dpm).

Characterization of antibody. (i) Determination of isotype. A commercially available mouse immunoglobulin isotype identification kit (Boehringer Mannheim) was used to determine the isotype of monoclonal antibody obtained above. A 1/10-ml sample of T-2-CMO-polylysine conjugate at a concentration of 0.3 μg/ml in carbonate buffer (0.05 M, pH 9.6) was coated onto the microplate for binding specific immunoglobulin. Identification of specific immunoglobulin was then carried out by using the protocol described by the manufacturer.

(ii) Analysis of monoclonal antibody specificity. The protocols for determination of specificity of the monoclonal antibody were essentially the same as for the antibody titer determination, except that unlabeled T-2 toxin, DAS, or various structurally related trichothecenes were present in the reaction mixture. Different derivatives were first dissolved in acetonitrile and then diluted in PBS. The final volume of the reaction mixture was 0.3 ml, and the acetonitrile concentration was 5% or less. The apparent association constants (K_{app}) of the monoclonal antibody were estimated from RIA calibration (competitive inhibition) curves by the method of Muller (16). The specificity of the monoclonal antibody with various trichothecenes was also determined by a competitive indirect ELISA for T-2 toxin as described before (8). Again, T-2-CMO-polylysine conjugate was coated to the microplate as the test ligand.

Determination of radioactivity. Radioactivity was determined in an LS-5801 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) using 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.).

RESULTS

Screening culture supernatant for anti-T-2 toxin antibody. In the initial screening, three clones were shown to produce antibody which was capable of binding 12 to 90% of both [^{3}H]T-2 toxin and [^{3}H]DAS at a 1:2 dilution. Background binding of the tritiated toxins with the culture supernatant was less than 2%. No further work was done on the two low-binding clones (12 and 45% binding) because they were unstable. One stable clone (H159-B1D5) which produced antibody that had the highest binding capacity with T-2 toxin (90% binding at a 1:2 dilution) was selected and expanded. This clone was subsequently used for the production of antibody both in ascites fluid as well as in cell culture supernatant by the standard methods (18). The supernatant and ascites fluid collected from this clone were further characterized.

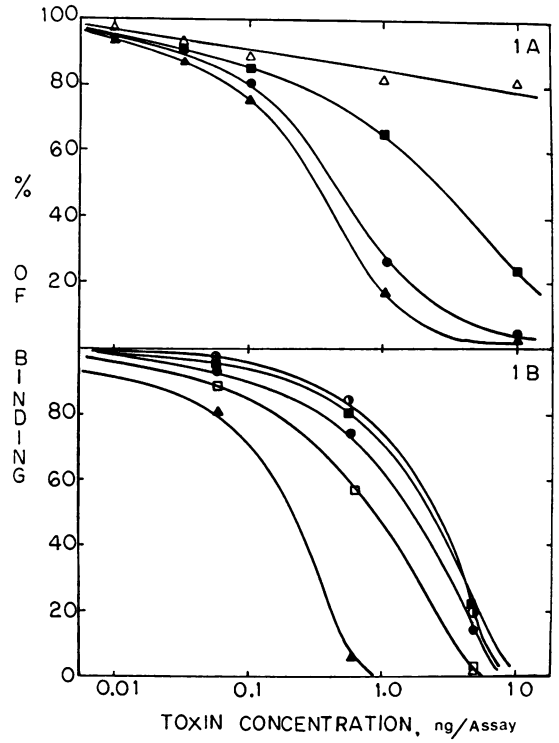

FIG. 1. Effects of different trichothecene mycotoxins on the binding of [^{3}H]T-2 toxin (A) and [^{3}H]DAS (B) with monoclonal antibody against T-2 toxin. Either [^{3}H]T-2 toxin (12,000 dpm) or [^{3}H]DAS (12,000 dpm) was used as the marker ligand. Separation of the free and bound ligands was achieved by an ammonium sulfate precipitation method. Results were the average of three sets of experiments. The concentrations shown on the x axis are in log scale. Symbols: ●, T-2; ◑, acetyl T-2; △, HT-2; ▲, T-2-4ol-4Ac; □, triacetoxyscirpenol; ■, DAS.

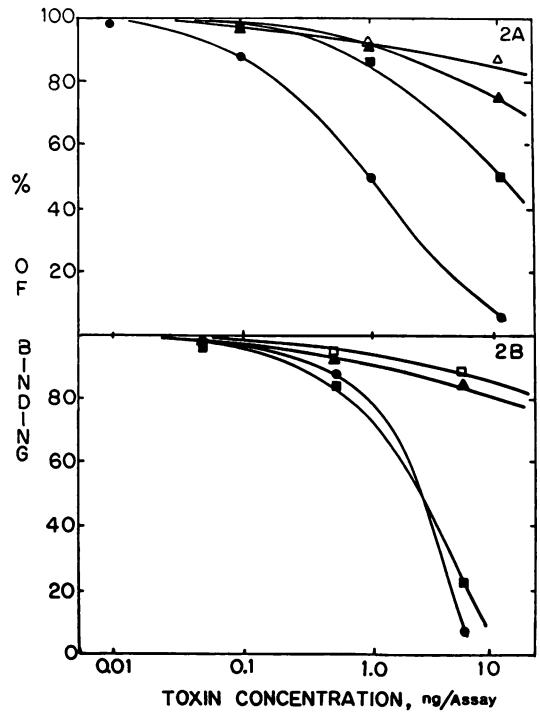

FIG. 2. Effects of different trichothecene mycotoxins on the binding of [^{3}H]T-2 toxin (A) and [^{3}H]DAS (B) with rabbit anti-3-Ac-NEOS-HS-BSA. Experimental conditions for this study were the same as for Fig. 1. Results were the average of three sets of experiments. The concentrations shown on the x axis are in log scale. Symbols: ●, T-2; △, HT-2; ▲, T-2-4ol-4Ac; □, triacetoxyscirpenol; ■, DAS.

Characterization of monoclonal antibody. The isotype of the monoclonal antibody as determined by using a sandwich-type ELISA (commercial kit) was found to be immunoglobulin G1, kappa light chain. The concentration of immunoglobulin G1 produced by the cell line was 0.4 μg/ml of culture supernatant solution.

Determination of antibody specificity by RIA analysis. A competitive RIA was used for the determination of the specificity of the monoclonal antibody. Results for the cross-reactivity of the monoclonal antibody with various trichothecenes using either [^{3}H]T-2 or [^{3}H]DAS are shown in Fig. 1. The concentration causing 50% inhibition of binding of [^{3}H]T-2 toxin (underlined values in parentheses are relative cross-reactivities, where inhibition by T-2 toxin is designated as 100%) to the monoclonal antibody by unlabeled T-2 toxin, DAS, T-2-4ol-4Ac, and HT-2 toxin were 0.50 (100), 2.4 (20), and 0.25 (200), and >10.0 ng per assay (less than 1%), respectively. The concentration causing 50% inhibition of binding of [^{3}H]DAS to the monoclonal antibody by unlabeled T-2 toxin (as 100%), DAS, triacetoxyscirpenol, T-2-4ol-4Ac, and acetyl T-2 toxin were 1.6 (100), 2.1 (73), 0.78 (206), 0.24 (667), and 2.3 (67) ng per assay, respectively. The apparent association constants (K_{app}) of the antibody for T-2-4ol-4Ac, T-2 toxin, and DAS, as calculated by the method of Muller (16), were 2.81×10^9, 1.05×10^9, and 1.57×10^8 liters per mole, respectively.

A comparison was made between the specificity of rabbit polyclonal antibody produced by using the same immunogen, 3-Ac-NEOS-BSA, and the monoclonal antibody. Again, two competitive RIAs (one with [^{3}H]T-2 toxin, the other with [^{3}H]DAS) were used. The results are shown in Fig. 2. The concentration causing 50% inhibition of binding of [^{3}H]T-2 toxin to the polyclonal antibody by unlabeled T-2 toxin (as 100%), DAS, T-2-4ol-4Ac, and HT-2 toxin were 1.0 (100), 10 (10), >50 (≤2), and 50 (≥2) ng per assay, respectively (Fig. 2A). The concentration causing 50% inhibition of binding of [^{3}H]DAS to the polyclonal antibody by either unlabeled T-2 toxin or DAS was 3.8 ng per assay. In the competitive RIA using tritiated DAS as the marker ligand, the polyclonal antibody showed almost no cross-reaction with T-2-4ol-4Ac, HT-2 toxin, and triacetoxyscirpenol. Less than 20% inhibition of binding was observed when 5 ng each of T-2-4ol-4Ac and triacetoxyscirpenol was tested in each assay.

Determination of antibody specificity by indirect ELISA. To avoid the use of radioactive ligand in the immunoassay, we also tested a competitive indirect ELISA of T-2 toxin. In this assay, polylysine-CMO-T-2 toxin was coated to the assay plate. Results indicate that both T-2 toxin and T-2-4ol-4Ac competed very effectively for the binding of the antibody with solid-phase ligand (Fig. 3). The concentration causing 50% inhibition of binding of the antibody (underlined values in parentheses are relative cross-reactivities, where inhibition by T-2 toxin is designated as 100%) with the polylysine-CMO-T-2 toxin by T-2-4ol-4Ac, T-2 toxin, and DAS were found to be 0.13 (85), 0.11 (100), and 0.80 (14) ng per assay, respectively.

For comparison purposes, we also tested the polyclonal antibody in the indirect ELISA under the same conditions.

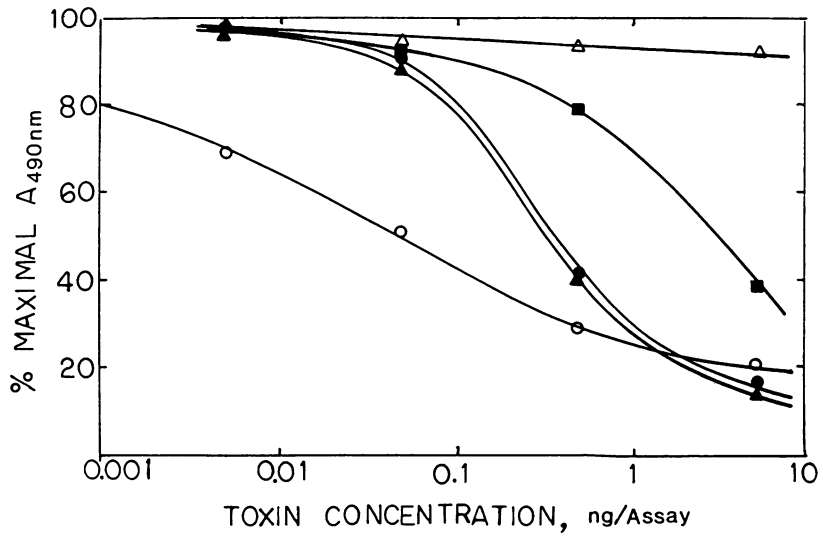

FIG. 3. Indirect ELISA of T-2 toxin using monoclonal antibody against T-2 toxin. Results were the average of three sets of experiments. The concentrations shown on the x axis are in log scale. Symbols: ●, T-2; △, HT-2; ▲, T-2-4ol-4Ac; ■, DAS. For comparison, results obtained from an experiment using polyclonal antibody and T-2 toxin are shown (○).

Although the competitive binding curve was less sharp, the assay system was more sensitive than the monoclonal antibody indirect ELISA system. The concentration causing 50% inhibition by T-2 toxin in the indirect ELISA using polyclonal antibody was found to be around 0.075 ng per assay.

DISCUSSION

In recognizing the advantages of using monoclonal antibodies in the diagnostic and therapeutic areas, several approaches have been used for production of monoclonal antibodies against T-2 toxin (11, 13, 14). However, the affinities of T-2 toxin to the monoclonal antibodies obtained from previous studies are generally lower than those to polyclonal antibodies. Those monoclonal antibodies have specificities toward T-2 toxin metabolites such as HT-2 toxin (13, 14) and 3'-OH-T-2 (11) rather than toward T-2 toxin. In the present study, a stable hybridoma which produced a monoclonal antibody cross-reactive with both T-2 toxin and DAS, two of the most important mycotoxins in the group A trichothecenes, was obtained. Our approach was to immunize mice with an immunogen which had been shown to produce polyclonal antibodies that cross-reacted with most group A trichothecenes (23). Results obtained from the present study, indeed, prove that the immunogen elicited a monoclonal antibody possessing good cross-reactivity with both T-2 toxin and DAS (Fig. 1 and 3).

Among three ligands tested, the highest affinity of the monoclonal antibody obtained from the present study was for T-2-4ol-4Ac, the next highest was for T-2 toxin, and the lowest was for DAS. The apparent binding constant of the monoclonal antibody for T-2 toxin obtained from the present study is about 20 times higher than those obtained in a previous study (14). Thus, this monoclonal antibody would be very useful both as a therapeutic agent and for analytical and diagnostic reagents. The capability of using the monoclonal antibody for the analysis of T-2 toxin has already been well demonstrated both in the competitive RIA and in the indirect ELISA in the present study. The linear ranges for RIA and a competitive indirect ELISA were found to be from 0.1 to 2.0 and 0.05 to 1.0 ng per assay, respectively. This kind of sensitivity was similar to or slightly higher than that of most other systems which have been reported earlier (3, 9, 13, 17). Since this monoclonal antibody has the highest affinity toward T-2-4ol-4Ac, higher sensitivity for detection of group A trichothecenes could be achieved by analysis for T-2-4ol-4Ac after converting all the related toxins to the acetate form, such as the one used for the analysis of deoxynivalenol (26). This approach can also be used for analysis of metabolites of T-2 toxin and DAS after converting them to the tetraacetate and triacetate forms. For example, the sample extract can directly be subjected to the ELISA or RIA after a mild alkali hydrolysis and acetylation.

Although the antibody had good cross-reactivity with DAS, the affinity of the monoclonal antibody toward T-2-4ol-4Ac and T-2 toxin was 10 times higher than that toward DAS. On the contrary, the polyclonal antibody had a higher affinity toward T-2 toxin, 10-fold less for DAS, and 100-fold less for T-2-4ol-4Ac, when [^{3}H]T-2 toxin was used as the marker ligand (23). These results are not to our surprise. Polyclonal antibodies are a population of antibodies, each group having different specificities. Thus, when [^{3}H]T-2-4ol-4Ac was used as the marker ligand in the RIA system, excellent cross-reactivity of the polyclonal antibody with the T-2 tetraol tetraacetate was observed (23). Consequently, the apparent specificity of the polyclonal antibody is also dependent on the specific marker used in the analysis.

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