Production of Monoclonal Antibodies Against Calmodulin by In Vitro Immunization of Spleen Cells

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ABSTRACT Monoclonal antibodies against the highly conserved ubiquitous calcium-binding protein, calmodulin (CaM), were produced by immunization of mouse primary spleen cell cultures. Dissociated spleen cells were cultured for 5 d in the presence of mixed thymocyte culture conditioned media (TCM) and purified bovine testes CaM (50 ng–1 mg). Following immunization, cells were fused with mouse myeloma cells (SP2/0, Ag 8.653) and cultured for 2–3 wk before initial screening for antibody. In five independent immunizations there was a range of 25–44% of the initial polyclonal cultures which produced antibodies reacting with purified CaM as determined by immunoassay. 80% of the cloned hybridoma produced IgM immunoglobulins while the remaining clones were IgG producers. This ratio was changed to 50% IgM and 50% IgG by subsequent extension of the in vitro immunization periods and reduced amounts of antigen and extended in vitro culturing. In vitro immunization introduces a new dimension to monoclonal antibody production where limited antigen or poorly antigenic proteins are of interest. The monoclonal antibodies produced in this study have enabled us to to selectively localize CaM in association with distinct subcellular structures, mitochondria, stress fibers, centrioles, and the mitotic spindle.

Production of antibodies against the calcium-binding protein, calmodulin (CaM), by conventional animal immunization regimens has required either large quantities (milligram range) of native CaM (1, 2) or chemical modification of the protein to increase its antigenicity (3, 4). These procedures result in antisera which consist of a pool of polyclonal antibodies that collectively recognize multiple antigenic determinants on the immunogen. In contrast, monoclonal antibodies represent individual, homogeneous classes of immunoglobulins which, if properly selected, may recognize discrete antigenic configurations of a given immunogen. Calmodulin has been shown to be capable of binding to many intracellular sites, presumably to proteins that are Ca^{2+}-calmodulin regulated (5). Depending upon which protein and where in the cell it is bound, different antigenic sites or segments of one antigenic site are exposed and accessible for antibody binding. Monoclonal antibodies to calmodulin (CaM) would permit the independent monitoring of intracellular CaM bound in different spatial or conformational states. Such differential localization would provide valuable information in further delineating the cellular function of CaM during various metabolic states.

In vivo immunization for the production of monoclonal antibodies against preselected antigens has become a routine laboratory procedure. However, relatively large quantities of the antigen are still required for injection into the host animal (6), and each animal must be screened as a producer. Alternatively, spleen cells have been activated with mitogen (7) and immunized with sheep erythrocytes (8) in vitro, resulting in specific monoclonal antibody production. In vitro immunization of spleen cell cultures permits a reduction in the amount of antigen required and enables enhanced recovery of specific antigen-activated clones (9, 10, 11, 12). In the present study, we have used an in vitro immunization protocol with a soluble protein antigen (bovine testes CaM) followed by fusion with mouse myeloma cells (SP2/0, Ag 8.653) to produce monoclonal antibodies. This technique was first reported by Luben and Mohler (9), Mishell and Dutton (11), and Click (12) and is the subject of recent review (13). The procedure utilizes a murine allogenic thymocyte culture to generate lymphokines that stimulate lymphocytes in the presence of antigen to promote antibody production. A preliminary report of this work has been presented (14).

MATERIALS AND METHODS

Preparation of Thymocyte-Conditioned Media: 10 (5 BALB/c, 5 C-57) mouse thymuses were surgically removed from 2–4-week-old animals and passed through a 50-mesh stainless steel screen. Thymus cells (4×10^6 cells/ml) were cultured in Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 4,500 mg glucose/l, 2 mM glutamine, 5×10^{-5} M-mercaptoethanol, 10 mM

MEM nonessential amino acids, 3 μM thymidine, 30 μM hypoxanthine, 1 mM pyruvate in 18 mM HEPES buffer with 12% type 100 rabbit serum (Quadroma, San Marcos, CA). Cells were cultured for 48 h at 37°C in 5% CO_2. The thymocyte-conditioned medium (TCM) was then harvested by centrifugation (500 *g* for 5 min to pellet the thymus cells) and the supernatant (TCM) stored at −70°C.

Isolation and Immunization of Spleen Cells: Individual spleens from nonimmunized BALB/c mice were surgically removed and washed three times in Hanks' balanced salt solution, pH 7.4. Spleen cells were mechanically dissociated as mentioned above. Individual in vitro immunizations consisted of the dropwise addition of the appropriate amount of antigen (calmodulin 1 mg, 500 μg, 200 μg, 100 μg, 500 ng, 200 ng, 50 ng, and 0 ng) directly to a 60-mm tissue culture dish containing 10 ml of serum-free DME and incubated at 22°C for 15 min. Following this initial incubation the 10 ml of antigen-exposed cells were transferred to a T-75 tissue culture flask containing an additional 10 ml of DME supplemented with 10 ml of TCM (SDME) and final serum concentration of 5%. The 30 ml of SDME with 5% rabbit serum containing 10^8 spleen cells and antigen were cultured for 4–5 d at 37°C, 5% CO_2. Following this 5 day in vitro immunization period, the individual cultures were centrifuged (5 min at 500 *g*) and the pelleted cells were mixed with SP2/0 or P3 × 63 Ag 8.653 mouse myeloma cells at a ratio 2:1 (spleen/myeloma cell) and co-pelleted by centrifugation. This myeloma and spleen cell mixture was fused in 47% polyethylene glycol (1,540 mol wt) and 3% dimethylsulfoxide according to previously published procedures (6). The fused cells were cultured in 80 ml of DME-HT media with 12% rabbit or fetal calf serum for 24 h. Aminopterin (10^{-5} M) was added to the media along with a feeder layer of nonimmune spleen cells (15) in 24-well tissue culture plates (Linbro) (1 ml/well) for 2–4 wk before screening was initiated.

Enzyme Immunosorbent Assay: 30 ng of bovine testes calmodulin in 100 μl of phosphate buffer saline, pH 6.9 was added to each well of a 96-well microtiter plate (Dynatech, Immunlon II) and incubated overnight at 4°C. Calmodulin was prepared as described according to Dedman et al. (1). Unbound calmodulin was removed from the wells by washing 10 times in 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl, 0.2% Triton X-100 (PBST). Nonspecific sites were then blocked for 1 h at room temperature by the addition of 100 μl of PBS with 10 mg/ml bovine serum albumin (Sigma Chemical Co.). Wells were then washed 10 times in PBST, and 50 μl of cell supernatant was added and incubated for 2 h at 37°C. The wells were again washed 10 times in PBST. 30 μl of peroxidase-tagged goat anti-mouse IgG or IgM (1/500 dilution) was added and incubated at 37°C for 1 h. After second antibody incubation, wells were washed 10 times in PBST, 200 μl of substrate, 10 ml of 0.1 M citrate buffer pH 4.5, 10 mg of orthophenyldiamine (Sigma Chemical Co.), 4 μl 30% H_2O_2. Reaction was allowed to proceed for 30 min and then was blocked with 5 μl of 1 M NaF. Optical densities were read on a Dynascan spectrophotometer (Dynatech, Inc.).

Fluorescence Microscopy: Swiss 3T3 fibroblasts were grown on 11 × 22 mm coverslips, fixed in 3% formaldehyde (20 min), lysed in 0.1% Triton X-100 (90 s), and stained with primary monoclonal antibodies to purified bovine testes calmodulin (14, 16). Lysed cells were incubated for 1 h in affinity-purified antibody (0.1 mg/ml) or a Na_2SO_4-precipitated fraction of hybridoma supernatant media, washed in PBS, pH 7.4, then incubated in fluorescein-conjugated goat anti-mouse immunoglobulin (Miles Laboratories Inc., Elkhart, IN), washed again in PBS, and mounted in glycerol:PBS, pH 9.0. Micrographs were taken on a Leitz Orthoplan epifluorescence microscope and recorded on Kodak Tri-X film, ASA 400.

Immunoblotting: Cross-reactivity of antibodies was determined by immunoblots on nitrocellulose paper in a Minimanifold block (Schleicher & Schuell, Inc., Keene, NH). Electrophoretically pure cellular antigens calmodulin, parvalbumin, troponin C, 6s tubulin, and actin were blotted on 0.2 -μm nitrocellulose paper (Schleicher & Schuell, Inc.) overnight at 4°C. Individual wells were then washed 10 times in PBS, pH 7.4. Nonspecific binding sites were blocked with 10 mg/ml BSA in PBS for 2 h at 22°C. 100 μl of antibody-containing supernatant medium was then placed in each well and incubated (4°C overnight). Following 10× washing in PBS, peroxidase-labeled goat anti-mouse immunoglobulins (Cappel Laboratories, Inc., Cochranville, PA) or rabbit anti-sheep IgG were incubated at room temperature. After 2 h the nitrocellulose sheet was removed from the block and washed in PBS, three changes, 20 min each. The entire nitrocellulose sheet was then placed in 100 ml of Tris-saline buffer, pH 7.4 containing 25 mg/100 ml O-dionisodine and 100 μl of 30% H_2O_2. The reaction was stopped after 2–3 min by washing in water, and the developed blots were then air dried.

Affinity Chromatography and Immunoprecipitation: Supernatant medium from clone C-101-4 was precipitated with 40% Na_2SO_4, and the pellet was resolubilized in PBS and applied to a CaM-affinity column prepared as in (1). Nonabsorbed protein was removed by washing with 0.1 M

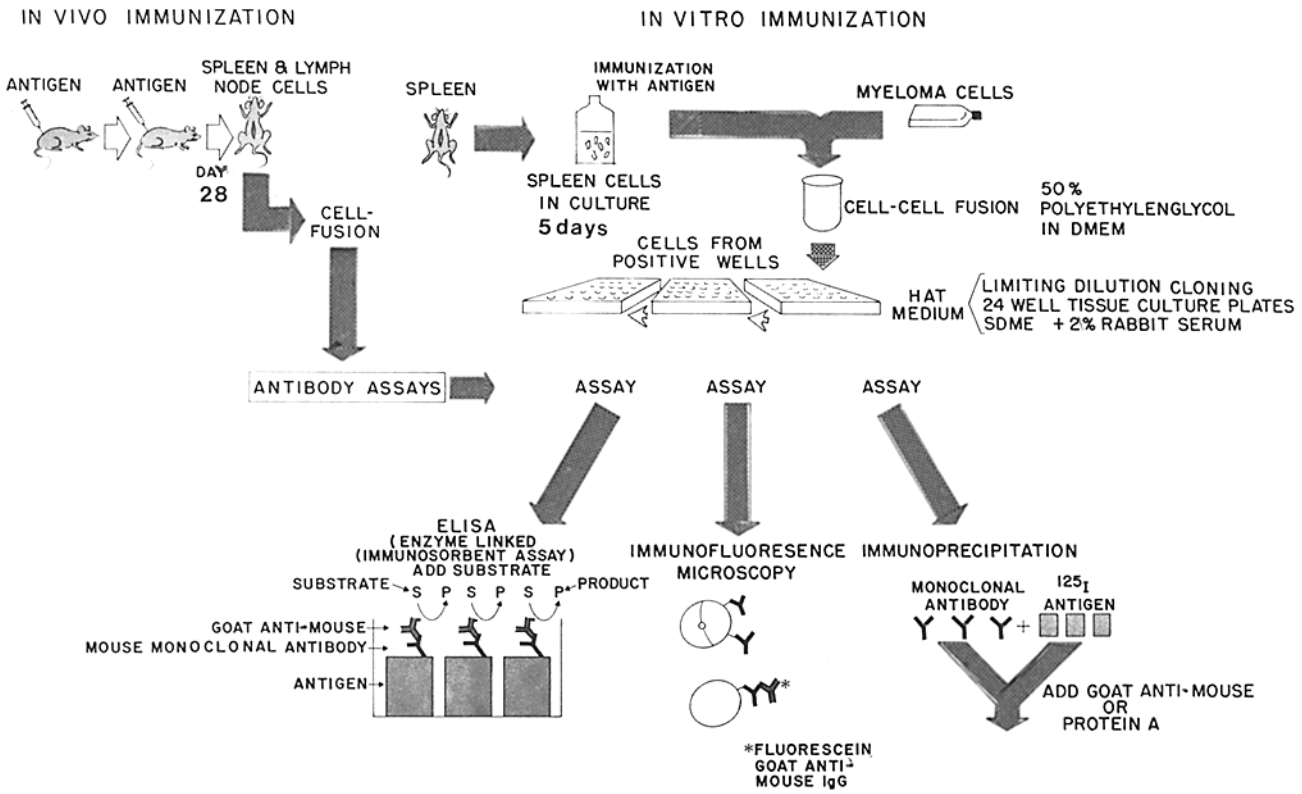

FIGURE 1 Strategy for *in vitro* immunization. In vivo monoclonal production involves a long incubation period and possible antigen degradation in situ before recognition by the host immune system. In vitro immunization permits β-lymphocyte binding of antigen with minimal degradation. Positive clones are screened by four criteria: EIA, immunofluorescence, affinity chromatography, and immunoprecipitation.

NaH_2CO_3, 0.5 M NaCl, pH 8.3. Specifically bound antibody was eluted with 200 mM glycine pH 2.7. Individual fractions of the flow-through and the eluate were then incubated with ^{125}I-CaM overnight at 4°C. After 24 h, 100 μl of a 1:30 dilution of goat anti-mouse antibody was added. Following incubation for 1 h at room temperature, *Staphylococcus aureus* was added for an additional hour. Samples were then centrifuged and washed twice in PBS, and radioactivity was counted in a gamma counter.

Cloning: 4–5 wks postfusion, positive wells were cloned by limiting dilution. Each 1-ml well was diluted to 10 ml in DME, 12% FCS. 100 μl was then added to each well of a 96-well microtiter plate and cells were grown to confluence before retesting. Those cells positive for anticalmodulin antibody production were then recloned at least two additional times by limiting dilution.

RESULTS

Polyclonal hybridoma cultures were screened for anti-CaM activity by four procedures: (*a*) enzyme immunosorbent assay (EIA), (*b*) indirect immunofluorescence, (*c*) antigen affinity chromatography, and (*d*) immunoprecipitation ^{125}I-CaM. Lack of cross-reactivity of various clonal antibodies with other cellular proteins (parvalbumin, troponin C, actin, and 6S tubulin) was verified by immunoblot procedures (Fig. 2). Initial screening of polyclonal culture immunizations (C-1 through C-5) demonstrated that 25–44% were positive. These positive cultures were then subcloned and rescreened for anti-CaM antibody production (Table I). In one spleen cell culture (C-6) immunized with 50 ng of CaM there were no detectable anti-CaM immunoglobulins. 10 different positive wells were cloned by limiting dilution and again assayed for anticalmodulin (Table II). Of the 10 cloned cultures, 9 survived and proliferated. Six of the nine clones gave positive results in all of the screening procedures; clone C101-10 died 5 d after subcloning. Three of the clones gave a positive reaction in EIA testing, but were negative in at least one of the other tests (C-101-2, C-101-6, C-101-19). Clone C-101-2 was unable to immunoprecipitate ^{125}I-CaM, using *S. aureus* or goat anti-mouse second Ab. C-101-6 was positive for EIA and negative for the three other tests. These results are most likely reflective of differences in antibody titer or antibody affinity among the different clones. Hybridoma supernatant media, Na_2SO_4 fraction or affinity-purified antibodies were used to stain monolayers of 3T3 fibroblasts by indirect immunofluorescence (16).

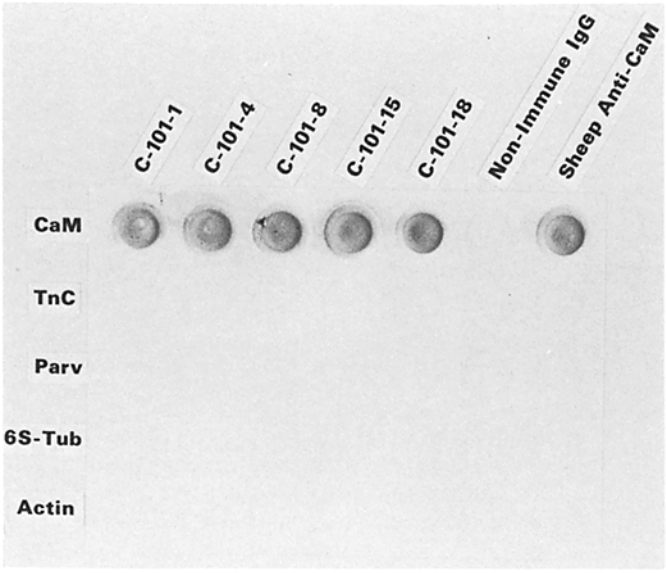

FIGURE 2 Immunoblots of calmodulin monoclonal antibodies. Immunoblots of calmodulin, troponin C, parvalbumin, 6S tubulin, and actin with supernatant media from selected clones. Polyclonal, sheep anti-CaM and nonimmune mouse IgG were used as controls.

TABLE I

Multiple In Vitro Immunizations

Spleen number	Immunization dosage of CaM	% EIA positive first polyclonal screening
C-1	1 mg	25%
C-2	500 μg	30%
C-3	100 μg	35%
C-4	500 ng	40%
C-5	200 ng	44%
C-6	50 ng	No detectable response

Six individual BALB/c mice spleens were separately immunized with varying amounts of purified bovine testes calmodulin (1 mg to 50 ng). Single immunizations in vitro followed by 4–5 d incubation in culture yielded 25–44% positive cultures upon first EIA screening of polyclonal cultures. Single immunizations in vitro produced clones secreting predominately IgM (80%) and some IgG's (20%) as tested with anti-mouse immunoglobulins, anti-mouse IgM and anti-mouse IgG (Miles).

Calmodulin-affinity chromatography elution of a 40% Na_2SO_4-precipitated fraction of hybridoma supernatant media yielded specific calmodulin antibodies (Fig. 3). Only the peak of protein eluted with 200 mM glycine specifically immunoprecipitated ^{125}I-CaM. The small number of counts precipitated indicated low binding affinity of the antibody similar to the original goat anti-CaM antibodies reported by Dedman et al. (1). Neither goat anti-mouse immunoglobulin, nonimmune mouse immunoglobulins, rabbit serum, nor affinity column flow-through were capable of immunoprecipitating ^{125}I-CaM in control experiments. When monoclonal antibodies were preabsorbed with 1 mg/ml bovine or rat calmodulin, no precipitation was detected. Preabsorption with parvalbumin (1 mg/ml) did not inhibit binding.

To evaluate whether the individual clones would recognize different aspects of CaM, antibodies from individual clones were used to stain cultured Swiss 3T3 cells by immunofluorescence. Purified media from clone C-101-1 resulted in the localization of CaM in the mitotic spindle (Fig. 4A_1, A_2) as previously demonstrated by Welsh et al. (17) and Andersen et al. (18) using goat and rabbit polyclonal anti-CaM. Clone C-101-4 antibody decorated stress fibers and centrioles, respectively, in interphase 3T3 cells (Fig. 4B_1 and B_2). Antibody from clone C-101-15 localized CaM bound to mitochondria and resulted in some faint staining of stress fibers (Fig. 4C_1 and C_2). No cellular structures other than those shown were stained with this set of monoclonal antibodies. Neither fetal bovine serum, rabbit serum, nonimmune mouse serum, nor supernatant medium from a nonimmunized mouse spleen hybridoma specifically stained cells or was capable of binding to isolated calmodulin by the three other test criteria. Preabsorption of monoclonal antibodies with excess of calmodulin (1 mg/ml) eliminated staining while antibodies preabsorbed with parvalbumin (1 mg/ml) had little or no effect on the specificity of the antibody. These immunofluorescence data suggest the unique specificity of monoclonal as opposed to polyclonal antibodies for protein localization. CaM has previously been localized to stress fibers with goat antibodies only after prolonged (6-h) incubation periods with primary antibody (17). Pardue and co-workers (19) utilizing rhodamine-labeled CaM demonstrated that CaM acceptor sites (CAPs) are associated with mitochondria while conventional polyclonal anti-calmodulin antibodies were unable to detect these sites unless permeabilized cells were first pre-incubated with exogenous calmodulin. The lack of staining of stress fibers and mitochondria by polyclonal anti-CaM may lie in the inability of these

antibodies to recognize partially masked antigenic sites. CaM when bound to various cellular structures may be totally or partially hidden by its conformational arrangement with the associated acceptor protein. This possibility is supported by the fact that most polyclonal CaM antibodies will not immunoprecipitate by intramolecular cross-linking, suggesting a restricted number of antigenic sites (1). In fact, Van Eldik and Watterson (3) have shown that multiple rabbit antisera produced against performic acid-oxidized calmodulin recognize a unique carboxy-terminal domain. Thus, the predominant antigenic sites normally seen in free calmodulin would not be available for antibody binding.

DISCUSSION

In vitro immunization of spleen cell cultures offers an opportunity to produce antibodies against highly conserved proteins that are poorly antigenic by in vivo standards. This procedure allows for the stimulation of β-lymphocytes with nanogram quantities of antigen in their native state. In our laboratory, tubulin (200 ng), actin (200 ng), and calmodulin (200 ng to 1 ng) have proven sufficiently antigenic to elicit specific antibody response in vitro. Luben and Mohler (9) have immunized in vitro with 50 ng of osteoclast activating factor (OAF), with similar positive results. More recently, picomole amounts of rat hypothalamic growth hormone-releasing factor (rGRF) have been used successfully for in vitro immunization and production of monoclonal antibodies (10). Thus, nanogram quantities of soluble protein antigens appear to be sufficient to stimulate β-lymphocytes to produce specific antibodies that can be detected within 2–4 wk after fusion. Mouse monoclonal antibodies produced by in vitro immunization with bovine calmodulin were also cross-reactive with mouse and rat calmodulin. The in vivo regulation of antigen recognition and processing through immunological tolerance and suppression mech-

TABLE II

Immunological Evaluation of Monoclonal Antibody from Several Clones

Clone no.	EIA* O.D.	Cell structures stained by immunofluorescence	Calmodulin affinity‡ elution peak $O.D._{280}$	Immunoprecipitation cpm§
C-101-1	0.38	Mitotic spindle	0.12	200
C-101-2	0.41	Mitotic spindle	0.14	0
C-101-4	0.51	Centrioles and stress fibers	0.10	250
C-101-6	0.42	None	0.00	0
C-101-8	0.39	Mitotic spindle	0.11	211
C-101-10	—	—	—	—
C-101-15	0.54	Mitochondria and stress fibers	0.16	201
C-101-18	0.48	Centrioles and stress fibers	0.13	190
C-101-19	0.40	None	0.09	182
C-101-27	0.40	Mitotic spindle	0.10	159
Fetal bovine serum	0	None	0	0
Rabbit serum	0	None	0	0
Nonimmune mouse serum	0	None	0	0
Media from nonimmune mouse spleen hybridoma	0	None	0	0
Monoclonal Ab to CaM absorbed with				
(1) CaM	0	None	0	0
(2) Parvalbumin	0.38	Mitotic spindle	0.12	205

* EIA background subtracted from above readings.
‡ Affinity column $O.D._{280}$ maximum reading for each clone.
§ Total counts of ^{125}I-CaM = 10,000; above counts represent total counts minus background.

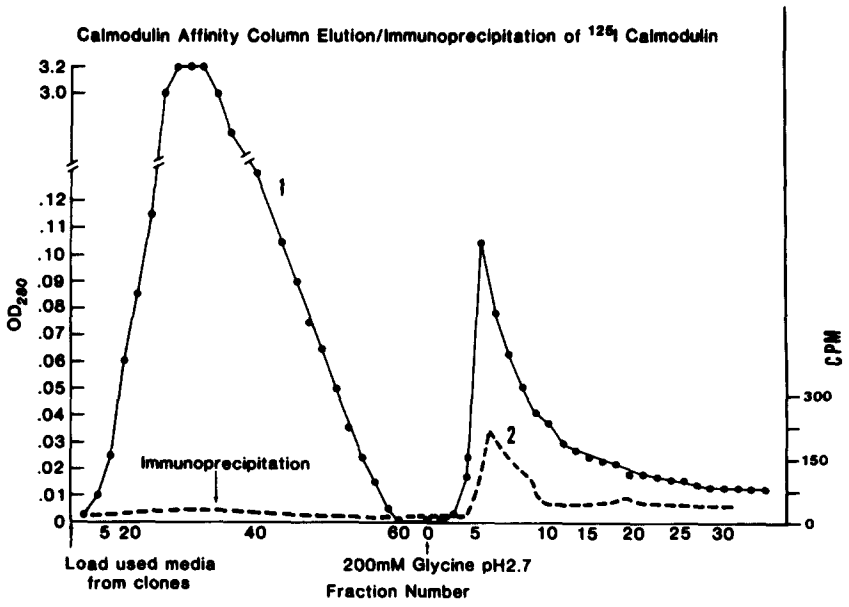

FIGURE 3 Determination of monoclonal anti-CaM activity via affinity chromatography and immunoprecipitation. Supernatant media from various clones were sodium sulfate precipitated and passed over a Sepharose 4B-CnBr calmodulin column. Peak 1 contained no detectable antibody capable of immunoprecipitating ^{125}I-CaM. Peak 2 was eluted with pH 2.7, 200 mM glycine and demonstrated calmodulin-binding specificity of low affinity. Unlabeled calmodulin (1 mg/ml) completely inhibited binding while 1 mg/ml parvalbumin did not. Second antibody alone did not immunoprecipitate ^{125}I-CaM.

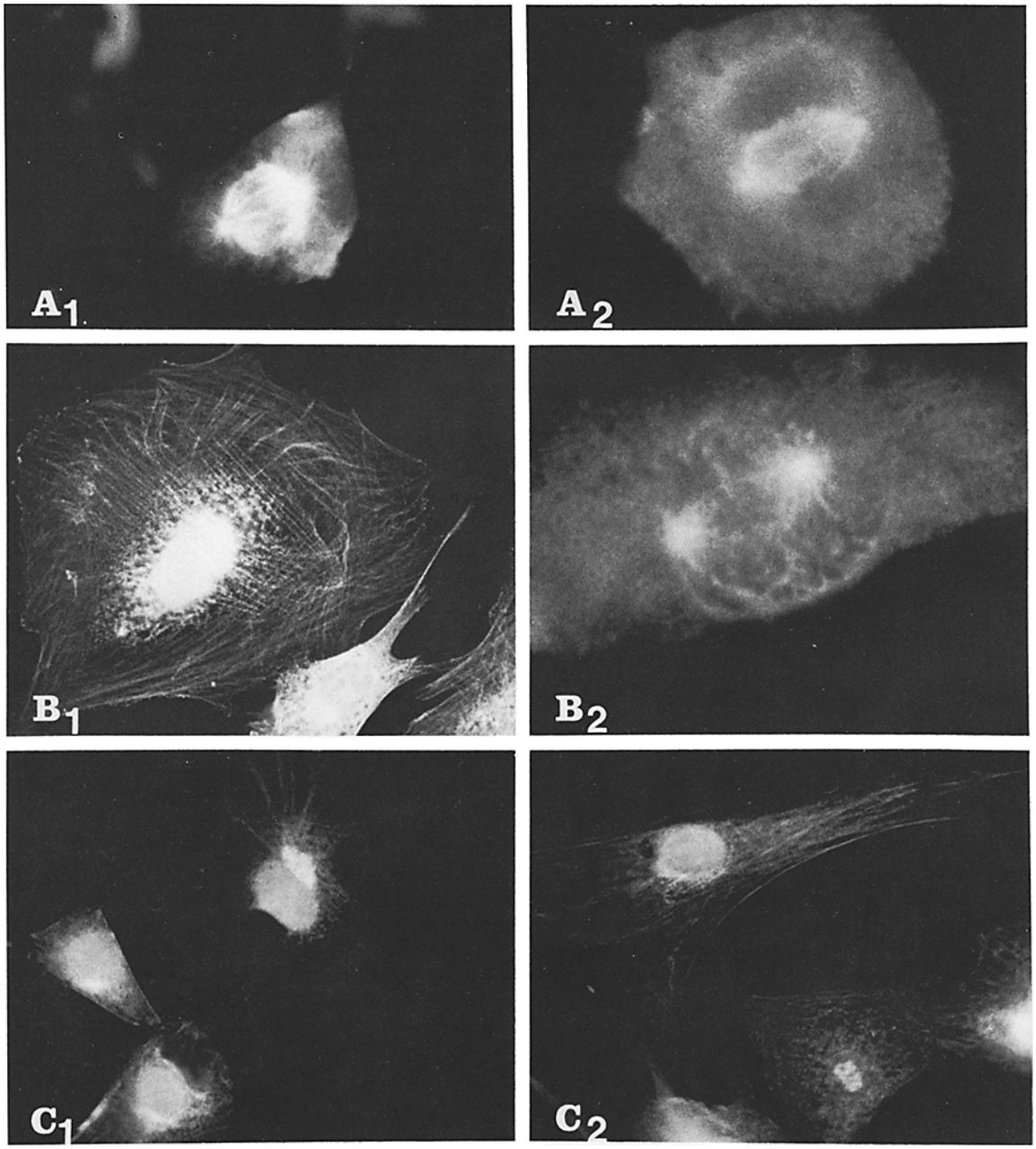

FIGURE 4 Panel A_1 and A_2 demonstrate typical half-spindle staining using monoclonal anti-calmodulin (clone C-101-1); this antibody has a high affinity for CaM associated with the asters and mitotic spindle. Anticalmodulin produced by clones C-101-4 and C-101-18 preferentially localized CaM in association with centrioles and stress fibers (B_1 and B_2). Clone C-101-15 yielded antibody which recognized CaM bound to mitochondria and some stress fibers but not to centrioles or spindles (C_1 and C_2). Neither normal mouse immunoglobulins, rabbit serum, nor monoclonal antibody absorbed with 1 mg/ml calmodulin stained cells by indirect immunofluorescence.

anisms appear to be altered under in vitro conditions (12). Murine autoreactive antibodies are the major products of polyclonal B-cell activation of mouse spleen cells (20), thus minimizing the potential autoantibodies to mouse calmodulin. Autoreactive murine monoclonal antibodies to angiotensin-converting enzyme have recently been reported after conventional in vivo immunization (Auerbach, R., L. Alby, J. Grieves, J. Joseph, C. Lindgren, L. W. Morrisey, Y. A. Sidky, M. Tu, and S. L. Watt, manuscript submitted for publication).

80% of the hybridomas produced in the present study secreted IgM (as opposed to IgG's) when single immunizations were used. When spleen cell cultures were exposed to secondary and tertiary in vitro immunizations during extended immunization periods, the ratio was altered to 50% IgM to 50% IgG's. The high percentage of IgM producers most likely represents an increased frequency of fusion of immature plasma cells with myeloma cells to form the hybridoma. The above data represent the generation of individual hybridoma clones that produce monoclonal antibodies which localize calmodulin in association with other intracellular proteins. These studies suggest that intracellular calmodulin may differentially expose antigenic sites or limited regions of one complex site, depending upon the cellular binding protein with which it is interacting. Thus, unlike polyclonal antibodies, monoclonal antibodies make it possible to study specific interactions of CaM with other cellular macromolecules. This techique should aid in

better understanding the role of calmodulin in regulation of various cellular functions.

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