RAPID COMMUNICATIONS

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 Ca2+-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 x 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 x 10^-5 M-mercaptoethanol, 10 mM...
**In Vivo Immunization**

**Antigen**

**Spleen & Lymph Node Cells**

**Cell Fusion**

**Antigen**

**In Vitro Immunization**

**Immunization with Antigen**

**Spleen Cells in Culture**

**Antibody Assays**

**ELISA (Enzyme-Linked Immunoabsorbent Assay)**

**Substrate**

**Product**

**Goat Anti-Mouse Monoclonal Antibody**

**Antigen**

**Immunofluorescence Microscopy**

**Goat Anti-Mouse IgG**

**Fluorescein**

**Add Goat Anti-Mouse or Protein A**

**Immunoprecipitation**

**Monoclonal Antibody**

**Anogen**

**I25**

**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 B-lymphocyte binding of antigen with minimal degradation. Positive clones are screened by four criteria: ELISA, immunofluorescence, affinity chromatography, and immunoprecipitation.
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. 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). 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. 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.

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 C101-1 resulted in the localization of CaM in the mitotic spindle (Fig. 4A, A1) as previously demonstrated by Welsh et al. (17) and Andersen et al. (18) using goat and rabbit polyclonal anti-CaM. Clone C101-4 antibody decorated stress fibers and centrioles, respectively, in interphase 3T3 cells (Fig. 4B, and B1). Antibody from clone C101-15 localized CaM bound to mitochondria and resulted in some faint staining of stress fibers (Fig. 4D, and D1). 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) did not inhibit binding.

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| 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).
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 mecha-

\[ \text{Table II} \]

| Clone no. | EIA* O.D. | Cell structures stained by immunofluorescence | Calmodulin affinity column 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  | 0.54      | Mitochondria and stress fibers                | 0.16                                          | 201                      |
| C-101-15  | 0.48      | Centrioles and stress fibers                  | 0.13                                          | 190                      |
| C-101-18  | 0.40      | None                                          | 0.09                                          | 182                      |
| C-101-19  | 0.40      | Mitotic spindle                               | 0.10                                          | 159                      |
| C-101-27  | 0.40      | Mitotic spindle                               | 0.12                                          | 205                      |

* EIA background subtracted from above readings.
† Affinity column O.D.280 maximum reading for each clone.
§ Total counts of 125I-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 125I-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 125I-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. Anti-calmodulin 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.

Mechanisms 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 technique should aid in
better understanding the role of calmodulin in regulation of various cellular functions.

The authors wish to thank Dr. B. R. Brinkley for his encouragement and support of this research project. In addition, we wish to thank Dr. C. L. Reading for his assistance in the initial monoclonal experiment.

This work was supported in part by National Institutes of Health grants GM29323 (to J. R. Dedman) and CA23022 (to B. R. Brinkley).

Received for publication 5 October 1982, and in revised form 29 December 1982.

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