Monoclonal Antibody to Chicken Fetal Antigens on Normal Erythroid Cells and Hematopoietic-Lymphoid Tumor Cell Lines

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ABSTRACT

Hybridoma cell lines secreting antibodies to chicken fetal antigens (CFAs) were generated by the fusion of mouse P3x63Ag8 myeloma cells with spleen cells from a mouse immunized with intact SC chicken strain one-day-hatched red blood cells. Immunodepletion studies show monoclonal anti-CFA to be detecting a subset of the M, 50,000 CFA molecules recognized by polyclonal anti-CFA. Monoclonal anti-CFA is erythroid specific against in vivo-derived hematopoietic-lymphoid cells. Exceptions to the erythroid specificity of monoclonal anti-CFA include failure to react with avian erythroblastosis virus-transformed erythroid cells both before and after butyric acid-induced differentiation and reactions with reticuloendotheliosis virus-transformed immature lymphoid cells and chicken embryo cells. Immunofluorescence and 125I binding analyses utilizing monoclonal anti-CFA show reticuloendotheliosis virus cells to possess high levels of CFA even though the CFA determinant does not appear to be a 125I-labeled immunoprecipitable M, 50,000 molecule. The unique property of monoclonal anti-CFA that permits it to distinguish among surface membrane antigens of normal and neoplastic cells of the same lineage makes it an important tool for future investigations of normal and abnormal cell differentiation.

INTRODUCTION

Two hematopoietic-lymphoid antigenic systems, CFAs and CAAs, are differentially expressed during normal erythroid maturation in the peripheral blood of the developing chicken (2, 3, 15, 26). Polyclonal antibodies identify 13 CFA determinants and 8 CAA determinants (7, 8, 15). CFA is immunoprecipitated from erythrocyte cell membranes as a major band with an apparent molecular weight of 50,000 and 2 minor bands with apparent molecular weights of 99,000 and 88,000 (15, 18). Antigens detected by antisera to CAA are associated with proteins having apparent molecular weight of 102,000, 81,000, 48,000 and 43,000 (15, 18).

Cells of the hematopoietic-lymphoid system proliferate and differentiate extensively, giving rise to the morphologically and functionally distinct erythrocytes, lymphocytes, monocytes, and platelets. In the chicken, there are 2 morphologically distinct erythrocyte series, primitive and definitive. The primitive erythrocyte series is present during the first 6 days of embryonic development, while the definitive erythrocyte series is expressed on the sixth day of embryonic development and constitutes the erythrocyte cell series produced throughout the remainder of chicken development. CFAs and CAAs have proven to be useful markers for examining hematopoietic-lymphoid cellular differentiation in the developing chicken (5, 6, 20–22). Three definitive erythrocyte populations are identified in the developing chicken based on CFA and CAA expression: definitive type I RBC which express CFAs, definitive type II RBC which express both CFAs and CAAs, and definitive type III RBC which express CAAs (2, 3, 15). CFA determinants have been shown to be differentially expressed on primitive RBC (23) and definitive RBC (22), as well as peripheral lymphocytes (5, 6) with chicken development. Cloned AEV-transformed erythroid cells differentially express CFAs and CAAs upon chemically induced cellular maturation in vitro. In addition to being useful as membrane markers for studies on cellular differentiation in the normal and neoplastic chicken, CFA determinant No. 9 has been demonstrated to be a cell surface receptor for Sindbis virus hemagglutinin (28).

Limitations on obtaining sufficient amounts of high-titered monospecific antibody by differential adsorptions and elutions of polyclonal anti-CFA sera with RBC from different avians prompted us to produce monoclonal antibodies to CFAs. Here, we describe the procedures for the production and screening of hybridomas to CFAs, and we outline the parameters used for correlating the monoclonally identified CFA determinant(s) to known polyclonally identified CFA determinants. Monoclonal antibody to CFA, produced by clone 190-4 and referred to in this paper as anti-CFA (clone 190-4) or monoclonal anti-CFA, was characterized as to: (a) isotype and functional properties; (b) specificity to hematopoietic-lymphoid cells; (c) ability to immunoprecipitate the M, 50,000 molecule referred to as CFA; and (d) ability to immune deplete CFA.

MATERIALS AND METHODS

Source of RBC. Chickens of the SC inbred strain were purchased from Hyline International (Johnston, Iowa) as fertile eggs, hatched, and raised to adulthood. Adult avians used as sources of RBC included 4-week-old, 81-week-old, 8-, 11-, 12-, 13-, 14-, 17-, 18-, and 19-days post-hatch development, respectively.

Received May 28, 1982; accepted July 14, 1982.

4532 CANCER RESEARCH VOL. 42
Japanese quail, mallard duck, goose, pigeon, and guinea fowl (all purchased locally). Collection of RBC from embryos was accomplished by dissecting a vein surrounding the embryo and collecting the blood in heparin with the aid of a Pasteur pipet. Blood from hatched chickens and adult avians was collected either by heart puncture or wing venipuncture using heparin as the anticoagulant. RBC were separated from lymphocytes on Ficoll-Hypaque and washed 3 times in PBS before use.

Source and Preparation of Various Cells and Tissues. Cells were flushed from pooled spleens, bursas of Fabricius, and thymuses of 17-day-old embryos or 12-day-old chicks (E17 and H12, respectively) by gentle teasing of the organs with tweezers and flushing with PBS as described previously (16). Lymphocytes were enriched by Ficoll-Hypaque treatment (technique of Boyum [4]), and the resulting lymphocyte suspensions were treated with Gey's hemolytic solution (9) to lyse any RBC contaminants. Bone marrow cells were obtained from the tibias and femurs of 14-day-old chicks. AEV-transformed erythroid cells, both before and after butyric acid induction, were prepared as described previously from 8-day-old chicks.4 REV-transformed and cloned cell lines and SPAFAS (specific pathogen free avian supplies) outbred virally free chicken primary fibroblasts (referred to as chicken embryo cells) were generous gifts of Drs. Robert F. Garry and Henry R. Bose, Jr. (Department of Microbiology, University of Texas at Austin). All cells were washed 3 to 4 times in PBS in prior to use in indirect IF. A binding study using chicken, liver, brain, and pectoral major Cryocut tissue sections (7 μm) were prepared from day-old chicks for use in IF studies.

Preparation of Polyclonal Anti-CFA Sera. The preparation of rabbit anti-CFA sera (polyclonal anti-CFA sera) which was prepared against 1-day-hatched SC chicken RBC and adsorbed with adult SC chicken (180 days or older) RBC has been described in detail elsewhere (26).

Hybridoma Production. A 3-month-old female BALB/c mouse was immunized with five 0.2-ml i.p. injections of a 20% suspension of 1-day-hatched SC chicken RBC over a period of 2 weeks. After ascertaining that the mouse was producing antibodies to 1-day-hatched SC chicken RBC, the mouse was sacrificed, and the spleen was taken under sterile conditions. The immune splenic lymphocytes were fused with P3 (17) as described by Getter et al. (11) with minor modifications. Spleen cells and P3 cells were washed 3 times in serum-free Dulbecco’s modified Eagle’s medium with high glucose (4.5 g/liter; [Grand Island Biological Co., Grand Island, N. Y.]) containing 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (Grand Island Biological Co.). Spleen cells (1 × 10^8) and 1 × 10^7 P3 cells were combined and pelleted. Cells were resuspended in 0.2 ml of 30% polyethylene glycol (1000; Koch-Light Laboratories, Ltd., Colnbrook Bucks, England) and centrifuged for 4 min. After a total of 8 min, 5 ml serum-free Dulbecco’s modified Eagle’s medium supplemented with 20% heat-inactivated fetal calf serum (Grand Island Biological Co.) and gentamicin (50 μg/ml; Schering Corp., Kenilworth, N. J.), were added, and the cells were pelleted. Cells were resuspended in 50 ml IM-DME and incubated overnight at 37°C in a humid atmosphere containing 5% CO₂. The cells were then pelleted and resuspended in IM-DME containing hypoxanthine-aminopterin-thymidine and divided equally among 480 wells in 96-U-well microtiter plates. The cells were cultured for 3 days, and 25% of the cells were resuspended in 50 ml of MRS or undiluted P3 media. The samples were incubated 30 min at RT. Samples were then washed 3 times in PBS in order to remove nonreacting antibody. Next, 50 μl of rabbit anti-mouse Ig (1:2000 dilution) were added to each sample, followed by the addition of 25 μCi of 125I-protein A (105 cpm/50 μl) and 20% bovine serum albumin (5 mg/ml) and 0.1% sodium azide, pH 7.4. Cells at 1 × 10^7/ml (50 μl aliquots) were placed in triplicate in flexible 96-U-well microtiter plates and were reacted with serially diluted polyclonal or monoclonal anti-CFA and appropriate controls (either undiluted NRS or undiluted P3 media). The samples were incubated 30 min at RT. Samples were then washed 3 times in PBS in order to remove nonreacting antibody. Next, 50 μl of rabbit anti-mouse Ig (1:2000 dilution) were added to each well, and the samples were incubated 30 min at RT and then washed 3 times in PBS. 125I-Protein A was eluted from a Sephadex G-25 column with PBS containing bovine serum albumin (5 mg/ml) and 0.1% sodium azide, pH 7.4. Cells were then pelleted and resuspended in IM-DME and incubated overnight at 37°C in a humid atmosphere containing 5% CO₂. The cells were then pelleted and resuspended in IM-DME containing hypoxanthine-aminopterin-thymidine and divided equally among 480 wells in 96-U-well microtiter plates. The cells were cultured for 3 days, and 25% of the cells were resuspended in 50 ml of MRS or undiluted P3 media. The samples were incubated 30 min at RT. Samples were then washed 3 times in PBS in order to remove nonreacting antibody. Next, 50 μl of rabbit anti-mouse Ig (1:2000 dilution) were added to each well, and the samples were incubated 30 min at RT and then washed 3 times in PBS. 125I-Protein A (105 cpm/50 μl/well) was added, and the mixtures were incubated for 30 min at RT, followed by 3 washes in PBS, and air dried. The flexible plastic plates were cut, and each well was counted directly in a Beckman gamma counter or the microtiter plates were exposed on Kodak SRP-1 film.

Radiolodination of Cells with the Lactoperoxidase-Glucose Oxidase Technique. Cells were labeled following the procedures of Keski-Oja et al. (12) with minor modifications as outlined previously (15). Briefly, all cells were washed 3 times in D-PBS followed by a single wash in D-PBS containing 1 μM potassium iodide. Packed RBC (0.1 ml) and 1 × 10^11 in 0.1 ml of all other cell types were brought up to a total volume of 0.5 ml in D-PBS. Next, 10 μl of lactoperoxidase (Sigma; 1 μg/1 μl in D-PBS) and 0.2 IU glucose oxidase (Sigma) were added to each sample, followed by the addition of 250 μCi of 125I in 0.5 of 10 mM glucose. Samples were mixed well and incubated at RT for 15 min with frequent gentle mixing. The reaction was quenched by adding 10 μl of 16% sodium azide in D-PBS and washing the cells 3 times in ice-cold D-PBS containing 5 mM potassium iodide. Cells were resuspended in 0.5 ml of D-PBS and lysed by adding 0.5 ml of 1.0% NP-40 in 10 mM PBS.

Characterization of Monoclonal Anti-CFA (Clone 190-4). Immunglobulin isotype was determined by immunodiffusion using antisera specific for mouse immunoglobulin classes, immunoglobulin subclasses, and light-chain types (Litton Bionetics, Kensington, Md.) The ability of hybridoma 190-4 supernatant to bind guinea pig complement was determined by a direct hemolytic assay described previously by Sanders (26). Determination of the ability of monoclonal anti-CFA to bind murine Fc receptors involved the formation of Fc rosettes as described previously (10).

Indirect Immunofluorescence. Tissue sections from lung, liver, brain, and pectoralis major as well as bursa, thymus, peripheral blood and splenic lymphocytes, bone marrow cells, AEV-transformed erythroleukemia cells, REV-transformed immature lymphoid cells, and chicken erythrocytes were examined for CFA using polyclonal anti-CFA, monoclonal anti-CFA (clone 190-4), P3 media control, or NRS by IF using techniques described previously (22). Two hundred cells per assay were scored for positive or negative fluorescence on a modified Leitz epifluorescence microscope. Identification of erythroid cells positive for CFA undergoing cellular maturation in the bone marrow was performed as described previously (22).

125I-Protein A Binding Assays. Protein A was radiolabeled with 125I by the chloramine-T technique described by Romani et al. (25). Briefly, 25 μl of protein A (1 mg/ml) was added to 1.0 μl of 125I in 25 μl of 0.4 M Tris-hydrochloric acid buffer containing 4 mM disodium EDTA (pH 7.4). Next, 10 μl of chloramine-T (2.5 mg/ml) was added. The reaction mixture was gently agitated at RT for 1 min and then terminated with the addition of 25 μl of sodium metabisulfite. The 125I-protein A was eluted from a Sephadex G-25 column with PBS containing bovine serum albumin (5 mg/ml) and 0.1% sodium azide, pH 7.4. Cells at 1 × 10^7/ml (50 μl aliquots) were plated in triplicate in flexible 96-U-well microtiter plates and were reacted with serially diluted polyclonal or monoclonal anti-CFA and appropriate controls (either undiluted NRS or undiluted P3 media). The samples were incubated 30 min at RT. Samples were then washed 3 times in PBS in order to remove nonreacting antibody. Next, 50 μl of rabbit anti-mouse Ig (1:2000 dilution) were added to each well, and the samples were incubated 30 min at RT and then washed 3 times in PBS. 125I-Protein A (105 cpm/50 μl/well) was added, and the mixtures were incubated for 30 min at RT, followed by 3 washes in PBS, and air dried. The flexible plastic plates were cut, and each well was counted directly in a Beckman gamma counter or the microtiter plates were exposed on Kodak SRP-1 film.

Monoclonal Antibody to CFAs
Tris, pH 7.3. After 30 min on ice, the lysates were spun at 40,000 × g for 20 min to remove insoluble materials.

**Indirect Immune Precipitation and SDS:PAGE.** Immunoprecipitation was carried out using heat-killed formalin-fixed Staph A prepared as described by Kessler (14). Cell extracts (1.0 ml) were incubated for 1 hr with 0.25 ml 10% Staph A in wash buffer (0.01 M Tris-HCl, pH 8.0, containing 0.15 M NaCl, 1 mM disodium EDTA, and 0.5% NP-40). After removal of the bacteria by centrifugation (7000 × g; 1 min), 100 µl of the cleared extracts were incubated (4°C; 16 hr) with polyclonal anti-CFA sera, monoclonal anti-CFA, P3 media control, or NRS. Immune complexes were then collected by incubation with 100 µl of 10% Staph A for rabbit sera or with 100 µl of 10% Staph A previously loaded with an excess amount of rabbit anti-mouse Ig (referred to as rabbit anti-mouse immunoglobulin Staph A) for mouse sera for 1 hr. The Staph A was harvested by centrifugation (7000 × g; 1 min) and then washed 3 times with the wash buffer. Bound antigens were then eluted by resuspension of the Staph A in 50 µl of SDS sample buffer (0.06 M Tris-HCl, pH 6.7, containing 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and incubated at 100°C for 5 min. The antigens were then analyzed by SDS:PAGE on 10% polyacrylamide (10% acrylamide:0.26% bisacrylamide) as described (19). Slab gels were stained with Coomassie Brilliant Blue R-250, dried on a slab drier, and exposed at -70°C on Kodak XAR-5 film using Dupont-Cronex Lightning-Plus intensifying screen. The following proteins were used as molecular weight markers: bovine serum albumin (M, 68,000); ovalbumin (M, 43,000); and carbonic anhydrase (M, 29,000) (all from Sigma).

**Immunodepletion.** Aliquots (50 µl) of cleared, radiolabeled, definitive type I RBC (obtained from 19-day embryos) solubilized extract and polyclonal anti-CFA sera, monoclonal anti-CFA, P3 media control, or NRS. Immune complexes were then collected by incubation with 100 µl of the reciprocal polyclonal or monoclonal anti-CFA sera for 1 hr. The Staph A was harvested by centrifugation (7000 × g; 1 min) and then was washed 3 times with the wash buffer. Bound antigens were then eluted by resuspension of the Staph A in 50 µl of SDS sample buffer (0.06 M Tris-HCl, pH 6.7, containing 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) and incubated at 100°C for 5 min. The antigens were then analyzed by SDS:PAGE on 10% polyacrylamide (10% acrylamide:0.26% bisacrylamide) as described (19). Slab gels were stained with Coomassie Brilliant Blue R-250, dried on a slab drier, and exposed at -70°C on Kodak XAR-5 film using Dupont-Cronex Lightning-Plus intensifying screen. The following proteins were used as molecular weight markers: bovine serum albumin (M, 68,000); ovalbumin (M, 43,000); and carbonic anhydrase (M, 29,000) (all from Sigma).

**RESULTS**

**Screening and Characterization of Hybridomas.** Initial screening of hybridoma supernatants with H1 and adult chicken RBC revealed 84 hybridomas secreting antibody that gave positive hemagglutination reactions with H1 RBC but failed to react with RBC obtained from adult chickens. On the basis of strength and speed of hemagglutination reactions, 8 hybridomas were selected for further testing. Supernatants from the 8 hybridomas were screened using RBC from several different avian species possessing different CFA combinations as previously determined with polyclonal antibody (27). Data from the second screen are depicted in Table 1. Seven of the supernatants were negative to all test cells except H1 chicken RBC. Hybridoma 190-1 secreted antibody that gave positive reactions with H1 chicken RBC as well as adult goose and adult Japanese quail RBC. On the basis of the reaction pattern of supernatant from hybridoma 190-1, it was concluded that this hybridoma was producing either antibody to CFA Determinant No. 13 or to some heretofore undetermined CFA determinant shared by goose, Japanese quail, and H1 RBC. Unfortunately, hybridoma 190-1 was lost during further cloning attempts.

Supernatants from hybridomas 190-2, -3, -4, -5, -6, -7, and -8 failed to bind protein A or to fix guinea pig complement. Immunoprecipitation of radiolabeled H1 RBC NP-40 extracts when analyzed by SDS:PAGE revealed that hybridoma 190-4 was the only hybridoma that secreted antibody that immunoprecipitated a labeled protein. Hybridoma 190-4 immunoprecipitated a molecule with an apparent molecular weight of 50,000.

On the basis of specificity to H1 RBC and the immunoprecipitation of a M, 50,000 molecule, hybridoma 190-4 was further cloned by limiting dilution and soft agar cloning.

**Characterization of Anti-CFA (Clone 190-4) for Immunoglobulin Class and Secondary Antibody Functional Properties.** Monoclonal anti-CFA (clone 190-4) is an IgG1, (y1 heavy, k light chain). It does not bind protein A, fix complement, or recognize the Fc receptor of murine lymphocytes.

**Comparison of the Cellular Distribution of CFA Detectable by Polyclonal and Monoclonal Anti-CFA.** When monoclonal anti-CFA was assayed for hematopoietic-lymphoid specificity by IF or 125I-protein A binding assays on normal erythroid and lymphoid cells, it was found to be erythroid specific. However, analysis of REV-transformed immature lymphoid cell lines and chicken embryo cells showed monoclonal anti-CFA to also be reactive with these nonerythroid cell types (Table 2; Chart 1).

Monoclonal anti-CFA failed to react with thymus or bursa lymphocytes from different aged chickens. Nor did it react with peripheral blood lymphocytes or splenic lymphocytes. Mature

### Table 1

<table>
<thead>
<tr>
<th>Source of test RBC</th>
<th>CFA expression by test RBC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hybridoma supernatants from 190-&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Controls&lt;sup&gt;c&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Adult chicken</td>
<td>None</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>9, 10, 11, 12</td>
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<tr>
<td>1-day-hatched chicken</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
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<tr>
<td>Adult Japanese quail</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
</tr>
<tr>
<td>Adult goose</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
</tr>
<tr>
<td>Adult turkey</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
</tr>
<tr>
<td>Adult duck</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
</tr>
<tr>
<td>Adult guinea fowl</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
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<tr>
<td>Adult pigeon</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12</td>
<td>+, +, +, +, +, +, +, +, +</td>
<td>+, +, +, +, +, +, +, +, +</td>
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<sup>a</sup> CFA is a serologically complex antigen composed of 13 distinct, serologically detectable, antigenic determinants. All 13 CFA determinants are expressed on 1-day-hatched chicken RBC, whereas RBC from other avians expressed unique subsets of CFA determinants. Thus, monoclonal antibody specificity to individual CFA determinants can be established by performing a series of hemagglutination assays using a variety of RBC possessing different subsets of CFA determinants.

<sup>b</sup> 9, P3 media; 10, normal mouse serum; 11, mouse anti-1-day-hatched chicken RBC, unadsorbed; 12, polyclonal anti-CFA sera.

<sup>c</sup> Negative hemagglutination reaction.

<sup>d</sup> Positive hemagglutination reaction.
granulocytes, lymphocytes, and monocyte-macrophages derived from H14 bone marrow did not react with monoclonal anti-CFA (Table 2). All stages of erythroid maturation (erythroblast, early polychromatophilic erythrocytes, midpolychromatophilic erythrocytes, late polychromatophilic erythrocytes, reticulocytes, and mature erythrocytes) in the bone marrow of H14 were positive when tested by IF with monoclonal anti-CFA (Table 2). However, the immature erythroid cells (erythroblasts and early polychromatophilic erythrocytes) exhibited weak fluorescence when compared to cells in the latter stages of erythroid maturation. Control and butyric acid-induced AEV-transformed erythroleukemia cells were negative for CFA when tested by IF with monoclonal and anti-CFA.

Peripheral RBC from E5 (representing primitive RBC which express CFAs), peripheral RBC from E18 (representing definitive type I RBC which express CFAs), and peripheral RBC from H83 (representing definitive type II RBC, which express a subset of CFA determinants and a subset of CAA determinants) (15) gave positive results with monoclonal anti-CFA when analyzed by IF or 125I-protein A binding assays. Peripheral RBC from adult chickens (representing definitive type III RBC which express CAAs) (15) were negative with monoclonal anti-CFA with these assays (Table 2).

IF analyses of normal non-hematopoietic-lymphoid tissues (including chicken lung, liver, brain, and muscle-pectoralis major tissues) with monoclonal anti-CFA showed these tissues to not possess CFA (Table 2).

Analysis of CFA on Definitive Types I and III RBC, REV-transformed Immature Lymphoid Cells and Chicken Embryo Cells by 125I-Protein A Binding Studies. 125I-Protein A binding assays using serially diluted monoclonal anti-CFA and definitive type I RBC, definitive type III RBC, REV-transformed immature lymphoid cells [cloned cell lines designated KBMC, C(1), and C4(1)], and chicken embryo cells were assayed. The average 125I cpm of 3 determinations is given for each cell type at each dilution of monoclonal anti-CFA assayed.

H83 (representing definitive type II RBC, which express a subset of CFA determinants and a subset of CAA determinants) (15) gave positive results with monoclonal anti-CFA when analyzed by IF or 125I-protein A binding assays. Peripheral RBC from adult chickens (representing definitive type III RBC which express CAAs) (15) were negative with monoclonal anti-CFA with these assays (Table 2).

Molecular Profiles of Antigens Immunoprecipitated with Polyclonal and Monoclonal Anti-CFA from Radioiodinated Membranes from Definitive Types I, II, and III RBC, AEV-transformed Erythroblasts, REV-transformed Immature Lymphoid Cells, and Chicken Embryo Cells. SDS-PAGE analyses of antigens immunoprecipitated with polyclonal and monoclonal anti-CFA from solubilized membrane extracts of radioiodinated E19 RBC (definitive type I RBC; No. 1), H96 (definitive type II RBC; No. 2), adult chicken RBC (definitive type III RBC; No. 3), chicken embryo cells (CE; No. 4), REV-transformed immature lymphoid cells (cloned cell lines designated KBMC, C(1), and C4(1)), and chicken embryo cells are depicted in Chart 1. All cell types reacted with the monoclonal anti-CFA with the exception of definitive type III RBC. Definitive type I RBC and the REV-transformed cloned cell lines showed similar 125I-protein A binding abilities at the different monoclonal antibody dilutions. A reduced pattern of 125I-protein A binding was exhibited by the chicken embryo cells.
formed immature lymphoid cells (the REV-transformed cloned cell line KBMC, both pre-No. 5 and post-No. 6 butyric acid induction), and AEV-transformed erythroid cells (both pre-No. 7 and post-No. 8 butyric acid induction) are depicted in Fig. 1. NRS and polyclonal anti-CFA immunoprecipitates are depicted in Fig. 1A, and P3 and monoclonal anti-CFA immunoprecipitates are depicted in Fig. 1B. NRS and P3 controls (run in the first of 2 lanes given for each cell type) did not immunoprecipitate any labeled proteins. Polyclonal anti-CFA immunoprecipitated M, 99,000 and 50,000 molecules from definitive type I RBC, M, 50,000 molecules from definitive type II RBC, and no molecules from definitive type III RBC. Polyclonal anti-CFA immunoprecipitated molecules with apparent molecular weight of 87,000, 83,000 and 44,000 from chicken embryo cells and molecules with apparent molecular weight of 108,000, 89,000, 80,000, 59,000 and 50,000 from preinduced KBMC cells and molecules with apparent molecular weight of 80,000 and 50,000 from induced KBMC cells. Pre- and postinduced AEV-transformed erythroid cells exhibited M, 110,000, 94,000, 87,000, 59,000, and 50,000 molecules with polyclonal anti-CFA immunoprecipitation.

Monoclonal anti-CFA immunoprecipitated M, 50,000 molecules from definitive types I and II RBC (note the low levels of M, 50,000 molecules immunoprecipitated from definitive type II RBC) and did not immunoprecipitate anything from definitive type III RBC. Low levels of 110,000 and 48,000 molecules were immunoprecipitated from chicken embryo cells and 2 molecules of 50,000 and 48,000 were immunoprecipitated from pre- and post-butyric acid-induced KBMC cells while no molecules were immunoprecipitated from pre- or post-butyric acid-induced AEV-transformed erythroid cells. The low-molecular-weight molecules immunoprecipitated by controls and anti-CFA sera in Fig. 1 probably represent degradation products.

Immunodepletion of CFA from Definitive Type I RBC by Monoclonal and Polyclonal Anti-CFA. Immunodepletion studies were conducted to ascertain whether the same or different M, 50,000 molecules of CFA were being detected with monoclonal as with polyclonal anti-CFA sera. Aliquots of solubilized membrane extracts of radioiodinated peripheral E19 RBC were immunodepleted with NRS (Fig. 2A), polyclonal anti-CFA (Fig. 2B), P3 (Fig. 2C), or monoclonal anti-CFA (Fig. 2D), and any resulting antigen-antibody complexes were removed by follow-

DISCUSSION

Hybridoma cell lines secreting antibodies to CFAs were generated by the fusion of mouse P3 myeloma cells with spleen cells from a mouse immunized with intact SC strain 1-day-
hatched chicken RBC. The possibility that monoclonal antibodies might recognize a subset of the different antigenic moieties comprising the antigenerically complex CFA was demon-

Based on specificity of erythroid membrane antigen detection and the ability to immunoprecipitate a M, 50,000 molecule, hybridoma 190-4 was subjected to rigorous cloning and further study. Monoclonal anti-CFA (clone 190-4) is of the IgG1 (κ) isotype. Monoclonal anti-CFA (clone 190-4) does not bind protein A, fix complement, or recognize the Fc receptor of murine lymphocytes.

Comparisons between reactivity of polyclonal and mon-

In comparing polyclonal and monoclonal anti-CFAs with CFAs on different cell types show monoclonal anti-CFA to be erythroid specific with the exceptions of reactions with REV-transformed immature lymphoid cells and chicken embryo cells cultured in vitro. While polyclonal and monoclonal anti-CFAs give similar reaction patterns for eryth-

In implementing these studies, all initial studies involving in vivo-derived cells suggested that monoclonal anti-CFA was recognizing an erythroid-specific CFA determinant. However, analyses of AEV-transformed cells, REV-transformed cells, and chicken embryo cells showed monoclonal anti-CFA to not be erythroid specific and to give a unique reaction pattern. Although it has been shown that
Monoclonal Antibody to CFAs

erythroleukemia cells derived from AEV transformation possess CFA, monoclonal anti-CFA did not react with erythroleukemia cells as measured by immunofluorescence nor was it able to immunoprecipitate a M, 50,000 molecule from erythroleukemia cells. Therefore, we conclude that the CFA determinants expressed by AEV-transformed cells or by AEV-transformed cells induced to undergo cellular maturation with butyric acid are not recognized by monoclonal anti-CFA even though monoclonal anti-CFA is capable of recognizing CFA determinants which are expressed by cells undergoing normal, in vivo, cellular maturation as early as the erythroblast stage.

Perhaps even more surprising than the inability of monoclonal anti-CFA to react with AEV-transformed erythroid cells was the discovery that monoclonal anti-CFA did react with REV-transformed immature lymphoid cells. REV-transformed immature lymphoid cells are described as possessing both B- and T-cell characteristics (1). When REV-transformed immature lymphoid cells were analyzed by IF or 125I-protein A binding studies using monoclonal anti-CFA, they were shown to possess high levels of CFA. Low levels of M, 50,000 and 48,000 molecules were immunoprecipitated by monoclonal anti-CFA from the REV-transformed cells. Polyclonal anti-CFA immunoprecipitated molecules with apparent molecular weight of 108,000, 89,000, 80,000, 59,000, and 50,000 from pre-butyric acid-induced REV-transformed cells. The diminution and loss of CFA molecules from butyric acid-induced REV-transformed cells (comparison between Figs. 1A, Lanes 5 and 6) may reflect membrane changes associated with cellular maturation. The discrepancy between the high amounts of CFA detected by IF and 125I-protein A binding and the low amounts of CFA immunoprecipitated is unknown. Perhaps the M, 50,000 CFA determinant recognized by monoclonal anti-CFA does not label well, or it is possible that CFA detectable with monoclonal anti-CFA is associated with glycolipids or glycopeptides that are not detectable using these techniques. The ability of monoclonal anti-CFA to react with REV-transformed immature lymphoid cells but not to react with a variety of normal B- and T-lymphocyte populations may reflect: (a) quantitative CFA differences between normal lymphocytes and REV-transformed cells; (b) qualitative differences between REV-target cell membrane antigens and the membrane antigens of the B- and T-lymphocyte populations tested; (c) quantitative or qualitative differences in CFA expression due to viral transformation; or (d) may represent an immunological cross-reactivity that may or may not be functionally significant.

IF and 125I-protein A binding assays show polyclonal anti-CFA to react with chicken embryo cells. Molecular profiles of chicken embryo cell antigens immunoprecipitated with polyclonal anti-CFA show molecules with apparent molecular weights of 87,000, 83,000, and 44,000. Molecular profiles of CFAs immunoprecipitated with monoclonal anti-CFA show chicken embryo cells to possess low levels of M, 110,000 and 48,000 molecules. The relationships among the M, 110,000 and 48,000 molecules immunoprecipitated with monoclonal anti-CFA and the M, 87,000, 83,000, and 44,000 molecules immunoprecipitated with polyclonal anti-CFA remain to be determined. Perisic et al. (24) demonstrated that an antisera that immunoprecipitates a M, 48,000 molecule from RBC obtained from embryonic chickens also immunoprecipitates a M, 48,000 molecule from chicken embryo cells. Subpopulations of CFAs on RBC were postulated when it was shown that polyclonal anti-CFA could be made nonreactive to definitive type II RBC by repeated adsorptions with definitive type II RBC while still retaining activity to definitive type I RBC (15). The immunodepletion studies reported here are indicative of 2 distinct populations of M, 50,000 molecules: (a) those that possess CFA determinants reactive with monoclonal anti-CFA; and (b) those that do not possess CFA reactive with monoclonal anti-CFA. Studies by Krsmanovic et al. (8) show 2 distinct populations of M, 48,000 molecule immunoprecipitated from chicken RBC.

The ability of polyclonal anti-CFA to remove all CFAs reactive with monoclonal anti-CFA suggests that polyclonal and monoclonal anti-CFA recognize the same CFA determinant, perhaps CFA determinant No. 8 or a subset of CFA determinant No. 8. Alternatively, monoclonal anti-CFA recognizes a unique CFA determinant not recognized by polyclonal anti-CFA. In the latter case, to explain the immunodepletion studies, one must postulate that the M, 50,000 molecule recognized by the monoclonal antibody must contain a polyclonally recognized antigen in addition to the unique antigen recognized by the monoclonal antibody. Although both polyclonal and monoclonal anti-CFA immunoprecipitate M, 50,000 molecules, 2-dimensional isoelectric SDS-PAGE studies now in progress show the M, 50,000 molecule to be comprised of at least 9 isoelectrically distinct M, 50,000 molecules suggestive of sialic acid differences.

The unique property of monoclonal anti-CFA that permits it to distinguish among surface membrane antigens of normal and neoplastic cells of the same lineage makes it an important tool for future investigations of normal and abnormal cellular differentiation.

REFERENCES


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immunoprecipitation methods, and the procedures for electrophoresis including molecular weight standards are detailed in ‘Materials and Methods.’

Fig. 1. An autoradiograph comparison of the molecules immunoprecipitated by polyclonal anti-CFA (A) and monoclonal anti-CFA (B) is given. Solubilized antigens from different cell types are designated by Numbers 1 through 8. Cell types examined were: definitive type I RBC (No. 1), definitive type II RBC (No. 2), definitive type III RBC (No. 3), chicken embryo cells (No. 4), pre- and post-butyric acid-induced REV-transformed immature lymphoid cells (Nos. 5 and 6), and pre- and post-butyric acid-induced AEV-transformed erythroid cells (Nos. 7 and 8). For comparative purposes, sera or media control data is given in the left lane and anti-CFA data is given in the right lane for each cell number. NRS (first lane of each cell number in A) served as control for polyclonal anti-CFA while P3 cell culture media (first lane of each


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A

B

C

D

99Kd

50Kd

99Kd

50Kd

1 2 3 4

1 2 3 4

1 2 3 4

1 2 3 4

A

B

C

D

Monoclonal Antibody to CFAs

B

Monoclonal Antibody to CFAs

C

Monoclonal Antibody to CFAs

D

Monoclonal Antibody to CFAs
Monoclonal Antibody to Chicken Fetal Antigens on Normal Erythroid Cells and Hematopoietic-Lymphoid Tumor Cell Lines

Bob G. Sanders, James P. Allison and Kimberly Kline


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