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Hybridoma cell lines secreting monoclonal antibodies against equine infectious anemia virus

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A monoclonal anti-equine infectious anemia virus (anti-EIAV) antibody (1B15) has been generated by fusion of X63 Ag 8.653 myeloma cells and spleen cells from mice hypersensitized with viral antigen p29. Ouchterlony double-diffusion analysis indicated that antibody 1B15 is of the IgG class. The specificity of the immune reaction for p29 was confirmed by cross-over immunoelectrophoresis and disc-gel electrophoresis. MAb 1B15 was used to devise a solid-phase 'capture' RIA for EIAV-p29 antigen.

The antigen, bound by 1B15 adsorbed onto wells of flexible microtitre plates, was detected using a rabbit anti-p29 serum followed by a ^{125}I-labelled tracer. The assay was applied to detect the presence of virus in horse serum and infected cell culture fluids.

Equine infectious anemia; Monoclonal antibody; Antigen detection

Introduction

The finding of the group-specific antigen p29 as major reacting equine infectious anemia (EIA) viral component has provided virological laboratories with useful tests for the detection of viral antigens and antibodies (Coggins et al., 1972). The level of virus antibodies is currently detected in the infected horse by the agar gel immunodiffusion (AGID) test, although it is 10^2- to 10^4-fold less sensitive than radioimmunoassay (Horenstein and Feinstein, 1985; Shen et al., 1979). Perhaps more importantly, there is a critical need for the development of a sensitive and practical assay for viral antigens to replace horse infectivity and equine leukocyte culture tests (Kobayashi and Kono, 1967; Malnquist et al., 1973) in order to discriminate between the extent of humoral immunity to EIA virus (EIAV) antigen p29 and viremic states (McConnell and Katada, 1981). On the other hand, it remains

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to be seen whether human cells are definitively nonpermissive for EIAV (Hyslop, 1966; Peters, 1954).

Standard radioimmunoassays (RIA) for viral antigen quantification suffer from the disadvantage that labelling of viruses might be followed by loss or alteration of antigenicity as a consequence of oxidation and radiation damage (Andres, 1982). Moreover, viral proteins possess antigenic determinants shared by other viruses as well as species-specific epitopes. In order to obtain large amounts of homogenous antibodies against these specific epitopes free from antigenic variations (Montelaro et al., 1984), we resorted to the hybridoma technology for preparation of clones of hybrid cell lines which secrete monospecific anti-viral antibody (Kohler and Milstein, 1975).

In the present study, we describe the isolation of cell lines producing monoclonal antibodies (MAb) against the p29 protein of EIAV, the characterization of one of the antibodies and how this antibody proved to be useful for specific antigen detection.

Materials and Methods

Antisera preparations

EIAV antiserum C1D3 was obtained by immunizing a rabbit with EIA viral antigen p29 as described previously (Horenstein and Feinstein, 1985). Antibodies to rabbit (GARIG) and mouse immunoglobulins (RAMIG), raised in goats and rabbits, respectively (Horenstein and Feinstein, 1985), were purified by salting out and chromatography on Sephadex G200 (Pharmacia) and iodinated with 50 μCi $Na^{125}I$ (NEN) and Iodo-Gen (Pierce Chemical) as solid-phase oxidant (Fracker and Speck, 1978).

Production of hybridomas

Balb/c mice were hyperimmunized with p29 antigen emulsified with complete Freund's adjuvant (50 μg p29, i.p.), followed by four booster injections in incomplete adjuvant over a period of 32 weeks. A similar dose (i.v., 0.9% NaCl) was given 3 days before fusion. Cell fusion was carried out using 10^8 splenic lymphocytes and 10^7 mouse myeloma cells X63 Ag 8.653 by means of polyethylene glycol (PEG 1500, 30% wt/vol). After fusion, the cells resuspended in RPMI 1640 medium containing 20 mM HEPES (pH 7.2), 15% FCS (GIBCO), 2 mM glutamine, 2.2 mM sodium bicarbonate, 2 mM sodium pyruvate, 100 IU/ml penicilin, 100 μg/ml streptomycin (HM, hybridoma medium) were distributed in 1-ml aliquots into 2 × 24-well Falcon trays containing feeder layers of 5×10^4 Balb/c peritoneal macrophages established one day before the addition of the cells. After 24-h incubation, one-half of the medium was replaced by HM containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT medium) on days 2, 3 and 4. Starting on day 21, HAT medium was gradually replaced by HM-containing

hypoxanthine and thymidine (HT medium). When clones had grown to about 40% confluence, aliquots of the supernatants were removed and tested for activity using an indirect RIA (see below). Positive cultures were cloned at limiting dilutions in 96-well microtiter plates (Nunc) in the presence of macrophage feeder layers. After repetitive minicloning and expansion, the hybridomas were frozen in liquid nitrogen.

To obtain larger amounts of antibody, hybrid cell lines were derived as ascitic tumors and the collected fluids were analysed by electrophoresis in cellulose acetate for immunoglobulin production. The strips were stained with Coomassie Blue and scanned at 280 nm. The Ig class of the MAb was determined by a standard Ouchterlony immunodiffusion against class-specific rabbit anti-mouse IgG and IgM sera (Sigma).

Solid-phase radioimmunoassay methods

Four systems of RIA were used (Fig. 1A–D). Details of each are given below.

(A) Heterologous-competitive RIA

1. Flexible PVC microplates (Linbro, Flow Laboratories) sensitized with 50 µl per well of antigen at 0.1 mg/ml optimal protein concentration determined previously (Horenstein and Feinstein, 1985) for 2 h at room temperature.
2. Wells washed 3 × with RIA buffer prior and after incubation for 30 min at 37°C with RIA buffer.

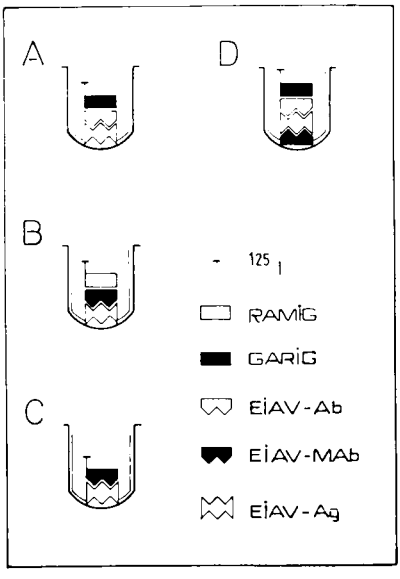

Fig. 1. Schematic assay protocols for EIAV antibody (A, B, C) and p29 antigen (D) detection. For details, see Materials and Methods.

3. Mouse test sera and normal control sera in serial two-fold dilutions in RIA buffer, 50 μl per well incubated for 2 h at 37°C.
4. Wells washed 3 × with RIA buffer.
5. Rabbit anti-p29 antiserum C1D3 diluted in RIA buffer, 50 μl per well incubated for 2 h at 37°C.
6. Wells washed 5 × with RIA buffer.
7. ^{125}I-GARIG, approximately 50000 cpm per 50 μl diluted in RIA buffer, incubated ON at 4°C.
8. Wells washed 5 × with RIA buffer.
9. Bound radioactivity counted.

(B) Indirect RIA

1. Performed as described in Step A1
2. Wells washed 3 × with RIA buffer prior and after incubation for 30 min at 37°C with RIA buffer.
3. Hybridoma supernatants, neat or diluted 1:10 in RIA buffer incubated at a rate of 50 μl per well for 4–6 h at 37°C.
4. Wells washed 3 × with RIA buffer.
5. ^{125}I-RAMIG diluted in RIA buffer at 50 μl per well incubated ON at 4°C.
6. Wells washed 5 × with RIA buffer.
7. Bound radioactivity counted.

(C) Direct-binding RIA

1. Flexible PVC microplates were sensitized with 50 μl per well of varying concentrations (μg protein per ml) of antigen in PBS for 2 h at room temperature.
2. Wells washed 3 × with RIA buffer prior and after incubating for 30 min at 37°C with RIA buffer.
3. ^{125}I-labelled hybridoma antibody in RIA buffer at 50 μl/well, incubated for 2 h at room temperature.
4. Wells washed 4 × in RIA buffer.
5. Bound radioactivity counted.

(D) Antigen capture RIA

1. Flexible PVC microplates sensitized with 50 μl per well of a solution 10^{-2} to 10^{-5} of the MAb in PBS incubated at room temperature ON.
2. Wells washed 3 × with RIA buffer prior and after incubation for 30 min at 37°C with RIA buffer.
3. Several sources of antigen p29 diluted in RIA buffer at 50 μl/well incubated for 6 h at room temperature.
4. Wells washed 3 × with RIA buffer.
5. Performed as described in Step A5–9.

Results

Specific immunoglobulin antibody in the sera from injected mice, assayed by heterologous-competitive RIA (Fig. 1A) could be detected by 8 weeks after the injection. Three mice with titers at or greater than 1:800 were boosted and killed 3 days later, and a pool of spleens was prepared for cell fusion. After hybridization and plating in HAT medium, anti-p29 antibody activity in culture fluids of growing hybridomas was detected by the indirect RIA procedure in 10 out of 36 wells (Fig. 1B). The number of antigen-specific hybridomas per total number of hybridomas (specific efficiency) was 27%. Cells from positive wells were cloned and 192 wells containing cells were tested, yielding 45 positives. Thus, from the number of positive wells in the two-culture step, it was calculated that about 1 in 15 hybrid cells was a specific antibody producer. Positive wells were recloned and one of the subclones, named 1B15, which bound 20 times the control value (3900 cpm vs 195 cpm) was used for further studies. The karyotype analysis confirmed that 1B15 cells grown in culture for over four months were chromosomally hybrids by the increase in chromosome number (modal no. 72) as compared to the X63 Ag 8.653 karyotype (modal no. 51).

The hybridoma 1B15 induced a vigorous ascitic tumor containing 10–12 mg/ml of antibody protein (E 280 mg/ml = 0.71). The immunological activity of MAb

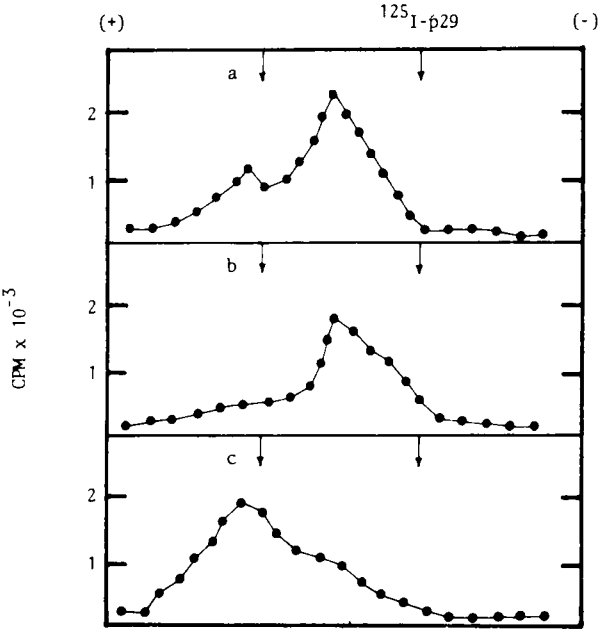

Fig. 2. Radioactive profile of ^{125}I-p29 antigen subjected to cross-over immunoelectrophoresis carried out on agar gel in the presence of: (a) MAb 1B15, (b) equine test serum, and (c) equine normal serum; placed in parallel slots and electrically driven toward each other (210 V; 4 mA per slide) in Veronal buffer (pH 8.6) in a Shandon apparatus.

1B15, purified from ascitic fluid by addition of ammonium sulphate to 40% saturation and further purified by gel filtration through a Sephadex G-200 column, and subsequently iodinated, was assessed by a simple direct-binding RIA (Fig. 1C).

The double-diffusion assay carried out with ascitic fluid 1B15 showed the presence of immunoglobulin of the IgG class. The immunoprecipitation complex detected by cross-over immunoelectrophoresis revealed the specificity of the immune reaction for p29 (Fig. 2A–C). Additional evidence comes from disc-gel electrophoresis analysis. Pre-electrophoresis of antibody 1B15 on 12% acrylamide gel was overlayed with ^{125}I-p29 and run through the same gel to detect p29 binding activity (Fig. 3). The detection of labelled material at the top of the gel indicated the presence of anti-p29 antibodies (Clevinger et al., 1979).

Based on the reliability of antigen capture methods with polyclonal antibodies used for diagnosing viral diseases (Yolken and Stopa, 1980), we employed the MAb 1B15 in a 'capture' immunoassay (AgC–RIA) for EIAV antigen p29 detection in test serum and infected cell culture fluids as sources of antigen (Fig. 1D). The MAb 1B15 was adsorbed on flexible microplates to serve as 'capture' antibodies. The antigen bound by the MAb was then detected by a double antiglobulin technique using C1D3 rabbit anti-p29 serum follow by ^{125}I-GARIG as labelled tracer. Following this procedure, titrations of p29 EIA viral antigen were performed in microplates coated with a series of 10-fold MAb 1B15 dilutions from 10^{-2} to 10^{-5} for the determination of the optimal concentration of 'capture' MAb. As the same MAb could not be used for both binding and labelling, due to competition for the same binding site, the combination with conventional rabbit antiserum C1D3 was suitable for the quantitation of 'captured' antigen p29. From the results, it was

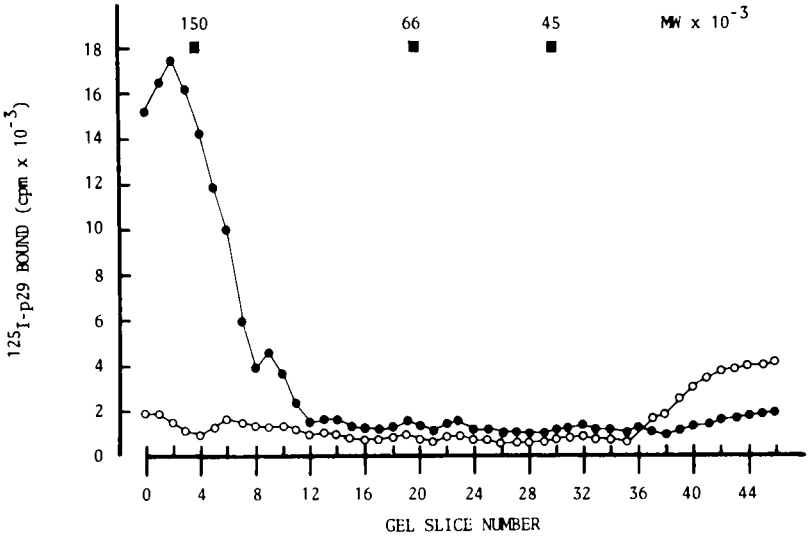

Fig. 3. Polyacrylamide tube gel electrophoresis of ^{125}I-p29 carried out in the presence (•—•) and absence (○—○) of MAb 1B15 previously run on the same gel. The radioactivity associated with each gel was determined in 2-mm slices. The running position of the molecular weight markers ovalbumin (45 000), bovine serum albumin (66 200), and IgG (150 000) is shown at the top.

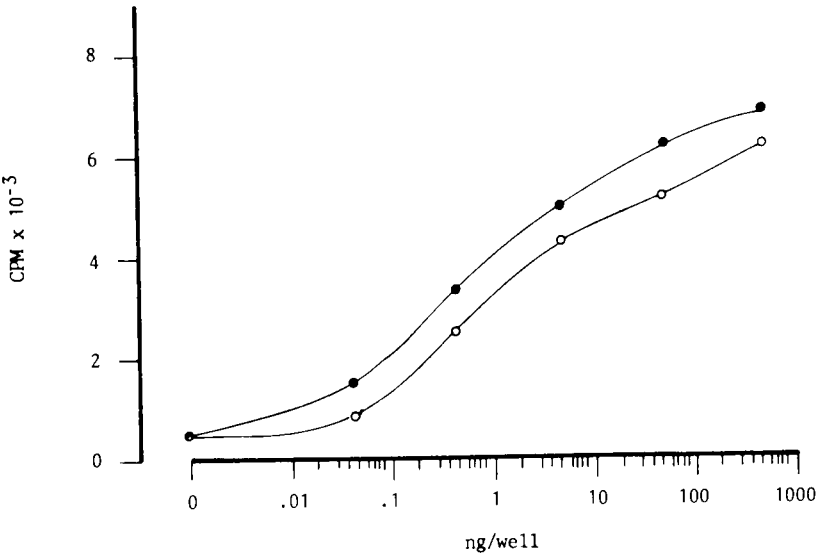

Fig. 4. Curve from antigen capture radioimmunoassay with 10^{-2} (•—•) and 10^{-3} (○—○) dilutions of monoclonal antibody 1B15 against varying amounts of p29 (points are means of two values).

concluded that with 10^{-2} and 10^{-3} dilutions of 1B15, similar titration curves were obtained (Fig. 4). Significantly lower responses were obtained with the highest 1B15 dilution. Practically no response was obtained with 10^{-3} dilution of normal control serum used instead of the MAb. The extent of binding of p29 to 1B15 adsorbed onto PVC wells was saturable and affected by the antigen concentration. The sensitivity threshold of EIA viral p29 detection was about 0.5 ng/ml. To calculate the affinity binding of MAb 1B15, the obtained saturation curve was transformed into a double reciprocal plot. The plot was linear, as expected for homogenous antibodies (not shown). From the data given in Fig. 4, the apparent affinity constant (K_a) of the MAb was 1.1×10^9 l/mol.

MAb 1B15 was further applied to detect the presence of antigen p29 in horse sera, containing antibodies against EIAV p29 measured by the heterologous-competitive RIA (Fig. 1A) and in culture fluids from EIAV grown in persistently infected equine fetal kidney (kindly provided by Dr. Nossetto, Institute of Virology, UNLP, Argentina). The preliminary experiments do not detect the presence of antigen p29 in horse sera (see Discussion). Nevertheless, the antigen p29 was detected in virus culture fluids after disruption of viral particles by repeated freezing and thawing.

Discussion

This report documents the successful generation of a stable line of a myeloma–spleen cell hybrid producing a monoclonal antibody with a high degree of

sensitivity for EIAV antigen p29. The value of continuous hybrid cell lines producing MAb for diagnosis and analysis of viral infections is obvious: MAbs ensured better specificity, reagent continuity and minimal nonspecific serum effects over conventionally raised antisera.

Several technical points arising from the development of the hybrid clones deserve mention. The high efficiency of the applied culture conditions appears to be due to the optimization of cellular culture by the use of macrophage feeder layers, obtained from peritoneal washings of young Balb/c mice (4–5-week-old) to avoid the isolation of activated cells. They were extremely beneficial to the developing hybrids owed to their growth supportive effect and the removal of cell debris formed after HAT selection. The frequency of antibody producing clones (1 in 15) was rather high as compared with those reported by others (Stahli et al., 1980). The extended mouse immunisation regimen and the sensitivity of our screening method are possible explanations. While it cannot be proved that they were solely responsible, they were highly important (Horenstein et al., 1985b).

The specificity of the MAb determined by the indirect RIA screening procedure was substantiated by cross-over immunoelectrophoresis experiments and disc-PAGE (Horenstein et al., 1985a; Clevinger et al., 1979). The reaction of labelled p29 with MAb 1B15 demonstrated by the presence of a high-MW radioactive band on the top of the gel (Fig. 3) and the lack of cross reaction with normal control serum (Fig. 2A–C) indicated that the antigenic determinant(s) recognized by 1B15 was associated with EIA viral antigen p29.

The detection of the viral antigen p29 required its adsorption directly onto a solid phase (Fig. 1A–C). However, such a system was not applicable to EIAV in tissue culture or antigen p29 in clinical specimens, since irrelevant proteins bind also, limiting the amount of p29 antigen bound and reducing the sensitivity of the detection. The device of the AgC-RIA (Fig. 1D) using the MAb 1B15 as 'capture antibody' facilitated by the production of an already proved polyclonal antisera C1D3 (Horenstein and Feinstein., 1985), gives the necessary specificity. On the other hand, the conventional antiserum C1D3 has at least one theoretical advantage. Due to its polyclonal nature, C1D3 contains antibodies of different avidity and antibodies to different epitopes of p29 (Horenstein and Feinstein, 1985). This allowed its use as a second antibody avoiding the most obvious threats that involve the use of MAb in place of polyclonal antisera: the possible presence of identical epitopes on unrelated proteins, and the possible lability of a particular epitope within the structure of the antigen.

The minimum level of crude EIA viral p29 protein detected by the AgC–RIA was approximately 50 pg (Fig. 4), which proved to be far more sensitive than other methods. Polyclonal antisera C1D3 were quite active but with a substantial difference in the detection limit under the conditions tested (Horenstein and Feinstein, 1985).

In this work, we have dealt mainly with crude antigen p29 and EIA virus culture fluids as models. We have not been able yet to detect the viral antigen p29 in antibody positive horse sera. The antigen may be present in horse sera in a cryptic form bound to antibodies and, thus, may elude detection in our assay conditions.

Our preliminary observations suggest that EIA viral antigen p29 attached to PVC well even when solubilized in buffer containing thyocyanate as a chaotropic agent (Hart and Broussard, 1973). Thus, a solid-phase technique for physical separation of antigen p29 from circulating antibodies is in progress.

In conclusion, our MAb represents an excellent investigative tool for EIAV research and it seems to be the most sensitive antibody to EIAV described to date.

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