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Production of Monoclonal Antibodies against the Challenge Strain of Infectious Laryngotracheitis Virus of Chickens and Their Use in an Indirect Immunofluorescent Diagnostic Test

Ferhat Abbas*, James Andreasen[1], Rockey Becker[1], Masroor Ahmed, M Arif Awan, Abdul Wadood and Anita Sonn[1]

Center for Advanced Studies in Vaccinology and Biotechnology, University of Balochistan, Quetta, Pakistan; [1]Oregon State University, USA
*Corresponding author: ferhatcasvab@yahoo.com

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ABSTRACT

The objective of the present research was to produce monoclonal antibodies (MCAs) against the USDA challenge strain of infectious laryngotracheitis virus and to perform an initial investigation of their use in an indirect immunofluorescence diagnostic test. Fourteen-day old chicken embryo liver cells were grown in tissue culture plates. Confluent monolayers were obtained after 48 hours. Monolayers were infected with the USDA challenge strain of infectious laryngotracheitis virus (ILTV). Cytopethic effect of the virus in the form of syncytial formation and clumping of cells was observed after 24 hours. The virus from the tissue culture flasks was collected and purified using discontinuous sucrose gradient. A clear band of the virus from sucrose gradient was obtained. The refractory index and the density measured were 1.410 and 1.20 g/cm^3, respectively. Spectrophotometry of the purified virus showed 68.117 ug/ml of protein and 9.8948 ug/ml of nucleic acid concentration. Spleen cells from immunized mice with pure virus were fused with myeloma cells and hybridomas were obtained after 10 days. Screening was performed using indirect immunofluorescence antibody test (IFAT) using rabbit anti-mouse immunoglobulins as secondary antibodies. Three hybridomas, 2D1D8, 2E11G2 and 2C6C7 were found producing antibodies against ILTV. All monoclonal antibodies were of isotype IgM and reacted with different strains of ILTV (ILTV USDA, S 88 00224, 86-1169) in IFAT. None of the monoclonals reacted with Parrot herpesvirus and avian adenovirus 301 in IFAT.

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INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is assigned to the subfamily alphaherpesvirinae, of the herpesviridae family on the basis of its rapid replication and latency in neurons (Ahmed *et al*., 2009). It is an enveloped virus, icosahedral, with double stranded DNA which has a density of 1.704 g/ml, which is similar to the DNA of other herpes viruses (Plummer *et al*., 1969). Four major glycoproteins with molecular weights of 205, 115, 90 and 60 kD have been described in the SA-2 strain of ILTV (York *et al*., 1987). These glycoproteins are considered to be the major immunogens of ILTV and are present on the virus envelope and virus-infected cells. Infectious laryngotracheitis virus DNA has a molecular weight of about 100 x 10^6 daltons, with the genome having two isomeric forms (Kotiw *et al*., 1982; Leib *et al*., 1987). Some workers have reported that ILTV has a guanine plus cytosine percentage of 45% which is lower than other animal herpesviruses (Plummer *et al*., 1969).

Chicken antisera and rabbit antisera raised against intact ILTV have been used to immunoprecipitate four major glycoproteins of 205, 115, 90 and 60 kD molecular weight present in the SA-2 strain of ILTV. Some additional glycoproteins also have been recognized by immmune chicken and rabbit antisera in western blotting using a glycoprotein fraction which was purified from virus-infected cells. In addition, monoclonal antibodies (MCAs) have been produced and have been characterized by immunoprecipitation and western blotting (York *et al*., 1987). Monoclonal antibodies have been reported to react with cytoplasmic and nuclear antigens in immunofluo-escence tests (York *et al*., 1990).

All strains of ILT virus isolated throughout the world so far are antigenically homogenous on the basis of their reaction in virus neutralization tests (York *et al*., 1989). However, restriction endonucleases have been used to study the DNA patterns and differences have been reported among some strains of ILT virus (Kotiw *et al*., 1982).

Monoclonal antibodies (MCAs) are a homogenous population of identical antibodies with a defined specificity secreted by a B-cell after fusion with tumour cell. Somatic cell hybridization can be used to generate a continuous hybridoma cell line producing a monoclonal antibody (Kohler and Milstein, 1975). The primary advantage of MCAs as diagnostic reagent is their specificity of binding to a single epitope, producing highly specific diagnostic test reagent. Monoclonal antibodies have been used to separate individual antigens from a complex mixture (Antczak, 1982). The objective of the present research was to produce MCAs against the USDA challenge strain of infectious laryngo-tracheitis virus and to perform an initial investigation of their use in an indirect immunofluorescence diagnostic test.

MATERIALS AND METHODS

Source of virus

Infectious laryngotracheitis virus strains included in the study were United States Department of Agriculture (USDA) challenge strain, provided by the National Veterinary Services laboratory in Ames, Iowa; 86-1169, a Georgia field isolate; and S 88 00224, a California field isolate. Other viruses were: a Parrot herpesvirus (Pacheco's disease virus) and avian adenovirus 301, obtained from The Avian Serology Laboratory, Veterinary Diagnostic Laboratory, Oregon State University, USA.

Virus growth and purification

Chicken liver cells were grown according to the method described earlier (Chomiac *et al*., 1960) with slight modification. Chicken embryo liver cells were taken from 14-day old chicken embryos. For this purpose, the livers were removed aseptically and placed in calcium and magnesium free phosphate-buffered saline (PBS) in a beaker. After chopping with scissors, livers were washed 5-6 times with PBS to eliminate the red blood cells and other fibrous tissue. Versene-trypsin (0.25%) was used to separate the cells. Trypsinization was stopped by adding sufficient chilled fetal bovine serum (FBS) to produce an 8% concentration, and cells were centrifuged at 750 X g for 5 minutes. Packed cells were diluted at the rate of 1:150 in minimal essential medium (MEM) containing 10% FBS, 1% gentamicin (50 ug/ml), and 1% amphotericin-B (2.5 ug/ml) at pH 7.0-7.1.

Liver cells were grown in tissue culture plates at 37^0C. Cell monolayers were infected with 300 ul of USDA challenge strain of ILTV ($TCID_{50}$ $10^{6.5}$/ml) and the cells infected with this particular strain were used for virus purification according to the method of Andreasen *et al*. (1990). Briefly, infected cells were collected and virus particles were released by sonification. The output frequency of sonicator was 20 KHz and sonification was conducted for 1 minute at the interval of 5 seconds. The solution was centrifuged at 6000 X g for 25 minutes to separate the cellular debris. The supernatant was layered onto discontinuous sucrose gradient and centrifuged at 100,000 X g for 1 hour. Visible virus bands were collected from the 30-65% sucrose interface and further centrifuged through continuous sucrose gradient at 100,000 X g for 20 hours at 4^oC. Visible virus bands were collected, refractory index and the density of the virus sucrose solution were calculated. Virus sucrose solution was placed in a concentrator (100,000 MW) and centrifuged at 1250 X g for 45 minutes to eliminate sucrose. Dialysis was performed by keeping dialysis tubing containing virus-sucrose solution in TEN buffer (Tris 0.01M, EDTA 0.001M, NaCl 0.1M in distilled water, pH 7.2) for 48 hours to completely eliminate sucrose. Scanning electron microscopy was performed to see virus particles (Watrach *et al*., 1959). Spectrophotometry was conducted at 260 nm wavelength to measure protein and nucleic acid concentration of the purified virus. Blank was run in spectrophotometry with TEN buffer at the same wavelength. Purified virus was stored at -70^oC.

Mouse immunization

Five female BALB/c mice were immunized intraperitoneally with an emulsion containing live virus and Freund's complete adjuvant. About 0.8 ml of pure virus was emulsified using sonicator (20 KHz output frequency for 1 minute) with 0.8 ml of Freund's complete adjuvant and centrifuged at 25000 rpm for 5 minutes. About 0.2 ml of this virus was given intraperitoneally to each mouse. Three weeks later, mice were immunized through the same route with an emulsion containing live virus and incomplete Freund's adjuvant. An intravenous booster injection of 1 ml of the live purified virus without any adjuvant was given to the mice 4 days before fusion (Kohler and Milstein, 1975). Five unimmunized female BALB/c mice were also kept for a source of negative control sera.

Fusion and cloning

SP2/0-Ag14 myeloma cells were obtained from the Monoclonal Antibody Facility, Oregon State University, USA. These cells were fused with spleen cells of the immunized mice for production of monoclonal antibodies (Kohler and Milstein, 1975). Briefly, the spleen cells were mixed with SP2/0-AG14 mouse myeloma cells at the ratio of 1:1 (Schulman *et al.*, 1978). One ml of 50% polyethylene glycol (PEG) was added to the cells as a fusion reagent. Cells were mixed in Dulbecco's Modified Eagle's medium (DMEM), centrifuged and resuspended in hypoxanthine aminopterin thymidine (HAT) medium. Cells were then grown in 96-well tissue culture plates at 37^oC in a 5% CO_2 atmosphere. Supernatant fluids from wells were screened by indirect immunofluorescene antibody test (IFAT) for specific ILTV antibodies. Each well was checked for the presence of antibodies against ILTV by indirect immunofluorescence test (Wilks and Kogan, 1979). Briefly, 100 ul of supernatant fluid from each well was added on a slide coated with ILTV antigen (5 ul of ILTV infected liver cells). Rabbit anti-mouse immunoglobulin was used for indirect immuno-fluorescence test for the detection of mouse antibodies against ILTV. The hybridomas that were found to be

producing antibodies were cloned by limiting dilution calculated to give <1 cell/well (Anonymous, 1991).

Immunoglobulin subclass

The isotypes of the monoclonal antibodies were determined by a mouse monoclonal antibody isotyping kit (Sigma, ISO-1) following manufacturer's procedure. The precoated strip (nitrocellulose membrane coated with specific isotype antigens) captured the relevant mouse immuniglobulin isotype by self-description.

RESULTS

Virus growth and purification

The monolayer from chicken embryo liver cells was developed in 48 hours. After infection with the ILTV, cytopathic effect (CPE) was developed in 24 hours. The CPE was syncytial formation with the clumping of the cells due to the virus. No bacterial or fungal contamination was observed in control cells, as their was intact monolayer with healthy cells observed in uninfected flasks. The cells infected with Parrot herpesvirus and avian adenovirus 301 also produced CPE in the form of damage and clumping of the cells after 24 hours.

After centrifugation through discontinuous sucrose gradient, visible virus band was obtained and the refractory index and density were measured, which were 1.410 and 1.20 g/cm^3, respectively. Icosahederal virus particles were also seen by electron microscopy (Electron micrograph not taken). Spectrophotometry of the purified virus sample showed 68.117 ug/ml of protein and 9.8948 ug/ml of nucleic acid concentration.

Mouse immunization

Serum of the immunized mice obtained from conjuntival venous sinuses showed a positive reaction against ILTV in indirect IFAT and their spleens were used for fusion. Serum from unimmunized mice did not show any reaction with ILTV in indirect IFAT.

Fusion and cloning

The hybridomas in the form of growing clump of cell were observed after 10 days of fusion in 96-well plates. Positive wells were identified by using their supernatant against ILTV in indirect IFAT.

Monoclonal antibodies

Of 234 growing hybridomas, only 15 showed positive reaction with ILTV infected cells but showed no reaction with uninfected control cells. Five of the 15 hybridomas died during the process of expansion and cloning. Four of the remaining 10 proved to give negative reactions, and 3 reacted with both infected and non-infected cells on the second screening after cloning by limiting dilution. Three hybridomas were positive against the USDA challenge strain of ILTV and were designated as 2D1D8, 2E11G2, and 2C6C7. Monoclonal antibodies secreted by three hybridomas were isotyped using the isotyping kit, and all three hybridomas 2D1D8, 2E11G2, and 2C6C7 were found to secrete IgM.

Indirect immunofluorescence assay

All 3 monoclonal antibodies 2D1D8, 2E11G2 and 2C6C7 reacted in an identical manner, with fluorescence was found mainly along the surface of the infected cells (Fig. 1). Same results were observed against the two ILT viruses including S88 00224 ILTV, and 86-1169ILTV. Parrot herpes virus and the avian adenovirus 301 did not show any fluorescence against these MCAs (Fig. 2).

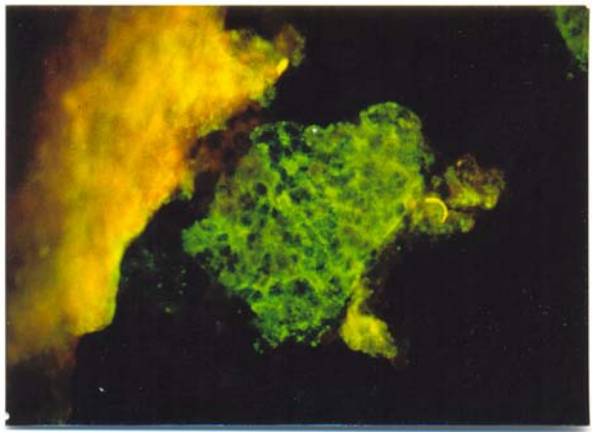

Fig. 1: Indirect immunofluorescence staining of cells infected with USDA challenge strain of infectious laryngotracheitis virus with 2D1D8 monoclonal antibody showing a positive reaction.

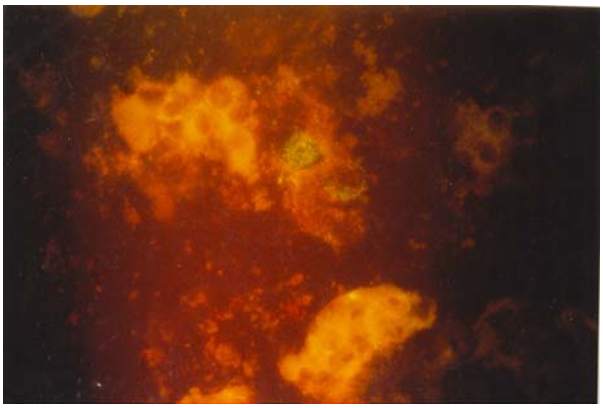

Fig. 2: Indirect immunofluorescence staining of cells infected with avian adenovirus 301 with 2D1D8 monoclonal antibody showing a negative reaction.

DISCUSSION

In the present study, three MCAs designated as 2D1D8, 2E11G2 and 2C6C7 were obtained. These MCAs are directed against proteins and glycoproteins which are considered as major immunogens (York *et al*., 1987). All three MCAs produced nearly identical reactions with ILTV-infected cells. Each of the three MCAs cross-reacted with the other strains of ILTV and the fluorescence was nearly identical to that observed against the USDA challenge strain. All three MCAs were found to not react with the avian adenovirus 301 or Parrot herpes virus. Fluorescence was observed mainly at the surface of the virus-infected cells and fluorescence was also observed within the nucleus. These patterns of

fluorescence by the MCAs suggest the reaction against glycoproteins and against viral proteins because cytoplasmic fluorescence suggests the presence of viral antigens expressed on the surface of virus-infected cells, whereas nuclear fluorescence suggests the reaction of MCAs against viral structural proteins (York *et al*., 1987). Cytoplasmic fluorescence in ILTV has also been reported by Fuchs *et al*. (2007). Immunofluorescence test has also been reported to show nuclear reaction and these studies have revealed that the UL31 gene product of ILTV is predominantly localized in the nucleus (Helferich *et al*., 2007). Monoclonal antibodies against ILTV epitopes using immunofluorescence have also been reported to study its antigenic structures (Veits *et al*., 2003b). Moreover, MCAs specific for glycoproteins have been used to investigate the role of glycoproteins in adsorption and penetration of cells, cell fusion and neutralization (Spear, 1985). Monoclonal antibodies have been reported to identify some virulence factor of ILTV such as glycoprotein G (Devlin *et al*., 2006). Expression of proteins in ILTV can also be studied using immunofluorescence test (Veits *et al*., 2003a).

While screening, some of the hybridoma supernatants were found to react both with infected and non-infected cells, which indicates that some spleen cells were also producing antibodies against chicken embryo liver cells or cell proteins. This suggests that some cell antigens remained after the virus purification and were injected with the virus at the time of immunization. This non-specific reaction was also observed when the immunized mouse serum was checked using the indirect immunofluorescence test before the mice were sacrificed.

Monoclonal antibodies 2D1D8, 2E11G2 and 2C6C7 were each found to be of isotype IgM. It is possible that the cells that were responsible for producing antibodies other than IgM could not fuse or could not survive during the process of fusion, expansion and cloning.

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