The Isolation, Detection, and Cross-Reactivity of Asian Elephant IgG for the Development of Serological Diagnostic Tests

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Few serological assays are available to study the exposure of Asian elephants to infectious organisms. To address this problem we isolated Asian elephant IgG and prepared antisera reactive with it. Using enzyme linked immunosorbent assays, it was determined that the anti-Asian elephant sera has strong reactivity with Asian elephant IgG and African elephant IgG, moderate reactivity with manatee IgG and weaker reactivity with IgG from hyrax and black rhinoceros. Commercially available antisera produced against cattle, deer, and rabbit IgG react weakly with Asian elephant IgG.

Key Words: Asian elephant, serology, elephant immunoglobulin G, ELISA

Introduction

Serological tests of elephant serum have been limited due to a lack of suitable detecting reagents. This has restricted the repertoire of assays for diagnosis as well as determination of species relatedness. This problem was recently highlighted by disseminated herpes-like infections in Asian elephants in North American zoos and wildlife parks.[1] This fatal disease may contribute to the 30% mortality rate seen in the first year of life of captive Asian elephants born in North America. In the absence of detecting antisera (i.e., conjugated anti-Asian elephant immunoglobulin), however, serological testing is restricted primarily to virus neutralization tests and virus isolation, which are expensive, time consuming, and laborious. To address this problem we isolated IgG from *Elephas maximus* (Asian elephant), prepared antisera reactive with it, and coupled an IgG fraction of anti-elephant IgG to biotin.

The binding of this antisera to elephant IgG as well as the cross-reactivity of anti-Asian elephant IgG with IgG of other species was determined. Conversely, the reactivity of commercial antisera prepared against IgG of other species was examined to determine their usefulness as diagnostic reagents for Asian elephant assays.

Materials and Methods

ISOLATION OF ASIAN ELEPHANT IGG

A recombinant protein A and protein G (protein A/G) affinity matrix (Pierce, Rockford, IL) was equilibrated with IgG binding buffer (Pierce) and serum from an Asian elephant, diluted in an equal volume of IgG binding buffer, was applied to the column. Unbound material was rinsed from the column with IgG binding buffer and bound IgG was removed with IgG elution buffer, pH 2.8 (Pierce). The major protein peak, determined by optical density at 280 nm, was pooled and dialyzed against 0.02M Tris-HCl, pH 8.0 containing 0.028 M sodium chloride (DEAE buffer). This material was applied to diethylaminoethyl beads containing immobilized cibacron blue dye (Bio-Rad, Hercules, CA) equilibrated in DEAE

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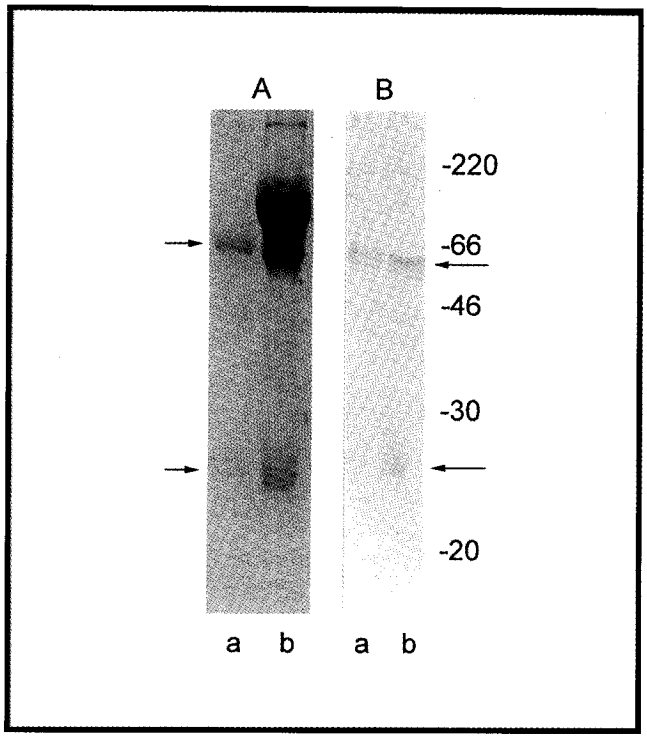

FIG. 1 — Asian elephant IgG, 20 mg per lane (lane a) and 5 ml of Asian elephant serum (lane b) were reduced with Beta-mercaptoethanol and separated by SDS-PAGE in 7.5% resolving gels. One set of samples (Panel A) was stained with Coomassie blue. Identical samples (Panel B) were examined by western blot. Proteins (Panel A) and antigens (Panel B) corresponding by M.W. to the heavy (upper arrows)and light chains (lower arrows) of mammalian immunoglobulin G are visible in panel A row a and Panel B rows a and b. The light chain is, however, more faint than the heavy chain.

buffer. The flow-through, containing IgG, was concentrated using an ultrafilter with a 100 kDa cut-off (Amicon, Beverly, MA). The purity of the IgG was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis[2] (SDS-PAGE) using a 7.5 % resolving gel.

PRODUCTION OF RABBIT ANTI-ASIAN ELEPHANT IGG

New Zealand white rabbits were inoculated by intramuscular injection with 300 µg of elephant IgG emulsified in incomplete Freund's adjuvant. The rabbits

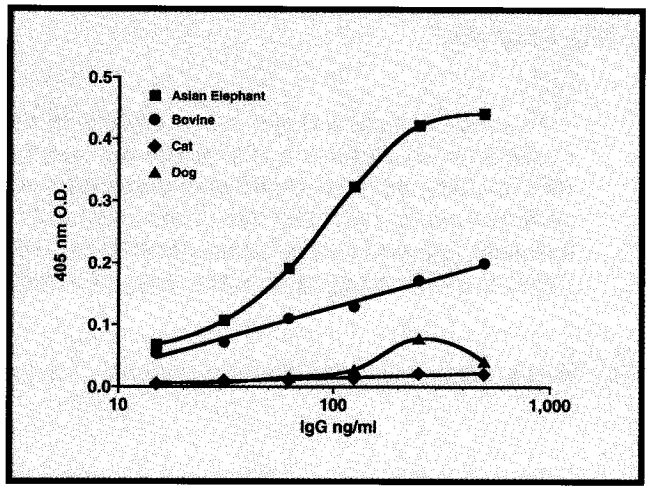

FIG. 3 — The binding of Asian elephant, bovine, feline, and canine purified IgG was measured with a capture ELISA.

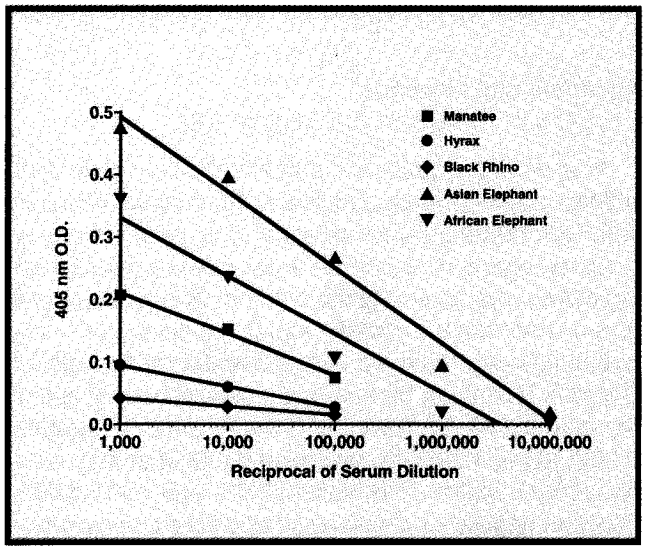

FIG. 2 — IgG from 5 Asian elephants, 2 African elephants, 5 manatees, 5 rock hyrax, and 2 black rhinoceros sera were detected with a capture ELISA. Values represent the mean optical densities obtained from duplicate samples of each sera.

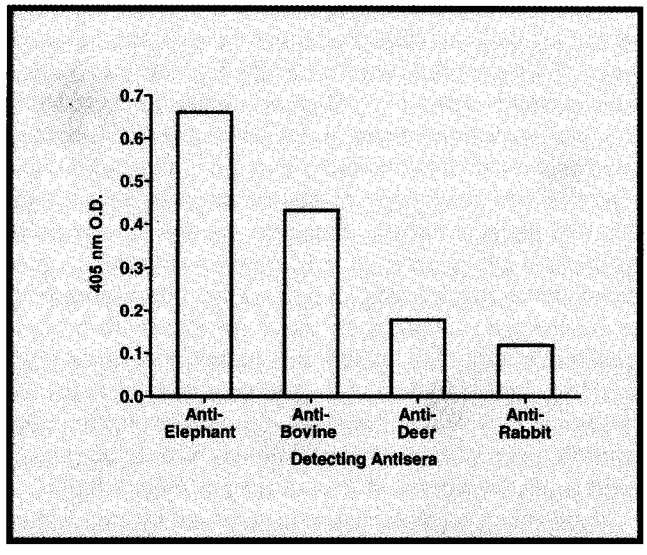

FIG. 4 — The cross-reactivity of commercially available peroxidase labeled antisera with Asian elephant IgG was measured with a direct ELISA. The homologous reactivity of anti-Asian elephant IgG with Asian elephant IgG was determined with biotin conjugated antibody.

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received 3 additional injections at 14-day intervals. Serum was obtained from the rabbits 10 days after the last inoculation.

WESTERN BLOTS OF RABBIT ANTI-ELEPHANT IGG

Asian elephant serum was separated by electrophoresis on 7.5% SDS-PAGE[2] and transferred to nitrocellulose.[3] The nitrocellulose was washed 3 times with phosphate buffered saline containing 0.05% polyoxyethylenesorbitan monolaurate (PBSTW) and incubated for 1 hour at room temperature with rabbit-anti-Asian elephant IgG diluted in PBSTW. Bound rabbit antibody was detected with peroxidase-conjugated affinity isolated anti-rabbit IgG diluted in PBSTW and incubated for 1 hour at room temperature. After 3 washes in PBSTW color was developed using 4-chloro 1-napthol substrate prepared in 128 mM sodium chloride and 418 mM triethanolamine (pH 7.5) containing hydrogen peroxide.

PREPARATION OF BIOTIN CONJUGATED RABBIT ANTI-ELEPHANT IGG

The IgG fraction of rabbit anti-elephant IgG was obtained from sera using immobilized protein A/G as described above for elephant IgG. The IgG fraction was coupled to succinimidyl-6-[biotinamido] hexone (biotin) essentially as described by Berger[4] for the third component of complement.

ENZYME-LINKED IMMUNOSORBENT ASSAY

A capture ELISA was performed by coating 96 well plates (Immulon 2, Dynex Technologies, Inc., Chantilly, VA) overnight at 4 degrees C with 2 µg/ml of an IgG fraction of rabbit anti-Asian elephant IgG diluted in PBS. The plates were washed 3 times with PBSTW for this and all subsequent steps. Serum or purified IgG from various species, diluted in PBSTW, was then added. Serum samples were obtained from 5 Asian elephants, 2 African elephants, 5 rock hyrax, 5 manatees, and 2 black rhinoceros. Reagent grade bovine, feline, and canine IgG was obtained from a commercial source (Sigma Chemical Co., St. Louis, MO). After a 1 hour incubation at 37 degrees C, bound antibody was detected with biotin labeled anti-Asian elephant IgG at a dilution of 1:200 in PBSTW. This concentration was determined to be optimal in titration experiments. This was followed by a 1 hour incubation at 37 degrees C with peroxidase conjugated avidin (Sigma Chemical Co.) diluted to 250 ng/ml in PBSTW. The plates were washed 3 times with

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PBSTW and once with PBS and then received 2,2'-azino-bis(2-ethylbenzthiazoline-6-sulfonic acid(ABTS) substrate dissolved in 0.05 M phosphate-citrate buffer (pH 5.0). Each 100 ml of substrate buffer contained 20 mg of ABTS and 40 ml of 30% hydrogen peroxide. The optical density of the samples was measured at 405 nm.

A direct ELISA was performed similar to the capture ELISA except that 2 mg/ml of IgG diluted in PBS was coated directly onto 96 well plates and the reactivity of peroxidase or biotin conjugated antisera with the purified IgG was determined.

Results

The purification scheme employing protein A/G and cibicron blue/DEAE resulted in the enrichment of Asian elephant IgG as determined by Coomassie Blue stained SDS-PAGE gels (Figure 1). Inoculation of rabbits with this immunogen produced antibody with detectible reactivity only against Asian elephant IgG heavy and light chains and not against other Asian elephant serum components (Figure 1).

The reactivity of anti-Asian elephant IgG with Asian elephant sera and sera from various species of mammals was determined in the capture ELISA with anti-Asian elephant IgG and biotin anti-elephant IgG (Figure 2). Anti-Asian elephant IgG gave strong reactivity with Asian elephant IgG and was readily detectible at a 1:1,000,000 dilution of Asian elephant sera. Relative to the homologous reactivity of anti-Asian elephant IgG with Asian elephant sera, the greatest cross-reactivity was with African elephant (*Loxodonta africana*) followed by manatee (*Trichechus manatus latirostris*), rock hyrax (*Procavia capensis*) and black rhinoceros (*Diceros bicornis*). At low IgG concentration, when heterologous sera were diluted 1:100,000, the intensity of the cross-reactivity was diminished. When 250 ng/ml of purified IgG was captured with anti-Asian elephant IgG, the reactivity of anti-Asian elephant IgG to bovine IgG (Figure 3) was 40.7%, canine IgG 18.5%, and feline IgG 5.2% relative to Asian elephant IgG.

For the direct ELISA, purified IgG was coated on 96 well plates and detected with conjugated anti-IgG prepared against various IgGs (Figure 4). The reactivity to elephant IgG of anti-bovine IgG was 65.3%, anti-deer IgG 26.9%, and anti-rabbit IgG 17.8% compared to the homologous Asian elephant IgG reaction. The reactivity of anti-bovine IgG with elephant IgG was only about one third as intense, however, as the homologous bovine reaction (anti-bovine IgG with bovine IgG).

Discussion

A 2 step approach for the purification of Asian elephant IgG using protein A/G and DEAE cibicron blue chromatography resulted in highly enriched IgG. Recombinant Protein A/G was chosen for this work because it has strong binding to elephant IgG.[5] Recombinant protein G has reduced binding to non-immunoglobulin serum proteins compared to native protein G and protein A but can bind to other classes of immunoglobulins. DEAE/cibicron blue chromatography medium binds most proteins except IgG; therefore, the flow-through is highly enriched for IgG and depleted of albumin and other serum proteins.[6]

The close phylogenetic relationship of elephants with hyrax and manatees has been hypothesized because of similar anatomical features.[7,8] Our study indicates a strong antigenic similarity between manatee and elephant IgG and a more distant relationship between the elephant and rock hyrax IgG.

At low serum concentration there is diminished reactivity of the anti-Asian elephant IgG even with African elephant IgG. This probably indicates a lower avidity binding to the African elephant IgG than to the Asian elephant IgG. Since diagnostic serological tests are often required to detect minute quantities of antibody bound to organisms or antigens, this antisera is probably not useful for serological assays with the manatee and a separate antisera for the diagnosis of African elephant diseases may be necessary. Commercially available antisera produced against domestic animals and deer react poorly with Asian elephant IgG and, therefore, are not likely to be useful for diagnostic serology with elephant samples. The biotin conjugated anti-Asian elephant antisera described in this study and a fluorescein conjugate of the same antibody should allow an expansion of the tests currently available to determine exposure to microbial pathogens. Tests may include indirect fluorescent assays using fluorescein conjugated antibody and ELISA. ■

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