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DEVELOPMENT AND VALIDATION OF TWO COMPETITIVE ELISAs FOR DETECTING HUMAN AND CANINE ADENOVIRUS ANTIBODY IN ONTARIO WILDLIFE SERA

A Thesis

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Department of Biochemistry, Microbiology, and Immunology

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by

Laura Maxwell

ABSTRACT

A human adenovirus type 5-rabies recombinant vaccine (Ad5RG) is a potential candidate for the oral immunization of skunks, foxes, and raccoons against rabies. Adenovirus seroprevalence studies in these species were required before using the vaccine in the field, as part of an environmental risk assessment and for determining if a barrier to immunization exists. For this purpose, two competitive enzyme-linked immunosorbent assays (cELISAs) measuring antibodies (Ab) against Ad5 and canine adenovirus type one (CAV-1) were developed and validated. Reference sera used for validation were derived from laboratory skunks, foxes, and raccoons given Ad5RG by either the oral or intra-muscular route. For CAV-1 cELISA validation, additional sera were used from dogs vaccinated intra-muscularly with CAV-2, which protects against CAV-1 and CAV-2 infection. Sera were examined for either Ad5 or CAV-1 neutralizing antibodies using plaque reduction neutralization tests (PRNT). As part of cELISA development, 10 Ad5 and 10 CAV-1 mAbs were characterized according to their viral protein specificities, their neutralizing abilities, and their cross-reactions with other adenoviruses. An Ad5 non-neutralizing hexon mAb (15G6) that cross-reacted with Ad1-30 was selected for the Ad5 cELISA. A CAV-1 neutralizing hexon mAb (M1260) that was non-cross-reactive was selected for the CAV-1 cELISA. cELISA performance was described in terms of its ability to discriminate between neutralization-positive and negative reference samples. This was accomplished using Receiver Operating Characteristic (ROC) plots, which demonstrated sensitivity and specificity for every % inhibition decision threshold relative to PRNT results. The Ad5 cELISA was 80% sensitive and 75% specific when an 8% inhibition positivity cutoff was used. The CAV-1 cELISA was 83% sensitive and 96% specific when a 17% inhibition positivity cut off was used. Contributing to the low Ad5 specificity

in particular were raccoons that generally lacked Ad5 neutralizing activity but had Ab which bound purified Ad5 in the cELISA and Western blot. It is possible that these animals were previously exposed to an adenovirus which shares the same epitope as the one recognized in both cELISA and Western blot but not in the PRNT. Sera collected from approximately two thousand field raccoons were then analyzed for Ad5 and CAV Ab using the validated cELISAs. Between 8-25% of the field raccoon sera had human adenovirus Ab depending on the % inhibition cut off used. None of the sera had Ad5 neutralizing activity, but the majority of sera with high cELISA titers bound purified Ad5 in a Western blot similar to findings with experimental raccoon sera. The CAV-1 survey revealed 38/1943 (2%) sera had Ab to CAV-1 based when a 17% inhibition cut off was used. The sera with the highest cELISA titers (37-79% inhibition) were capable of neutralizing CAV-1 in a PRNT and bound purified CAV-1 in a Western blot. These findings do not significantly impact the planned usage of Ad5RG for oral rabies immunization in wildlife.

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LIST OF ABBREVIATIONS

Ab	antibody
ABTS	2,2'-azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid]
Ad1-30	adenovirus serotype 1-30
Ad5E1RG	Ad5 (early gene 1-deleted)-rabies glycoprotein recombinant
Ad5E3RG	Ad5 (early gene 3-deleted)-rabies glycoprotein recombinant
Ad5RG	Ad5-rabies glycoprotein recombinant
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
β-ME	β-mercaptoethanol
BAV	bovine adenovirus
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BES	bovine equine serum
bp	base pair
CAV-1, 2	canine adenovirus serotype 1, 2
cELISA	competitive ELISA
CI	confidence interval
CPE	cytopathic effect
CsCl	cesium chloride
CTL	cytotoxic T lymphocyte
DF	degrees of freedom
DIOC	direct inoculation into the oral cavity
DK	dog kidney
DNA	deoxyribonucleic acid
E1-4	early gene region 1-4
EAV	equine adenovirus
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ERA	Evelyn Rokitniki Abelseth
FBS	fetal bovine serum
FITC	fluorescein-5-isothiocyanate
g	force of gravity
GAV	goat adenovirus
GPAV	guinea pig adenovirus
HIV	human immunodeficiency virus

HRP	horse radish peroxidase
IF	immunofluorescence
Ig	immunoglobulin
IM	intra-muscular
ITR	inverted terminal repeat
kD	kilo Dalton
L1-5	late gene region 1-5
mAb	monoclonal antibody
MAPS	monoclonal antibody purification system
MAV	murine adenovirus
MDBK	Madin Darby bovine kidney
MEM	minimal essential media
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NBT	nitroblue tetrazolium
OAV	ovine adenovirus
OD	optical density
OIE	Office International des Epizooties
OMNR	Ontario Ministry of Natural Resources
ORF	open reading frame
P	probability
PAGE	polyacrylamide gel electrophoresis
PAV	porcine adenovirus
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PFU	plaque forming unit
PRNT	plaque reduction neutralization test
PSCID	primary severe combined immunodeficiency disease
RIPA	radio immunoprecipitation antibody
ROC	Receiver Operating Characteristics
r_r	rank correlation coefficient
SAD	Street Alabama Dufferin
SAV	simian adenovirus
SDS	sodium dodecyl sulfate

SMP	skim milk powder
SNA	serum neutralizing antibody
STE	saline tween EDTA
SV40	simian virus 40
TBST	tris buffered saline tween
$TCID_{50}$	tissue culture infectious dose 50%
USA	United States of America
VRG	vaccinia virus-rabies glycoprotein recombinant
WHO	World Health Organization

INTRODUCTION AND RATIONALE

Wildlife rabies in North America and Europe continues to be a significant public health concern and economic burden. In spite of the control of domestic rabies and advances in rabies diagnosis, post-exposure treatment, and vaccine production for human and veterinary use, the virus continues to be transmitted to humans at an enormous cost to the health care system. Rabies virus transmission to people can occur directly through the bite of an infected wild animal, or indirectly via unvaccinated pets and livestock who have been bitten by an infected wild animal.

Early attempts at controlling wildlife rabies, which involved selective population reduction, were largely unsuccessful and became culturally unacceptable (Debbie 1991). In the quest for progression, Baer and colleagues reported in the early 1960's that laboratory foxes could be orally vaccinated with ERA (Evelyn Rokitniki Abelseth), an attenuated rabies virus vaccine (Black and Lawson 1970; Baer et al 1971; Black and Lawson 1973). ERA was derived from the SAD (Street Alabama Dufferin) rabies street virus isolated originally from a dog in 1935. Steck and Wandeler applied the concept of oral vaccination to the field for the first time in the late 1970's (Steck et al 1982). By delivering baits that contained another SAD derivative, SAD-Bern, in the Swiss Rhone Valley, their efforts to control an advancing fox rabies front succeeded (Steck et al 1982). The concept of orally immunizing wild animals by luring them into eating baits containing rabies vaccines has since proven to be the most effective method of reducing the number of susceptible hosts in Europe and North America.

Virtually all mammalian species are susceptible to infection with rabies viruses; however, terrestrial wildlife carnivores and bats represent the principal hosts in North

America. The predominant terrestrial species which maintain the enzootic and therefore represent targets for oral rabies immunization are arctic foxes (*Alopex lagopus*) (Blancou et al 1991), red foxes (*Vulpes vulpes*) in subarctic and north-eastern parts of North America (Crandell 1991), grey foxes (*Urocyon cinereoargenteus*) and coyotes (*Canis latrans*) in the southern USA, striped skunks (*Mephitis mephitis*) in central North America and California (Charlton et al 1988; Charlton et al 1991), and raccoons (*Procyon lotor*) along the eastern USA seaboard and most recently in eastern Ontario (Winkler and Jenkins 1991). Raccoons frequent urban as well as rural domains, increasing the risk of exposure to people.

In rural areas of southern and eastern Ontario, large-scale aerial distribution of baits containing ERA vaccine seems to be leading towards the elimination of rabies in red foxes, Ontario's principal host vector. Since the Ontario Ministry of Natural Resources (OMNR) implemented this vaccination campaign in 1989, the incidence of positive wildlife rabies cases has dropped significantly, and periodic prevalence peaks and advancing rabies epizootics into vaccinated areas have not been observed (unpublished observations, Wandeler 1999).

Although attenuated SAD derivatives, including ERA, effectively immunize foxes (Lawson et al 1987; Lawson et al 1992; Lawson et al 1997), they are less effective in raccoons (Rupprecht 1989) and are ineffective and often pathogenic in skunks (Tolson et al 1988; Rupprecht et al 1990; Tolson et al 1990). Also, problems of safety exist with SAD derivatives like ERA, which have been shown to be pathogenic in non-target species such as rodents (Lawson et al 1987; Lawson et al 1989; Artois et al 1992) and may potentially revert to virulence in susceptible hosts. Presently, trap-vaccinate-release procedures with inactivated rabies vaccines are the only safe and effective means of vaccinating raccoons and

skunks in Ontario (Rosatte et al 1990; Rosatte et al 1992; Rosatte et al 1993).

Vaccine inefficacies are not directly related to the existence of different rabies variants in different species, since all current rabies vaccines including SAD derivatives and others induce cross-protective immunity against all rabies virus variants when given by a parenteral route (WHO Expert Committee on Rabies Eighth Report 1992). Inefficacy is probably linked to the refractiveness of certain species to oral vaccination with rabies viruses in general. Protective immunity is achieved only when the immunizing virus has access to susceptible host cells and can induce sufficient expression and cell surface presentation of rabies glycoprotein. This is essential, since the rabies glycoprotein is the target for neutralizing Ab and cytotoxic T lymphocytes (CTLs), the main elements of the protective immune response.

There has since been an interest in recombinant vectors for use in wildlife rabies vaccine development, mainly for improved efficacy and safety in target and non-target species. The ERA glycoprotein gene has been inserted into the genomes of a variety of infectious vectors including adenoviruses and poxviruses (Paoletti 1996; Dietzschold et al 1996). These may be advantageous over attenuated rabies virus vaccines since they may better access target cells in the gut and oral cavity leading to glycoprotein expression and presentation in species which are normally refractive to ERA vaccination.

The vaccinia virus-rabies glycoprotein (VRG) vaccine was the first rabies recombinant vaccine to be exploited in the laboratory and in the field, and has shown some success in controlling fox rabies in parts of Western Europe (Brochier et al 1995; Fu et al 1997). It is also being used in the USA to help prevent the spread of rabies in raccoons and coyotes (Fu et al 1997). It has demonstrated efficacy in foxes (Tolson et al 1988; Brochier

et al 1991; Pastoret and Brochier 1996), skunks (Tolson et al 1987), and raccoons (Rupprecht et al 1986) and is apathogenic in target and non-target species (Brochier et al 1989; Artois et al 1990; Artois et al 1992). For these reasons VRG has also been considered as a potential alternative to ERA vaccination in Ontario.

Although effectiveness in skunks was initially demonstrated, subsequent laboratory trials with VRG showed low rates of rabies seroconversion suggesting that other recombinant vaccines be examined. Also, the field application of VRG instigated theoretical concern for potential animal reservoir establishment, genetic recombination between the vector and endogenous poxviruses, and accidental contact spread of VRG to humans. The latter concern emerged in the light of previous reports of occasional but severe adverse reactions in immunocompromised individuals following vaccinia virus immunization. Unfortunately, our knowledge of the ecology of these sophisticated viruses in wildlife is still limited.

Adenoviruses likewise show great potential as vaccine vectors and the availability of over 100 different adenovirus serotypes, almost 50 of which are of human origin, provide great versatility for vaccine developers. Adenoviruses are common and usually innocuous human and animal pathogens that primarily infect naso/oropharangeal and intestinal tissues making them useful candidates for oral vaccines (Shenk 1996). Human adenovirus types 4 (Ad4) and 7 (Ad7) have provided safe oral vaccines, effective in preventing acute respiratory disease in military recruits, for the past 30 years (Top et al 1971a; Top et al 1971b; Dudding et al 1972; Dudding et al 1973). The widespread popularity of adenoviruses in vaccine development is also due to their extensively characterized molecular biology (mostly the lower numbered adenoviruses), genomic stability, ability to grow to high titers in cell culture, ease of manipulation by recombinant DNA techniques, and ability to infect a broad

spectrum of cells (Graham and Prevec 1992). They are currently being investigated as potential vaccine vectors for HIV, cytomegalovirus, respiratory syncytial virus, herpes simplex virus, parainfluenza virus, hepatitis B virus, and rabies virus among others. They have shown to induce humoral and cell-mediated immune responses to the foreign gene products expressed in infected cells, providing protection against disease in vaccinated individuals (Graham and Prevec 1992).

Replication-competent Ad5 with the ERA glycoprotein gene inserted into its non-essential E3 (early gene region 3) transcriptional unit have been developed as potential alternatives to attenuated ERA and VRG vaccines (Prevec et al 1990; Yarosh et al 1996). Ad5-rabies glycoprotein recombinant vaccines (Ad5RG) are particularly amenable to wildlife immunization as they can be effectively administered by the oral route and may induce mucosal as well as systemic immunity (Xiang and Ertl 1999). They are effective at inducing rabies virus neutralizing antibodies in animals normally semi- or non-permissive to human adenoviral infection, such as mice, dogs (Prevec et al 1990; Yarosh et al 1996), foxes and skunks (Charlton et al 1992), and raccoons (Dr.Wandeler, personal communication). Mice, foxes, and skunks given the vaccine orally survive peripheral challenge with street rabies virus (Charlton et al 1992). Rabies antibody titers in skunks have been shown to be effective over a wide range of vaccine doses and can persist for more than one year (Charlton et al 1992). Virus shedding in skunk feces and oral fluids has been observed for up to 4 days post-immunization and only occasionally in oral fluids up to 10 days post-immunization, suggesting a reduced probability of adenovirus transmission to other animals (Lutze-Wallace et al 1995b). Genetic stability of Ad5RG1, including glycoprotein gene integrity, after passage through skunks has been confirmed (Lutze-Wallace

et al 1995a; Lutze-Wallace et al 1995b).

The main concerns surrounding the release of genetically engineered microorganisms like Ad5RG into the environment are their potentially harmful effects in non-target animals, animal reservoir establishment, and participation in genetic recombination either as donors or recipients of genetic material (Tiedje et al 1989). The likelihood of these events occurring depends on if and for how long the vaccine vector is excreted from the vaccinated host, vaccine vector survival and genetic stability in the environment; whether a susceptible host makes contact somehow with the virus, whether productive infection occurs as a result of this contact, and whether viruses related to the vaccine vector are naturally prevalent in wildlife species. These questions will be addressed in the literature review and the latter concern is addressed experimentally.

Despite the lack of official regulatory guidelines, the World Health Organization and other veterinary biologics advisory groups recommend that the prevalence of viruses related to the recombinant vaccine vector in the species targeted for vaccination be established before field use as part of the general assessment of risk (WHO Meeting 1990; Office International des Epizooties (OIE) 1993). The overall goal of this project was, therefore, to design and validate serological assays (as will be described) for measuring the occurrence of human and canine adenoviruses in the terrestrial mammals of Ontario targeted for rabies immunization. In addition to complying with WHO recommendations, the purpose of this study was to collect information necessary for tracking potential Ad5 dissemination and to assess whether an immune barrier exists to vaccination with Ad5RG.

LITERATURE REVIEW: THE ADENOVIRUSES

Similarities in adenovirus properties and patterns of transmission, pathogenesis,

disease, epidemiology and epizootiology are observed in humans and animals respectively.

Classification. Adenoviruses belonging to the family *Adenoviridae* are divided into two genera based on the presence of a genus-specific antigen: the *aviadenoviruses* with 5 groups infecting birds (duck, fowl, goose, pheasant, turkey) and the *mastadenoviruses* with 10 groups infecting mammals (human, dog, cow, pig, sheep, goat, horse, mouse, monkey, and tree shrew) (Ishibashi and Yasue 1984; Russell 1998). Recent comparisons of viral genes suggest a third phylogenetically distinct group, the proposed *atadenoviruses*, comprising the avian Egg Drop syndrome virus-76, bovine adenovirus serotype 7 (BAV-7), and ovine adenovirus (OAV) strain 287 isolated from sheep in Western Australia (Harrach et al 1997; Venktesh et al 1998; Matiz et al 1998). Additionally, circumstantial evidence of adenovirus infection has been demonstrated in the white-footed deer mouse (*Peromyscus maniculatus*) (Reeves et al 1967), opossum (Morales-Ayala et al 1964), rabbit (Bodon et al 1979; Bodon 1980), California sea lion (*Zalophus californianus*)(Britt et al 1979; Dierauf et al 1981), and frog (Clark et al 1973; Jensen and Block 1980). It is difficult to say in these cases, however, whether the viruses involved are actually new serotypes or whether these animals simply represent aberrant hosts. Also, a new deer adenovirus has been implicated in a large epizootic of hemorrhagic disease in mule deer (*Odocoileus hemionus*) in northern California, causing high mortality (Woods et al 1996; Woods et al 1997; Lapointe et al 1999; Woods et al 1999).

Over 120 different adenovirus serotypes have been identified, 47 of which are human in origin and represent the group most extensively studied (Russell 1998). Human adenovirus serotypes have been classified into subgroups based on hemagglutinating activity, guanine and cytosine content of viral DNAs, common antigens and genetic sequences, host

specificity, tissue tropism, clinical disease, pathogenicity, oncogenic potential in rodents and ability to transform rodent cells (Table 1) (Shenk 1996; Horwitz 1996).

Table 1: Classification of human adenoviruses into subgroups.

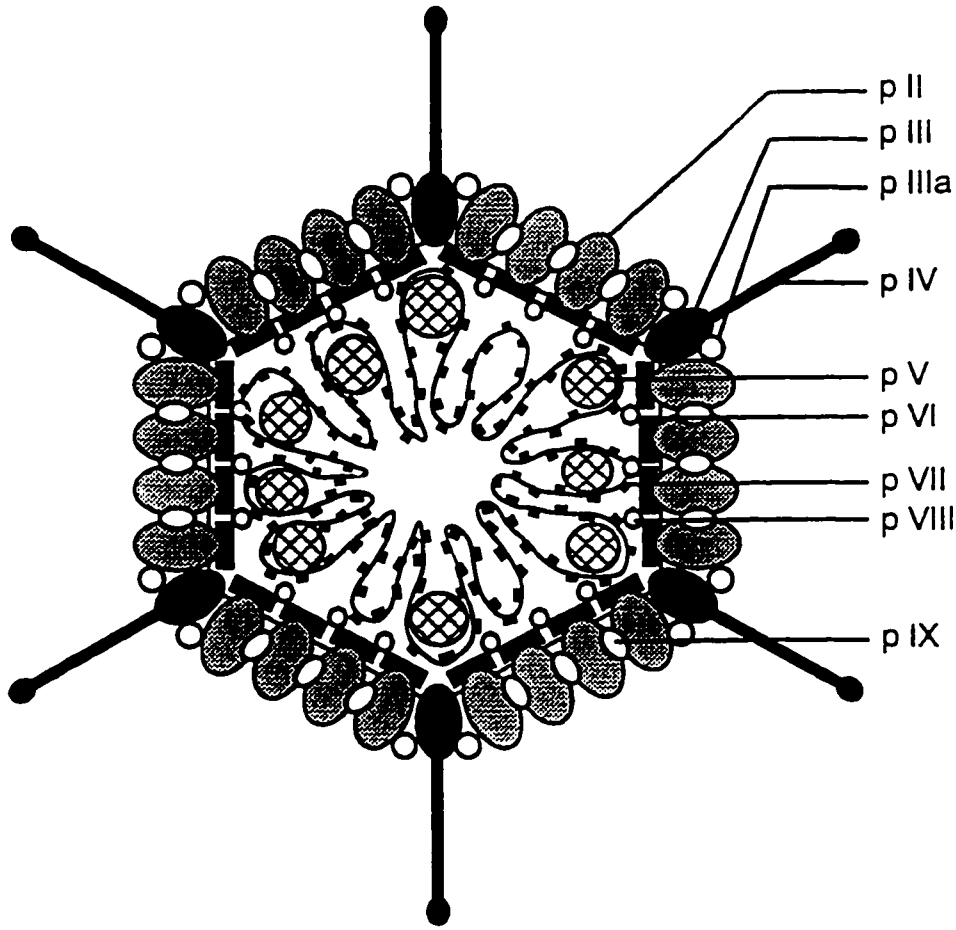
Group	Sub Group	Serotypes	Oncogenicity in hamsters	Transformation in cell culture	Site of Infection
Human	A	12, 18, 31	High	+	GI Tract
	B	3, 7, 11, 14, 16, 21, 34, 35	Weak	+	Resp Tract, Eye
	C	1, 2, 5, 6	No	+	Resp Tract, adenoids, tonsils
	D	8-10,13,15,17,19,20,22-30,32,33,36-39,42-47	No	+	Eye
	E	4	No	+	Resp Tract, Eye
	F	40, 41	No	+	GI Tract

Bovine adenoviruses (BAV) comprise 10 serotypes to date, divided into two subgroups based on differences in their antigenic properties, gene sequence similarities, and their replication in either calf kidney and testicle cells (Horner et al 1989; Bürki 1990). Further studies, however, suggest that BAV-10 be considered the first member of a third subgroup (Matiz et al 1998). It has been suggested that BAV-7, BAV-4 and perhaps BAV-6 are phylogenetically distinct from the *mastadenoviruses* and warrant classification in the proposed *atadenovirus* genus (Matiz et al 1998).

Five distinct porcine adenovirus (PAV) serotypes have been recognized and evidence for at least one other has been presented (Derbyshire 1989; Kadoi et al 1995). Six different ovine adenovirus (OAV) serotypes have been isolated from sheep (Belak 1990), as well as one which is phylogenetically distinct from the others (Harrach et al 1997), two goat adenovirus serotypes (GAV) (Ishibashi and Yasue 1984), two equine adenovirus (EAV) serotypes isolated from horses (Studdert 1996), approximately 27 simian adenovirus serotypes (SAV), 7 isolates from chimpanzees and 20 isolates from monkeys, and one

prosimian adenovirus (phylogenetic position between primates and rodents) isolated from a kidney culture of a healthy tree shrew (*Tupaia belangeri*) (Ishibashi and Yasue 1984). Two murine adenovirus (MAV) serotypes have been isolated from laboratory mice (Kraft 1994) and may be implicated in adenovirus infections of rats (Ward and Young 1976; Kraft 1994) and hamsters (Gibson 1990). A guinea pig adenovirus (GPAV) is implicated as the main etiologic factor in spontaneous guinea pig bronchopneumonia and does not appear to belong to the same subgroup as MAV-1 (Naumann et al 1981; Kraft 1994; Pring-Akerblom et al 1997).

Structural properties. All adenoviruses are 70-100 nm non-enveloped icosahedral particles, 11-13% of which is linear double-stranded DNA with the remaining portion made up of protein, 1% of which is carbohydrate (Nermut 1984; Shenk 1996). Genomes synthesize approximately 40 different polypeptides, at least 11 of which are structural, ranging from 5 to 120 kD and making up the outer capsid and inner core proteins (Shenk 1996). The outer capsid is composed of 240 non-vertex capsomeres (hexons) and 12 vertex capsomeres (pentons), each with one base and one protruding fiber, whose length depends on the serotype (Figure 1). The penton base and fiber interact non-covalently and fibers can dissociate from the capsid after freezing and thawing or ultracentrifugation (Pettersson 1984). The hexon and fiber proteins are trimers, comprising three identical polypeptides each (Shenk 1996). Minor capsid proteins help cement the hexons and anchor the capsid to the core (Russell 1998). Although less variation is seen with internal core polypeptides, the electrophoretic patterns and molecular weights of adenovirus polypeptides differ with respect to the phylogenetic relationship of the corresponding host species (Ishibashi and Yasue 1984).



Designation	Protein	MW
II	hexon	120 kD
III	penton base	85 kD
IIIa	penton base-associated	66 kD
IV	fiber	62 kD
V	core protein	48.5 kD
VI	hexon-associated	24 kD
VIII	core protein	18.5 kD
VIII	hexon-associated	13 kD
IX	hexon-associated	12 kD

Figure 1. Cross-section representation of an adenovirus particle. The main structural proteins, their designations, and their molecular weights are listed in the table. Reprinted with permission from the Doctoral Thesis of Oksana Yarosh (1994).

Genome structure and function. Adenovirus genome sizes may differ, but all contain virus-coded terminal proteins covalently linked to each 5' end in association with three other polypeptides, 50-200 base pair (bp) inverted terminal repeat (ITR) sequences, and a guanine-cytosine content of 48-61% (Nermut 1984; Shenk 1996). The mastadenovirus genome is approximately (20-25) x 10^3 kD and the prototype Ad2 is approximately 36 000 bp (Russell 1998). Nuclear transcription of the adenoviral genome making use of cellular transcription factors involves the synthesis of five early mRNAs (E1A, E1B, E2, E3, and E4) coding for early proteins important for virus replication, such as the virus DNA binding protein, proteins which modulate host cell functions, and proteins which counteract the host immune response (Russell 1998). Minor intermediate transcripts are also synthesized as is one major late transcript, which is processed to generate five families of late mRNAs (L1-5) coding for most of the virus structural and assembly proteins (Russell 1998). Adenoviruses replicate well in the nuclei of primary kidney or testicular cells of the presumptive natural host animal, giving a characteristic pattern of nuclear staining. Infection generally causes characteristic cytopathic alterations which consist of rounding granular cells with increasing refractility, aggregating into grape-like clusters.

Physical and chemical properties. Adenoviruses are generally resistant to chloroform, fluorocarbon, ether, and to some degree to trypsin and heat (Nermut 1984). Most are destroyed at 60°C after 3-5 minutes and can be inactivated by ultra-violet light, Lysol™, bleach, and Virkon™. All adenoviruses can endure pH 6-9, while BAV and OAV can resist pH 2-11 (Bürki 1990; Belak 1990). All are stable from 4-36°C for several weeks, and survive freezing with minimal loss of infectivity suggesting their potential survival in the environment (Shenk 1996).

Antigenic properties. The antigenic relationships among adenoviruses are very complex but have more recently been aided by the application of monoclonal antibodies to serological techniques such as immunofluorescence (IF) and enzyme-linked immunosorbent assay (ELISA) (Russell et al 1981; Adam et al 1986; Adam et al 1992; Adam et al 1995a; Adam et al 1995b). Adenovirus serotypes are identified and classified based on neutralization of viral infectivity and hemagglutination of erythrocytes from different species, except in mice (Kraft 1994) and certain BAV serotypes for which classification by the hemagglutination test does not figure prominently (Bürki 1990). The top part of the hexon proteins and the knob region of fiber proteins contain most of the type-specific epitopes recognized by neutralizing Ab (Norrby 1969); however, penton base proteins also have some epitopes recognized by neutralizing Ab and which are probably responsible for subgroup specificity (Horwitz 1996). Fibers also contain domains responsible for hemagglutination and domains responsible for attachment to cellular receptors. Antibodies to type-specific determinants on the fiber neutralize virus by sterically preventing cell attachment (Toogood et al 1992). Hemagglutination and neutralization results are usually concordant.

The complex array of antigenic determinants on hexons demonstrate genus, type, intertype, intersubgroup, and intrasubgroup specificities (Norrby 1969; Norrby and Wadell 1969). Hexon genes show a distinct middle segment containing intermediate and hypervariable regions, and two highly conserved flanking regions at the - and C-termini (Pring-Akerblom and Adrian 1993; Crawford-Miksza and Schnurr 1996). The majority of variable residues, comprising type-specific antigenic determinants, occur in two amino acid loops which protrude from the hexon surface (Toogood et al 1992; Horwitz 1996; Crawford-Miksza and Schnurr 1996). Variable sequences in a fourth loop have been implicated in the

host species specificity of human adenoviruses (Crawford-Miksza and Schnurr 1996). Neutralization of these epitopes allows virus attachment and entry into the host cell; however, further events are inhibited once the virus is inside the cell endosome (Wohlfart et al 1985; Wohlfart 1988). Non-neutralization-associated conserved residues, comprising group-specific antigenic determinants, are located to the interior base of the hexon (Norrby 1969; Norrby and Wadell 1969; Horwitz 1996). Classification of an adenovirus isolate as a member of the *mastadenoviruses*, except in the case of a few bovine adenovirus serotypes, is based on the presence of a shared non-neutralization-associated genus-specific antigenic determinant located on the hexon (Norrby 1969; Ishibashi and Yasue 1984; Shenk 1996).

Genome comparisons. The genomes of human Ad2 (Roberts et al 1984), Ad5 (Chroboczek et al 1992), Ad12 (Sprengel et al 1994), Ad40 (Davison et al 1993), CAV-1 (Morrison et al 1997), and some OAV isolates (OAV287) (Vrati 1996) have been completely characterized and murine, porcine, and bovine adenoviruses have been partially characterized, with the observation that basic genome organization and identity of the structural proteins appear to be conserved. The analysis of DNA sequence homologies has helped elucidate evolutionary relationships between adenovirus serotypes and is generally in good accordance with previous classification schemes (Bailey and Mautner 1994). For example, Ad2 and Ad5 (subgroup C) genomes are 95% identical, except for type specific residues in the fiber coding region (Chroboczek et al 1992). In general human adenovirus subgroup serotypes exhibit 90% homology, except among pairs of serotypes from subgroup A whose homology index ranges from 48-69%, and the homology index between subgroups is less than 20% (Green et al 1979). Analysis of human and animal adenovirus gene sequences indicate that the hexon, fiber, and E3 coding regions are subject to the greatest variation, especially the E3

region which is implicated in differences in serotype pathogenicity (Bailey and Mautner 1994; Khatri 1997).

A 75% nucleotide identity was determined for CAV-1 and CAV-2 E1 sequences (Spibey et al 1989), which corresponds with the previously reported 70% DNA hybridization homology index (Marusyk and Hammarskjold 1972), although their restriction endonuclease patterns were very different (Assaf 1983). The CAV-2 E3 gene also carries an extra sequence of about 500 nucleotides that is speculated to contribute to differences in pathogenicity (Linne 1992). Open reading frames (ORFs) in the late and E2 regions of CAV-1 show greatest identity with published human adenovirus sequences, whereas ORFs in E1, E3, E4, and fiber do not (Morrison et al 1997).

A homology index of 78-95%, 80-100%, and 64-87% has been reported for simian adenovirus serotypes within subgroups I, II, and III respectively, while a 28% to 55% homology index is reported to exist between different subgroups (Burnett et al 1972). Phylogenetic studies of human and some simian adenovirus serotypes demonstrated clustering of subgroup A and F serotypes and SAV-16 and 8 which are associated with intestinal disease, suggesting an evolution independent of the other human adenoviruses which generally infect the respiratory tract (Bailey and Mautner 1994; Kidd et al 1995). Subgroup B, D, and E serotypes appear most related to each other and to simian adenovirus serotypes infecting apes (chimpanzees) (Kidd et al 1995). Human subgroup C adenoviruses, however, share both monkey and chimpanzee adenovirus gene similarities, suggesting a possible origin from recombination between ancestral ape and monkey adenoviruses (Kidd et al 1995). A region of nucleotide similarity within the E1 region was observed between a PAV and Ad5 sequence (Kleiboèker 1995) and polypeptides encoded by this PAV region

are homologous to other adenovirus polypeptides, with the strongest homology to BAV-3 E1 products (Kleiboeker 1995). Hexon sequence comparisons from many human and non-human adenoviruses have been used to deduce phylogenetic relationships between adenoviruses (Reubel 1997).

Genetic recombination. Recombination between adenoviruses and closely-related serotypes, other virus sequences, and host cellular DNA is a natural phenomenon contributing to the evolution and diversity of adenovirus serotypes and serotype variants (Johansson et al 1994; Doerfler 1995; Doerfler 1996; Shenk 1996). Homologous recombination in cells coinfected with adenoviruses from the same subgroup, such as Ad2 and Ad5, is well established (Takemori 1972; Williams et al 1975; Lippe 1989). Recombination between subgroups has been implicated in the origin of Ad4, the result of a probable crossover event between the E2 coding region of a group B virus and fiber coding region of a group C virus (Gruber et al 1993). As a result, new serotypes may possess hexon specificities of one type and fiber specificities of another, complicating the antigenic picture (Norrby 1969; Norrby and Wadell 1969). Ad1-5 and Ad7 can recombine with simian virus 40 (SV40), a simian papovavirus known for its oncogenic potential, allowing adenoviruses to extend their host range into monkey cells (Rowe and Baum 1964; Kelly and Lewis 1973). Recombination between Ad12 DNA and human KB cellular DNA in cells productively infected with Ad12 has been documented (Deuring and Doerfler 1983; Gahlmann et al 1984; Doerfler et al 1984). Integration of Ad12 DNA into the DNA of Ad12-transformed hamster and Ad12-induced rat brain tumor cells has also been observed (Doerfler et al 1980). Recombination events *in vitro* between human 293 cell E1 gene sequences or wildtype virus and E1-deleted replication defective adenoviruses can yield replication competent

adenoviruses which have recovered their E1 region (Lochmuller et al 1994; Oualikene et al 1995). Similar events, however, have not been observed in *vivo* (Oualikene et al 1995).

Inter-animal transmission. Adenoviruses usually cause respiratory, intestinal, or ocular infections or a combination thereof depending on the route of transmission and the tissue tropism of the particular infecting serotype. For instance, serotypes causing respiratory or intestinal infections are acquired either through the mouth and nose by inhalation of infected aerosols spread by coughing, ingestion of or contact with infected food, urine, feces, water, saliva, or nasal secretions, although the fecal-oral route is probably the most common mode of transmission in both humans and animals (Derbyshire et al 1966; Sharp et al 1976; Ishibashi and Yasue 1984; Grate et al 1987; Appel 1987a; Derbyshire 1989; Bürki 1990; Belak 1990; Kraft 1994). Feral dogs may contribute to transmission in nature, but viral spread may also occur through ingestion of contaminated meat of sick or dead animals, although there is no available data on the survival of the virus in small animal carcasses (Grate et al 1987; Appel 1987a). Ocular infections in humans are acquired through contact with contaminated hands, swimming pool water (Foy et al 1968), or ophthalmologic instruments (Mueller and Klauss 1993), and in animals through contact with infected lacrimal or conjunctival fluids.

Environmental transmission. Animal excrement and sewage sludge provide valuable fertilizers for farmers, but may contain adenoviruses which can lead to environmental contamination (Derbyshire et al 1986; Hurst 1989; Snowdon et al 1989; Strauch 1991). Excreted animal adenoviruses can often withstand complete inactivation in manure stored anaerobically for extended periods (Pesaro 1995). Human adenoviruses have been isolated from domestic sewage, as well as treated primary and secondary effluents (Irving and Smith

1981; Payment et al 1983) and have been shown to survive saltwater for a few days to several weeks (Metcalf and Stiles 1967; Gerba et al 1975). Shellfish, such as oysters, clams, and mussels, which can concentrate viruses and bacteria in sewage-contaminated waters even at a distance from the point of sewage discharge, can become contaminated as a result (Gerba and Goyal 1978; Goyal et al 1979; Yamashita et al 1992). Human infection with enteric viruses and illness have occurred when contaminated shellfish are consumed, especially when eaten raw (Portnoy et al 1975; Mackowiak et al 1976; Gunn et al 1982; Gill et al 1983) and have been associated with Ad3 related outbreaks in children (Yamashita et al 1992). Experimental studies on the survival of Ad5 under ambient conditions in fox and skunk fecal suspensions, however, indicate a rapid loss of virus infectivity during the first 8 hours after deposition, and a remaining 3% infectivity after 72 hours (Kalicharran et al 1992). Low levels of adenoviruses have also been detected in urban river water without any apparent seasonal distribution (Tani et al 1995) as well as in drinking water (Nestor et al 1989).

Pathogenesis. Adenoviruses are frequently isolated from apparently normal adenoids and tonsils, implicating tissues of the oropharynx as the site of primary replication (Horwitz 1996). Depending on the serotype, adenoviruses will then either replicate preferentially in the epithelium lining the respiratory tract, sometimes establishing infection in the lung, or in the intestinal tract epithelium, establishing persistent infections in some cases (Horwitz 1996). General respiratory pathology includes rhinitis, tracheitis, bronchopneumonia and interstitial pneumonia, and conjunctivitis (Ishibashi and Yasue 1984; Horwitz 1996). Some infecting serotypes will be taken up into local lymph nodes, disseminating quickly into the blood, replicating in vascular endothelial cells and in other organs in severe cases, such as the lungs, liver, kidney, spleen, brain, adrenals and heart (Collier et al 1966; Shadduck et al

1968; McChesney et al 1974; McChesney and England 1975; Grate et al 1987; Appel 1987a; Kraft 1994), often causing massive hemorrhaging in these tissues (Appel 1987a). Following recovery from CAV-1 infection in dogs, complications may arise due to immune complex deposition in the kidneys and eyes ("blue eyes") (Appel 1987a).

Virus from fecal and oropharyngeal swabs is often recovered during acute disease and shedding may persist with subclinical infection for months to years. Bladder infections observed after viraemic spread will often result in long term virus excretion in urine (Kraft 1994). Other evidence of adenovirus persistence comes from the frequent isolation of adenoviruses in cell cultures (e.g. adenoids, tonsillar, kidney, testicle, and lung cells) from apparently healthy humans and animals (Ishibashi and Yasue 1984). Also, Ad1, 2, 5, and 6 DNA have been detected in peripheral lymphocytes of otherwise healthy individuals (Straus 1984; Horvath et al 1986; Ginsberg et al 1987; Ginsberg and Prince 1994).

Disease signs. During adenovirus infection, infected cells may be damaged or die causing disease to the host. Most human and non-human adenovirus infections are subclinical and not generally associated with any serious morbidity (Appel 1987b; Derbyshire 1989; WHO Meeting 1990; Bürki 1990; Belak 1990; Kraft 1994; Russell 1998). In some humans and animals, however, adenoviruses will cause mild to severe symptomatic respiratory, ocular, or intestinal disease, depending on the infecting serotype and its associated pathogenicity. Respiratory symptoms, which may become chronic in some animals, include nose and eye secretions, coughing, sneezing, conjunctivitis and lacrimation, and superficial and accelerated breathing (Powell et al 1974). The most common enteric infection symptom is diarrhea (Derbyshire 1989; Horwitz 1996), often accompanied by salivation, fever, anorexia and weight loss, leukocytosis, and kidney involvement as seen by protein, blood, and pus in

urine (Bürki 1990; Belak 1990; Studdert 1996). BAV-10, in contrast to the other BAV serotypes, seems to cause a well-defined hemorrhagic enterocolitis condition in calves (Adair et al 1996). CAV-1 in dogs and foxes causes infectious canine hepatitis, encephalitis, chronic hepatitis, neonatal disease, respiratory disease, interstitial nephritis, and ocular lesions (Appel 1987a). CAV-2 causes a milder illness in dogs, characterized by rhinitis, tonsillitis, bronchitis, and interstitial pneumonia, and laryngotracheitis or "kennel cough" (Appel 1987b). These serotype differences in pathogenicity are also observed with human adenoviruses (Horwitz 1996), MAV (Kraft 1994), and EAV (Studdert 1996). Most adenovirus infections in domestic livestock or horses, are rarely single etiological agents of clinical disease, and involve multiple pathogens like reovirus, parainfluenza-3 virus, *Pasteurella haemolytica*, corynebacteria, *Mycoplasma hyopneumoniae* or *Mycoplasma ovipneumoniae*, which may cause pneumonia in pigs and sheep respectively, coronavirus, and cryptosporidium in the intestinal tract (Stipkovits et al 1975; Ishibashi and Yasue 1984; LeaMaster et al 1987; Derbyshire 1989; Belak 1990).

In the laboratory, the successful induction of disease caused by infection with animal adenoviruses is only typically observed in young calves, foals, lambs, suckling mice, weaning pigs, and other young animals reflecting the natural disease situation. Likewise infants, young children, and canine puppies are most susceptible to illness during natural infection. Some human adenovirus serotypes have been associated with disease in other organs such as the bladder, liver, and pancreas as well as the central nervous system (Horwitz 1996) and often act as opportunistic pathogens in immunocompromised individuals such as bone marrow transplant and AIDS patients (Zahradnik et al 1980; Yolken et al 1982; Krilov et al 1990). Ad1, 2, and 5 in particular can cause acute hepatitis and death in severe cases

associated with liver transplants in children (Carmichael et al 1979; Koneru et al 1987; Michaels et al 1992; Horwitz 1996). Ad5 infection in immunocompromised individuals has been shown to cause prolonged and undulating fever and diarrhea (Johansson et al 1990). Animal adenovirus serotypes also act as opportunistic pathogens, for example EAV-1 which causes a progressive fatal bronchopneumonia in Arabian foals born with the primary severe combined immunodeficiency disease (PSCID) (about 3% of all purebred Arabian foals) (Thompson et al 1975; Perryman and Torbeck 1980).

The immune response in an otherwise healthy individual will usually allow spontaneous and rapid recovery from disease, except with CAV-1 in dogs and foxes, which can be fulminating and fatal in its early stages (Appel 1987a). Dogs that survive infection or have been vaccinated usually have lifelong immunity (Appel et al 1975; Appel 1987a; Appel 1987b). General immune responses to adenovirus infection usually involve production of high titers of specific antibody; however, the cell mediated immune response plays a critical role as evidenced by fatal disease in athymic (nude) mice (Kraft 1994), AIDS patients lacking T cells, and PSCID foals who severely lack T and B lymphocytes. Neutralizing Ab titers seem to gradually decrease with time, but increase following boosting with another serotype. With most human and animal adenovirus infections there is a good correlation between maternal Ab in newborns and protection against disease with the same serotype (Aldasy et al 1965).

Epidemiology. Major findings from longitudinal "virus watch" studies suggest that human adenoviruses are ubiquitous in individuals of all ages, races, nationalities, and sex (except in the case of predominantly male military recruits), as well as in tropical and temperate climates throughout the year but exhibit a midwinter and midsummer peak of infections in

temperate climates (Fox et al 1969; Brandt et al 1969; Fox et al 1977). These studies also suggest that adenovirus infections can be divided for clinical purposes into endemic, epidemic, and sporadic infections. Ad1, 2, 5, and 6 (subgroup C) appear to be the most common and are endemic in most parts of the world, probably due to their persistent intermittent excretion in feces (Fox et al 1977; Horwitz 1996). Infection rates of these endemic adenoviruses are highest in infants (>90% for Ad 1 and 2), decrease with age in older children but increase again in parents (Fox et al 1977). By three years of age, 37% of children in Sweden, 80% in the USA, and 100% in Taiwan have adenovirus neutralizing antibodies (Fox et al 1977). Antibody studies indicate exposure to Ad5 in 50-100% of the population depending on the region, and homotypic immunity was shown to be 85% protective against infection (Fox et al 1969). Epidemic infections more often involve Ad3, 4, 7, and 8 and occasionally involve Ad11, 14, 19, and 21. Antibody prevalence in children to these types is lower than that of the endemic Ad1, 2, and 5 (Huebner et al 1954; Jordon et al 1956; Brandt et al 1969).

Only half of human adenovirus infections cause illness (Brandt et al 1972) and the average individual undergoes a minimum of 2-3 clinical episodes of adenoviral infection during childhood (Chanock 1974). Children living in institutions, or in situations of crowding and poor hygiene, tend to become infected at a younger age. Adenoviruses are the cause of 5-10% of respiratory tract infections in infants and children in North America (Fox et al 1969; Brandt et al 1972; Makela et al 1998) and are responsible for a number of infant deaths (Simla et al 1971) and certain morbidity in adult populations. Ad4 and Ad7-induced ARD is observed in approximately 80% of military recruits, 20% to 60% whom need hospitalization (Dudding et al 1973; Horwitz 1996) and these infections usually peak once

a year during the cooler months (Hilleman 1957). A recent national seroprevalence survey indicated that nearly 90% of US army recruits lacked Ab to either Ad4 or Ad7 (Ludwig et al 1998). Adenoviruses are associated with approximately 4-17% cases of gastroenteritis in children, a rate second only to that of rotavirus as a cause of pediatric diarrhea (Uhnoo et al 1990). One study suggested that Ad48 and 49 may be endemic in some part of the homosexual population and that sexual transmission may be the primary source of continuous exposure (Crawford-Miksza and Schnurr 1996).

Epizootiology. Most animal adenoviruses have a worldwide distribution, are endemic in most farm populations, and are more likely to cause disease under conditions of crowding, poor ventilation, and heat stress which often prevail in fattening plants and large industrialized farms designed for intensive breeding (Derbyshire 1989; Bürki 1990; Belak 1990; Lehmkuhl et al 1993; Lehmkuhl et al 1998). For this reason, commercial vaccines for BAV and OAV are currently employed in some countries. For calves, these may consist of either BAV-3 or a combination of BAV-1, 3, and 5, even though no definitive proof exists that BAV-3 causes severe enzootics in the field (Bürki 1990). Serological studies indicate that any one particular BAV serotype may dominate a given region for a given period of time before it is replaced by another and multiple seroconversions have been observed in cattle indicating parallel infections by more than one serotype (Lehmkuhl et al 1998). OAV-related disease can occur in as many as 30-40% of suckling lambs and 10-15% of fattening lambs; however, economic losses incurred from diseased animals and death have been significantly reduced with effective sheep vaccination (Belak 1990). In adult swine, high Ab titers indicate that PAV-4 is the most common serotype in pigs with respiratory problems, with prevalences between 15-78% (Harkness et al 1971; Dea and El Azhary 1984; Elazhary

1985), but the virus is not responsible for any great economic burden (Derbyshire 1989). At the very most, there may be minor losses in production due to poor weight gain and reduced efficiency of food conversion in some animals (Derbyshire 1989). EAV-1 is endemic in most equine populations and Ab prevalence in adult horses suggest that the majority of foals tolerate adenoviral infection (Studdert et al 1974). EAV-1 Ab prevalence can vary from 2-100% depending on the age, breed, activity, size and region of the sample population (Studdert et al 1974; Harden et al 1974; Powell et al 1974; Farina et al 1978; Herbst et al 1992). Although parenterally administered live and attenuated EAV-1 preparations have been shown to reduce or abrogate disease in foals, no commercial vaccine has been developed. Simian and vervet monkey (*Cercopithecus aethiops*) adenovirus Ab have generally not been detected in captive apes such as gorillas, orangutans, and gibbons, but were detected in captive chimpanzees, rhesus monkeys, some baboons and vervets; however, the captive scenario may not reflect the wildlife scenario (Kalter and Heberling 1971). The prevalence of murine adenoviruses in laboratory colonies appears to be low (Kraft 1994). Serological studies on MAV prevalence revealed only 4 positive mouse colonies out of 34 examined (Parker et al 1966; Van Nunen et al 1978), while other studies failed to detect any infection (Descoteaux et al 1977; Carthew and Verstraete 1978; Kraft and Meyer 1990). Adenoviruses in hamster and guinea pig laboratory colonies, however, appear to be ubiquitous (Gibson 1990) with low morbidity and high mortality in guinea pigs (Brandon 1995). A serological study on the prevalence of murine viruses in field populations of house mice (*Mus domesticus*) detected MAV Ab in 46% and 26% of mice captured near a permanent water source and in crops, respectively, with seroprevalence at its highest in July, and corresponding with increases in population density (Singleton et al 1993). Low MAV

Ab titers in another study suggest that MAV is probably not present in wild meadow voles (*Microtus pennsylvanicus*) (Descoteaux and Mihok 1986).

The prevalence of CAV in domestic dogs, estimated at 30-60% in various parts of the world with a 10-30% mortality rate, has dropped since the introduction of attenuated CAV vaccines (Cabasso 1953; Sasaki et al 1956; Bass et al 1980; Appel 1987a). There is little evidence of animal adenoviruses causing disease in wildlife populations except CAV-1, which represents a natural pathogen in red foxes (Green 1925). The study of fox fur farm epizootics revealed that mortality in young pups is twice that in adult foxes and can be as high as 80% in breeding units (Green et al 1930; Green 1931). CAV-1 has also been isolated from coyotes (*Canis latrans*) (Gier et al 1978), wolves (*Canis lupus*) (Pursell et al 1983), striped skunks (Karstad et al 1975), black bears (*Ursus americanus*) (Kapp and Lehoczki 1966; Pursell et al 1983; Dunbar et al 1998), and polar bears (*Thalarctos maritimus*) (Chaddock and Carlson 1950). Although the existence of CAV wildlife reservoirs is unknown, serological surveys have demonstrated varying levels of exposure to CAV in raccoons (6-16%) (Parker et al 1961; Jamison et al 1973; Bolin et al 1982; Sumner et al 1988; Hable et al 1992), skunks (32-63%) (Alexander et al 1972), wild and captive giant pandas (*Ailuropoda melanoleuca*) (50%) (Mainka et al 1994), and mongoose (77%) (Sumner et al 1988). High Ab titers and frequent exposure in wolves (13-95%) (Choquette and Kuyt 1974; Stephenson et al 1982; Zarnke and Ballard 1987), coyotes (33-100% depending on age) (Trainer and Knowlton 1968; Davidson et al 1992; Gese et al 1997; Cypher et al 1998), red foxes (4-100%) (Davidson et al 1992; Truyen et al 1998), gray foxes (56%) (Davidson et al 1992), and island foxes (72-96%)(*Urocyon littoralis*) (Garcelon et al 1992) suggest that CAV-1 might be enzootic in these species. Minks (*Mustela vison*) and ferrets (*Mustela*

putorius) experimentally infected with CAV-1 showed no signs of disease (Grate et al 1987) and no Ab titers were detected in opossums (*Didelphis marsupialis*) or woodchucks (*Marmota monax*) (Jamison et al 1973). Mortality and morbidity data for susceptible wild carnivores other than foxes is unknown; however, case reports suggest that mortality in non-domestic carnivores is high (Grate et al 1987). The high frequency of CAV exposure as described previously would suggest otherwise.

Cross-species infection. Human and animal adenoviruses typically demonstrate a narrow host range and generally do not or weakly replicate in other species (Betts et al 1962; Prevec et al 1989; Oualikene et al 1994; Horwitz 1996). Certain evidence exists, however, that adenoviruses can cross natural species barriers. Some bovine adenovirus serotypes, for example BAV-3, -4, and -8, have also been isolated from free-living African buffaloes (Baber and Condy 1981). On another occasion, BAV-6 infection was associated with bronchiolitis in a red deer (*Cervus elaphus*) in New Zealand (Horner and Read 1982) and was isolated from the lung of a fallow deer (*Dama dama*) in Budapest exhibiting similar histopathology (Boros et al 1985). BAV-2 infection has been implicated in a natural outbreak of respiratory disease in sheep (Belak and Palfi 1974; Belak et al 1983) and a virus isolated from a sheep in New Zealand was more antigenically similar to BAV-7 than with any of the other recognized ovine adenoviruses (Adair et al 1982). Ab to BAV-3, BAV-7, and BAV-8 have been detected in sheep (8%-83%) (Adair et al 1984; Goyal et al 1988; Belak 1990), goats (0.02-9%) (Fulton et al 1982), Idaho mule deer and pronghorn antelopes (*Antilocapra americana*) (34-41%) (Stauber et al 1977; Stauber et al 1980). Ovine adenovirus Ab have conversely been detected in goats and cattle (Dubey 1985; Dubey et al 1985).

Adenoviruses have also demonstrated cross-species infection under experimental conditions. Cotton rats and New Zealand rabbits have provided fully permissive animal models of human adenovirus ocular (Ad5 and Ad8) and lung infections (Ad1, 2, 5, and 6) (Tsai et al 1992; Gordon et al 1992; Prince et al 1993; Ginsberg and Prince 1994). Some human and animal adenoviruses may produce abortive infections in non-host species, resulting in the production of early adenovirus proteins and possible transformation, but little or no progeny virus (Russell 1998). As such, mouse cells and live mice have provided semi-permissive models of Ad5 infection for the study of early protein synthesis, pulmonary pathology, cytokine responses (Duncan et al 1978; Ginsberg et al 1991; Graham and Prevec 1992; Ginsberg and Prince 1994), the role of single or groups of Ad genes (Fejer et al 1994), efficacy of Ad5-recombinant vaccines (Prevec et al 1990; Xiang et al 1996), and gene transfer by adenoviruses (Yang et al 1996). Also, Ad5 infection has on occasion demonstrated persistence in guinea pigs (Faucon et al 1974) and rabbits (Pereira and Kelly 1957; Reddick and Lefkowitz 1969). Ad12, 18, and 31 are highly oncogenic in hamsters and inoculation of Ad9 into rats has been shown to induce fibroadenomas and mammary sarcomas (Horwitz 1996). Ad2 and Ad5 can replicate in monkey cells; however, the process requires co-infection with SV40 or the use of an adenovirus host range mutant (Klessig and Grodzicker 1979; Horwitz 1996). Animal adenoviruses have demonstrated full and limited replication in cells of other species (Gehle and Smith 1969; Ishibashi and Yasue 1984; Appel 1987a; Appel 1987b; Bürki 1990) and CAV-1 strains have been shown to experimentally produce tumors in newborn hamsters (Sarma et al 1967; Kinjo et al 1968).

Although practically no evidence exists concerning the existence of human adenoviruses in animal populations, serological studies of primates including humans imply

that an interchange of organisms between humans and animals can readily occur (Kalter et al 1967). Serum neutralizing Ab to Ad12 have been detected in chimpanzees, and Ab to a vervet monkey adenovirus isolate have been detected in laboratory personnel and Africans (Kalter et al 1967; Kalter and Heberling 1971). A high prevalence (77% and 74%) of CAV1 neutralizing Ab was demonstrated in veterinary and non-veterinary worker sera respectively (Gehle et al 1973); however, only a small fraction of dogs develop Ab to human adenoviruses despite close contact of dogs with humans (Sinha et al 1960; Carmichael and Barnes 1961; Carmichael and Barnes 1962; Lundgren et al 1969; Winters 1979). Human adenovirus proteins, however, are produced and accumulate to greater amounts in inoculated canine tumor cells than in normal canine cells (Winters and Young 1980), and levels of Ad5 Ab were significantly higher in sera collected from tumor-bearing dogs than from normal dogs (Winters 1979). Exposure to human adenovirus was suspected in a brown bear (*Ursus arctos*) in Croatia (Madic et al 1993). Despite evidence of cross-species adenovirus exposure, which may be more likely a function of cross-reacting Ab, no animals have been shown to be reservoirs for any adenovirus strains causing human disease.

SEROPREVALENCE TOOLS AND OBJECTIVES

Seroprevalence surveys of wildlife population samples have been employed extensively for measuring the occurrence of known pathogens, especially since mortality and morbidity data are usually unavailable and case reports are infrequent. The detection of serum Ab to a particular organism usually provides evidence of exposure to that organism, so that Ab prevalence can be used to generalize the occurrence of an organism in a population. Seroepidemiology has been accomplished using complement fixation, immunodiffusion, or ELISA, which measure group-reactive Ab (Anderson et al 1983;

Rossmanith and Horvath 1988; Studdert 1996). Positive sera can then be assayed for type specificity by serum neutralization, hemagglutination inhibition, or serotype-specific ELISA (Bürki 1990; Belak 1990).

ELISAs, first described by Engvall and Perlmann (1971), provide highly sensitive and precise methods for measuring Ab in serum, with the added advantage that large numbers of samples can be processed in only a few hours. As well, they circumvent the costs, time, and labor intensiveness of serum neutralization cell culture assays. Many ELISAs employing serotype-specific monoclonal antibodies, which detect adenovirus serotype-specific Ab, have been described (Mockett and Cook 1983; Florent and de Marneffe 1986; Lahdeaho et al 1992; Adam et al 1996; Liebermann et al 1996; Akalu et al 1998).

There are a wide range of ELISA configurations available to choose from. This study focuses on the competitive ELISA (cELISA) format, involving the competition between a specific monoclonal Ab and test serum Ab for binding to Ag which has been passively coated onto a solid support. This approach is potentially highly specific and amenable to testing sera from multiple animal species, which is desirable for wildlife surveillance. Conducting Ad5 and CAV-1 seroprevalence surveys prior to field application of Ad5RG will provide information necessary for tracking the potential spread of the vaccine in the environment, for predicting the likelihood of recombinatorial events, and for assessing whether an immune barrier exists to vaccination. Hence, the four main objectives of this study were as follows: to determine neutralizing Ab activity in a collection of "reference sera" using a "gold standard" reference assay (while generally measuring Ab responses to Ad5RG vaccination in experimental animals); to develop two cELISAs capable of detecting human and canine adenovirus Ab respectively; to statistically validate the developed

cELISAs with the data generated using the reference assay system; to apply the validated cELISAs to a large, representative sample from the field in order to estimate the seroprevalence of human and canine adenovirus Ab in the population to be targeted for oral rabies immunization.

MATERIALS AND METHODS

CELL CULTURES

A seed culture of 293 cells, Ad5-transformed primary human embryonal kidney cells (Graham et al 1977), was obtained from Dr. Lud Prevec (McMaster University; Hamilton, Ontario). A seed culture of dog kidney (DK) cells was obtained from Connaught Laboratories Ltd. (Willowdale, Ontario) and Madin-Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (ATCC CCL #22). All cells were maintained in 80 cm^2 flasks (Nalge Nunc International, Denmark) with Eagle's minimal essential media (MEM) (Life Technologies; Burlington, Ontario) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent Inc.; St.Bruno, Quebec), 0.03M sodium bicarbonate (Fisher Scientific; Fair Lawn, NJ), antibiotic/antimycotic solution (100 units/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) (Sigma Chemical Co.; St.Louis, MO), at 37°C and 4.5% CO_2.

VIRUSES

Wild type Ad5 was obtained from Dr. Lud Prevec and official prototype strains of all other human adenoviruses (Ad1-30) were obtained from Dan McLeod (National Biorepository Center, Laboratory Center for Disease Control; Health Canada). CAV-1 Toronto strain and CAV-2 Manhattan strain were obtained from Dr. Jim Campbell (University of Toronto; Toronto, Ontario). BAV-3 WBR-1 strain was obtained from the Ontario Veterinary College (Guelph, Ontario).

PREPARATION OF VIRUS STOCKS

Infected cell supernatant. Confluent 293 and DK cell monolayers were infected with Ad5 and CAV-1 respectively at a multiplicity of infection (MOI) of 0.1. Virus was adsorbed for

1 hour at 37°C followed by addition of maintenance media. Five days post-infection, when viral cytopathic effect (CPE) was apparent in approximately 80% of each monolayer, infected cells were scraped into the supernatant and the suspension was centrifuged for 15 minutes at 500 x the force of gravity (g) (fixed angle GSA rotor; Sorvall® RC-5B Refrigerated Centrifuge; Du Pont Canada Inc.). Infected cell pellets were pooled, re-suspended in a small volume of MEM, and stored at -70°C until needed for virus purification and cELISA development (Ad5 only). Infected cell supernatants were pooled and virus was quantified by monolayer plaque assay (Hierholzer and Killington 1996). Pooled supernatants were stored at -70°C until needed for plaque reduction neutralization tests (PRNT), indirect IF, and cELISA development (Ad5 only).

Pelleted virus. Virus from infected cell supernatant (Ad5 only) was pelleted by ultracentrifugation for 2 hours at 160 000 x g (swinging bucket SW28 rotor; Beckman Optima L-70 Ultracentrifuge) and quantified by monolayer plaque assay (Hierholzer and Killington 1996) for Ad5 cELISA development.

Infected cell lysate. Infected cell pellets were disrupted with a Dounce tissue homogenizer, then treated for 30 minutes at 4°C with 200µL/mL 10% v/v non-ionic detergent (IGEPAL™, Sigma) in STE buffer (0.13 M NaCl, 0.03 M Tris[hydroxymethyl]aminomethane, 0.001 M EDTA, pH 7.8). An equal volume of fluorocarbon (Arcton™; Sigma) was vigorously mixed with the suspension for 5 minutes followed by centrifugation for 5 minutes at 3000 x g (fixed angle SS-34 rotor; Sorvall® RC-5C Refrigerated Centrifuge). The fluorocarbon phase and cell material at the interphase were re-extracted with an equal volume of STE buffer and the aqueous extracts were combined for virus purification and cELISA development (Ad5 only).

Purified virus. The aqueous extracts derived from treating infected cell pellets, as described above, were centrifuged through a sucrose gradient (1.1-1.2 g/mL STE buffer) and concentrated onto a caesium chloride (CsCl) (ICN Pharmaceuticals Inc.: Irvine, CA) cushion (1.4 g/mL) at 120 000 x g for 2 hours (SW40 rotor: L-70 Optima Preparative Ultracentrifuge®; Beckman Instruments Inc., Palo Alto, CA). Fractions containing virus were collected and virus was further purified by ultracentrifugation in a continuous self-forming CsCl density gradient (1.1-1.4 g/mL STE buffer) at 120 000 x g for 18 hours. Visible virus bands (upper band, density 1.25 g/mL and lower band, density 1.28 g/mL) were harvested and the CsCl was exchanged with STE buffer by dialysis for 2 days at 4°C and then with 0.01 M phosphate buffered saline (PBS) (145 mM NaCl, 3 mM NaH_2PO_4, 8 mM $Na_2HPO_4.7H_2O$, pH 7.2) for 1 day. The purified products were confirmed to be adenoviruses by the Electron Microscopy (EM) Unit at ADRI, and stored at -70°C until needed for gel electrophoresis, Western Blotting, cELISA development, and immunization of mice for mAb production (CAV-1 only).

SERUM

Approximately 500 and 600 reference sera, respectively, were used in the validation Ad5 and CAV-1 cELISAs. These were obtained from animals used in Ad5RG immunization experiments conducted previously by Dr. Alex Wandeler and staff, who conducted rabies neutralizing Ab tests with the sera (Table 2). These sera, when pooled, were also used for positive and negative controls for the PRNT and cELISAs. Sera (unpaired), used additionally in CAV-1 cELISA validation and obtained from dogs immunized with CAV-2, were donated by Dr. Sue Armstrong (Ottawa Veterinary Hospital: Ottawa, Ontario) and the Ontario Veterinary College (Guelph, Ontario).

Table 2: Ad5RG immunization experiments in skunks, foxes, and raccoons.

Species	Immunogen	Route of Immunization	Dose ($TCID_{50}$/mL)	No. of animals	Bleed days post-immunization
Skunk	Ad5E1RG (E1 gene deletion	DIOC	10^8	7	day 0, 21, 35, 67, 109
			10^7	8	
			10^6	8	
	Ad5E3RG (E3 gene deletion)	DIOC	10^8	8	
			10^7	8	
			10^6	7	
	none (control group)			8	
Fox	Ad5E3RG	DIOC	10^8	7	day 0, 14, 30 (boost), 44, 60, 91, 116, 161
		IM	10^8	7	
	none (control group)			7	
Raccoon	Ad5E3RG	DIOC	10^8	8	day 0, 30, 60, 90
		vaccine-inoculated bait	10^9	8	

$TCID_{50}$/mL = tissue culture infectious dose 50 (i.e. dose which infects 50% of cells)
DIOC = direct instillation into the oral cavity
IM = intra-muscular

A polyclonal serum sample obtained from a rabbit immunized intra-muscularly with purified Ad5 was used in part of the Ad5 cELISA development. Survey sera were derived from wild raccoons trapped and bled by Ontario Ministry of Natural Resources (OMNR) staff in the Niagara and St.Lawrence regions of Ontario from June to November, 1996.

DETERMINING NEUTRALIZING Ab ACTIVITY IN SERA BY PRNT

Neutralizing Ab activity was determined by plaque reduction neutralization tests (PRNT), based on a standard Ad5 plaque assay system suggested by Kjellen (1961). Sera were heat inactivated for 30 minutes at 56°C and serially diluted 10-fold four times in MEM. Each dilution was mixed with an equal volume of either Ad5 or CAV-1 (1000 PFU/mL of MEM). Two virus controls (no serum), one pooled negative (diluted 1:10 in MEM) and one pooled positive (diluted 1:10 in MEM) serum control were included in each assay. The

virus-serum mixtures were incubated for 2 hours at 37°C. Confluent cell monolayers in polystyrene 6 well cell culture plates (Corning Costar Corp.; Costar, NY) were inoculated with 0.2 mL virus-serum mixture per well in triplicate (i.e. three wells for every serum dilution) so that virus control wells had approximately 100 PFU each. Virus was adsorbed for 1 hour at room temperature on a rocking platform and cell monolayers were then overlayed with 1.6% Seakem® agarose (FMC Bioproducts; Rockland, MA) diluted 1:2 with 2xMEM containing 20% FBS and 2% antibiotic/antimycotic solution. Ad5-infected cell plates were incubated for 6 days and CAV-1-infected cell plates were incubated for 4 days at 37°C prior to overnight staining with 200 µL/well 0.01% w/v neutral red (BDH Laboratory Supplies; Poole, England). Plaques were counted macroscopically using a light board and neutralizing Ab titers were calculated based on 50% endpoints (Reed and Muench 1938). A neutralizing Ab titer was expressed as the reciprocal of the serum dilution that resulted in a 50% reduction in plaque number when compared to the mean plaque number in virus control wells (Appendix 1).

MONOCLONAL ANTIBODIES (mAbs)

Prior to the initiation of this project, Ad5 mAbs were produced and protein specificities determined at the Animal Diseases Research Institute (ADRI). For CAV-1 mAbs, the production, cloning, and propagation of hybridomas in cell culture, and mAb isotyping were carried out by the ADRI MAb Unit, while immunization of mice, hybridoma screening, and additional mAb characterization were carried out here as follows.

Immunizing mice with CAV-1. Five 6-week-old BALB/c mice (Charles River Canada Co.; Montreal, Quebec) were immunized subscapularly with 100 µL purified CAV-1 suspended in an equal volume of Freund's Incomplete Adjuvant (Sigma). Mice were bled four weeks

post-immunization and serum Ab responses to CAV-1 were measured by indirect immunofluorescence (IF) as described below. Four months post-immunization, two of the mice were boosted intra-peritoneally with 300 µL purified CAV-1 and intra-venously through the tail with 50 µL purified CAV-1.

Screening CAV-1 hybridomas by indirect IF. DK cells were mixed in suspension with CAV-1 at a MOI of 0.1 PFU/cell. This mixture was inoculated onto 96 mini-well Terasaki plates (Robbins Scientific Corp.; Sunnyvale, CA) and incubated for 48 hours at 37°C at which time visible CPE was detected in each well. Cells were fixed in 75% cold acetone at room temperature for 10 minutes, air dried, and stored at -70°C until used. Supernatants of growing parent hybridoma colonies were screened for CAV-1 Ab by incubating 12 µL of hybridoma supernatant fluid with CAV-1-infected cells and uninfected cell controls on Terasaki plates for 1 hour at 37°C. Plates were rinsed twice and washed 3 times (10 minutes per wash) with tris buffered saline tween (TBST) (50 mM Tris, 200 mM NaCl, 0.5% v/v Tween-20), and incubated with FITC (fluorescein-5-isothiocyanate)-conjugated goat anti-mouse immunoglobulin (Ig) (ICN/Cappel) for 1 hour at 37°C. Plates were again rinsed and washed in TBST, counter stained with 0.5% w/v Evan's Blue (Sigma) and examined for fluorescence of infected cells using a Leica fluorescence microscope. Supernatants from first and second time cloned hybridomas were also screened for CAV-1 Ab in the same manner.

Selecting CAV-1 hybridomas. Ten CAV-1 clones were selected for ascites production in mice based on the degree and cellular localization of fluorescence in CAV-1-infected cells, the isotype produced, and their cross-reactions with CAV-2, Ad5, and BAV-3. The main objective was to select a panel of strongly staining IgG mAbs localizing to viral structures in either the host cell nucleus or cytoplasm, and exhibiting different cross-reactivity patterns

so that a wide array of epitopes might be represented. For cross-reactivity studies, supernatants were assayed for binding to DK, MDBK, and 293 cells infected in suspension with CAV-2, BAV-3, and Ad5 respectively prepared as above.

Radioimmunoprecipitating CAV-1 proteins with CAV-1 mAbs. Confluent DK cell monolayers were washed twice with PBS and infected with CAV-1 at a MOI of 10 PFU/cell. Confluent 293 cell monolayers were infected with Ad5 at a MOI of 2 PFU/cell. Virus was adsorbed for 1 hour at 37°C, and maintenance media was added back to tissue culture flasks for overnight incubation at 37°C. At 17 hours post-infection, maintenance media was replaced with warm methionine-cysteine-free MEM (ICN) for 1 hour. Infected cells and uninfected cell controls were then labeled with 250 microcurie (μCi) or 9.25 megabecquerel (MBq) /T162 flask Tran ^{35}Sulfur-Label (ICN) for 2 hours. Medium was removed and cells washed twice with PBS followed by lysis with cold RIPA buffer (50 mM TRIS, 150 mM NaCl, 0.1% w/v sodium dodecyl sulfate (SDS), 1% w/v sodium deoxycholate, 1% v/v TRITON X-100, pH 8.0) for 20 minutes on ice. Cell extracts were collected and kept at 4°C for 30 minutes then passed through a 22 gauge needle to shear cellular DNA, and centrifuged for 5 minutes at 1500 x g (#216 swinging bucket rotor in Centra-8R centrifuge; IEC, Needham Heights, MA). For immunoprecipitations, each mAb (2.5 μL of ascites fluid) was added to 500 μL cell extract and rotated at 4°C for 1.5 hours. Positive Ab controls (Ad5 hexon and fiber mAbs incubated with CAV-1 and Ad5 cell extracts respectively), negative Ab control (rabies glycoprotein mAb incubated with Ad5 and CAV-1 cell extracts), and mock Ag control (all mAbs incubated with uninfected cells) were included. Protein A Sepharose® CL4B beads (Pharmacia Biotech Inc.; Uppsala, Sweden) were added to each mixture (100 μL beads/sample) and rotated overnight at 4°C. Immune complexes bound to

beads were washed three times with cold RIPA buffer spinning for 20 seconds at 12 000 x g in between washes (Eppendorf 5415C Microcentrifuge), and dissociated by boiling in Laemmli 2x sample buffer with β-mercaptoethanol (β-ME) (Bio-Rad Laboratories; Hercules, CA) (50 µL/cell extract). Adenovirus polypeptides were resolved on 10% SDS-polyacrylamide gels (Laemmli 1970). Gels were fixed in 20% methanol containing 10% glacial acetic acid, treated with Amplify™ fluorographic reagent (Amersham International; Amersham, UK), and dried under vacuum at 80°C (Gel Dryer Model 583 and HydroTech™ Vacuum Pump; Bio-Rad) onto Whatman filter paper. Dried gels were exposed to Kodak® X-OMAT AR scientific imaging film in a closed cassette at room temperature for 2 days. Film was developed and fixed according to manufacturer's instructions.

Determining additional CAV-1 and Ad5 mAb cross-reactions. Supernatants from available Ad5 hybridomas and selected CAV-1 hybridomas were also tested for cross-reaction with Ad1-30 by indirect IF. 293 cells were infected in suspension with each Ad, inoculated onto Terasaki plates, incubated at 37°C, and monitored daily for visible CPE. Cells were then fixed with acetone and stained with Ad5 and CAV-1 hybridoma supernatants as described previously, and examined for fluorescence in infected cells.

Determining CAV-1 and Ad5 mAb neutralizing abilities. Ad5 and CAV-1 mAbs (ascites fluid) were also examined for their neutralizing activity against Ad5 and CAV-1 respectively. Neutralizing titers were determined by PRNT as described previously.

PURIFICATION OF MONOCLONAL ANTIBODIES

Ascites fluid was clarified by filtration in Spin-X® centrifuge tube filters (Corning Costar Corp.) for approximately 1 hour at 12000 x g (Eppendorf 5415C Microcentrifuge). A portion of the filtrate was diluted in 50% glycerol and stored at -20°C for cELISA

development. Purification of mAbs from the remaining filtrate was carried out by protein A affinity chromatography. Filtrate pH was adjusted to pH 7.6, diluted 1:5 in mAb purification system (MAPS) II binding buffer (Bio-Rad), and applied to an equilibrated Econo-Pac® Protein A Cartridge (Bio-Rad). After washing the column with 15 bed volumes of binding buffer, mAbs were eluted using 5 bed volumes of MAPS II elution buffer (Bio-Rad), desalted in PBS and concentrated by ultra filtration at 1220 x g using a Centriprep-30 protein concentrator (Amicon Canada Ltd., Oakville, Ontario). Sample protein concentration was determined using the Bio-Rad Protein Assay, based on the method of Bradford (1976). Protein dye reagent (Bio-Rad) diluted 1:5 in sterile water was added to serial dilutions of sample followed by measurement at 595 nm with a microplate reader (Multiskan® MCC/340; Labsystems Inc., Finland). The relative protein concentration was calculated by comparing to a bovine gamma globulin standard (Bio-Rad) curve. The concentration was adjusted to 1 mg/mL PBS for labeling.

LABELING MONOCLONAL ANTIBODIES

The procedure for labeling of mAbs was adapted from Nakane and Kawaoi (1974). 0.1 M sodium meta-periodate was mixed 1:20 with horseradish peroxidase (HRP), Type VI (Sigma) (10 mg/2.5 mL H_2O) for 20 minutes and dialyzed against 1 mM acetate buffer, pH 4.4, for 2 days at 4°C. One part carbonate buffer was added to 60 parts HRP aldehyde and immediately mixed with purified Ad5 fiber-specific mAb (1C5-5-2) (2 mg protein/3 mL HRP aldehyde) followed by gentle stirring for 2 hours. The same was repeated for Ad5 hexon-specific mAbs (8B12-4-11 and 21E7-4-5) and each reaction was stopped by 250 µL ascorbic acid (4 mg/mL H_2O). The mixtures were left for 2 hours at 4°C, then dialyzed against PBS for 2 days at 4°C. Conjugate was stored in 50% glycerol at -20°C.

Note: Clarified ascites was selected during cELISA development to be used unpurified and unlabeled in all subsequent cELISAs, resulting in the incorporation of a labeled secondary Ab for mAb detection.

VALIDATED cELISAs

Ad5 cELISA. Purified Ad5 diluted 1:1600 in 0.5 M carbonate/bicarbonate buffer, pH 9.6, was passively coated onto certified polystyrene Maxisorp™ Immuno Plates (Nalge Nunc International) (100 µL/well) by incubating for 20 hours at 4°C. Plates were stored at -70°C until used. For each assay, plates were thawed on the bench and washed five times with PBST (145 mM NaCl, 3 mM NaH_2PO_4, H_2O, 8 mM Na_2HPO_4, $7H_2O$, 0.05% Tween-20) using a Microplate EL 403 Autowasher (Bio-Tek Instruments Inc.; Winooski, VT). Unreacted sites on Ad5 plates were blocked with 5% pooled fetal bovine-equine serum (BES) (Gibco BRL; Grand Island, NY) in PBST(200 µL/well) for 2 hours at 28°C. Followed by another wash cycle, Ad5 mAb (15G6-7 clarified ascites stored in 50% glycerol at -20°C) diluted 1:400 in 3% BES/PBST was then added to the plates (50 µL/well). Sera diluted 1:10 in 3% BES/PBST were immediately added in duplicate (50 µL/well) to the plates giving a 1:20 final serum dilution. A high positive serum control (C++), low positive serum control (C+), and negative serum control (C-) each diluted 1:10, as well as a buffer control (Cc) were added to each plate in quadruplicate. Sample placement in quadrants was used in order to minimize between-well variation often observed in perimeter wells (Stemshorn et al 1983). Reagents were mixed by agitation on an orbital plate shaker (Lab-Line Instruments Inc., Melrose Park, IL) for 1 minute and incubated for 2 hours at 28°C. Followed by another wash cycle, HRP-conjugated goat-anti-mouse IgG, $F(ab')^2$ fragments (Jackson ImmunoResearch Laboratories Inc.; West Grove, PA), diluted 1:2000 in 3%

BES/PBST was then added (100 µL/well) and plates were incubated for 1 hour at 28°C. After the final wash cycle, plates were developed with 100 µL/well of chromagen-substrate solution (4 mM 2,2'-azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid] (ABTS), 1mM H_2O_2 (3% solution), in 0.05 M citrate buffer, pH 4). The reaction was allowed to take place with agitation on an orbital plate shaker and optical density (OD) values were assessed at exactly 4 minutes after chromagen/substrate addition at 414 nm in a microplate reader (Labsystems Inc.). Based on a standard curve, the "target reading time" (time required for the negative control (C-) to reach an optical density of 1.0) for each plate was calculated using an ELISA computer program (Version 2.20; Copyright© Agriculture Canada 1993). Plates were read again at their target time for final OD results. This 4-minute targeting protocol was used in order to minimize minor fluctuations caused by day-to-day environmental conditions. The ELISA titer for each competing test serum was expressed as the degree to which the mAb (15G6-7) was inhibited by that serum:

% inhibition = 100 - (OD test sample / OD negative control x 100)

CAV-1 cELISA. The cELISA was similarly carried out except for the use of a different blocking agent. Purified CAV-1 was diluted 1:1000 in 0.5 M carbonate buffer, unreacted sites on CAV-1 plates were blocked with 5% skim milk powder in PBST, and all other diluents included 3% skim milk powder. CAV-1 mAb (M1260 clarified ascites stored in 50% glycerol at -20°C) was diluted 1:1600 in 3% SMP/PBST, and the HRP-conjugated goat-anti-mouse secondary antibody was diluted 1:4000 in 3% SMP/PBST.

SEMI-DENATURING SDS-PAGE AND Western BLOTTING

Ad5 and CAV-1 proteins were resolved in the cold under non-reducing conditions in order to retain conformational epitopes, since standard SDS-PAGE conducted under

reducing conditions proved unsuccessful for detecting raccoon Ab and all mAbs by Western blot. Purified Ad5 and CAV-1 were diluted in Laemmli 2x sample buffer (Bio-Rad) without β-ME. Samples were immediately loaded, without boiling, onto 6% SDS polyacrylamide gels and electrophoresed at 50 V for 5 hours at 4°C. Western blotting was performed by equilibrating gels in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) followed by electrophoretic transfer to nitrocellulose membrane (Amersham Life Science; England) in a Trans-Blot® Semi-Dry apparatus (Bio-Rad). Membranes were airdried overnight and strips were cut and blocked in 5% SMP/TBST for 2 hours while rocking at room temperature (note: TBST was used for all rinsings, washings, and as diluent with 3% SMP). Strips were rinsed and incubated individually with experimental or field raccoon sera diluted 1:50 (1 mL/strip) for 2 hours at room temperature. Two positive Ab controls (Ad5 polyclonal rabbit hyperimmune serum diluted 1:500 and either Ad5 or CAV-1 hexon mAb ascites fluid, each diluted 1:100), a negative Ab control (a rabies glycoprotein mAb ascites fluid diluted 1:100), and a secondary Ab control (alkaline phosphatase (AP)-conjugated goat anti-dog (KPL; Gaithersburg, MD) diluted 1:1000) were included in each assay. Strips were rinsed 2 times and washed 4 times (5 minutes each wash) then incubated for 2 hours at room temperature with the appropriate conjugate (AP-goat anti-mouse diluted 1:5000, anti-rabbit diluted 1:5000, or anti-dog for detecting raccoon sera diluted 1:1000) (all KPL). Strips were rinsed 2 times and washed 4 times followed by development in 0.1 M Tris buffer with 8% v/v BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and 8% v/v NBT (nitroblue tetrazolium) substrate (KPL). All enzymatic reactions were terminated with the addition of deionized water.

STATISTICAL ANALYSES

Validation of cELISAs using Receiver-Operating Characteristics (ROC) was executed using a medical statistics software program (MedCalc® Version 4.20.021) (Schoonjans et al 1995). Reference serum neutralizing titers obtained in PRNT were classified as either positive or negative and compared with their corresponding cELISA titers. From this data, MedCalc® assigned sensitivity and specificity pairs to every possible % inhibition positivity cut-off, indicating the highest sensitivity-specificity pair. Spearman's rank correlation coefficients, Student's paired, and unpaired t-tests were also calculated when necessary using MedCalc®. The degree of association between two variables when using Spearman's rank correlation coefficient was interpreted as follows: r = 0-0.25 defines little or no relationship; r = 0.25-0.5 defines a fair degree of relationship; r = 0.5-0.75 defines a moderate to good relationship; 0.75-1.0 defines a very good to excellent relationship (Colton 1974).

RESULTS

NEUTRALIZING Ab IN REFERENCE SERA (FOR VALIDATION)

Approximately 500 sera were obtained from animals (serial bleeds from skunks, foxes, and raccoons) involved in Ad5RG immunization experiments conducted previously by Dr. Alex Wandeler and staff (Table 2-Materials and Methods). All sera were examined for Ad5 and CAV-1 neutralizing activity in the PRNT, representing the "gold standard" reference assay. These sera, therefore, served as reference sera in the validation of cELISAs. Sera from approximately 100 dogs (unpaired) vaccinated with CAV-2 were additionally used for validating the CAV-1 cELISA and were therefore examined for CAV-1 neutralizing activity. Calculation of Ab titers is demonstrated in Appendix 1.

With respect to neutralizing Ab results, there were sufficient numbers of positives and negatives for statistical validation of cELISAs, with 170/489 (35%) Ad5 neutralizing and 319/489 (65%) Ad5 non-neutralizing sera (Table 3A) and 184/609 (30%) CAV-1 neutralizing and 425/609 (70%) CAV-1 non-neutralizing sera (Table 3B). Ad5 neutralizing Ab titers, measured among skunks, foxes, and only a few raccoons, were well represented across Ab titer ranges with 36% having titers between 1:10-1:100 and the remainder having titers between 1:100-1:3000 (Table 3A), enhancing the power of statistical validation. CAV-1 neutralizing sera, measured in 24% of skunks and all dogs, were not distributed as evenly with only 4% having titers between 1:10-1:100, while 26% had titers between 1:100-1:500, and 70% had titers greater than 1:500 (Table 3B).

cELISA DEVELOPMENT - MAb SELECTION

CAV-1 hybridoma screening and selection. The main objective during initial screening of CAV-1 hybridomas was to pick clones secreting mAbs which were mainly of the IgG

Table 3A. Distribution of Ad5 neutralization-positives and negatives in reference sera.

Species	No. Sera Tested	Positives No. (%)	Negatives No. (%)	Distribution of Ad5 Positives by Titer - No. (%)					
				1:10-50	1:50-100	1:100-500	1:500-1000	1:1000-3000	1:3000+
Skunk	267	59/267 (22)	208/267 (78)	22/59 (37)	8/59 (14)	18/59 (30.5)	5/59 (8.5)	6/59 (10)	0/59 (0)
Raccoon	64	10/64 (16)	54/64 (84)	5/10 (10)	3/10 (30)	2/10 (20)	0/10 (0)	0/10 (0)	0/10 (0)
Fox	158	101/158 (64)	57/158 (36)	12/101 (12)	11/101 (10)	29/101 (29)	13/101 (13)	33/101 (33)	3/101 (3)
TOTALS	489	170/489 (35)	319/489 (65)	39/170 (23)	22/170 (13)	49/170 (29)	18/170 (10)	39/170 (23)	3/170 (2)

Table 3B. Distribution of CAV-1 neutralization-positives and negatives in reference sera.

Species	No. Sera Tested	Positives No. (%)	Negatives No. (%)	Distribution of CAV-1 Positives by Titer - No. (%)					
				1:10-50	1:50-100	1:100-500	1:500-1000	1:1000-3000	1:3000+
Skunk	268	65/268 (24)	203/268 (76)	0/65 (0)	0/65 (0)	10/65 (15)	4/65 (6)	12/65 (19)	39/65 (60)
Raccoon	64	0/64 (0)	64/64 (100)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)
Fox	158	0/158 (0)	158/158 (100)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)
Dog	119	119/119 (100)	0/119 (0)	3/119 (2.5)	3/119 (2.5)	38/119 (32)	8/119 (7)	29/119 (24)	38/119 (32)
TOTALS	609	184/609 (30)	425/609 (70)	3/184 (2)	3/184 (2)	48/184 (26)	12/184 (6)	41/184 (22)	77/184 (42)

A neutralizing Ab titer represents the reciprocal serum dilution required to neutralize 50% of viral PFU in the virus-serum mixture.

isotypes and exhibiting differences in the cellular localization of fluorescence (nuclear vs. cytoplasmic staining) and cross-reactivity patterns. Ten clones were, therefore, selected for ascites production in mice based on their strong staining of infected cells (Table 4). Supernatant from one clone (M1272) cross-reacted with CAV-2, Ad5, and BAV-3; another (M1243) cross-reacted with CAV-2 and Ad5; four clones (M1249, M1263, M1245, M1259) cross-reacted with CAV-2; and four others (M1260, M1241, M1248, M1262) were non-cross-reactive.

CAV-1 radioimmunoprecipitation. In order to then determine the specificity of these clones for specific viral proteins, ascites fluid representing the 10 clones was used to immunoprecipitate CAV-1 proteins from radiolabeled CAV-1-infected cells. Analysis of immunoprecipitated proteins by SDS-PAGE indicated that M1272, M1243, M1249, M1263 (cross-reactive clones) and M1260 (a non-cross-reactive clone) bound a subunit approximately 103 kD, corresponding with the reported molecular weight of the CAV-1 hexon subunit (Verkhovskaia et al 1990) (Figure 2A, lanes 2-6). Hexon protein specificity was confirmed by comparison with a similar band generated from the immunoprecipitation of radiolabeled CAV-1 proteins with an Ad5 hexon mAb, 21E7-4-5, which cross-reacts with CAV-1 by indirect IF (Figure 2A, lane 7). M1245, M1259 (CAV-2 cross-reactive clones), M1241, M1248, and M1262 (non-cross-reactive clones) bound a subunit approximately 57 kD, corresponding with the reported molecular weight of the CAV-1 fiber subunit (Dragulev et al 1991) (Figure 2B, lanes 2-6). Four of these CAV-1 fiber-specific mAbs also co-precipitated a lower molecular weight polypeptide, probably a fiber cleavage product (Figure 2B, lanes 2, 4-6). Fiber protein specificity was confirmed by comparison with a similar band generated by the immunoprecipitation of radiolabeled Ad5 fiber with an Ad5 fiber mAb.

Table 4. CAV-1 clones with their mAb isotypes and cross-reactions.

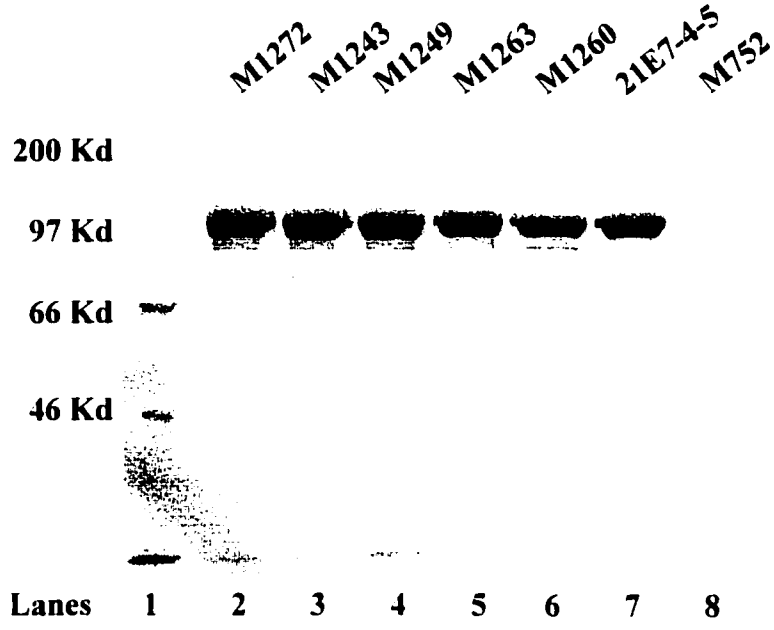
PARENT HYBRIDOMA		FIRST TIME CLONE		SECOND TIME CLONE		SECOND TIME CLONE (final cell culture supernatant)				
NAME	ISOTYPE	NAME	ISOTYPE	NAME	ISOTYPE	NAME	CROSS-REACTION			
							CAV-1	CAV-2	Ad5	BAV-3
24A6	IgG2b	24A6-1	IgG2b	24A6-1-8	IgG2b	M1272	3N/C	3N/C	3N	3N/C
11A1	IgG2b	11A1-7	IgG2b	11A1-7-12	IgG2b	M1254	3N/C	3N/C	3N	2N
22C1	IgG1	22C1-11	IgG1	22C1-11-11	IgG1	M1243	3N/C	3N/C	3N	
15D1	IgM>2a>G1[a]	15D1-2	IgG1	15D1-2-7	IgG1	M1256	3N/C	3		
22F3	IgG2a	22F3-3	IgG2a	22F3-3-4	IgG2a	M1259	3N/C	2		
25D11	IgG1	25D11-F2	IgG1	25D11-12-10	IgG1	M1245	3N/C	1		
15C6	IgG1	15C6-13	IgG1	15C6-13-1	IgG1	M1255	3N	3		
29G11	IgG2a	29G11-18	IgG2a	29G11-18-4	IgG2a	M1247	3N	3		
38D3	IgG1>>G3	38D3-18	IgG1	38D3-18-8	IgG1	M1267	3N	3		
3A10	IgG2b	3A10-8	IgG2b	3A10-8-8	IgG2b	M1251	3N	3		
4B2	IgG2a	4B2-2	IgG2a	4B2-2-5	IgG2a	M1240	3N	3		
9A5	IgG2b	9A5-6	IgG2b	9A5-6-1	IgG2b	M1270	3N	3		
31F8	IgG1	31F8-12	IgG1	31F8-12-12	IgG1	M1263	3N	3		
38C6	IgG2b	38C6-17	IgG2b	38C6-17-6	IgG2b	M1277	3N	3		
28G6	IgG1>G2a>>M	28G6-10	IgG2a	28G6-10-8	IgG2a	M1274	3N	3		
28H7	IgG2a	28H7-11	IgG2a	28H7-11-3	IgG2a	M1261	3N	3		
12F3	IgG2a	12F3-14	IgG2a	12F3-14-5	IgG2a	M1242	3N	2		
36B4	IgA	36B4-10	IgG1	36B4-10-2	IgG1	M1249	3N	2		
38E9	IgG1	38E9-8	IgG1	38E9-8-10	IgG1	M1268	3N	1		
21F11	IgG2a>G1	21F11-17	IgG2a	21F11-17-1	IgG2a	M1258	3N	1		
28A5	IgA	28A5-12	IgA	28A5-12-6	IgG>>M	M1246	2N	1		
10A1	IgA	10A1-24	IgA	10A1-24-12	IgA	M1257	2N/C	2		
34D6	IgG2a	34D6-16	IgG2a	34D6-16-9	IgG2a	M1266	3N/C			
5D4	IgG2a	5D4-1	IgG2a	5D4-1-9	IgG2a	M1253	3N/C			
29D6	IgG1	29D6-6	IgG1>>G2a	29D6-6-2	IgG1	M1262	3N/C			
39C1	IgG1	39C1-2	IgG1	39C1-2-7	IgG1	M1269	3N/C			
39C10	IgG1>>M	39C10-1	IgG1	39C10-1-8	IgG1	M1278	3N/C			
28G6	IgG1>G2a>>M	28G6-5	IgG1	28G6-5-2	IgG1	M1273	3N/C			
8F10	IgG1>>M	8F10-2	IgG1	8F10-2-1	IgG1	M1241	3N			
28H4	IgG2a	28H4-19	IgG2a	28H4-19-12	IgG2a	M1260	3N			
29H10	IgG1	29H10-17	IgG1	29H10-17-7	IgG1	M1248	3N			
30C9	IgG1	30C9-24	IgG1	30C9-24-2	IgG1	M1275	3N			
33F8	IgG2b	33F8-11	IgG2b	33F8-11-8	IgG2b	M1276	3N			
36C1	IgG1>>G3	36C1-2	IgG1	36C1-2-2	IgG1	M1265	3N			
17B8	IgG1	17B8-2	IgG1	17B8-2-7	IgG1	M1271	3N			
24B1	IgG3	24B1-9	IgG3	24B1-9-6	IgG3	M1279	3N			
36E5	IgG2a	36E5-23	IgG2a	36E5-23-3	IgG2a	M1250	3N			
39B4	IgG2b	39B4-12	IgG2b	39B4-12-7	IgG2b	M1280	3N			
31G12	IgG3>G1	31G12-1	IgG3	31G12-1-2	IgG3	M1264	2N			
25C8	IgG2a	25C8-1	IgG2a	25C8-1-12	IgG2a	M1244	1N			
4B4	IgG1	4B4-12	IgG1	4B4-12-3	IgG1	M1252	1N			

[a] Isotypes detected are listed in decreasing order of concentration

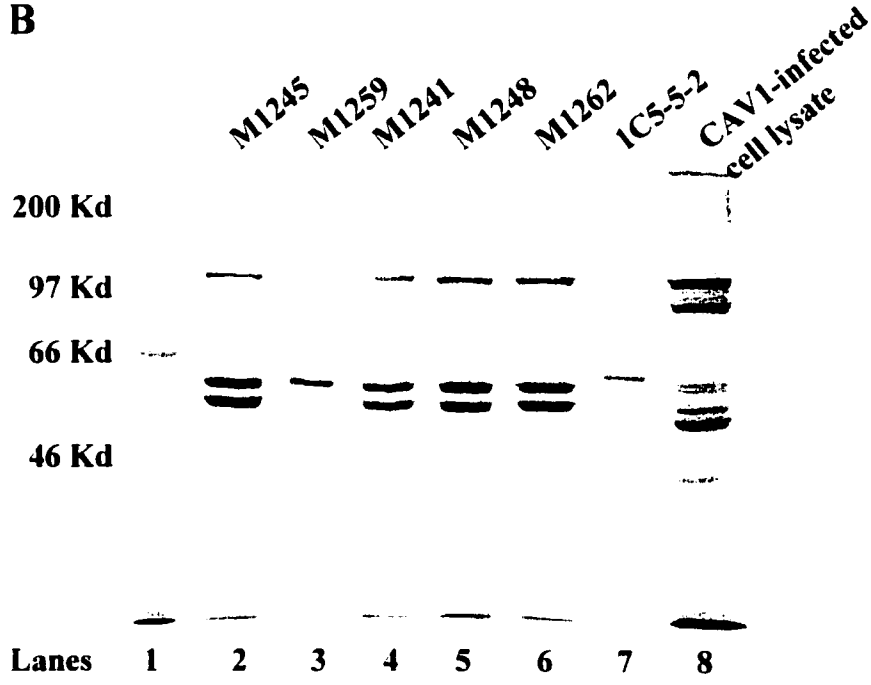
Supernatants from parent hybridomas, first time, and second time clones were incubated with CAV-1, CAV-2, Ad5, and BAV-3-infected cells. CAV-1 specific and cross-reacting Ab were detected with an FITC-goat anti-mouse conjugate. A scale of 1-3 was used for grading fluorescence.
1 = dim; 3= bright
N = nuclear staining; N/C = nuclear and cytoplasmic staining
Clones shaded in gray were selected for ascites production.

Figure 2. A. Autoradiograph showing electrophoresis of radioimmunoprecipitated CAV-1 hexon polypeptides. B. Autoradiograph showing electrophoresis of radioimmunoprecipitated CAV-1 fiber polypeptides. Radiolabeled infected cell extracts were reacted with mAbs (ascites fluid) and Protein A Sepharose® CL4B beads. Immune complexes bound to beads were dissociated under reducing conditions and adenovirus polypeptides were resolved on 10% SDS-polyacrylamide gels. Gels were dried, exposed to X-ray film, and developed. Viral proteins were immunoprecipitated by the mAbs indicated for each lane, except in Figure B-lane 8, which represents a CAV-1 control without mAb.

A


M1272
M1243
M1249
M1263
M1260
21E7-4-5
M752
200 Kd
97 Kd
66 Kd
46 Kd
Lanes 1 2 3 4 5 6 7 8

B


M1245
M1259
M1241
M1248
M1262
1C5-5-2
CAV1-infected cell lysate
200 Kd
97 Kd
66 Kd
46 Kd
Lanes 1 2 3 4 5 6 7 8

1C5-5-2 (Figure 2B, lane 7). None of the CAV-1 mAbs immunoprecipitated radiolabeled mock Ag (uninfected DK cells). A negative control Ab (M752, a rabies mAb) did not immunoprecipitate CAV-1 proteins (Figure 2A, lane 8).

CAV-1 mAb cross-reactions and neutralizing activity. In order to additionally characterize CAV-1 mAbs, they were further tested for their cross-reactions with a panel of human adenoviruses as well as their ability to neutralize homologous virus. Two CAV-1 hexon mAbs (M1272 and M1243), which were the only two to initially cross-react with Ad5 during the hybridoma screening process, were also the only two which cross-reacted with all other human adenoviruses tested by indirect IF (Ad1-31) (Table 5). M1272 additionally cross-reacted with BAV-3. Interestingly, these two hexon mAbs were also the only CAV-1 mAbs not capable of neutralizing CAV-1, in addition to M1259, a fiber mAb, which cross-reacted with CAV-2 and Ad4. All other CAV-1 fiber and hexon mAbs, which were not cross-reactive with any human adenoviruses, could neutralize CAV-1.

Ad5 mAb cross-reactions and neutralizing activity. The ten Ad5 mAbs were also tested for their cross-reactions with human adenoviruses and ability to neutralize homologous virus. Similar to CAV-1 mAb findings, Ad5 hexon mAbs were the ones which cross-reacted with other human adenovirus serotypes and did not have Ad5 neutralizing activity (Table 5). Three of these hexon mAbs (19G11, 21E7, and 29F5) additionally cross-reacted with CAV-1, CAV-2, and BAV-3. All of the fiber mAbs were non-cross-reactive could neutralize Ad5. Interestingly, one Ad5 mAb specific for a hexon-associated protein (pIX) cross-reacted with Ad1, 2, and 6 (all serotypes belonging to subgroup C) and was non-neutralizing.

cELISA DEVELOPMENT - Ag SELECTION

Once mAbs were characterized, one of the initial aspects of cELISA development

Table 5. Summary of Ad5 and CAV-1 mAb characteristics.

Adenovirus Serotype	Ad5 mAbs										CAV-1 mAbs									
	Neutralizing FIBER				HEXON ASSOC	HEXON					Neutralizing FIBER				FIBER	Neutralizing HEXON			HEXON	
	7G1	22B3	1C5	8C6	2C12	8B12	15G6	19G11	21E7	29F5	M1241	M1248	M1262	M1245	M1259	M1260	M1249	M1263	M1243	M1272
	164	4037	1767	1557						70	1506	165	7988	12426		31623	242170	30516		
HUMAN GROUP																				
SUBGROUP A																				
Ad12																				
Ad18																				
Ad31																				
SUBGROUP B																				
Ad3																				
Ad7																				
Ad11																				
Ad14																				
Ad16																				
Ad21																				
SUBGROUP C																				
Ad1																				
Ad2																				
Ad5																				
Ad6																				
SUBGROUP D																				
Ad8-Ad10																				
Ad13																				
Ad15																				
Ad17																				
Ad19-Ad20																				
Ad22-30																				
SUBGROUP E																				
Ad4																				
CANINE GROUP																				
CAV-1																				
CAV-2																				
BOVINE GROUP																				
BAV-3																				

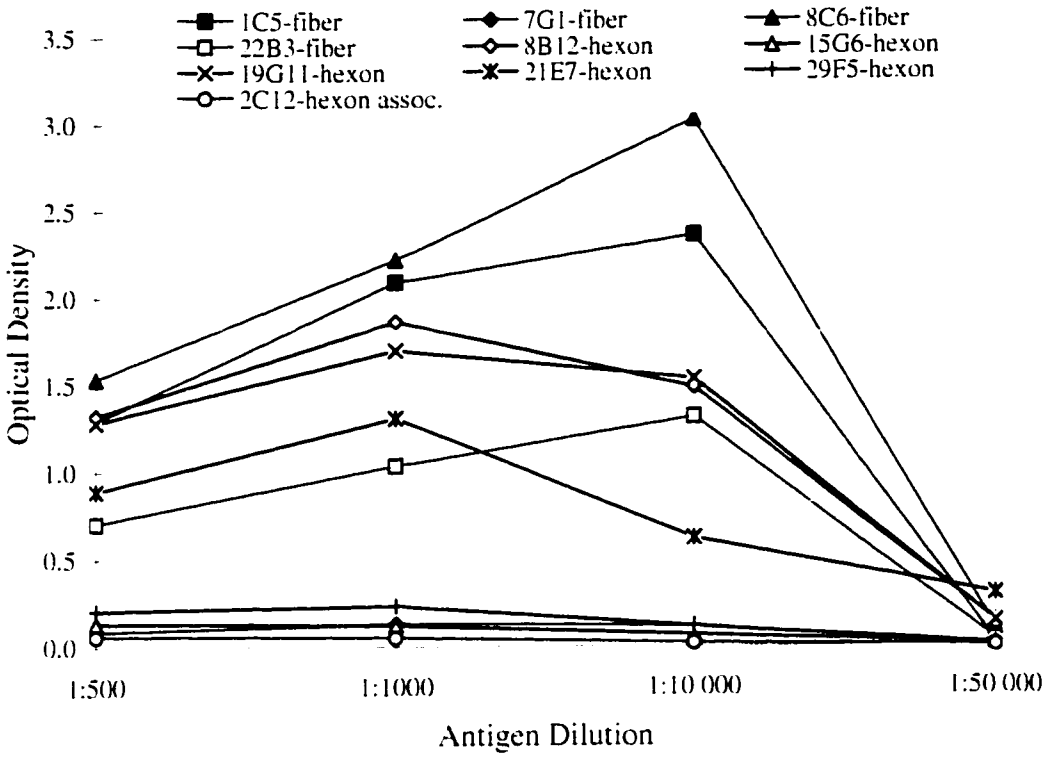
Ad5 and CAV-1 mAbs (ascites fluid) were tested in PRNTs for their abilities to neutralize homologous virus. Cross-reactions with other adenoviruses were detected by staining infected cells with Ad5 and CAV-1 hybridoma supernatants and observing fluorescence under a microscope. Gray shaded areas indicate positive fluorescent staining and open spaces indicate a negative reaction. Numbers shaded in gray represent neutralizing Ab for the mAbs indicated above them.

involved selecting an appropriate virus preparation as coating Ag. Ad5 preparations (as described in Materials and Methods) tested for suitability in order of increasing concentration and purity were Ad5-infected cell supernatant, pelleted Ad5 from infected cell supernatant, Ad5-infected cell lysate, and purified Ad5 (later in development). In order to determine the most suitable coating Ag, all Ad5 preparations were titrated on microtiter plates and reacted with each Ad5 mAb (hybridoma supernatant undiluted) without competing serum. After necessary washing steps, incubation with a labeled secondary Ab (HRP-goat anti-mouse Ig), and addition of the enzyme substrate-chromagen (H_2O_2-ABTS), coating efficiencies and Ag-Ab binding activity were assessed "by-eye" based on the intensity of the colored product in each well. Ad5-infected cell supernatant was discarded due to its poor plate binding. The best Ad5 preparation was the Ad5-infected cell lysate since it generated the highest signals (highest optical density readings and most intense color development) upon dilution, in comparison with the others (Figure 3A). Also, Ad5 fiber mAbs (which were initially preferable over hexon mAbs as will be described below) demonstrated improved binding to this Ag, over pelleted Ad5 (Figure 3B). Ad5-infected cell lysate was, therefore, selected initially for coating plates even though the detergent used during treatment had an inhibitory effect on plate binding at the lower dilutions. All subsequent mAb-Ag reactions described in this thesis were carried out using clarified mAb ascites fluid (unlabeled or purified and labeled) instead of hybridoma supernatant.

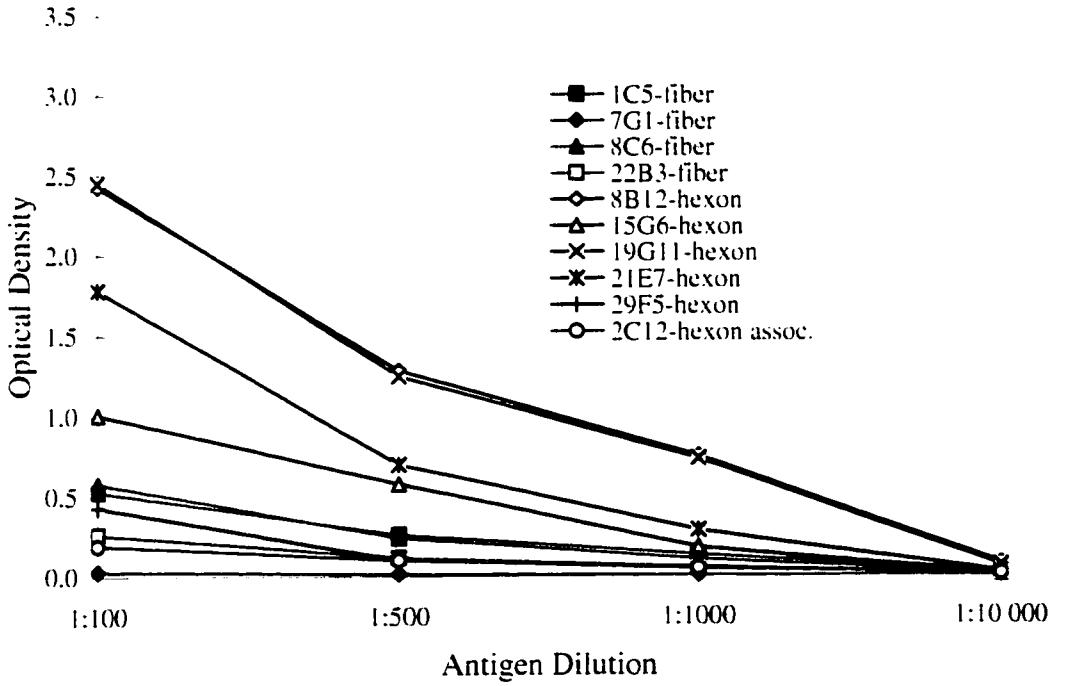
Purified Ad5 demonstrated good coating efficiency (better than pelleted virus and equivalent to Ad5-infected cell lysate) and hexon mAb binding when tested later in development. After selecting mAbs (as described below), purified Ad5 and CAV-1 were chosen over infected cell lysate for coating microtiter plates, including those for validation

Figure 3. A. Ad5-infected cell lysate titration. B. Pelleted Ad5 titration. Each Ad5 preparation was serially diluted and coated onto microtiter plates. Ad5 mAbs (undiluted hybridoma supernatants) were allowed to react with Ag. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Optical densities were plotted for each mAb-Ag reaction. Red lines indicate reactions with fiber mAbs.

A. Ad5-infected cell lysate



B. Pelleted Ad5



and survey. All Ad5 preparations demonstrated improved binding to certified Maxisorp™ Immuno Plates (100 µL/well) (a higher protein-binding plate) while maintaining low backgrounds (results not shown). These plates were, therefore, selected over regular microtiter plates for subsequent usage in development, validation, and survey.

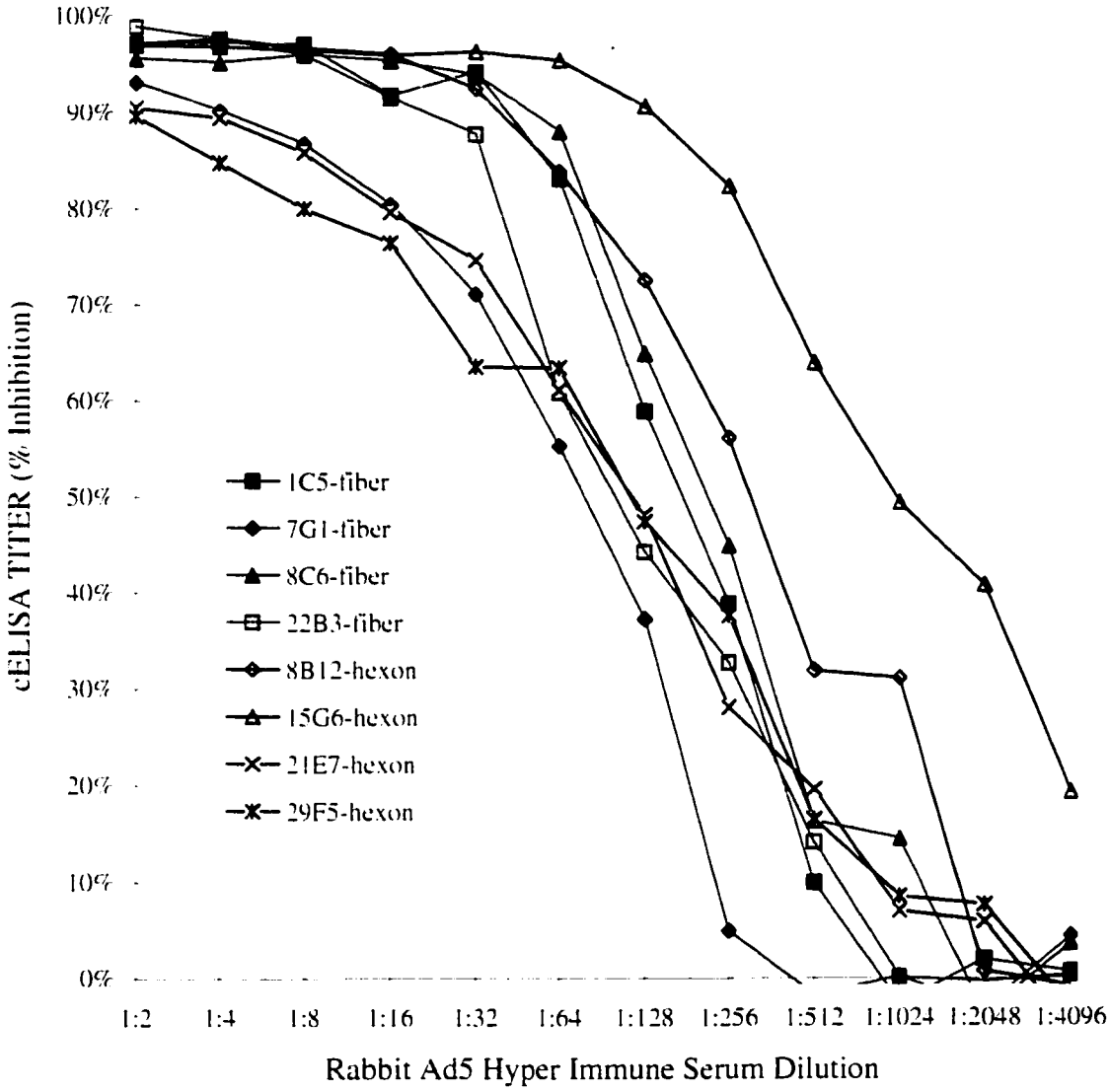
MAb SELECTION (Ad5)

The selection of an Ad5 mAb specific for an epitope immunodominant in experimental animals orally-vaccinated with Ad5RG was critical to cELISA sensitivity and specificity. It was also desirable for this epitope to be neutralization-associated and serotype-specific so that reference sera tested by cELISA would have titers comparable to their corresponding positive or negative neutralizing Ab titers determined by PRNT.

Rabbit serum. Ad5 mAbs (clarified ascites fluid) were first tested in an cELISA with a serially titrated serum sample obtained from a rabbit immunized intra-muscularly with purified Ad5. The rabbit serum successfully competed with all mAbs (i.e. the serum was capable of blocking all mAbs) at low serum dilutions (high level of Ab) (Figure 4). At higher serum dilutions (low level of Ab), such as at 1:128 and 1:256, a non-neutralizing hexon mAb (15G6) appeared to be the most sensitive (Figure 4). Nevertheless, another mAb (1C5) was pursued for Ad5 cELISA development because it was neutralizing and non-crossreactive. Also, it was comparatively sensitive with respect to higher dilutions of the rabbit serum, and ascites fluid for this mAb was available in abundance.

Skunk sera (from orally immunized skunks). 1C5 was then tested in a cELISA with some neutralizing and non-neutralizing reference sera from skunks (given Ad5RG by the oral route as described previously) to ensure that Ad5 neutralizing Ab in the test serum could in fact

Figure 4. Competitive inhibition of Ad5 mAbs by serum from a rabbit immunized intra-muscularly with purified Ad5. All Ad5 mAbs (optimal dilutions of clarified ascites - data not shown) were allowed to compete for binding with a 2-fold serially diluted rabbit serum. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Optical densities were plotted for each mAb-Ag reaction. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition = 100 - (OD test sample / OD negative control x 100). Red lines indicate reactions with fiber mAbs.


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
cELISA TITER (% Inhibition)
1C5-fiber
7G1-fiber
8C6-fiber
22B3-fiber
8B12-hexon
15G6-hexon
21E7-hexon
29F5-hexon
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
1:4096
Rabbit Ad5 Hyper Immune Serum Dilution

compete for 1C5's neutralization-associated epitope and block 1C5 from binding (seen as a decrease in color development since less of the enzyme-labeled secondary Ab which recognizes mouse mAbs is binding). None of the five skunk sera positive for Ad5 neutralizing activity could block 1C5 from binding (i.e. could not inhibit greater than 20%). To test the possibility that HRP-labeled goat anti-mouse Ig was non-specifically binding to the skunk Ab, 1C5 ascites was purified and labeled with HRP and the experiment was repeated. Again, none of the five positive skunk sera could block labeled 1C5 from binding.

All Ad5 mAbs (clarified ascites fluid) were then tested in a cELISA with the same five neutralizing and five non-neutralizing skunk sera. None of the five neutralizing sera could block 1C5 or any of the other neutralizing fiber mAbs; however, 3/5 (60%) neutralizing sera could block non-neutralizing hexon mAbs (Table 6). These 3 sera which had blocking potential also had CAV-1 neutralizing Ab titers.

Fox sera (from orally immunized foxes). In order to eliminate the possibility of cross-reacting CAV-1 Ab, Ad5 neutralizing fox sera were tested with Ad5 mAbs (clarified ascites fluid) since none of the sera had CAV-1 neutralizing activity. Like skunk sera, none of the five neutralizing fox sera could inhibit any of the three fiber mAbs; however, 5/5 (100%) were capable of blocking hexon mAbs (Figure 5). This demonstrated that Ad5 specific Ab in sera from animals given the vaccine orally were in fact capable of blocking hexon mAbs, but were still unable to block fiber mAbs.

Fox sera (from intra-muscularly immunized foxes). Since the only serum thus far capable of blocking all fiber and hexon mAbs was from a rabbit immunized by the intra-muscular route, seven neutralizing sera from foxes also immunized by the intra-muscular route were tested with unlabeled Ad5 fiber and hexon mAbs. Like the rabbit serum, 7/7 (100%)

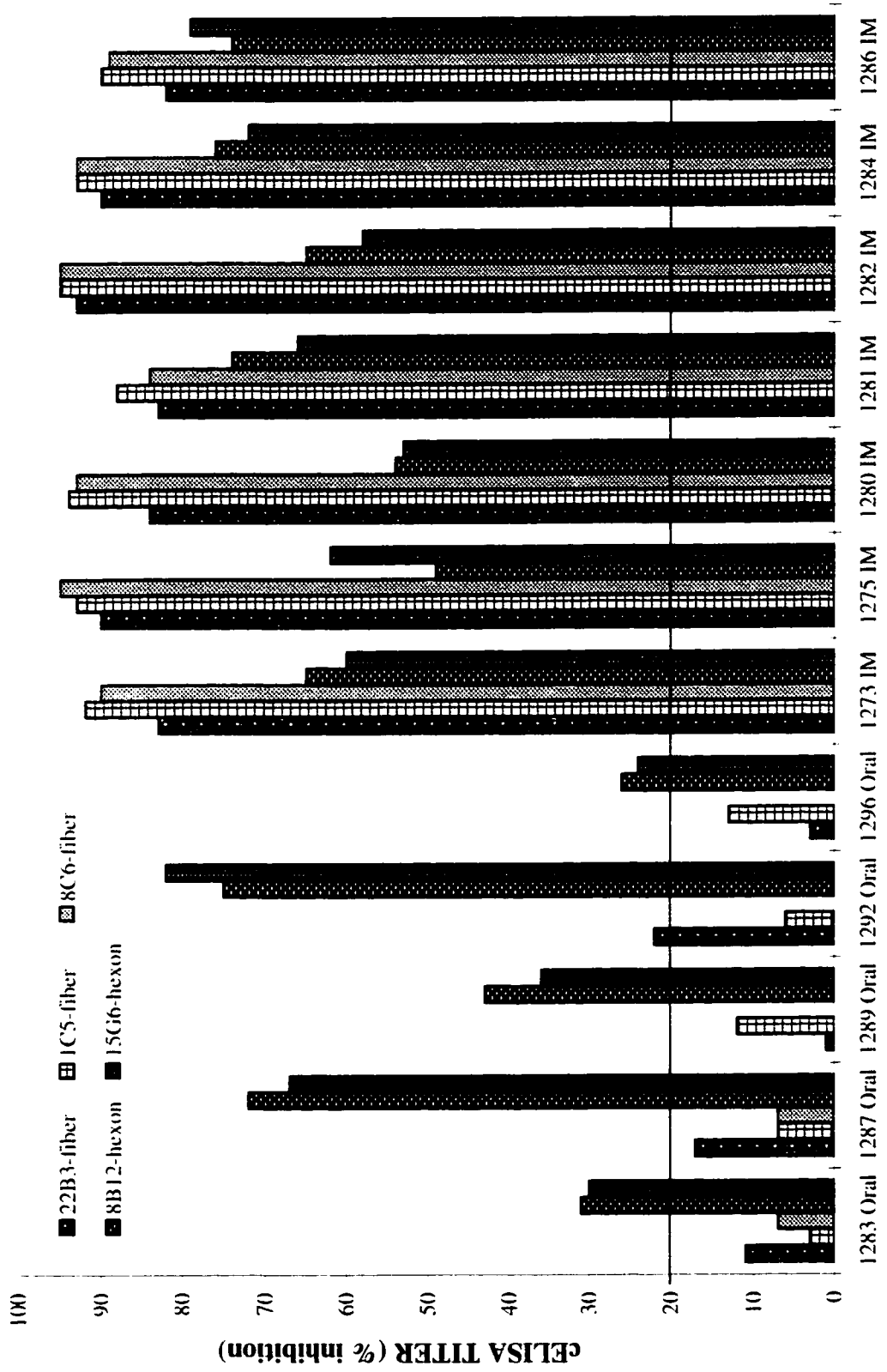
Table 6. Competitive inhibition of Ad5 mAbs by various skunk sera.

SKUNK SERA	PRNT NEUTRALIZING Ab TITER		Ad5 cELISA % INHIBITION OF Ad5 mAbs							
			Neutralizing FIBER				Non-neutralizing HEXON			
	Ad5	CAV-1	7G1	22B3	1C5	8C6	8B12[a]	15G6[a]	21E7[b]	29F5[b]
			164	4037	1767	1557				70
1869R	2521	6218	23%	13%	14%	13%	73%	84%	85%	33%
1869R	1808	5575	32%	0%	0%	0%	61%	76%	75%	24%
1870R	2164		24%	0%	5%	0%	0%	0%	0%	28%
1871R	2681		35%	0%	2%	5%	2%	23%	15%	31%
1873R	2364	8710	32%	0%	11%	0%	62%	78%	60%	20%
1826R		3814	3%	0%	17%	0%	0%	5%	10%	20%
1827R			25%	0%	0%	0%	0%	0%	0%	9%
1828R			15%	0%	2%	0%	0%	0%	9%	21%
1828R			27%	0%	3%	0%	12%	53%	42%	40%
1830R			39%	7%	0%	0%	0%	0%	0%	37%

[a] cross-react with Ad1-30; [b] cross-react with Ad1-30, CAV-1,-2, BAV-3

Ad5 fiber and hexon mAbs (optimal dilutions of clarified ascites) were allowed to compete for binding with 5 Ad5 neutralization-positive and 5 negative skunk sera diluted 1:20. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Optical densities were plotted for each mAb-Ag reaction. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100).

Figure 5. Competitive inhibition of Ad5 mAbs by various fox sera. Ad5 fiber and hexon mAbs (optimal dilutions of clarified ascites) were allowed to compete for binding with 5 Ad5-neutralization positive sera from foxes immunized orally and 7 from foxes immunized intra-muscularly diluted 1:10. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Optical densities were plotted for each mAb-Ag reaction. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100). Red bars indicate reactions with fiber mAbs. Horizontal line indicates hypothetical 20% inhibition positivity cut-off.



Sera From Foxes Immunized with Ad5E3RG by the Oral or IM Routes

neutralizing fox sera were capable of blocking all Ad5 fiber and hexon mAbs (Figure 5). All fiber mAbs, despite their neutralizing capabilities, were eliminated for use in cELISAs since they would be unable to detect positive samples from field animals that would most likely have encountered the virus by the oral route. Two non-neutralizing hexon mAbs which cross-reacted with other human adenoviruses (15G6 and 8B12) were the most specific since the other hexon mAbs additionally cross-reacted with CAV-1, 2, and BAV-3. Unlabeled 15G6 was finally selected as the mAb system of choice because it was sensitive and specific when tested against the rabbit serum (Figure 4), positive and negative reference sera (Table 6), and it titrated well against either purified Ad5 or Ad5-infected cell lysate (data not shown for titrations with clarified ascites on these Ab preparations). 15G6 did not bind mock Ag (uninfected 293 cell lysate and purified rabies virus) or to uncoated microtiter plates, nor did a control mAb (M752, a rabies mAb) bind to Ad5 or uncoated microtiter plates (data not shown). Also, the enzyme-labeled secondary Ab (HRP-goat anti-mouse Ig) did not bind non-specifically to Ab from other species, any other serum component, or microtiter plates.

MAb SELECTION (CAV-1)

All ten unlabeled CAV-1 mAbs were tested in an indirect cELISA with 12 reference skunk sera known to be positive for CAV-1 neutralizing activity and 12 negative skunk sera. Again, this was to ensure that test serum Ab could in fact block neutralizing CAV-1 mAbs from binding neutralization-associated epitopes on purified CAV-1. M1260 was selected since it appeared sensitive and specific when tested against these sera, and it was the only CAV-1 hexon mAb which was neutralizing and non-cross-reactive (Table 7).

ASSAY OPTIMIZATION

Assay parameters such as dilutions of Ag (purified Ad5 or CAV-1), mAb (15G6 for

Table 7. Competitive inhibition of CAV-1 mAbs by various skunk sera.

SKUNK SERA	NEUTRALIZING PRNT Titer		CAV-1 NEUTRALIZING cELISA Titers (% INHIBITION OF CAV-1 mAbs)						
			Neutralizing FIBER		Neutralizing HEXON			Neutralizing HEXON	
	CAV-1	Ad5	M1262	M1245	M1260	M1249[a]	M1263[a]	M1243[b]	M1272[c]
			7988	12 426	31 623	42 170	30 516		
1841R	330		0%	6%	0%	7%	9%	8%	10%
1873R	710	2364	22%	25%	6%	61%	63%	83%	76%
1823R	360		10%	25%	73%	68%	69%	42%	18%
1825R	385	812	11%	1%	16%	37%	29%	33%	21%
1826R	428	52	9%	20%	78%	74%	78%	88%	49%
1862R	276	65	20%	8%	48%	67%	63%	84%	51%
1853R	3162		28%	25%	90%	84%	87%	74%	53%
1855R	5240		32%	18%	74%	72%	70%	48%	24%
1874R	2453	15	ND	ND	54%	ND	ND	83%	55%
1835R	5839		ND	ND	79%	ND	ND	85%	63%
1872R	785		ND	ND	67%	ND	ND	57%	30%
1843R	1885		ND	ND	30%	ND	ND	23%	ND
1821R			0%	11%	0%	1%	7%	10%	0%
1822R			0%	0%	0%	7%	3%	8%	0%
1849R			10%	13%	0%	3%	5%	0%	0%
1850R			0%	5%	0%	21%	19%	18%	10%
1845R			0%	0%	0%	0%	0%	0%	0%
1856R			0%	8%	0%	5%	2%	0%	0%
1864R		107	16%	9%	0%	5%	2%	9%	3%
1870R		2164	14%	21%	0%	2%	7%	8%	4%
1838R			ND	ND	0%	ND	ND	0%	0%
1876R			ND	ND	11%	ND	ND	6%	9%
1839R			ND	ND	0%	ND	ND	0%	0%
1840R			ND	ND	0%	ND	ND	8%	0%

[a] cross-react with CAV-2; [b] cross-react with CAV-2, Ad1-30; [c] cross-react with CAV-2, Ad1-30, BAV-3

CAV-1 fiber and hexon mAbs (optimal dilutions of clarified ascites) were allowed to compete for binding with 12 CAV-1 neutralization-positive and 12 negative skunk sera diluted 1:20. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Optical densities were plotted for each mAb-Ag reaction. Each cELISA titer was based on the inhibition of mAb by competing test serum : (% inhibition = 100 - (OD test sample / OD negative control x 100).

Ad5 cELISA; M1260 for CAV-1 cELISA), test serum, and enzyme-labeled secondary Ab (HRP-goat anti-mouse Ig) were critical to optimization and easily established using checkerboard titrations (Figure 6). When optical densities from Ag-mAb titrations were plotted, each curve (representing a different Ag dilution) was generally sigmoidal with non-linear or "plateau" regions at the top and bottom of the graphs and a linear middle region. A 1:1600 dilution of purified Ad5 (Figure 7A) and a 1:800 dilution of purified CAV-1 (not shown) was considered optimal since these curves had their upper plateau regions in a range accurate for spectrophotometric readings (i.e. between 1.0-1.5 OD units) coupled with low background levels (i.e. less than 0.1 OD unit).

Optimization of 15G6 (clarified ascites) and conjugate (HRP-goat anti-mouse Ig) for the Ad5 cELISA involved titration on purified Ad5 diluted 1:1600. A conjugate dilution between 1:1000 and 1:2000 (i.e. each curve represented a different conjugate dilution) was in the desired range; therefore, 1:1400 was chosen (Figure 7B). A mAb dilution of 1:400 was found to be optimal since it corresponded with a signal of approximately 1.0 OD, near the top linear region of the optimal conjugate curve, after 10 minutes of development (Figure 7B). Choosing mAb dilutions in this region ensured saturation of all free virus epitopes, making possible the measurement of mAb inhibition by very low amounts of test serum Ab (i.e. assay sensitivity). Aside from convenience, a short development time (approximately 10 minutes) minimizes residual substrate colour development which is independent of any enzymatic activity and usually as a result of oxidation owing to the air and effects of light. Likewise, optimization of M1260 (clarified ascites) and conjugate (HRP-goat anti-mouse Ig) for the CAV-1 cELISA involved titration on purified CAV-1 diluted 1:1000. A conjugate dilution of 1:4000 was considered optimal for detecting M1260 diluted 1:1600 (not shown).

Figure 6. An example of a checkerboard titration. One virus preparation is shown titrated vertically and each of four mAbs (hybridoma supernatant) is shown titrated horizontally. Shaded areas represent color reaction in microtiter plate wells. As Ag and mAb concentrations decrease (dilutions get bigger), the signal decreases (i.e. the color lessens and optical densities decrease).

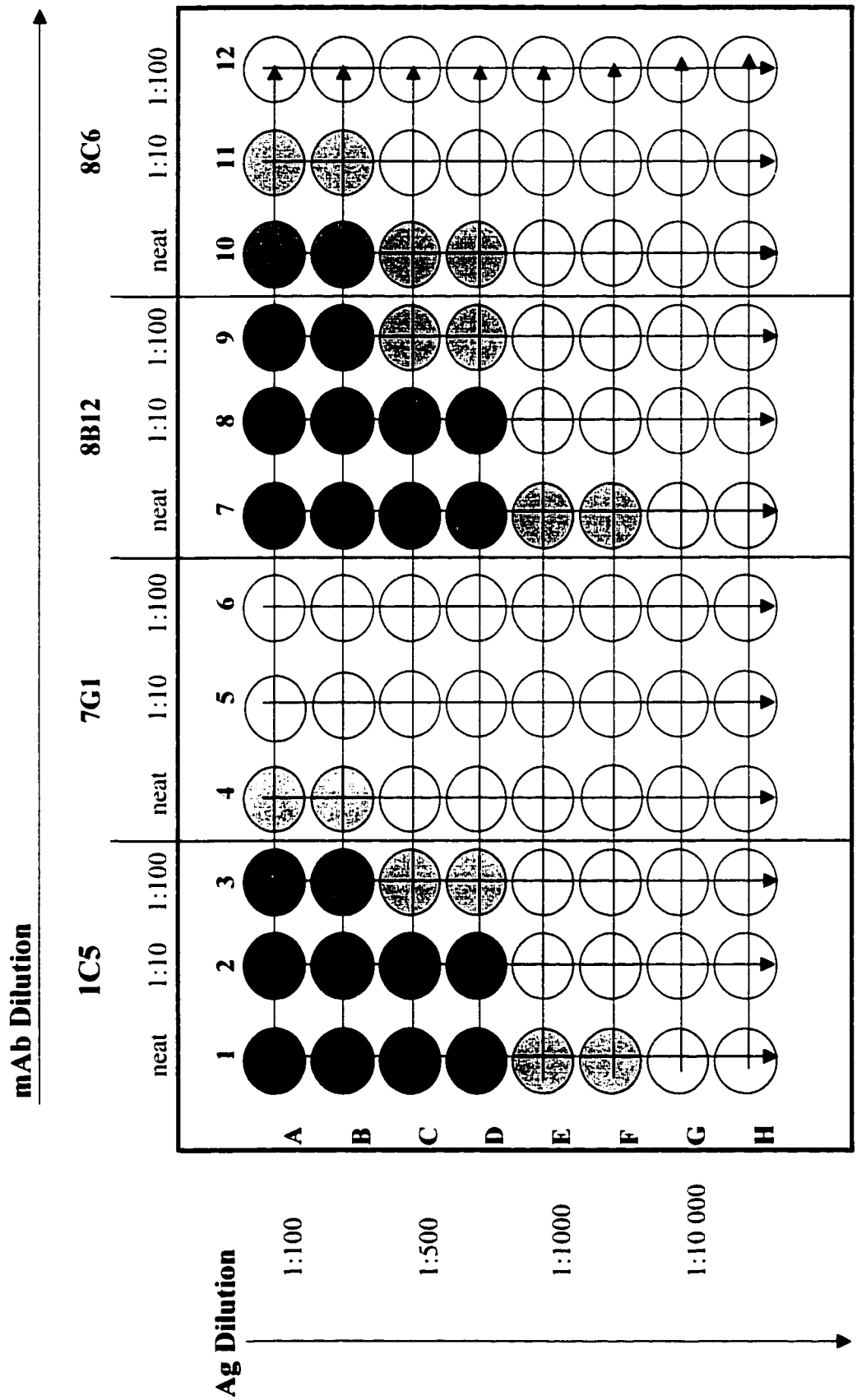
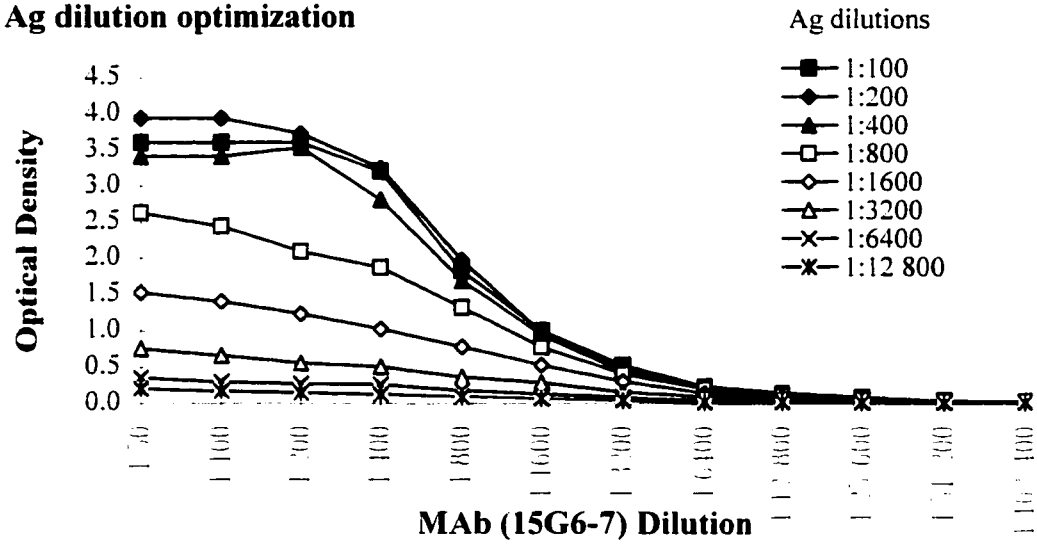
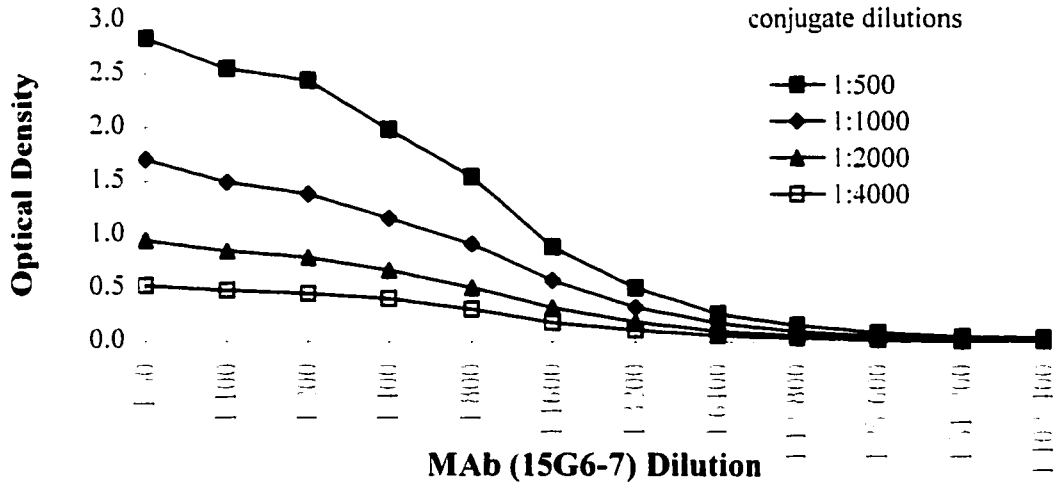

mAb Dilution
1C5
7G1
8B12
8C6
neat
1:10
1:100
1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
Ag Dilution
1:100
1:500
1:1000
1:10 000

Figure 7. A. Ag dilution optimization (checkerboard titration of Ad5 non-neutralizing hexon mAb ascites fluid, 15G6-7, and purified Ad5). **B. MAb and conjugate dilution optimization** (checkerboard titration of 15G6-7 and HRP-goat anti-mouse Ig on plates coated with purified Ad5, 1:1600). **C. Control serum dilution optimization** (titration of cELISA control serum in competition with 15G6-7, 1:400 detected with conjugate, 1:1400). Color development was assessed, after enzymatic reaction with substrate/chromagen (H_2O_2/ABTS), at 414 nm in a spectrophotometer.

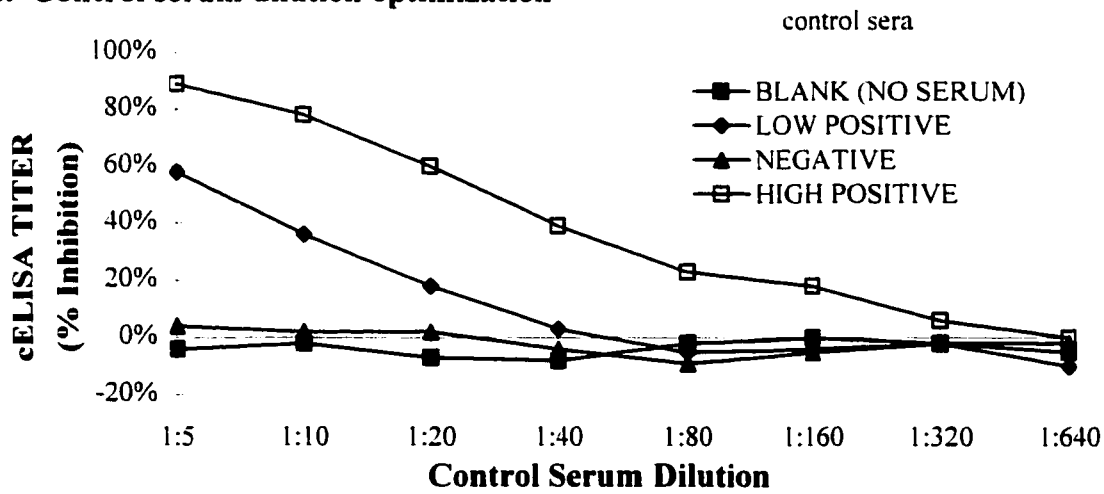
A. Ag dilution optimization



B. MAb and conjugate dilution optimization



C. Control serum dilution optimization



The optimal test serum dilution was more difficult to establish since every test serum contains different quantities of antibody. A 1:10 dilution of test and control serum (resulting in a 1:20 final dilution when mixed with competing mAb) for both cELISAs was selected after titrating Ad5 negative, high, and low positive cELISA controls, which were made up of a pooled collection of experimental skunk, fox, and raccoon sera (Figure 7C). This dilution appeared to balance sensitivity with minimal background in the presence of other serum proteins.

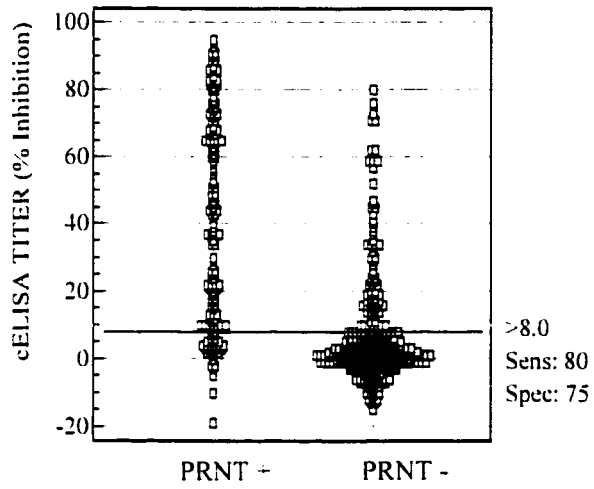
STATISTICAL VALIDATION OF cELISAs

Ad5. For validation of the Ad5 cELISA, which measured non-neutralizing and possibly cross-reacting Ab to other human adenoviruses, reference serum results from the PRNT were compared with their corresponding cELISA titers. In Figure 8A, the negative and positive distributions partially overlap and a horizontal line marks one of many possible % inhibition positivity cut-offs. Receiver-Operating Characteristics (ROC) was employed to assign sensitivity (i.e. the probability that the cELISA test result will be positive when Ad5 neutralizing Ab is present) and specificity (i.e. the probability that the cELISA test result will be negative when Ad5 neutralizing Ab is absent) pairs to a whole range of possible % inhibition cut-offs. ROC cited an 8% cut-off, corresponding with an 80% sensitivity and 75% specificity, which most accurately discriminated between positivity and negativity (minimizing false positive and negative cELISA results) (Figure 8A).

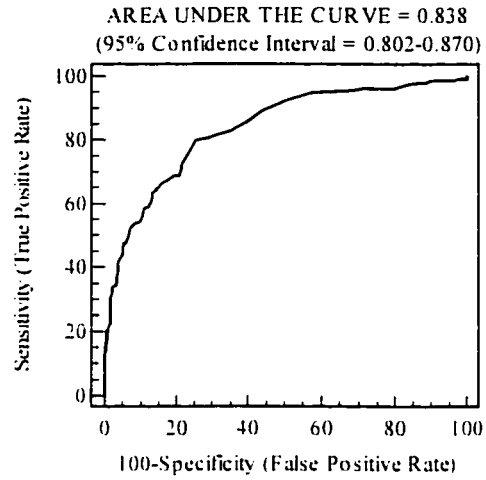
ROC also plotted all sensitivity-specificity coordinates and quantified test accuracy based on the area under the ROC curve, which can range from 1.0 (perfect discrimination between positivity and negativity; curve coincides with the upper left-hand corner of the plot) to 0.5 (no discrimination; curve coincides with the diagonal)

Figure 8. A. Ad5 cELISA titer distribution for all reference sera. cELISA titers were plotted according to their PRNT status (i.e. either neutralization-positive or negative). The horizontal line marks the % inhibition cut-off which most accurately discriminates between positivity and negativity. **B. ROC curve demonstrating Ad5 cELISA performance.** cELISA performance or accuracy is expressed as the area under the ROC curve. **C. Distribution without raccoon data. D. ROC curve without raccoon data. E. Distribution without raccoon or CAV-1 neutralizing positive skunk sera. F. ROC curve without raccoon or CAV-1 neutralizing positive skunk sera.**

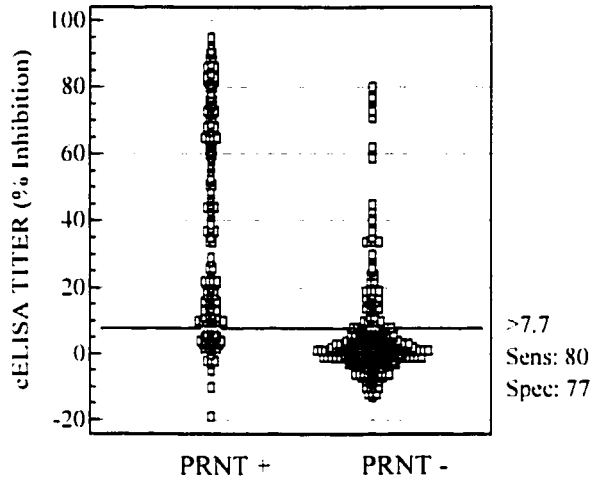
A. All reference sera



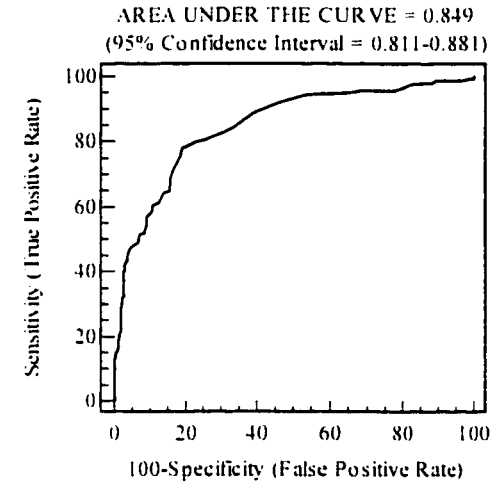
B. All reference sera



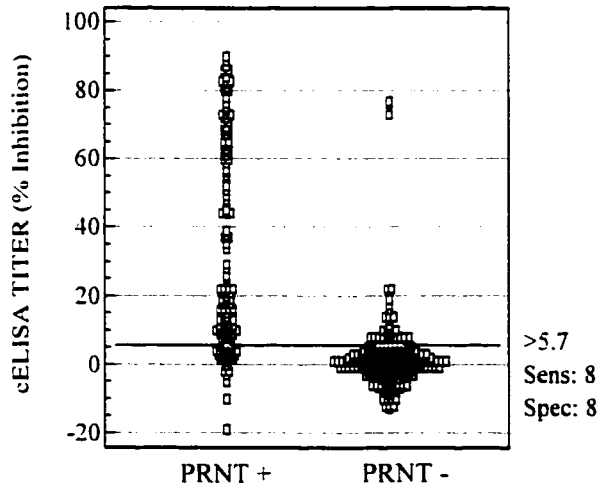
C. No raccoon sera



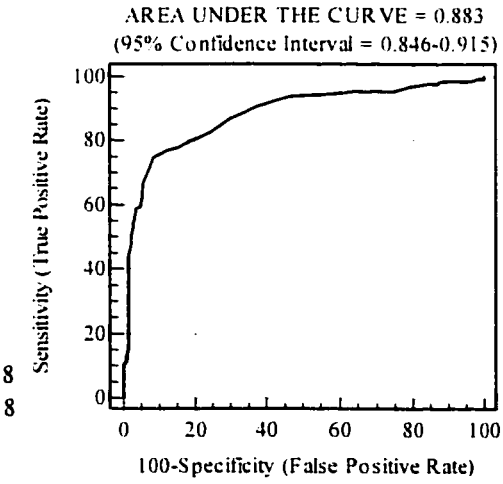
D. No raccoon sera



E. No raccoon or skunk sera



F. No raccoon or skunk sera



(Hanley and McNeil 1982; Zweig and Campbell 1993). The area under the Ad5 cELISA ROC curve was equal to 0.838 (95% CI 0.802-0.880; positive sera n=172, negative sera n=316), representing Ad5 cELISA performance in terms of a single number (Figure 8B).

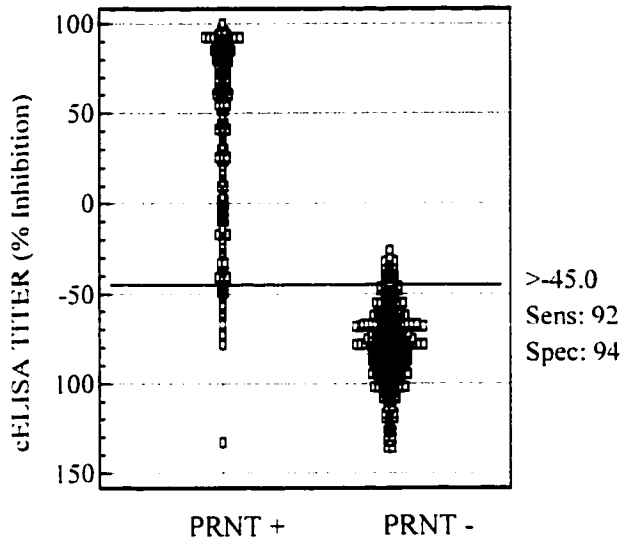
Since the immune status of experimental raccoons to adenovirus was debatable (i.e. no neutralizing activity but Ab detected by cELISA and Western blot), raccoon serum results (n=64) were eliminated from ROC analysis. This elimination resulted in improved discrimination of positive and negative results (Figure 8C) and assay performance based on a ROC curve area of 0.849 (95% CI 0.811-0.881) (Figure 8D). Also, since CAV-1 neutralizing Ab in 24% of skunks may have interfered with the neutralizing Ab response to Ad5RG vaccination, these serum results (n=63) were additionally eliminated from ROC analysis. This elimination resulted again in improved discrimination of positive and negative results (Figure 8E) and assay performance based on a ROC curve area of 0.883 (95% CI 0.846-0.915) (Figure 8F).

CAV-1. For validation of the CAV-1 cELISA, which measured neutralizing Ab to CAV-1, there was initially very good discrimination of CAV-1 neutralization-positive and negatives. This was based on a - 45% inhibition cut-off, corresponding with a 92% sensitivity and 94% specificity (Figure 9A). The area under the ROC curve constructed from all possible sensitivity and specificity pairs was equal to 0.979 (95% confidence interval 0.964-0.989; positive sera n=184, negative sera n=413) (Figure 9B).

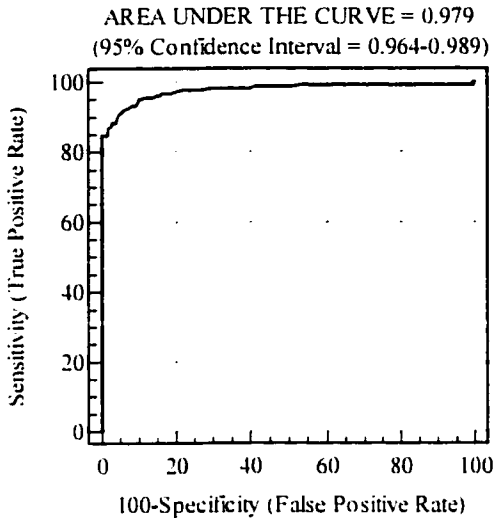
Due to the extremely low % inhibition cut-off, blocking reagents and diluents were adjusted to suit the needs of this assay. Under new conditions using a blocking buffer containing 5% skim milk powder and diluent containing 3% skim milk powder, CAV-1 cELISAs testing all reference sera and statistical validation were repeated. Discrimination

Figure 9. A. CAV-1 cELISA titer distribution for all reference sera. cELISA titers were plotted according to their PRNT status (i.e. either neutralization-positive or negative). The horizontal line marks the % inhibition cut-off which most accurately discriminates between positivity and negativity. **B. ROC curve demonstrating CAV-1 cELISA performance.** cELISA performance or accuracy is expressed as the area under the ROC curve. **C. Distribution after technical modifications. D. ROC curve after technical modifications.**

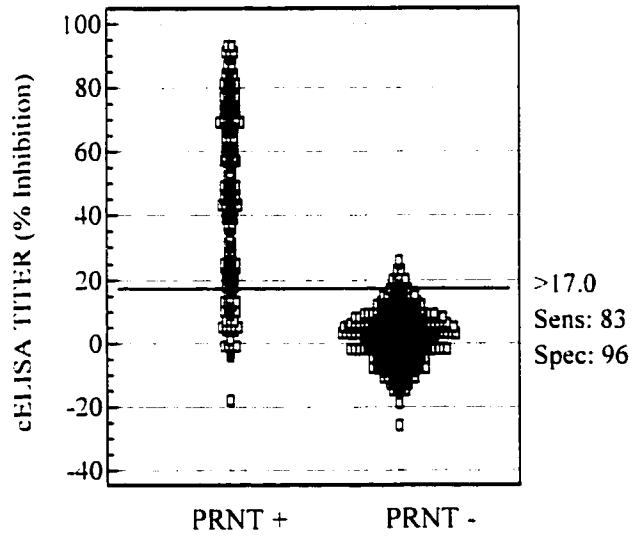
A. All reference sera



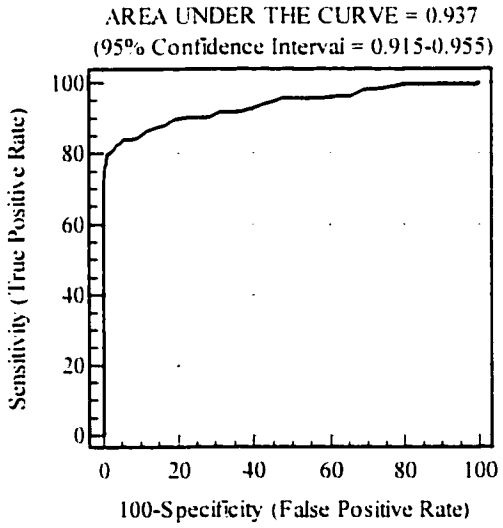
B. All reference sera



C. With assay modifications



D. With assay modifications



of CAV-1 neutralization positives and negatives was slightly compromised for a more realistic 17% inhibition cut-off, corresponding with an 83% sensitivity and 96% specificity (Figure 9C). Assay performance was based on a ROC curve area of 0.937 (95% confidence interval 0.915-0.955; positive sera n=184, negative sera n=413) (Figure 9D).

SERUM Ab RESPONSES IN Ad5RG VACCINATED ANIMALS

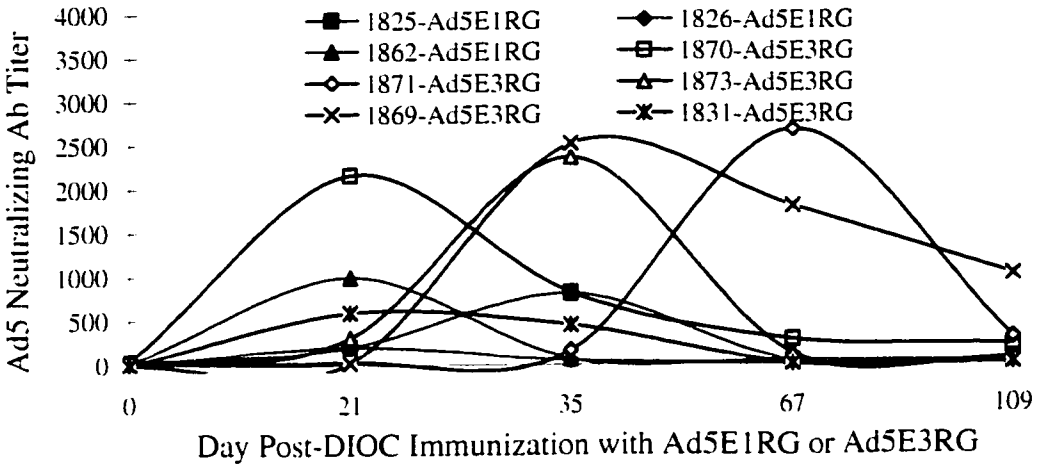
Neutralizing Ab responses to Ad5RG vaccination were measured in experimental skunks, foxes, and raccoons (i.e. sera used for validation) by PRNT. Later, non-neutralizing Ab responses were measured by cELISA using a cross-reactive non-neutralizing Ad5 mAb.

Skunks. Among skunks vaccinated orally with the E1-deleted vaccine (Ad5E1RG), 4/7 (57%) in the highest dose group seroconverted (i.e. had at least one out of five serum samples taken throughout the course of immunization with a 1:100 neutralizing titer), while none of the skunks in the two lower dose groups and control group seroconverted. Among skunks vaccinated orally with the E3-deleted vaccine (Ad5E3RG), 6/8 (75%) in the highest dose group, 3/8 (37.5%) in the next highest dose group, and 2/7 (29%) in the lowest dose group seroconverted, and neutralizing Ab titers were generally higher than in skunks vaccinated with the E1-deleted vaccine (Figure 10A). In general, neutralizing activity among skunks peaked at different times post-immunization (Figure 10A).

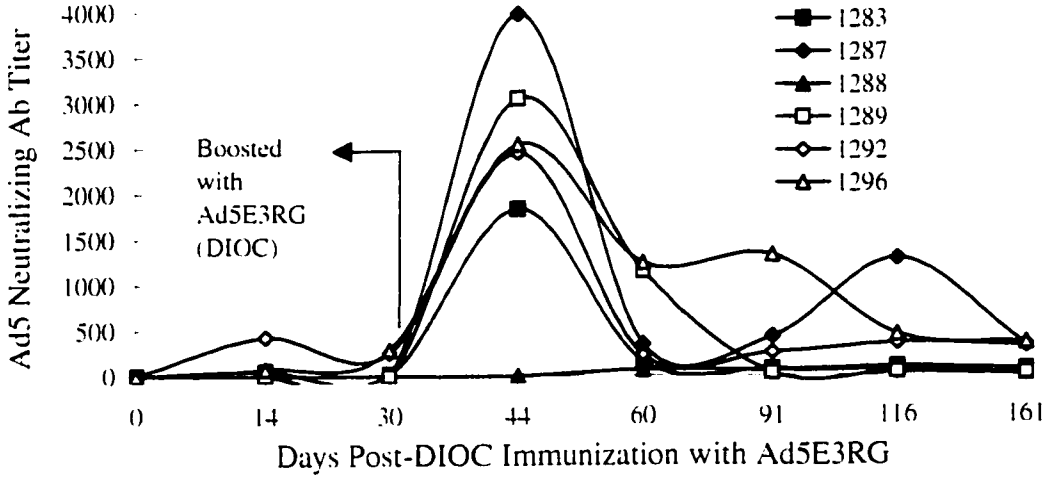
A moderate relationship was observed between Ad5 and pre-existing CAV-1 PRNT neutralizing titers; the rank correlation coefficient was 0.53 (P = 0.00, 95% CI 0.43-0.61, n = 268). A stronger relationship was observed between Ad5 cELISA titers (which measured non-neutralizing Ab cross-reacting with Ad1-30) and CAV-1 PRNT neutralizing titers; the rank correlation coefficient was 0.71 (P = 0.00, 95% CI 0.65-0.77, n = 267). Also, the Student's t-test statistic calculated for two groups of sera with Ad5 Ab titers (one group with

Figure 10. A. Neutralizing Ab responses in selected skunks that seroconverted after oral Ad5RG immunization. B. Neutralizing Ab responses in selected foxes that seroconverted after oral Ad5RG immunization. C. Neutralizing Ab responses in selected foxes that seroconverted after IM Ad5RG immunization. Ad5 neutralizing Ab in sera obtained over a series of bleeds was measured by PRNT. A neutralizing Ab titer represents the reciprocal serum dilution required to neutralize 50% of viral PFU in the virus-serum mixture. Red lines indicate immune responses to E1-deleted vaccine (AdE1RG).

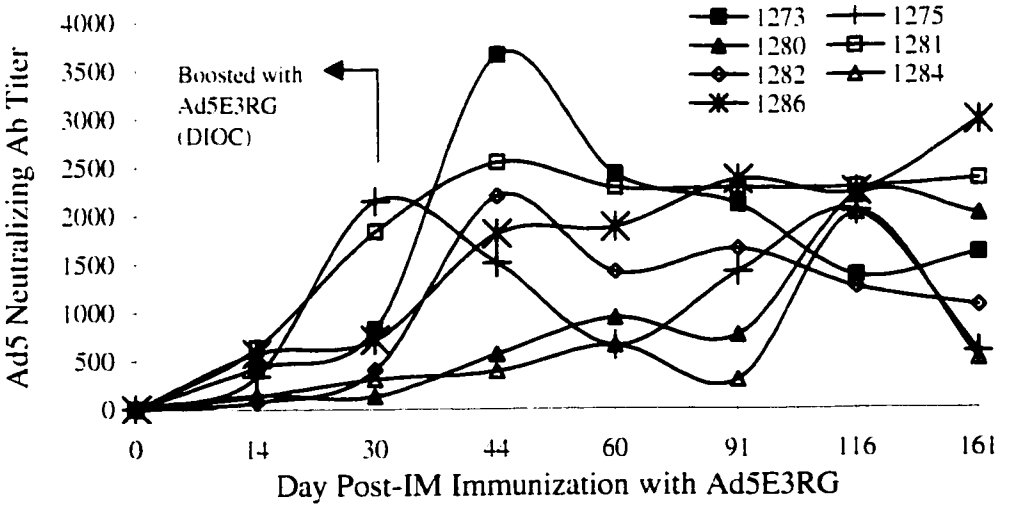
A. Skunks (orally vaccinated)



B. Foxes (orally vaccinated)



C. Foxes (IM vaccinated)

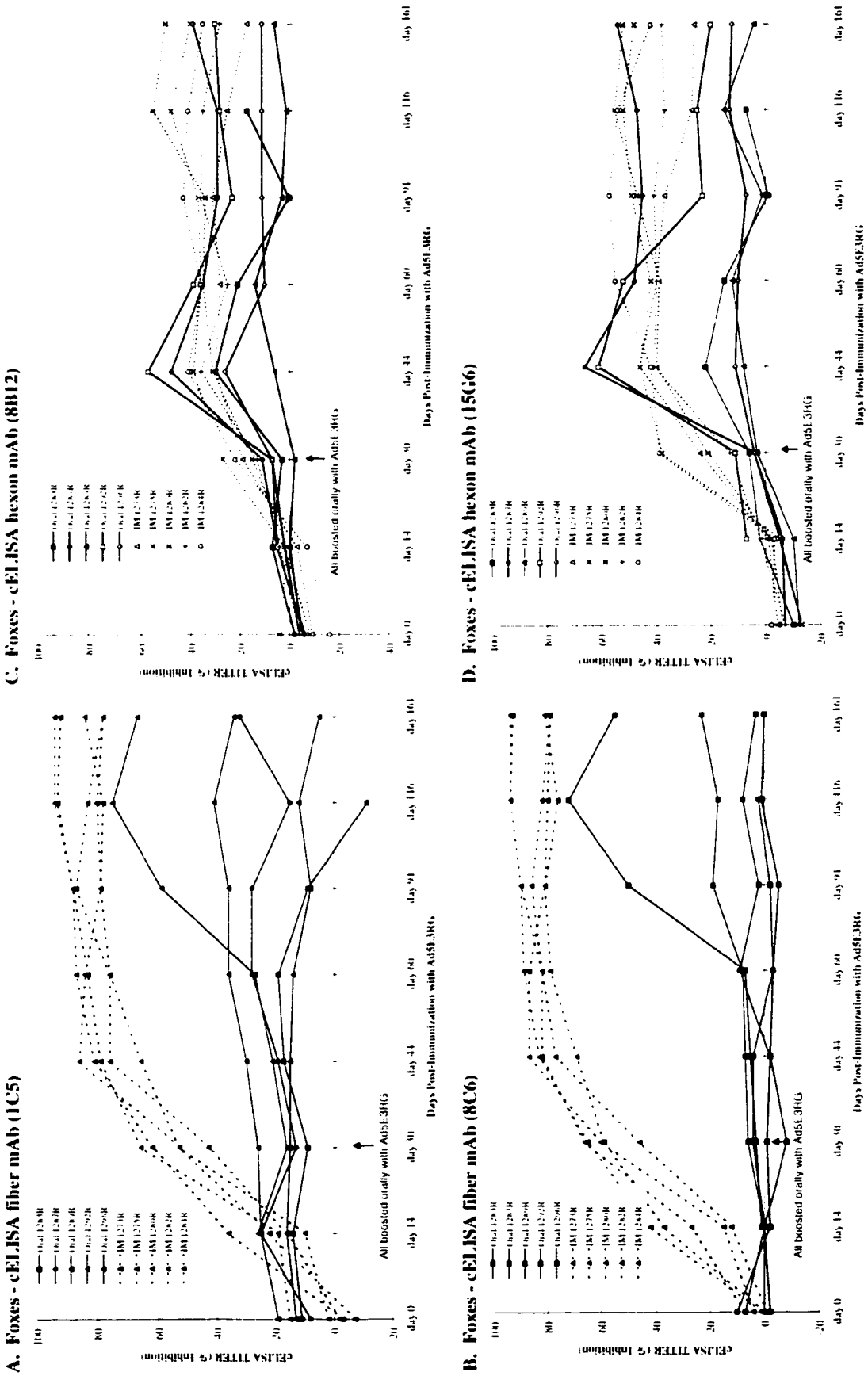


CAV-1 Ab, and the other group without CAV-1 Ab) indicated that there was a significant difference between the mean Ad5 titers in these two groups (for Ad5 PRNT results, t test statistic was 2.4, with 267 degrees of freedom and an associated P value of P=0.0106; for Ad5 cELISA results, t=-14.832, DF=265, P=0.000). This suggests that the presence of CAV-1 Ab may be affecting the production of Ad5 Ab in response to the vaccine.

Foxes. Among foxes vaccinated and boosted (on day 30 post-vaccination) orally with Ad5E3RG, 5/7 (71%) seroconverted, while 7/7 (100%) of foxes vaccinated intra-muscularly and boosted orally seroconverted. Of controls that were not initially immunized but boosted orally, 2/7 (29%) seroconverted. In general, neutralizing activity among foxes vaccinated and boosted by the oral route were uniform, with responses in most individuals peaking at day 44 (Figure 10B). Neutralizing activity among foxes vaccinated by the intra-muscular route and boosted orally on day 30 was much less uniform, with some individuals demonstrating a rise and gradual fall in titer, some showing a rise and plateau in titer, some showing a gradual rise in titer, and one showing early and late peak activity (Figure 10C). Generally, neutralizing titers in IM-immunized foxes were higher and longer-lasting than in orally-immunized foxes.

Differences in Ab responses to specific Ad5 capsid proteins between orally and IM-immunized animals were discovered during cELISA development using fiber and hexon mAbs (as will be described in section on mAb selection). Foxes formed a steady, uniform, and much greater neutralizing Ab response to Ad5 fiber when IM-immunized than when orally immunized (except fox 1292R) (Figure 11A and B). Non-neutralizing Ab responses to Ad5 hexon were also steady and uniform in IM-immunized foxes but were much less pronounced than Ad5 fiber Ab responses (Figure 11C and D). Orally-immunized foxes

Figure 11. A. Neutralizing Ad5 fiber Ab responses in foxes given Ad5RG orally and intra-muscularly. Serum Ab responses were measured by cELISA using 1C5, an Ad5 fiber specific mAb. **B. (Same but using 8C6, another Ad5 fiber mAb). C. Non-neutralizing Ad5 hexon Ab responses in foxes given Ad5RG orally and intra-muscularly.** Serum Ab responses were measured by cELISA using 8B12, an Ad5 hexon mAb. **D. (Same but using 15G6, another Ad5 hexon mAb).** Serum Ab responses were measured by cELISA using either a fiber-specific or hexon-specific mAb. Serum was diluted 1:20 and allowed to compete with each mAb for binding to Ad5-infected cell lysate coated onto plates. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100). Red lines indicate reactions with fiber mAbs.


A. Foxes - cELISA fiber mAb (1C5)
B. Foxes - cELISA fiber mAb (8C6)
C. Foxes - cELISA hexon mAb (8B12)
D. Foxes - cELISA hexon mAb (15G6)
cELISA TITER (% Inhibition)
Days Post-Immunization with Ad5F.3HG
All boosted orally with Ad5F.3HG

generally formed a greater Ab response to Ad5 hexon than fiber (Figure 11A, B, C, and D).

Raccoons. Among raccoons vaccinated with Ad5E3RG by direct installation into the oral cavity (DIOC), only 2/8 (25%) seroconverted and among vaccine-bait fed raccoons, 0/8 (0%) seroconverted (Figure 12A). Interestingly, all raccoons in both groups seroconverted for rabies neutralizing Ab.

Despite lack of Ad5 neutralizing activity, 4/8 (50%) of the raccoons vaccinated DIOC responded to non-neutralization-associated Ad5 components based on rising cELISA titers following vaccination, including the two raccoons who seroconverted (Figure 12B). 1/8 (12.5%) had a cELISA titer on day 0 which persisted throughout the immunizing period (Figure 12B). Of vaccine-bait fed raccoons, 2/8 (25%) showed a rise in Ad5 cELISA titers following vaccination and 2/8 (25%) had cELISA titers on day 0 which persisted throughout the immunizing period (Figure 12C).

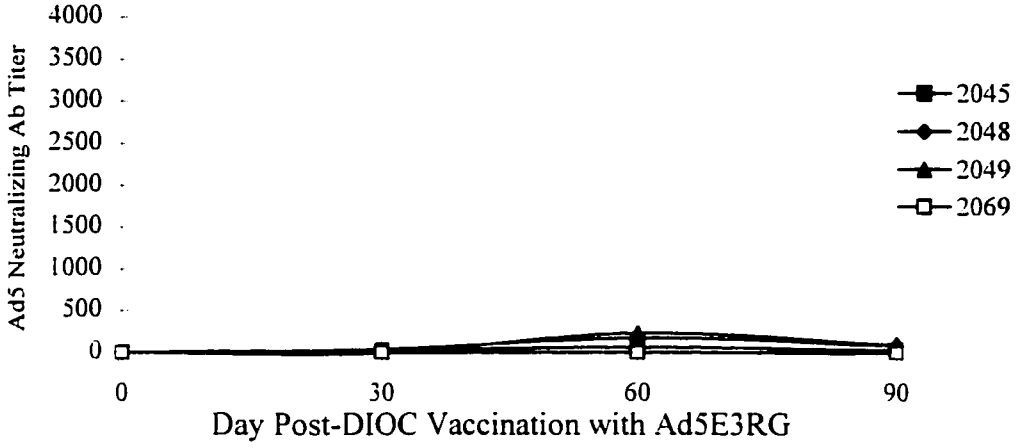
To further examine Ad5-specific responses in the raccoons, Western blots with raccoon sera were performed. Raccoon sera lacking Ad5 neutralizing activity but reacting in the Ad5 cELISA bound an Ad5 hexon epitope under non-reducing conditions (Figure 13). This indicated that the epitope these Ab are recognizing is conformational in nature and probably lost upon Ad5 denaturation under reducing conditions. None of the sera bound mock Ag (pelleted rabies virus). Primary Ab control (M752, a rabies mAb) and secondary Ab control (AP-goat anti-dog Ig) did not bind Ad5 components non-specifically.

ADENOVIRUS SEROPREVALENCE IN FIELD SAMPLES

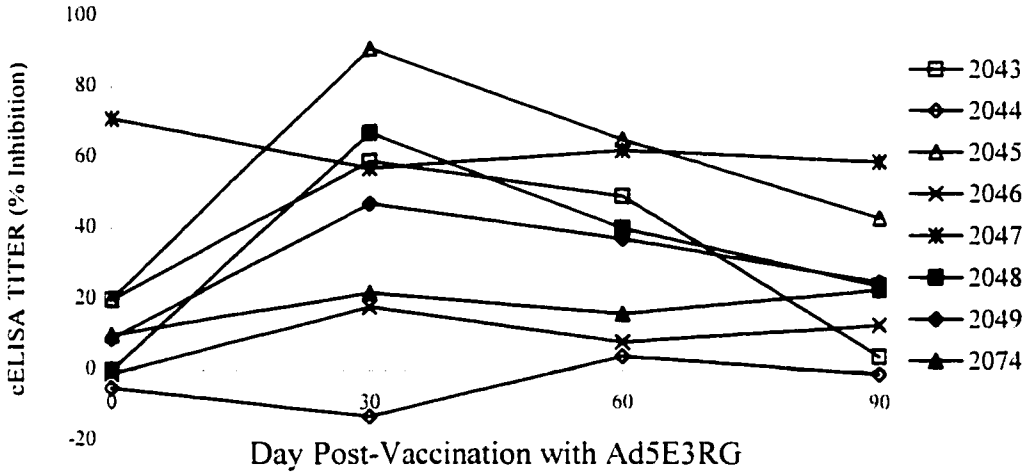
Ad5 seroprevalence. The validated human adenovirus cELISA, which measured non-neutralizing and possibly cross-reacting Ab to other human adenoviruses, was then applied to a representative sample from the field (sera from approximately 2000 raccoons). The

Figure 12. A. Ad5 neutralizing Ab responses in selected raccoons that seroconverted after oral Ad5RG immunization. Ad5 neutralizing Ab in sera obtained over a series of bleeds was measured by PRNT. A neutralizing Ab titer represents the reciprocal serum dilution required to neutralize 50% of viral PFU in the virus-serum mixture. **B. Ad5 Non-neutralizing Ab responses in raccoons after Ad5RG immunization (DIOC). C. Ad5 Non-neutralizing Ab responses in raccoons after Ad5RG immunization (vaccine-bait).** Non-neutralizing serum Ab responses were measured by cELISA using an Ad5 hexon mAb (15G6). Serum was diluted 1:20 in 3% BES/PBST and allowed to compete with mAb for binding to purified Ad5 coated onto plates. MAbs were detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100).

A. Raccoons (PRNT titers)



B. Raccoons given vaccine DIOC (cELISA titers)



C. Raccoons given baits containing vaccine (cELISA titers)

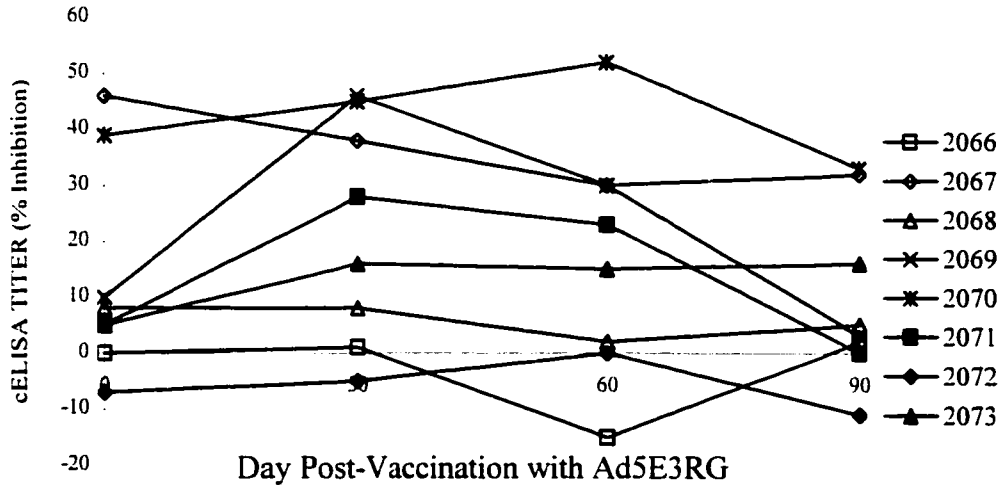


Figure 13. Western blot of some raccoon sera lacking Ad5 neutralizing activity but reacting in the Ad5 cELISA. Purified Ad5 was electrophoresed through 6% polyacrylamide gels under non-reducing conditions. Western blots were carried out with selected raccoon sera diluted 1:50, after blocking nitrocellulose strips with 5% SMP. Raccoon Ab were detected with AP-goat anti-dog conjugate, followed by enzymatic reaction with substrate/chromagen (NBT/BCIP). Reaction was stopped with distilled water after sufficient color development. Lanes:

1: negative Ab control (M752, rabies glycoprotein mAb)
2: conjugate control (AP-goat anti-dog Ig)
3: positive polyclonal Ab control (rabbit Ad5 immune serum)
4: positive mAb control (15G6, Ad5 hexon mAb)
5: raccoon serum 1 (cELISA titer=65% inhibition; PRNT negative)
6: raccoon serum 2 (cELISA titer=67% inhibition; PRNT negative)
7: raccoon serum 3 (cELISA negative; PRNT negative)
8: raccoon serum 4 (cELISA negative; PRNT negative)

1 2 3 4 5 6 7 8

objective was to estimate the seroprevalence of human adenovirus Ab in the raccoon population, which is to be targeted for oral rabies immunization. The investigation revealed that 503/2033 (25%) sera had Ab to human adenovirus based on the 8% inhibition cut-off cited by ROC analysis, lending an 80% sensitivity and 75% specificity to the assay (Figure 14). Raising the cut-off to 20% inhibition decreased the number of positive results so that 156/2033 (8%) sera had Ab to human adenovirus (Figure 14). The median (i.e. middle value) of the distribution was 1% inhibition (95% central range -21 to 38% inhibition).

Approximately 40 field raccoon sera with negative cELISA results and 40 field raccoon sera with inhibition values greater than 40% were tested for Ad5 neutralizing activity by PRNT. None of the sera had Ad5 neutralizing activity, but 88% of the raccoon sera from the latter group bound an Ad5 hexon epitope in Western blots conducted under non-reducing conditions (Figure 15A), a scenario similar to that found with experimental raccoon sera as described previously.

CAV-1 seroprevalence. The validated CAV-1 cELISA, measuring neutralizing Ab to CAV-1, was also applied to field raccoon sera. The investigation revealed that 38/1943 (2%) sera had Ab to CAV-1 based on the 17% inhibition cut-off cited by ROC analysis, lending an 83% sensitivity and 96% specificity to the assay (Figure 16). The median (i.e. middle value) of the distribution was -1% inhibition (95% central range -17 to 16% inhibition). All positives were also tested for CAV-1 neutralizing activity by PRNT. Only 11/38 (29%) were capable of neutralizing CAV-1 in a PRNT; however, six of these corresponded with the six highest cELISA titers (i.e. 37 to 79% inhibition) (Table 8). These six sera were also positive in a western blot conducted under non-reducing conditions (Figure 15B and Table 8). In fact, all eleven sera capable of neutralizing CAV-1 in a PRNT were positive in a western blot

Figure 14. Frequency distribution of Ad5 cELISA titers (% inhibition of 15G6-7) in field raccoons. Each sample diluted 1:10 was allowed to compete with 15G6-7 for binding to purified Ad5. 15G6-7 was detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100).

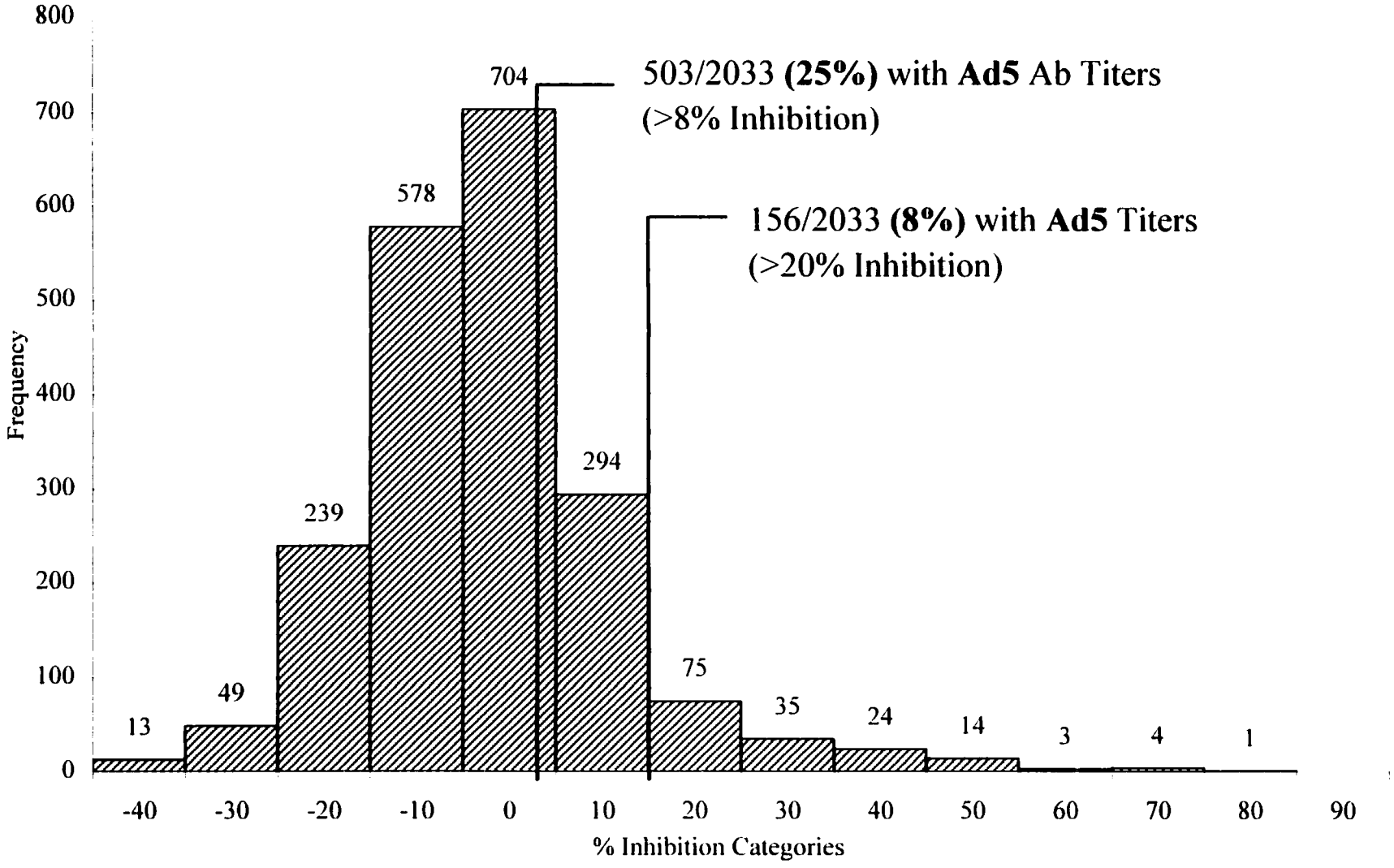

800
700
600
500
400
300
200
100
0
Frequency
704
578
294
239
75
49
35
24
14
13
3
4
1
503/2033 (25%) with Ad5 Ab Titers
(>8% Inhibition)
156/2033 (8%) with Ad5 Titers
(>20% Inhibition)
-40
-30
-20
-10
0
10
20
30
40
50
60
70
80
90
% Inhibition Categories

Figure 15. A. Western blot of some field raccoon sera testing positive and negative in the Ad5 cELISA. Purified Ad5 was electrophoresed through 6% polyacrylamide gels under non-reducing conditions. Western blots were carried out with selected field raccoon sera diluted 1:50, after blocking nitrocellulose strips with 5% SMP. Raccoon Ab were detected with AP-goat anti-dog conjugate, followed by enzymatic reaction with substrate/chromagen (NBT/BCIP). Reaction was stopped with distilled water after sufficient color development. Lanes:

1: negative Ab control (M752, rabies glycoprotein mAb)
2: conjugate control (AP-goat anti-dog Ig)
3: positive polyclonal Ab control (rabbit Ad5 immune serum)
4: positive mAb control (15G6, Ad5 hexon mAb)
5: positive raccoon serum control 1 (cELISA titer=65% inhibition; PRNT negative)
6: positive raccoon serum control 2 (cELISA titer=67% inhibition; PRNT negative)
7: negative raccoon serum control 3 (cELISA negative; PRNT negative)
8: negative raccoon serum control 4 (cELISA negative; PRNT negative)
9: field raccoon serum 1 (cELISA titer=89% inhibition; PRNT negative)
10: field raccoon serum 2 (cELISA titer=77% inhibition; PRNT negative)
11: field raccoon serum 3 (cELISA negative; PRNT negative)
12: field raccoon serum 4 (cELISA negative; PRNT negative)

B. Western blot of some field raccoon sera testing positive and negative in the CAV-1 cELISA. (same as above except using purified CAV-1) Lanes:

1: negative Ab control (M752, rabies glycoprotein mAb)
2: conjugate control (AP-goat anti-dog Ig)
3: positive polyclonal Ab control (rabbit Ad5 immune serum)
4: positive mAb control (M1260, CAV-1 hexon mAb)
5: field raccoon serum 1 (cELISA titer=79% inhibition; PRNT titer=1:3120)
6: field raccoon serum 2 (cELISA titer=59% inhibition; PRNT titer=1:3105)
7: field raccoon serum 3 (cELISA negative; PRNT negative)
8: field raccoon serum 4 (cELISA negative; PRNT negative)

A

1 2 3 4 5 6 7 8 9 10 11 12

B

1 2 3 4 5 6 7 8

Figure 16. Frequency distribution of CAV-1 cELISA titers (% inhibition of M1260) in field raccoons. Each sample diluted 1:10 was allowed to compete with M1260 for binding to purified Ad5. M1260 was detected with an HRP-goat anti-mouse conjugate, followed by enzymatic reaction with substrate/chromagen (H_2O_2/ABTS). Color development was assessed at 414 nm in a spectrophotometer. Each cELISA titer was based on the inhibition of mAb by competing test serum (% inhibition=100 - (OD test sample / OD negative control x 100).

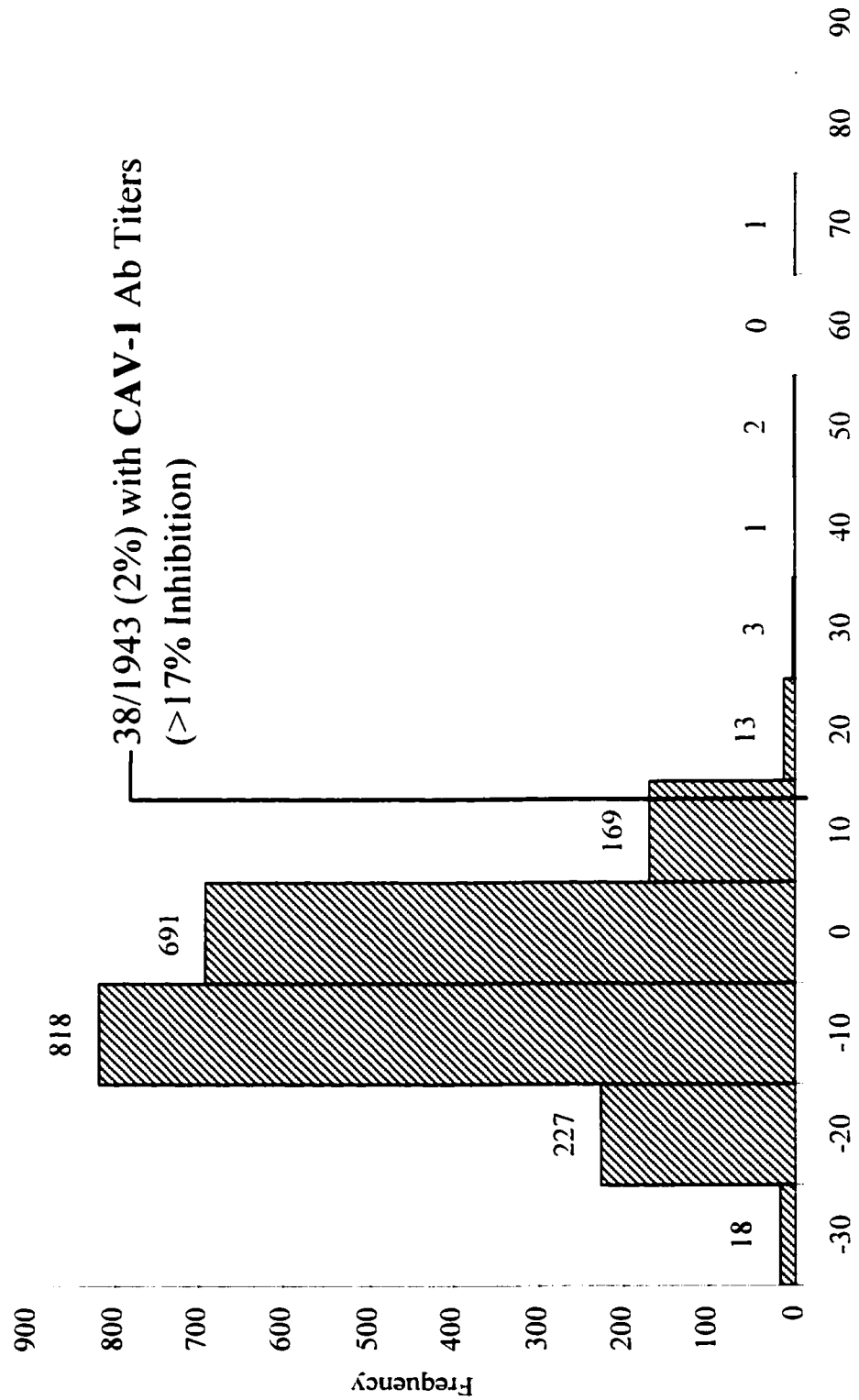

38/1943 (2%) with CAV-1 Ab Titers
(>17% Inhibition)
Frequency
% Inhibition Categories
900
800
700
600
500
400
300
200
100
0
818
691
227
169
18
13
3
1
2
0
1
-30
-20
-10
0
10
20
30
40
50
60
70
80
90

Table 8. Comparison of CAV-1 cELISA titers, CAV-1 PRNT titers, and CAV-1 western blot results for some field raccoon sera.

FIELD RACCOON ID	cELISA Titer	PRNT Titer	WESTERN BLOT RESULT	FIELD RACCOON ID	cELISA Titer	PRNT Titer	WESTERN BLOT RESULT
602781/782	79%	3120	+	604302/303	15%	0	ND
601108/109	59%	310	+	601056/057	15%	0	ND
600194/195	57%	316	+	601072/073	10%	0	ND
601421/422	47%	1050	+	609091/092	4%	0	ND
59995	38%	64	+	609070/071	4%	0	ND
960621-2	37%	21	+	609665/0914	1%	0	ND
601251/252	30%	0	+	609030/502959	1%	0	ND
604344/345	28%	0	-	601013/014	0%	0	ND
59605	27%	1700	+	601009/010	-1%	0	ND
609608/610	25%	0	-	601001/002	-3%	0	ND
603487/488	24%	0	-	601040/041	-3%	0	ND
601259/260	23%	0	-	601007/008	-4%	0	ND
606183/184	21%	626	+	601025/026	-4%	0	ND
601751/752	21%	33	-	600152/153	-5%	0	ND
600618/619	21%	0	-	600177/178	-6%	0	ND
610461/462	21%	0	-	600213/214	-6%	0	ND
57473	21%	0	-	609034/037	-7%	0	ND
602695/696	20%	0	-	601017/018	-7%	0	ND
607994/504539	20%	0	-	601019/020	-7%	0	ND
603275/276	20%	0	-	601027/028	-8%	0	ND
600292/293	19%	0	-	600200/201	-9%	0	ND
609602/603	19%	0	-	601033/034	-9%	0	ND
601079/080	19%	0	-	601035	-9%	0	ND
601127/128	19%	0	-	601038/039	-9%	0	ND
601255/256	19%	0	+(?)	601029/030	-10%	0	ND
601676/677	19%	0	-	609679/680	-11%	0	ND
621036/037	19%	0	-	609686/687	-11%	0	ND
608911/912	19%	0	-	600160/161	-12%	0	ND
603351/352	19%	0	-	609658	-12%	0	ND
612218/202	19%	0	-	609639/640	-12%	0	ND
96101802	19%	0	-	609678/502966	-12%	0	ND
960627-4	19%	0	+(?)	601023/024	-12%	0	ND
609163/164	18%	365	+(?)	601042/043	-12%	0	ND
603303/304	18%	0	-	609684/685	-13%	0	ND
610456/506597	18%	0	-	601005/006	-13%	0	ND
603331/332	18%	0	-	601011/012	-13%	0	ND
601973/974	18%	0	+(?)	601036/037	-13%	0	ND
55610	18%	0	-	601031/032	-14%	0	ND
600580/581	17%	190	(?)	609057/058	-15%	0	ND
602775/776	17%	0	-	601003/004	-15%	0	ND
603008/009	17%	0	-	609659/1233	-16%	0	ND
601689/690	17%	0	-	609068/069	-16%	0	ND
601920/921	17%	0	-	601015/016	-16%	0	-
606190/191	16%	0	ND	601021/022	-16%	0	-
602819/820	16%	0	ND	609002/646	-19%	0	-
601096/097	16%	0	ND	609681/682	-20%	0	-

Shaded areas indicate positive results, using a cELISA 17% inhibition cut-off. Some western blot results were difficult to interpret, as indicated with a question mark (?).

except one sample whose PRNT neutralizing titer was very low (1:33). A moderate to good relationship was generally observed between CAV-1 cELISA and PRNT neutralizing titers (r_r = 0.65, 95% CI 0.51-0.75 P = 0.000, n = 92).

There was no relationship observed between Ad5 and CAV-1 cELISA titers in field raccoon sera (r_r = 0.100, CI = 0.055-0.144, P = 0.000, n = 1942).

cELISA QUALITY CONTROL

Quality control, as defined here, refers to the effective control of factors contributing to cELISA variability. These factors include thermal gradients causing "edge effects", adsorption and desorption characteristics, sample placement, meniscus formation, day-to-day variability, different reagent and plate lots, deviations from protocols, and timing of reactions. Efforts were taken to manually control sources of variation, which were successful since excessive intra-plate variability was not observed (i.e. variability between replicates on the same plate which was denoted by an asterisk on the computer printout) and samples were, therefore, rarely repeated. Also, the intraplate coefficient of variation was calculated based on the variability between 80 replicates of negative control serum tested on one plate from an internal lot of plates. The coefficient of variation for Ad5 cELISA plates used for survey purposes was 6.5% and was 3.3% for CAV-1 plates.

Intertest variability (i.e. variability between replicates tested on different days) was monitored by continually inspecting control serum results over time. Quality control charts (Figures 17 and 18) indicated that both cELISAs generally functioned consistently over time with minor day-to-day fluctuations in assay performance. Ad5 cELISA intertest variation was also assessed by repeat testing of approximately 240 field raccoon sera, and likewise with approximately 40 field raccoon sera for the CAV-1 cELISA. Results from one Ad5 or

Figure 17. Ad5 cELISA quality control charts. Ad5 cELISA intertest variation was monitored based on buffer control, low and high positive serum control, and negative serum control values. The mean optical density of four replicates for each control was plotted for each plate tested. Upper and lower control limits representing 1 and 2 SD from the mean are indicated. Plates with any one control value exceeding 2 SD from the mean were rejected.

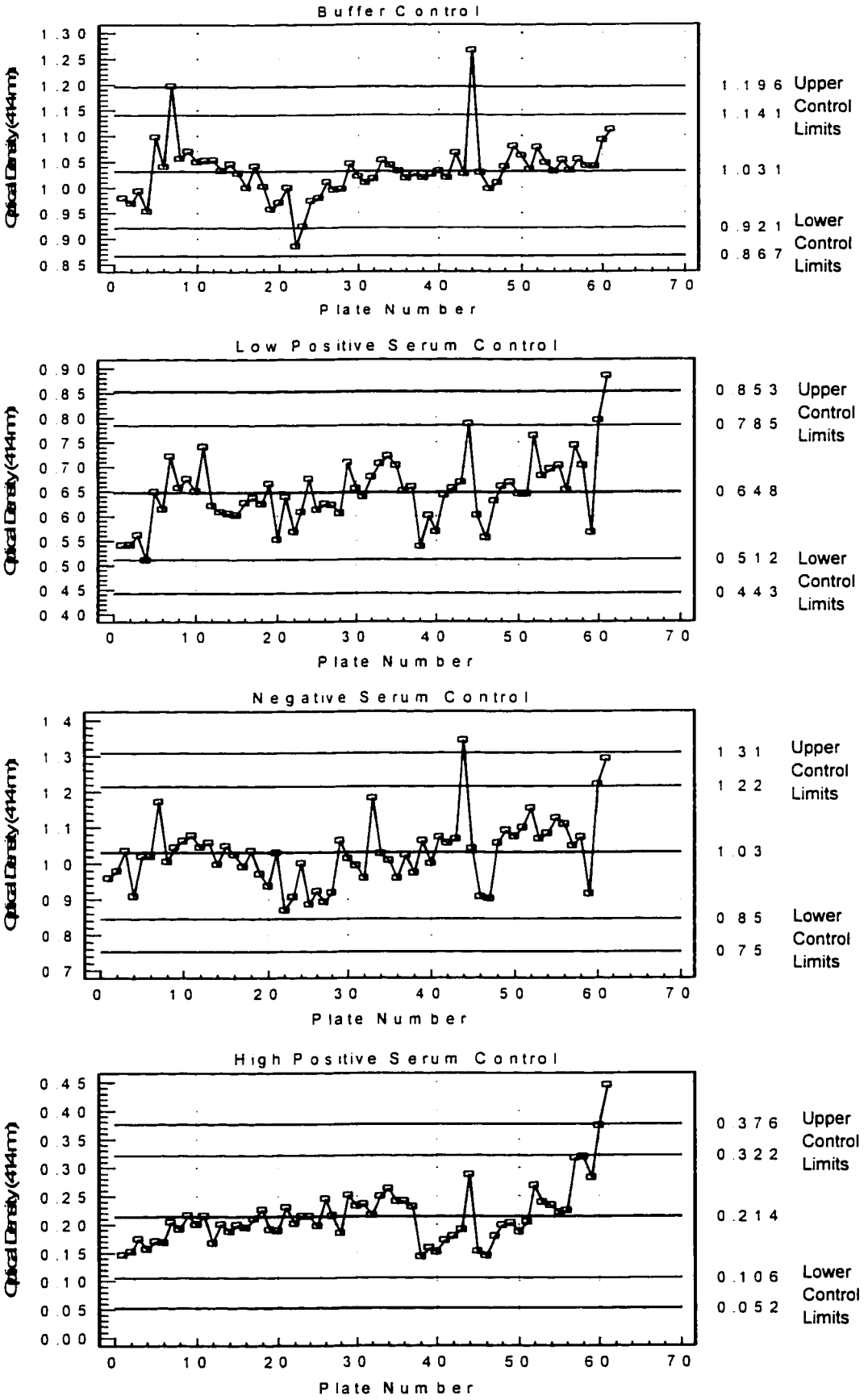
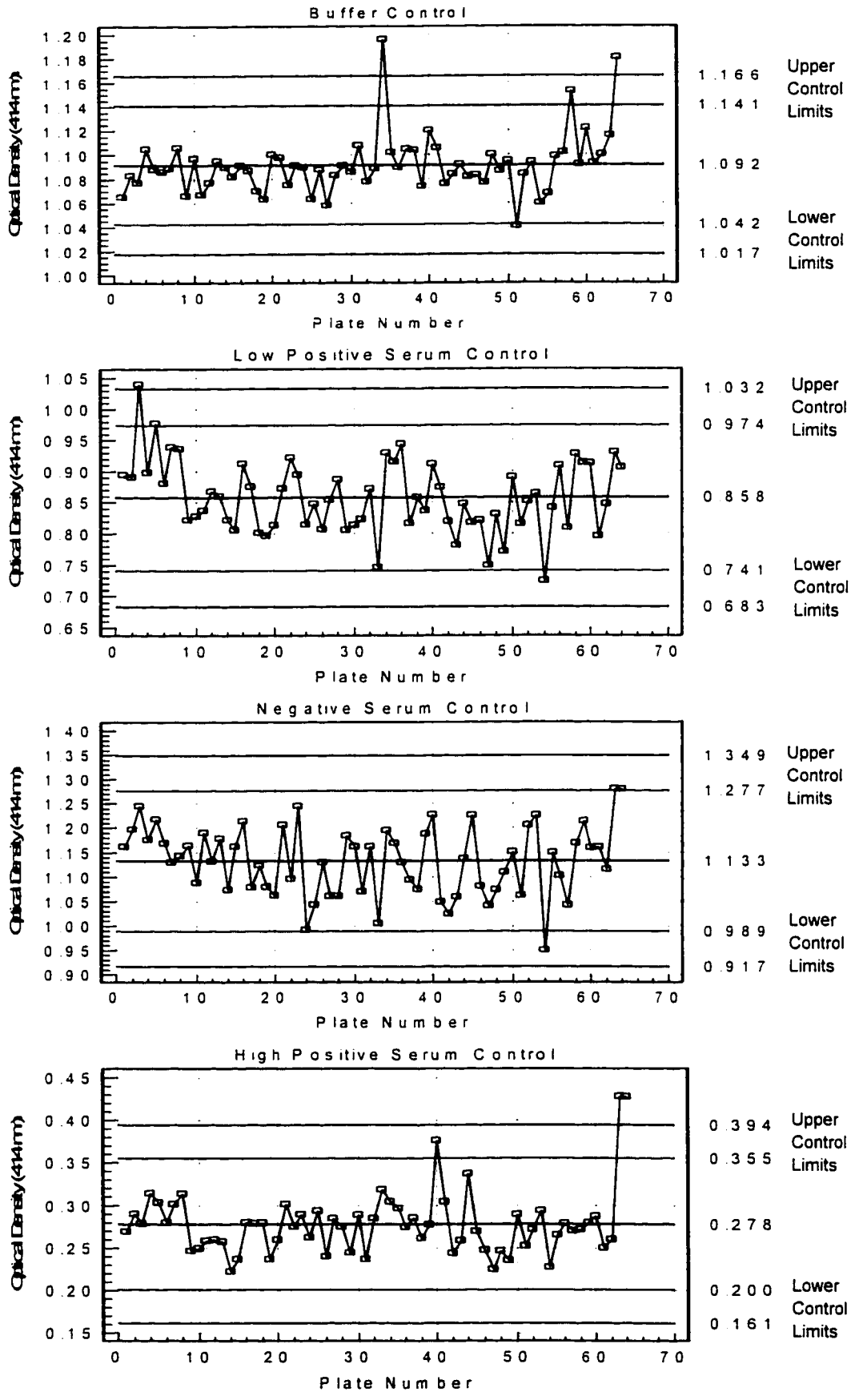

Buffer Control
Optical Density(414nm)
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0 10 20 30 40 50 60 70
Plate Number
1.196 Upper Control Limits
1.141
1.031
0.921 Lower Control Limits
0.867
Low Positive Serum Control
Optical Density(414nm)
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0 10 20 30 40 50 60 70
Plate Number
0.853 Upper Control Limits
0.785
0.648
0.512 Lower Control Limits
0.443
Negative Serum Control
Optical Density(414nm)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0 10 20 30 40 50 60 70
Plate Number
1.31 Upper Control Limits
1.22
1.03
0.85 Lower Control Limits
0.75
High Positive Serum Control
Optical Density(414nm)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 10 20 30 40 50 60 70
Plate Number
0.376 Upper Control Limits
0.322
0.214
0.106 Lower Control Limits
0.052

Figure 18. CAV-1 cELISA quality control charts. CAV-1 cELISA intertest variation was monitored based on buffer control, low and high positive serum control, and negative serum control values. The mean optical density of four replicates for each control was plotted for each plate tested. Upper and lower control limits representing 1 and 2 SD from the mean are indicated. Plates with any one control value exceeding 2 SD from the mean were rejected.


Buffer Control
Optical Density(414nm)
1.20
1.18
1.16
1.14
1.12
1.10
1.08
1.06
1.04
1.02
1.00
0 10 20 30 40 50 60 70
Plate Number
1.166 Upper Control Limits
1.141
1.092
1.042 Lower Control Limits
1.017
Low Positive Serum Control
Optical Density(414nm)
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0 10 20 30 40 50 60 70
Plate Number
1.032 Upper Control Limits
0.974
0.858
0.741 Lower Control Limits
0.683
Negative Serum Control
Optical Density(414nm)
1.40
1.35
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0.90
0 10 20 30 40 50 60 70
Plate Number
1.349 Upper Control Limits
1.277
1.133
0.989 Lower Control Limits
0.917
High Positive Serum Control
Optical Density(414nm)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0 10 20 30 40 50 60 70
Plate Number
0.394 Upper Control Limits
0.355
0.278
0.200 Lower Control Limits
0.161

CAV-1 cELISA always correlated positively with repeat results from second and third assays performed (not shown).

DISCUSSION

An adenovirus-rabies recombinant vaccine (Ad5RG) is a potential candidate for the oral immunization of skunks, foxes, and raccoons against rabies. Before using the vaccine in the field, the WHO and other advisory groups recommended that adenovirus seroprevalence studies in these species be conducted with the goal of assessing environmental risk. Additionally, information collected from these kinds of studies are useful for tracking the potential spread of vaccine virus and for determining if a barrier to immunization exists. For these purposes, two cELISAs measuring Ab against Ad5 and CAV-1 were developed, statistically validated, and applied to a representative sample from the field in order to determine human and canine adenovirus seroprevalence.

cELISA DEVELOPMENT - MAb characterization

The outer adenovirus capsid is composed of 240 non-vertex capsomeres (hexons), and 12 vertex capsomeres (pentons), each with one base and a protruding fiber (Figure 1). Minor capsid proteins, such as hexon-associated proteins (designated protein VI, VIII, and IX), help cement hexons and anchor the capsid to the core. Hexons, penton bases, and fibers are the primary antigens recognized by the immune system (Shenk 1996). The antigenic relationships among adenoviruses are very complex but have more recently been aided by the application of mAbs to serological techniques such as immunofluorescence (IF) and ELISA (Russell et al 1981; Adam et al 1986; Adam et al 1992; Adam et al 1995a; Adam et al 1995b). Studies conducted with the ten Ad5 and ten CAV-1 mAbs used in this investigation confirmed many antigenic relationships already reported in the literature. More important to the objectives of this project, these studies allowed the selection of mAbs suitable for use in Ad5 and CAV-1 cELISAs.

Viral protein specificities were first determined for CAV-1 mAbs by immunoprecipitation of radiolabeled CAV-1 proteins. MAbs were either specific for hexon or fiber proteins. Interestingly, immunoprecipitated CAV-1 fiber migrated to a slightly greater extent than Ad5 fiber in SDS-PAGE (Figure 6), which was expected since CAV-1 and Ad5 fiber molecular weights are reportedly different (57 kD and 62 kD respectively) (Dragulev et al 1991; Shenk 1996). The electrophoretic patterns and molecular weights of adenovirus capsid polypeptides are known to differ with respect to the phylogenetic relationship of the corresponding host species (Ishibashi and Yasue 1984).

Ad5 mAbs and CAV-1 mAbs were further characterized by their abilities to neutralize homologous virus in the PRNT and cross-react with other adenoviruses by indirect IF. Type-specific neutralization-associated epitopes were identified on both fiber and hexon proteins. These results were not unexpected since evidence supports the presence of these kinds of epitopes on the knob region of the fiber (Norrby 1969) as well as on the hexon where they are located to two amino acid loops protruding from the surface (Toogood et al 1992; Horwitz 1996; Crawford-Miksza and Schnurr 1996).

Cross-reactive neutralization-associated epitopes on fibers of both canine adenovirus serotypes (CAV-1 and 2), as well as on CAV-1 and 2 hexon proteins, were also identified, contradicting the general theory that neutralization-associated epitopes define serotype specificity. This finding was not surprising considering that CAV-1 hexon antiserum has been shown to cross-neutralize CAV-2 and a two-way cross-protection in dogs immunized with either CAV-1 or CAV-2 has been documented repeatedly (Appel 1987a).

Human adenovirus group-specific non-neutralization-associated residues were identified on hexon proteins. These conserved residues have been located to the interior base

of the hexon (Norrby 1969; Norrby and Wadell 1969; Horwitz 1996). Other cross-reactive non-neutralization-associated epitopes were identified on the hexons of human and canine adenoviruses and others on human, canine, and bovine adenoviruses, substantiating the possibility of a genus-specific epitope. All members of the *mastadenoviruses* (except in the case of a few bovine adenovirus serotypes) are known to share a non-neutralization-associated cross-reacting epitope that maps to the inner surface of the hexon, and forms the basis for initial classification (Norrby 1969; Ishibashi and Yasue 1984; Shenk 1996).

Also, a subgroup-specific non-neutralization-associated epitope was identified on the hexon-associated protein (pIX) of Ad1, 2, 5 and 6 (subgroup C). There are no known reports of a human adenovirus subgroup C-specific epitope on this protein, as was described here.

A non-neutralization-associated epitope was also identified on a CAV-1 fiber protein, which was also identified on CAV-2, and Ad4 (the only member in its subgroup). The fiber protein carries type-, group-, and subgroup-specific epitopes (Pettersson 1984). The type-specific epitopes, which can raise both neutralizing and non-neutralizing Ab, map to the carboxy-terminus knob of the fiber (Pettersson 1984). It is, therefore, possible that this unique non-neutralization-associated epitope maps to the fiber knob or to the penton base junction where a non-neutralization-associated subgroup determinant has been localized (Pettersson 1984).

cELISA DEVELOPMENT - MAb selection

Hexon mAbs were selected for use in cELISAs after discovering that fiber mAbs could not be blocked by neutralizing sera from animals given the Ad5RG vaccine orally, but could be blocked in the case of IM-immunized animals. This also implies that sera from the orally vaccinated animals neutralized Ad5 via a neutralization-associated hexon epitope

whereas sera from IM-immunized animals probably neutralized Ad5 via neutralization-associated hexon and fiber epitopes in the PRNT. This was an important finding since the cELISA would be applied to field samples, and general adenovirus transmission in wildlife is presumed to occur through the naso/oropharangeal route (Grate et al 1987; Appel 1987a).

Differences in serum Ab responses to adenoviral capsid proteins depending on the route of immunization were also observed by Gahery-Segard et al (1997), who reported that mice differentially responded to Ad5 fiber when two routes of immunization with an Ad5-recombinant vector were used. They observed systemic fiber recognition in mice only after intra-venous immunization and not after intra-peritoneal immunization. These differences in the Ab response to specific viral proteins must be related to differences in cell types available for infection, Ag processing, and presentation. Ab responses to viral proteins administered by parenteral vaccination can obviously be different than Ab responses obtained through mucosal vaccination, emphasizing the complex interactions of the mucosal and systemic immune systems.

STATISTICAL VALIDATION OF cELISAs

The sera of skunks, foxes, and raccoons experimentally vaccinated with Ad5RG and dogs vaccinated with CAV-2 served as reference sera. These were tested for neutralizing Ab in the PRNT and used to validate Ad5 and CAV-1 cELISAs. Plaque assays are generally considered by most virologists to be the easiest, most accurate, and sensitive method for quantitating virus (Hierholzer and Killington 1996). As such, the plaque reduction neutralization test (PRNT), which involved counting viral plaques, provided a "gold standard" reference system for quantifying Ad5 and CAV-1 neutralizing Ab in reference sera. There are a number of technical factors, however, which can affect assay

reproducibility and accuracy. One factor is the health status and confluency of cell monolayers. Dog kidney cells used for measuring CAV-1 neutralization were easy to manipulate; however, 293 cells, which were chosen because of their susceptibility to Ad5 infection, did not always form reliable monolayers. Cells damaged during inoculation or dying for other reasons can cause poor plaque formation leading to false positive Ab results (i.e. a reduction in plaque number normally indicates the presence of neutralizing Ab). Variation in plaque size and overlapping plaques can sometimes obscure plaque-counting results. Virus inactivation, depending on what stage in the process it occurs, can lead to false positive or negative results, although this was partially controlled for by including virus controls in each assay. Naturally-occurring substances present in animal sera may obscure the significance of the results. For instance, non-specific viral inhibitors such as interferons, cross-reacting heterologous antibodies, or substances toxic to cells may block viral replication and plaque formation leading to false positive results. The natural cell growth properties of serum (especially at the lower dilutions) may cause cell invasion of the plaque area created by viral CPE diminishing plaque visibility, and also leading to false positive Ab results.

Concerning the number of reference sera used for statistical validation of cELISAs, meaningful qualitative conclusions can be drawn from ROC analysis performed with approximately 100 observations, with at least 50 cases in each positive and negative group (Metz 1978). Approximately 500 sera were tested for each cELISA, with at least 180 positives (i.e. sera with neutralizing Ab) in each; therefore, the reference serum sample size used here was statistically appropriate. Ad5 neutralizing Ab titers, measured in experimental animals, were well represented across Ab titer ranges, enhancing the power of statistical

validation. The validity of low Ab titers (<1:100) in 36% of sera, however, is questionable. Factors inhibiting or obscuring plaque formation as discussed previously may have prompted false positive interpretations. Sera such as these may, therefore, be partly responsible for low cELISA sensitivity estimates. Non-specific serum factors which may alter the binding of test serum Ab, mAbs, or conjugate in the cELISA may have likewise contributed to aberrant results in the cELISA.

ROC methodology is based on statistical decision theory and has been used extensively in many areas of medicine (Van Steirteghem et al 1982; Carson et al 1985; Hermann et al 1986; Leung et al 1989; Kazmierczak et al 1991; Guyatt et al 1992). ROC can evaluate the ability of a test to discriminate between "diseased cases" and "normal cases" (Metz 1978; Zweig and Campbell 1993). Perfect discrimination, however, is rarely observed and the two populations often overlap as was observed in this investigation, in which the ability of each cELISA to discriminate between PRNT neutralizing (positive) and non-neutralizing (negative) sera was evaluated. In general, the Ad5 cELISA demonstrated poorer discrimination (i.e. a lower sensitivity and specificity) than the CAV-1 cELISA for several reasons. Since the mAb chosen for the Ad5 cELISA was non-neutralizing, the Ad5 cELISA actually measured non-neutralizing Ab in reference sera in contrast to the PRNT, which measured neutralizing Ab. For this reason alone, ROC analysis for the Ad5 cELISA may not be an accurate reflection of its true performance.

Since the reaction to an immunogen is polyclonal in nature, the assumption was made at the outset that animals making Ad5 neutralizing Ab would also make Ad5 non-neutralizing Ab. This assumption may not have necessarily been correct in all species, as demonstrated in raccoons. The fact that raccoons lacked Ad5 neutralizing activity but had

detectable Ab by cELISA and Western blot, may indicate that serum Ab responses in raccoons to oral Ad5RG vaccination may be towards non-neutralization-associated adenovirus epitopes only. Also, the type of Ab response that might be going on simultaneously at mucosal surfaces is unknown, and perhaps Ad5 neutralizing Ab of the IgA isotype is being produced here.

The other possibility is that raccoon Ab was not produced in response to oral Ad5RG vaccination, but was initially present in raccoons prior to vaccination. This Ab may have been the product of a previous exposure to another adenovirus serotype and simply cross-reacted with the epitope being measured in the cELISA and Western blot (especially since the Ad5 mAb used in the cELISA cross-reacted with Ad1-30 and the epitopes involved in the Western blotting were conformational in nature). Evidence of this possibility was observed in some vaccinated raccoons that had very high cELISA titers on day 0 of vaccination, with titers persisting throughout the immunizing period. cELISA responses in other raccoons, which increased in titer following oral Ad5RG vaccination, may possibly represent a cross-reactive anamnestic response to the epitope measured in the cELISA as a result of a previous exposure to another adenovirus. Therefore, for the reasons discussed, raccoon sera reacting in the Ad5 cELISA contributed to the low 75% specificity estimate.

Another factor possibly contributing to the poorer discrimination in the Ad5 cELISA was interference by CAV-1 neutralizing Ab detected in 24% of skunks before Ad5RG vaccination. A strong correlation was measured between Ad5 cELISA titers (non-neutralizing Ab) and CAV-1 PRNT titers (neutralizing Ab) in all skunk sera. It is possible that Ad5RG may boost immunological memory for some epitopes that are common to both Ad5 and CAV-1. Otherwise, CAV-1 neutralizing Ab itself may be cross-reacting in the Ad5

cELISA, causing positive cELISA results when Ad5 PRNT results are negative. Skunk sera may, therefore, have been incorrectly interpreted as false positive cELISA results, lowering assay specificity. When raccoon data and data from 24% of skunks that had CAV-1 titers before Ad5RG vaccination are systematically removed from ROC analysis, assay performance improves.

Other limits to cELISA validation include the possibility of low affinity test serum Ab, which may have been able to neutralize viruses in the PRNT but unable to compete with mAb in the cELISA, contributing to false negative cELISA results and low sensitivity. It is obvious from the previous discussion that the nature of the epitope involved in the cELISA competition is extremely important to the general interpretation of cELISA results, and can sometimes compromise test sensitivity and specificity.

All possible sensitivity-specificity pairs, each corresponding to a particular decision threshold (i.e. % inhibition), can be plotted as a ROC curve in order to provide a unique description of cELISA performance without depending on the arbitrary selection of a decision threshold (Metz 1978). Good assay performance or accuracy can be described by a ROC curve area greater than 0.80 (Dr. Klaus Nielsen, personal communication). Since areas under ROC curves representing both Ad5 and CAV-1 cELISA performance were 0.84 and 0.94 respectively, it can be concluded that both assays demonstrated good overall performance. Essentially these numbers indicate that a randomly selected sample from the PRNT positive group will have a cELISA result larger than that of a randomly selected sample from the PRNT negative group 84% of the time in the case of Ad5, and 94% of the time in the case of CAV-1.

Ad5 SERUM Ab RESPONSES IN Ad5RG VACCINATED ANIMALS

Neutralizing and non-neutralizing Ab responses to Ad5RG vaccination in experimental skunks, foxes, and raccoons were measured over time by PRNT and cELISA respectively. Differences in Ab responses seemed to depend on several factors, including the recombinant vector itself, the immunizing dose, the route of inoculation, species differences, and individual differences.

In skunks, given either Ad5E1RG (E1-deleted) or Ad5E3RG (E3-deleted) in different doses (Refer to Table 2-Materials and Methods), clearly the E3-deleted vaccine was generally more effective at inducing adenovirus Ab since it induced the highest rate of adenovirus seroconversion (i.e. at least one of five serum samples taken throughout the course of immunization with a 1:100 neutralizing Ab titer by PRNT). Since the E1 region in the adenovirus genome is necessary for viral replication, Ad5E1RG which lacks its E1 region probably experiences abortive infections where no infectious progeny are produced. This would effectively decrease viral load and the immune response to the vaccine. Also, because this vaccine retains its E3 gene region, it may be better overall at avoiding the host cytotoxic T lymphocyte (CTL) response since the E3 gene codes for a 19 kD protein that helps adenovirus-infected cells escape detection by CTLs (Horwitz 1990).

In foxes, the main observations involved the differences in fiber recognition depending on the route of immunization, which were previously discussed. Also, there was a higher rate of seroconversion in foxes given Ad5RG intramuscularly vs. orally, which is usually the case with any vaccine. Differences in the level of neutralizing activity observed with different routes of Ad5 immunization have previously been demonstrated (Gahery-Segard et al 1997).

In raccoons, there was a slightly higher rate of adenovirus seroconversion when animals were given Ad5E3RG directly into the oral cavity instead of when fed a vaccine-containing bait, but there was generally a lack of Ad5 neutralizing activity in both groups. Interestingly, Ab was detected against adenovirus by cELISA and Western blot (as discussed previously). Also, all raccoons demonstrated very high rabies neutralizing Ab titers (data not shown). The vaccine may be so effective at inducing expression of the early region rabies glycoprotein gene that an overabundance of glycoprotein is presented at the cell surface. On the other hand, the rabies glycoprotein may competitively inhibit presentation of other adenovirus proteins, leading to the lack of Ad5 neutralizing Ab.

Ab responses to Ad5RG vaccination, therefore, differed not only according to vaccine type, dose, and route of inoculation, but also according to species, with raccoons forming the greatest response to the rabies glycoprotein but the smallest response to adenovirus proteins. Ab responses to Ad5RG also differed between individuals. Even within the same vaccine dose group, some animals produced neutralizing Ab against rabies and Ad5, some produced neutralizing Ab to rabies only, and some did not respond to either Ag. Animals that did produce neutralizing Ab against Ad5, such as skunks, exhibited varying neutralizing Ab titers which peaked at different times post-immunization.

Relative degrees of sensitivity to viral infection (permissiveness) exist among cell types of the same species, and even within the same cell line. One can then imagine that the differences in sensitivity to adenoviral infection from one species to another, let alone one individual to the other would be very complex. The reasons for these individual differences are unknown and largely understudied. The block to viral replication in the majority of human lymphocytes (while a small fraction support replication) is in part due to the inability

of the virus to adsorb to these cells (Horvath et al 1986; Silver and Anderson 1988). Although skunks, foxes, and raccoons are considered generally non- or semi-permissive at best to human Ad infection, they all have been shown to produce rabies serum neutralizing Ab when vaccinated with Ad5RG, and skunks and foxes have been shown to resist peripheral rabies virus challenge (Charlton et al 1992; Dr.Alex Wandeler, personal communication). Conceivably, some level of abortive Ad5 infection, which perhaps varies depending on the species and even the individual, may occur in the cells of wild animals vaccinated with Ad5RG. Some species and/or individuals, therefore, might only express and present early proteins (i.e. rabies glycoprotein) before infection is aborted while others might express and present early and late proteins (i.e. both rabies glycoprotein and Ad5 hexon and fiber proteins). This has previously been observed in canine cells, in which early protein synthesis and DNA replication occurs, but late protein synthesis and production of viral progeny is inhibited (Carmichael 1965; Winters and Young 1980; Prevec et al 1989). Similar events have been reported for Ad5 infection of mouse cells except late proteins are synthesized in small amounts (Silverstein and Strohl 1986; Blair et al 1989).

CAV-1 SERUM Ab RESPONSES IN DOGS GIVEN CAV-2

Although serum neutralization tests are generally serotype-specific and minimally cross-reactive, they may detect heterologous responses within a subgroup, especially when adults are being studied (Horwitz 1996). Immediately after vaccination of dogs with CAV-2, antibody can be detected which neutralizes CAV-2 but not CAV-1; however, serum cross-neutralizes CAV-1 as the response matures (Fairchild and Cohen 1969). This effect was most likely the reason why all dog sera (unpaired samples), from dogs vaccinated with CAV-2, cross-neutralized CAV-1 in the PRNT. Dog sera were obtained at least a few months after

vaccination in most cases, so that cross-neutralization of CAV-1 was expected. In a cELISA, the majority of these sera was capable of inhibiting the CAV-1 mAb (M1260), which did not cross-react with CAV-2 by indirect IF. Immunization with CAV-2 must, therefore, induce some host Ab to CAV-1 epitopes.

CAV-1 SERUM Ab IN SKUNKS

High CAV-1 neutralizing Ab titers detected in 24% of skunk sera indicate recent natural infection with a canine adenovirus, since these animals were not given a CAV-1 or CAV-2 vaccine. Other studies report the detection of CAV-1 Ab in approximately 32-63% of skunks (Alexander et al 1972) and one report describes the isolation of CAV-1 from a skunk (Jamison et al 1973).

ADENOVIRUS SEROPREVALENCE IN FIELD SAMPLES

Published data concerning Ab prevalence obtained from serological surveys must be interpreted with caution for several reasons. Laboratory test validation is generally not stated and assumptions are often made concerning test performance and the specificity of results. Often, prevalence rates are predicted based on extremely small sample sizes and serum quality may be questionable. Serological data can consequently be misunderstood by investigators and readers. By determining the test accuracy in this investigation with well-characterized mAbs, quality survey information has been provided.

Ad5 seroprevalence. In the cELISA, a % inhibition cutoff for discriminating between positive and negative test results can be selected if the trade-off between test sensitivity and specificity is acceptable (Zweig and Campbell 1993). As the % inhibition cut-off is varied over the spectrum of possible "positive" and "negative" survey results, the test sensitivity and specificity move in opposite directions. The higher the cut-off, the lower the number of

"positive" raccoons, the lower the sensitivity. Since the assays described here were not used for diagnostic purposes, it was not critical to select one particular cut-off value. In fact, since Ad5 cELISA sensitivity and specificity was underestimated, it would be unfair to interpret survey results based on the single cut-off value determined by ROC analysis. By assuming the cut-off to be somewhere in between 8% and 20% inhibition, the proportion of "positive" raccoons was determined to be between 8-25%. All of these lacked Ad5 neutralizing activity but most bound a conformational Ad5 epitope by Western blot conducted under non-reducing conditions. Interestingly, this scenario was the same as that observed with experimental raccoon sera, where a lack of Ad5 neutralizing Ab but presence of other adenovirus Ab was demonstrated.

This proportion of "positive" field raccoons was greater than expected since human adenoviruses typically demonstrate a narrow host range and generally do not or weakly replicate in other species (Betts et al 1962; Prevec et al 1989; Oualikene et al 1994; Horwitz 1996). Although there have been no reports of human adenovirus Ab in wild animal populations, certain evidence exists that adenoviruses can cross natural species barriers. For example, experimental human adenovirus infections have been demonstrated in cotton rats and New Zealand rabbits (Tsai et al 1992; Gordon et al 1992; Prince et al 1993; Ginsberg and Prince 1994), mice and mouse cells (Duncan et al 1978; Prevec et al 1990; Ginsberg et al 1991; Graham and Prevec 1992; Fejer et al 1994; Ginsberg and Prince 1994; Yang et al 1996; Xiang et al 1996), guinea pigs (Faucon et al 1974), hamsters and rats (Horwitz 1996) and exposure to human adenovirus was suspected in a brown bear (*Ursus arctos*) in Croatia (Madic et al 1993). Serological studies of primates, including humans, imply that an interchange of organisms between humans and animals can readily occur, and human

adenovirus neutralizing Ab have been detected in chimpanzees (Kalter et al 1967). Despite close contact of dogs with humans, only a small fraction of dogs have demonstrated Ab to human adenoviruses, except in sera from tumor-bearing dogs where levels of Ad5 Ab were significantly higher (Sinha et al 1960; Carmichael and Barnes 1961; Carmichael and Barnes 1962; Lundgren et al 1969; Winters 1979). Serological studies must be interpreted with caution since cross-species adenovirus exposure may be more likely a function of cross-reacting Ab.

Upon speculation of adenovirus ecology and epidemiology, the existence of human adenoviruses in some urban animal species, such as the raccoon, can be contemplated. Human adenoviruses have been isolated from domestic sewage, treated primary and secondary effluents (Irving and Smith 1981; Payment et al 1983) and the consumption of shellfish from sewage-contaminated water has been associated with adenoviral gastroenteritis (Portnoy et al 1975; Gerba and Goyal 1978; Gill et al 1983; Yamashita et al 1992). Low levels of adenoviruses have also been detected in urban river water and drinking water (Nestor et al 1989; Tani et al 1995). Ad1, 2, 5, and 6 are the most common and endemic human adenoviruses with infection rates highest in children probably due to their persistent excretion in feces (Fox et al 1969; Brandt et al 1969; Fox et al 1977). It is quite possible that garbage containing contaminated diapers or other items handled by contaminated hands, sewage, water or shellfish represent sources of human adenovirus transmission to foraging raccoons, especially since these viruses may survive in the environment for an undefined period of time because of their relative stability at low and high temperatures and pH (Shenk 1996).

Wild mammals and birds may regularly visit or may even establish residence at

animal production facilities if possible, especially where food, water and shelter are available (Corn 1995). Daily activities, such as foraging, may bring raccoons into direct contact with livestock and poultry or with associated products, feed, water sources, urine, feces, bedding and litter, or even dead rodents or birds. Most animal adenoviruses are endemic in farm populations and are frequently transmitted through contact with contaminated urine, feces, water, saliva, or nasal secretions (Derbyshire 1989; Bürki 1990; Belak 1990; Lehmkuhl et al 1993; Lehmkuhl et al 1998) and perhaps through contact with carcasses contaminated with adenoviruses. Excreted animal adenoviruses have been shown to withstand inactivation in animal manure stored anaerobically for several weeks to months (Pesaro 1995). Adenovirus survival and transmission by these routes suggest that raccoons might represent aberrant hosts. It is also possible that an antigenically-related adenovirus has evolved separately in wildlife, becoming adapted to a specific wild host such as raccoons. All of this information implies that adenoviruses are probably widespread in nature. Hence we must consider the possibility that Ab detected in raccoon sera by cELISA represent cross-reacting heterologous antibodies to other human, animal, or avian adenovirus serotypes.

CAV-1 seroprevalence. Experimental infection of raccoons with CAV-1 has been demonstrated, indicating their relative permissiveness to CAV-1 infection (Green et al 1943; Bolin et al 1982; Sumner et al 1988). Also, several other serological surveys have detected CAV Ab in 6-16% of raccoons tested (Parker et al 1961; Jamison et al 1973; Bolin et al 1982; Sumner et al 1988; Hable et al 1992), which compares to the 2% CAV-1 Ab prevalence determined in this study. The pathogenicity of CAV-1 in dogs and foxes and the limited case reports of CAV-1 infection in non-domestic carnivores suggest that mortality is high (Grate et al 1987). This suggests low survivorship and hence the low CAV-1 Ab

prevalence determined in survey raccoons. This may also explain the lack of CAV-1 Ab in experimental foxes and raccoons. Approximately one third of "positive" raccoons, representing those with the highest cELISA titers, also had CAV-1 neutralizing activity and bound conformational CAV-1 epitopes in a Western blot conducted under non-reducing conditions. Ab detection to CAV-1 in three different assays strongly suggests that these raccoons have previously been infected with a canine adenovirus and that the Ab detected in this study is specific to this exposure. Since the sera which are positive in all three assays have the highest cELISA titers, the more accurate % inhibition cut-off may closer to 30% inhibition than 17% inhibition. Sera with titers close to the 17% inhibition cut-off that exhibit no or low neutralizing activity may, therefore, represent false positive cELISA results. Otherwise, these samples may contain low-level or low-affinity CAV-1 or CAV-2 Ab, or cross-reacting Ab produced from exposure to another adenovirus.

LIMITATIONS TO SURVEY RESULTS

Extrapolating these serological findings to the target population involves a certain element of uncertainty. Results are limited by the sample under study, which included only raccoons, and the time frame used for sampling. More information might be gathered if the survey could be extended to other vaccine target species (i.e. foxes and skunks), geographic regions, and over several years. The interpretation of survey results is also limited by the extent of the mAb cross-reactivity studies. There are many more mammalian and avian adenovirus serotypes which must be tested for cross-reactive epitopes in both neutralization assays and by indirect IF in order to more precisely define what we are measuring in the cELISA. Although Ab evidence usually indicates exposure to a particular organism, no definitive conclusions concerning viral prevalence in a sample population can be drawn

unless agents are actually isolated and identified.

UNRESOLVED ISSUES

There are still many questions concerning issues related to the oral immunization of wildlife with a recombinant vaccine that require further investigation. We do not fully understand how or why different wildlife species respond differently to the vaccine or what changes in host specificity or pathogenicity may have occurred after virus vector construction. Alteration in the E3 gene region during vector construction may contribute to host specificity, and variation in this region specifically in different human and animal gene sequences has been implicated in differences in serotype pathogenicity (Linne 1992; Bailey and Mautner 1994; Khatri 1997). Nor have the sites of virus vector infection, rabies antigen expression and presentation, or the full spectrum of immune response mechanisms involved been studied in any detail.

There are also many questions concerning issues related to adenovirus epidemiology in wildlife. Since mortality and morbidity data are for the most part unavailable and case reports are infrequent, it is difficult to assess the pathogenicity of wildlife adenoviruses. Seroprevalence surveys of wildlife population samples can help measure exposure to adenoviruses, but the information gathered in this study alludes to a more complicated picture. Adenovirus epidemiology in wildlife will always be very difficult to understand if it is based on serology alone. Virus isolations are, therefore, needed to provide insight into the role these organisms play in nature.

CONCLUSIONS

In this investigation, the process of developing and statistically validating two competitive ELISAs for detecting Ad5 and CAV-1 Ab was described. During the process

of Ad5 cELISA development, it was discovered that an animal's serum Ab response to Ad5 capsid components depends on the route of Ad5RG immunization. Specifically, animals given Ad5RG orally did not form a serum Ab response to Ad5 neutralization-associated fiber epitopes, whereas animals given the vaccine intra-muscularly did. The reasons why this phenomenon occurs are inconclusive; however, differences in the immune response after the mucosal and systemic introduction of Ag are certainly evident and have previously been documented.

This observation led to the selection of an Ad5 hexon mAb for use in the cELISA which measured non-neutralizing Ab. Despite the fact that the Ad5 PRNT measured neutralizing Ab, the Ad5 cELISA demonstrated good performance, with a single number of 0.84 in the ROC analysis representing accuracy. Likewise, the CAV-1 cELISA demonstrated very good performance using a neutralizing CAV-1 mAb, with a single number of 0.94 in the ROC analysis representing accuracy. These cELISAs can be applied to the future surveillance of adenoviruses in wildlife since they have demonstrated their abilities to discriminate between adenovirus Ab-positive and negative populations in multiple animal species. Results generated from cELISAs and other serological assays incorporating mAbs must, however, be interpreted with caution due to the limited ability of each mAb to recognize one specific epitope. Ab detected in an animal's serum does not always indicate prior exposure to a particular virus, but does indicate prior exposure to a specific epitope present on that virus and perhaps, others.

Using the Ad5 cELISA and PRNT to measure serum Ab responses to Ad5RG vaccination in experimental animals, it was observed that different species as well as individual animals within species can respond very differently to vaccination. Most notedly,

experimental raccoons form a very strong and consistent rabies serum neutralizing Ab response in light of a comparatively minute Ad5 serum neutralizing Ab response. The possibility of a cross-reactive memory response in these raccoons to one specific epitope present on Ad5, and possibly on other adenoviruses, cannot be ignored. These findings also suggest that a plaque reduction serum neutralization test is more narrowly specific than a cELISA employing cross-reactive mAbs, such as the human adenovirus cELISA, and may not be fit for validating these kinds of assays. Some of the differences between tests applied in this study are summarized in Table 9.

Upon practical application of cELISAs to a large, representative sample from the field, it was determined that approximately 8-25% of field raccoons had Ab against an epitope present on Ad5. These results were confirmed by Western Blot; however, these sera were unable to neutralize Ad5, suggesting that raccoons have not been exposed to this serotype. These findings do suggest, however, that raccoons may become infected in nature by other human or mammalian adenoviruses which share an epitope with Ad5. This study represents the first suggestion that raccoons may become exposed to adenoviruses other than those which typically infect dogs and foxes.

Approximately 2% of field raccoons had Ab against a canine adenovirus epitope. Approximately one third of these results were confirmed by Western Blot and neutralization of CAV-1, indicating that these animals have in fact been exposed to a canine adenovirus in nature. Other investigators have likewise reported the occurrence of canine adenoviruses in raccoons.

From these results, adenovirus seroprevalence can be extrapolated to the population to be targeted for oral rabies immunization, fulfilling recommendations made by the WHO

Table 9. Summary of differences between tests applied in this study.

ANTIGEN	CRITERIA	DIFFERENCES BETWEEN TESTS		
		PRNT	cELISA	WESTERN BLOT (confirmatory)
Ad5	SPECIFICITY OF Ab DETECTED	neutralizing Ad5	non-neutralizing Ad1-30, possibly others	neutralizing or non-neutralizing Ad1-30, possibly others
	SPECIES THAT Ab DETECTED IN	lab skunks lab foxes no no	lab skunks lab foxes lab raccoons field raccoons	nd nd lab raccoons field raccoons
CAV-1	SPECIFICITY OF Ab DETECTED	neutralizing CAV-1, 2	neutralizing CAV-1, 2, possibly others	neutralizing or non-neutralizing CAV-1, 2, possibly others
	SPECIES THAT Ab DETECTED IN	lab skunks dogs field raccoons	lab skunks dogs field raccoons	nd nd field raccoons
Ad5 CAV-1	LIMITATIONS	narrowly specific non-specific effects	cross-reactions epitope-dependent affinity-dependent	cross-reactions non-specific effects not optimized or validated

and other veterinary biologics advisory groups. Reports such as these are also important for political reasons and may be of use in the future, when issues concerning the impact of modified live virus vaccines on the environment will need responding to.

Presently, one of the biggest obstacles to successful gene therapy using adenovirus vectors is the pre-existing immunity to adenoviruses in many patients, allowing only transient expression of the foreign gene insert. Likewise, natural pre-existing immunity to adenoviruses in wildlife may also be a barrier to oral immunization of wildlife against rabies. The adenovirus prevalences demonstrated here will not likely impact the planned usage of Ad5RG; however, studies are still required to measure the effect of adenovirus Ab on rabies immunization via Ad5RG. Preliminary statistical analyses on experimental data describing rabies and CAV-1 neutralizing Ab titers in skunks indicate that the presence of CAV-1 neutralizing Ab does not inhibit the production of rabies glycoprotein neutralizing Ab.

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Appendix 1. Sample calculation for determining neutralizing Ab titer using method of Reed-Muench

EXPERIMENT NUMBER	SERUM SAMPLE	SERUM DILUTION	A VIRUS CONTROL COUNT[a]	B PLAQUE COUNT[a]	C "NON-PLAQUE" COUNT[a] A - B	D CUMULATIVE SUM DOWN B	E CUMULATIVE SUM UP C	PROPORTION OF PLAQUES RELATIVE TO A D/(D+E) X 100%
FOX 56	1273R	1:10	79	0	79	0	222	0%
	DAY 60	1:100		2	77	2	143	1%
	(90/1/26)	1:1000		15	64	17	66	**20%**[b]
		1:10 000		77	2	94	2	**98%**[b]

SAMPLE CALCULATION:

LOG 50% ENDPOINT = [(50% - 20%)/(98%-20%) x LOG 10] + LOG 1000
= (30/78 X 1) + 3
= 3.38

50% ENDPOINT = 2424

Therefore, a serum dilution of 1:2424 (or reciprocal 2424) is required to neutralize 50% of viral PFU in virus-serum mixture and the titer is expressed as the reciprocal (i.e. 2424)

[a] virus control counts and plaque counts represent and average of three independent results

[b] 50% endpoint (where 50% virus in virus-serum mixture is neutralized) falls somewhere between 20% and 98% and, therefore, between 1:1000 and 1:10 000 serum dilution

3851 Fallowfield Rd.
Nepean, Ontario, Canada
K2H 8P9
Tel: (613) 228-6698 Fax: (613) 228-6664

December 14, 1999

Ms. Laura Maxwell,
Rabies Centre of Expertise
ADRI-CPQP
3851 Fallowfield Rd.
Nepean, ON K2H 8P9

SUBJECT: Permission for use of Adenovirus figure

Dear Laura:

As discussed during one of our conversations in the fall of 1998, I have granted you permission to use my figure (Cross-section representation of an adenovirus particle; Ph.D. Thesis, 1994) in your M.Sc. thesis entitled "Development and Validation of Two Competitive ELISAs for detecting Human and Canine Adenovirus Antibody in Ontario Wildlife Sera".

Congratulations on the successful defence of your work and best of luck in your future endeavours.

Sincerely,

Oksana K. Yarosh, M.Sc. Ph.D.