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VIRUS DISEASES IN LABORATORY AND CAPTIVE ANIMALS

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PREFACE

The influence of basic science, particularly molecular biology, in human and veterinary medicine revolutionized thinking in many aspects and changed fundamentally and creatively the classical strategy for research and prevention of infectious diseases.

Genetic engineering and related disciplines have progressed to a remarkable degree over the last decade and now form the keystone supporting medicine. These are strong and efficient instruments for health and disease oriented research and their application gives the opportunity to receive more answers and not only more questions.

The prime objective of this book is to create new knowledge within the medical disciplines and inspire colleagues working in this field with the unity and unambiguous importance of this science and its technologies for identifying, clarifying and planning new strategies for curing and preventing disease.

This book contains original studies on the molecular biology of animal viruses. Some of the viruses discussed in this book are also hazardous to man. In this light it can be considered as a contribution to modern education on the human infectious diseases. From this point of view the book contains a chapter on Hantaan virus that causes no detectable disease in animals but hemorrhagic fever with renal syndrome has been attributed to infection of humans by this virus.

The book is addressed primarily to professional investigators, though I hope that junior and senior scientists who seek to know the actual progress in virology may also find it of interest. The study of this book will give the reader who is interested in understanding the molecular aspects and mechanisms of infectious viral diseases, a new tool for providing a clear perspective of the technology for search of the viral pathogen. Each chapter endeavoured to present concise data of those aspects of most interest to the scientist and key literature references are provided for those who wish to read further. There are some new topics that should be considered as pilot technology but their implications are not yet realized and need more investigations to be developed further.

I wish to thank all the contributors of this volume. I have the privilege of particularly thanking my friend Professor Yechiel Becker who gave me the opportunity to edit this volume in his series ''Developments in veterinary virology''. To my regret I did not receive two chapters which were promised. The assistance of Julia Hadar for the correction of the English text of the manuscripts, the valuable help of Jurgen Scholz and Paul Schnitzler for indexing the manuscripts, and the excellent secretarial help for careful retyping of some of the manuscripts by Barbara Holder are much appreciated.

Gholamreza Darai

VIRUS DISEASES IN LABORATORY AND CAPTIVE ANIMALS

1

YABA VIRUS

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ABSTRACT

Yaba virus causes a disease in nonhuman primates which is characterized by formation of tumors that regress slowly; the animals are then susceptible to reinfection. The virus is an unclassified member of the poxvirus family. The molecular weight of the viral DNA is 119 X 10^6 daltons. Assay is by foci or plaque formation. The virus grows slowly in tissue culture--the minimum replicative cycle is from 35 to 60 hours depending upon the cell line and the growth temperature. The virus replicates in the host cytoplasm, but viral DNA is present in the host cell nucleus late in the infection cycle. Virus infection does not inhibit host protein synthesis. Yaba virus initiates transformation of monkey kidney cells. Lipid granules accumulate in infected cells and in transformed cells.

INTRODUCTION

Yaba disease was first observed in 1958 as an outbreak of subcutaneous tumors in captive rhesus monkeys (*Macaca mulatta*) housed in open-air pends in Yaba, Nigeria (1). The disease spread to 20 of the 35 monkeys in the colony within a few weeks. One baboon (*Papio papio*) also became infected. The disease was confined to Asiatic monkeys.

Lesions could be produced in rhesus monkeys by injecting of extracts from lesions produced on infected animals. However, one species of African monkeys tested (*Cercopithecus aethiops tantalus*) became infected with the virus. The lesions consisted of large, pleomorphic cells, some of which contained intracytoplasmic, eosinophilic inclusions beneath the squamous epithelium in the dermis and subcutaneous tissues.

Andrewes, *et al*. (2) studied tumor material from infected monkeys in the Nigerian colony and found that the disease could be transmitted to rhesus monkeys by cell-free filtrates. Electron microscopy revealed

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a virus measuring 250 - 280 Mμ in the long axes, which morphologically resembled poxvirus particles.

The disease was transmitted by injection into Macaca irus and Cercopithecus aethiops tantalus monkeys but the lesions remained flat and regressed in a few weeks. Attempts to transmit the disease to other species of African monkeys were unsuccessful. A New World monkey (Cebus apella) was also not susceptible to subcutaneous injection of the virus.

Ambrus et al. (3) reported a spontaneous outbreak of Yaba disease in 11 monkeys housed in the Roswell Park Memorial Institute, Buffalo, New York. Infected species included M. mulatta, M. irus, and M. speciosa. Most tumors occurred on hairless areas of the face, on palms and interdigital areas, and on the mucosal surfaces of the nostrils, sinuses, lips and palate. These workers suspected that insect vectors transmitted the disease. Thus, the colony was sprayed with insecticides, cleaned daily, and sterilized weekly. Strict isolation was enforced. The spread of the disease was controlled by these methods.

VIRUS-CELL INTERACTIONS

Histology. Characterization of Yaba virus-cell interactions was begun by Niven et al. (4). The virus does not grow in mice, nor on the chorioallantoic membrane of embryonated chicken eggs, nor in rabbits. Monkeys inoculated with Yaba virus develop tumors after 5 days which grow to 25 to 45 mm in diameter and project up to 25 mm in diameter. Tumor growth proceeds steadily, reaching a maximum in six week, after which regression occurs and is completed by 12 weeks after inoculation (4).

The histological studies of tumors induced by Yaba virus show the transformation of fibrocytes of the dermis and subcutaneous cells to pleomorphic polygonal cells (4). The virus-induced alterations of tumor cells are characterized by the appearance of multinucleated cells, cytoplasmic granulation, nuclear enlargement, nucleolar hypertrophy, and the formation of numerous lipid vacuoles in the cytoplasm. The major alterations are observed in the cytoplasm, especially in the region around the nuclear membrane. The granular inclusions within the cytoplasm stain positively for DNA with acridine orange indicating that Yaba

virus contains DNA (5). Infected cells lose their normal orientation and form a regular mosaic with the intercellular spaces decreasing, leading to direct cell-to-cell contact. This suggests that viral transfer is directly from cell to cell, since no intercellular spaces eventually remain (4).

The pathogenesis of Yaba virus-induced tumors has been further characterized by Sproul _et al_. (5). These studies indicate that the histiocyte is the cell giving rise to tumor. In monkeys inoculated with Yaba virus, histiocytes migrate into the infected area by 48 hours post-inoculation. After three to five days, the histiocytes undergo striking morphologic alterations and proliferate rapidly leading to tumor formation. Mitotic activity is evident in the tumor. The nuclei of tumor cells are larger than normal, and chromatin material moves to the periphery of the nucleus. The nucleoli increase in size and number. Multinucleated cells with many cytoplasmic inclusions and globules of neutral lipids are common characteristics of tumor cells. Regression is an individual cell phenomenon, beginning while proliferation is still active, but becoming more prominent over a period of two to three months. There is no evidence that the tumor cells migrate (5). Intravenous inoculation of virus is followed by the appearance of many tumors in the heart, lungs, muscles, and subcutaneous tissues of the monkeys. No tumors develop when virus is injected subcutaneously into rats, guinea pigs, and rabbits (5).

Immunology. Circulating neutralizing antibody is ineffective in preventing growth of the established tumors (5). However, immunity to superinfection is present when tumors are present or regressing, but after total regression, reinfection results in new tumor formation (6).

Yohn _et al_. (7) demonstrated several antigenically distinct complement-fixing Yaba virus antigens both in subcutaneous tumor extracts and infected CV-1 cell cultures. Wolfe _et al_. (8) induced pulmonary and nasal tumors experimentally by aerosol transmission of Yaba virus. Wolfe _et al_. (9) reported comparable complement-fixing antibody titers with subcutaneous tumor and lung tumor extracts.

A convalescent-phase antibody distinct from the complement-fixing antibody detected during the clinical stages of infection was demonstrated by Hall _et al_. (10). Complement-fixing antibody was detected up to 35 weeks post-infection. The synthesis of Yaba virus-induced antigens has

been examined by immunodiffusion technique (11). Analysis of Yaba virions and virus induced tumor extracts has resulted in the detection of 9 structural and 17 nonstructural virus antigens (11).

Immunological studies of Yaba virus indicate no serological relationship to vaccinia, or monkeypox virus (4; 12). Based on these studies, Yaba virus is considered an unclassified member of the poxvirus family (13). Although Yaba virus is serologically distinct from orthopoxviruses, a host-dependent conditional lethal mutant of vaccinia virus strain can be resuced to form plaques in nonpermissive cell lines co-infected with Yaba virus (14).

Quantitative Assay. Yaba virus has a narrow host range in tissue culture. Prolonged replication cycle and low virus yield have been the major drawbacks in research with Yaba virus. Human embryonic kidney cells and continuous lines of cercopithecus monkey kidney cells (BSC-1, CV-1) are susceptible to Yaba virus infection (7; 15; 16; 17). Tsuchiya *et al*. (18) examined several cell lines for Yaba virus propagation. The lower passages of cynomolgus monkey kidney cell line, designated Jinet (19), are highly susceptible to Yaba virus but HeLa cells, rabbit kidney cells and chicken embryo fibroblasts are not (18).

The establishment of tissue culture cells susceptible to Yaba virus infection has greatly facilitated the study of virus-cell interactions as well as the propagation of the virus for physical and biochemical analysis. Quantitative assay of Yaba virus has been reported by different investigators. Focus formation (18; 20), and plaque assay (21) have been described. Confluent BSC-1 cells are infected with Yaba virus, incubated at 35°C, and focus formation units (FFU) are counted after two to three weeks postinfection (20).

Tsuchiya and Rouhandeh (21) described the Yaba virus plaque assay method under agar overlay medium with $MgCl_2$. Jinet cells are infected with Yaba virus and incubated at 35°C. After seven days, the maintenance medium is replaced with an agar overlay medium. Five days after the first overlayering, a second overlay medium is added. Two days after the second overlay, $MgCl_2$ solution (final concentration of 2.5 M) is added and cultures are reincubated for one day. Infected cells are killed with $MgCl_2$, resulting in the formation of plaques which are counted after staining with neutral red (21).

Focus or microtumor formation induced by Yaba virus in BSC-1 cells has been described (7; 16). The morphology of the foci is essentially of two types. In areas of low cell density, contact inhibition is decreased and the cells pile up, forming dense microtumors. The morphological alterations of infected cells are characterized by cytoplasmic inclusion bodies and accumulation of lipid granules in the cytoplasm of infected cells (7; 16; 20). Kato et al. (22) examined the inclusion bodies seen in the infected cell cytoplasm and found them to be B-type inclusions, that is, the site of viral DNA and protein synthesis.

Milo and Yohn (23) reported that the formation of foci in CV-1 cells following infection with Yaba virus is dependent upon cell passage level, temperature of incubation, and calcium concentration in the medium. They have also suggested that the type of cellular migration by uninfected cells into a foci is probably analogous to the in vivo migration of histiocytes (5), and that the migration of adjacent cells into a focus to form microaggregates is stimulated by a chemotactic factor. This factor has been isolated from the membranes of infected cells and tentatively identified as a glycoprotein (23).

Rouhandeh and Richards (24) showed that CV-1 cells which are productively infected with Yaba virus clearly exhibit plasma membrane alterations when treated with concanavalin A, a plant lectin capable of binding specifically to carbohyudrate-containing molecules. The marked alterations in cellular proteins and membrane-associated enzyme patterns of infected cells (23) suggest that structural alterations in membranes of Yaba virus-infected cells probably occur. These results indicate that focus formation can occur after alterations in the structure of the cystoplasmic membranes (23).

The relationship of physical particles of Yaba virus to infectivity has been studied (25). In lower passages of Jinet cells one focus-forming unit corresponds to 500 to 800 physical particles.

Morphogenesis. Yaba virus morphogenesis in tumor cells and infected tissue culture cells has been investigated by electron microscopy. Within three hours after infection, the adsorption and phagocytosis of virus particles by the cells are seen (26). This is followed by the disruption of phagocytic vacuole membrane, with the release of viral DNA into the cytoplasm. Twenty-four hours postinfection, large

cytoplasmic inclusions termed "factories" are observed (26; 27; 28). A typical factory contains a large number of viral particles, particulate glycogen, DNA-containing electron dense material, and small membranous spherical structures (40 nm in diameters), designated as "micelles."

The morphogenesis of Yaba virus has been divided into six stages, representing the steps in maturation of the virus (27). The first stage is the formation of short crescent or arc-shaped structures composed of a typical membrane bilayer. The micelles are frequently connected to the arc structures, suggesting that concurrent assembly of the micelles and surface subunits is necessary to form the surface layer of the immature viral particles and to regulate a constant curvature of the particle (28). In the second stage, the membrane is formed into a complete spheroid particle. In the third stage, viral particles contain DNA-containing electron dense material. By stage four, the immature viral particles are formed and assembly of new membrane structures appear inside the viral particles. In stage five, internal structures, such as the core and lateral bodies, are recognizable. The sixth stage represents the addition of an external membrane and formation of the mature virion (27).

Rouhandeh _et al_. (29) studied the morphogenesis of the virus in high-passage cell lines where it grows more slowly. During the early stages, membranous structures called "arcs" and micelles are formed. The arc structures become elongated to form incomplete immature viral particles. During the formation of the immature viral particles (Fig. 1 stages 2 to 5), electron dense material containing DNA becomes incorporated inside the viral particle. In the next several stages micelles migrate inside the viral particle, the immature viral particle is sealed, and the formation of the internal structures begins (Fig. 1, stages 6 to 9). A rectangular membrane viral core is formed (Fig. 1, stage 10). The transition of the viral core into a dumbell-shaped form is seen in Fig. 1, stage 11. Lastly, an additional external membrane is wrapped around the viral particle forming the mature virion (27). The outer surface consists of thread-like structures, called tubules (30). The tubules (about 10 μm thick) are seen in association with the viral particles in infected cells (Fig. 2c).

Physical characteristics of viral DNA. Yaba virus DNA has a density of 1.6905 in CsCl and its Tm value is 0.015 M citrate in saline is

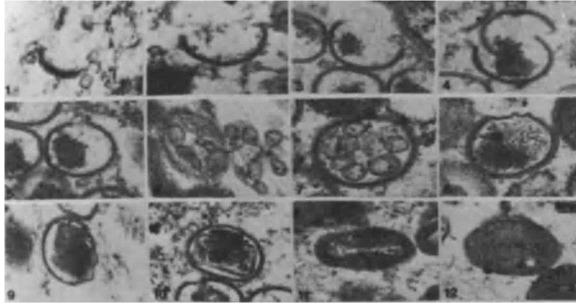

Fig. 1. Sequential development of Yaba virus morphogenesis detected in Yaba virus-infected cells. (The bar is (12) is equal to 83 nm.)

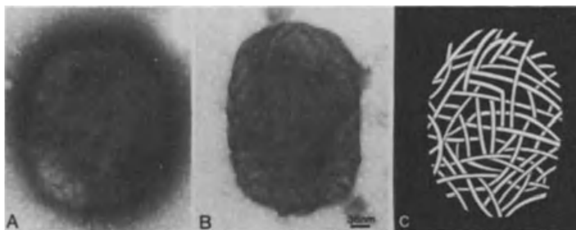

Fig. 2. Surface structure of Yaba virion consisting of thread-like structures which are randomly formed around the viral particle (B and C). A shows one ofthe extremities of the Yaba virion. Virions were negatively stained with 1% phosphotungstic acid and observed under an electron microscope. Magnification: A, X 21,000; B, X 36,000. The bar in B is equal to 36 nm.

82.3°C (31). The guanine plus cytosine content is estimated to be 32.5 ± 0.5% (31), a value 2 to 3% less than that of DNA from vaccinia virus (32). Rouhandeh (33) determined the molecular weight of Yaba virus DNA to be 119 x 10^6 daltons.

Virus synthesis. Yaba virus synthesis is BSC-1 cells has been studied by various histochemical techniques (7). The first evidence of infection is detected at 24 hours when nucleoli become hypertrophic, reflecting enhanced RNA synthesis. At 36 hours, DNA synthesis is detected in the cytoplasm. This is followed by the localization of Yaba virus antigens and enhanced DNA synthesis in the cytoplasm. The time required to complete the synthetic cycle from time of infection to production of infectious progeny virus is estimated to be 60 hours.

The growth kinetics of Yaba virus in CV-1 cells has been followed by Yohn et al. (34). At 35°C, the minimum replicative cycle is 35 hours; however, maximum virus yields are not obtained until 75 hours postinfection. Cytoplasmic viral DNA synthesis is detected three hours postinfection, preceded by synthesis of virus induced antigens. Synthesis of at least two virus structural antigens occurs in the presence of a DNA inhibitor, cytosine arabinofuranosyl, indicating potential transcription and translation of these antigens from parental DNA. The first progeny DNA is completed after 20 hours postinfection, but is not detected in infectious form until 35 hours postinfection. The maximum rate of progeny DNA synthesis occurs between 20 and 30 hours postinfection. Viral DNA synthesis continues 45 to 50 hours after infection.

Nucleic acid synthesis. The synthesis of Yaba virus nucleic acid in Jinet cells has been investigated (35), with particular emphasis on Yaba virus-specific RNA production. DNA is detected three hours after infection. Six hours postinfection, 7 to 10 S RNA is detected and this is present in greater amounts after 12 hours. Twenty-four hours after infection 14 to 15 S RNA, as well as 7 to 10 S RNA, are detected. The first and largest peak of mRNA synthesis occurs between 11 and 12 hours postinfection and a second, slightly smaller peak occurs between 21 and 23 hours after infection (35).

Taylor and Rouhandeh (36) reported the presence of Yaba virus specific DNA in the host cell nucleus. These studies show that Yaba virus-specific DNA is present in the host cell nucleus late in the infection cycle.

Protein synthesis. Analysis of Yaba virus structural proteins by polyacrylamide gel electrophoresis has resulted in the detection of 37 protein bands, of which 21 are core-associated (37). The molecular weights of these proteins range from 10,000 to 220,000. Three non-core proteins are labeled when Yaba virus is grown in the presence of ^{14}-C-glucosamine. Four enzyme activities have been identified with purified Yaba virion: deoxyribonuclease with pH optima at 5.0; deoxyribonuclease with pH optima at 7.8; RNA polymerase; and nucelotide phosphohydrolase (38).

Yaba virus-infection does not inhibit host protein synthesis (39). Two dimensional gel electrophoresis of Yaba virus and monkey poxvirus-infected cells reveals that while 60% of host protein synthesis is reduced by monkey pox virus infection, the majority (95%) of cell proteins are synthesized as long as three days post infection in Yaba virus-infected cells, and the synthesis of certain host protein appears to be increased after infection, as shown in Fig. 3.

Yaba virus induced proteins in infected cells are also synthesized at different times after infection and can be grouped into two classes, early and late. Early proteins are synthesized before the onset of viral DNA replication which begins at three hours post infection. Some of the proteins in this group are structural and continue to be synthesized in the presence of a DNA inhibitor (40). Late Yaba virus proteins are detected at six hours post-infection and continue to increase in number during the infection period.

Transformation. The ability of Yaba virus to transform cells has been shown by Rouhandeh and Vafai (41). (See Fig. 4.) Monkey kidney cells are morphologically transformed in vivo with uv-irradiated Yaba tumor pox virus. Cell lines established are virus nonproducers and exhibit biological characteristics typical of transformed cells. These characteristics include increased saturation density, reduced serum requirements for growth, and ability to grow in soft agar. The morphological alterations of transformed cells are similar to Yaba virus-induced tumor cells and are characterized by loss of contact inhibition, multinucleated cells, and cytoplasmic lipid droplets. Southern blot hybridization reveals that sequences homologous to low-molecular-weight viral DNA (5.1, 4.8, 3.9 kbp) are present in the transformed cells (41). (See Fig. 5.) Yaba virus-specific antigens detected by

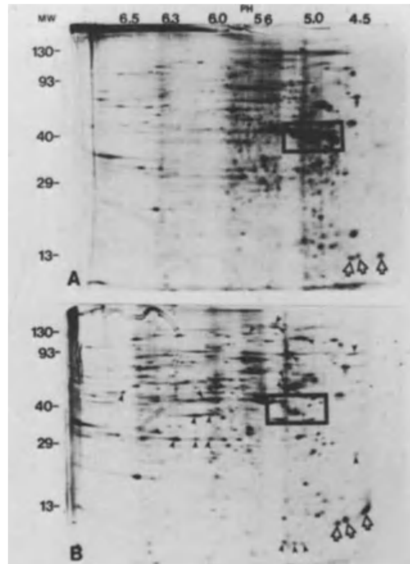

Fig. 3. Autoradiograms of two-dimensional analysis of (A) uninfected cells, (B) Yaba virus-infected cells. Gels containing app. 300,000 ct/min were exposed for 30 days. Open arrows represent the same host proteins in (A) or (B). Solid arrows represent virus-specific proteins. Numbers on left of the gels are mol. wt. x 10^{-3}.

immunofluorescence assays are found in the cytoplasm of transformed cells. (See Fig. 6.) Four virus-specific proteins, with molecular weights of 160,000, 140,000, 107,000, and 74,000 daltons, are contained in transformed cells immunoprecipitated with sera from tumor bearing monkeys. (See Fig. 7.)

Lipid accumulation. The accumulation of a large number of lipid droplets in the cytoplasm of Yaba virus-infected cells is of interest because of the diseases associated with localized accumulations of fat. Globules of neutral fat are common within tumors induced by Yaba virus (5; 6). Lipid granules accumulate as infection progresses and the presence of cytosine arabinofuranoside during infection does not stop the production of the granules (39). This suggests that if the virus is responsible for the stimulation and/or control of these lipids, it

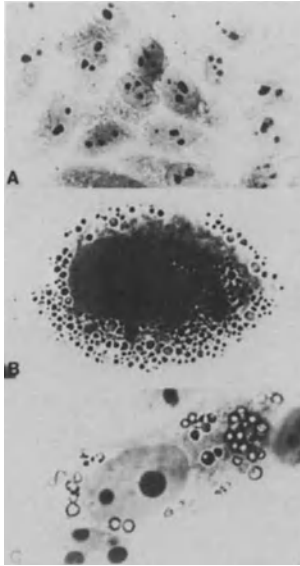

Fig. 4. Photomicrographs of (A) normal cells, (B) Yaba virus-infected cells, and (C) cells transformed with virus exposed to UV irradiation for 1 min. Magnifications X400, X950, and X950, respectively.

is an "early" function of the virus genome. The accumulation of lipid droplets is also observed in cells transformed by Yaba virus. (Fig. 4.)

Cloning and physical mapping of viral DNA. The physical map positions for the BamHI, EcoRI, and SalI restriction fragments of Yaba monkey tumor poxvirus DNA were determined using cloned virus DNA fragments as probes for hybridization as well as for analyzing the secondary digests of larger DNA restriction fragments. Digests of EcoRI A and B fragments and SalI A and B fragments with BamHI allowed for the orientation of most of the BamHI restriction map. These secondary digest products were confirmed and the map positions for the EcoRI fragments were established using cloned BamHI fragments. Fig. 8 shows recombinant plasmid containing PstI inserts, HindIII inserts, and BamHI inserts analyzed by cleaving the extracted plasmid DNA with PstI, HindIII, and BamHI restriction enzyme, respectively and running the samples with native

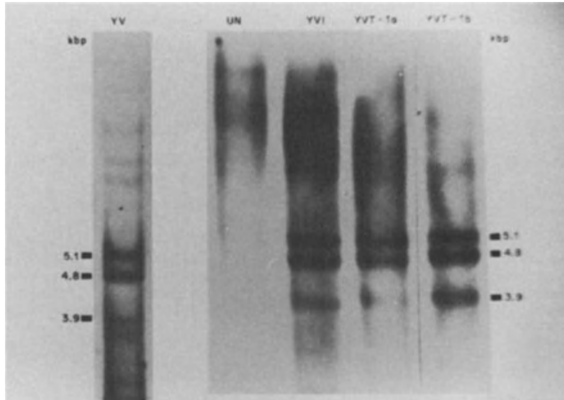

Fig. 5. Southern blot analysis of Yaba virus DNA from cells transformed with uv-irradiated virus. YV, Yaba virus; UN, uninfected cells; YVI, Yaba virus-infected cells; YVT-la and YVT-lb, two separate clones derived from cells exposed to uv-irradiation for 1 min.

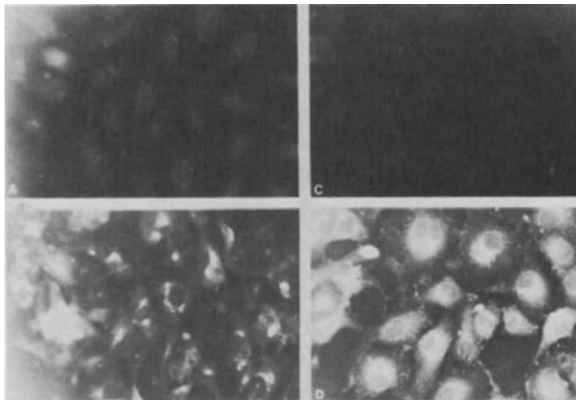

Fig. 6. Immunofluorescence photomicrographs of (A) normal JINET cells reacted with monkey anti-Yaba virus serum, (B) Yaba virus-infected cells reacted with monkey anti-Yaba virus serum (C) transformed cells reacted with normal monkey serum, and (D) transformed cells reacted with monkey anti-Yaba virus serum.

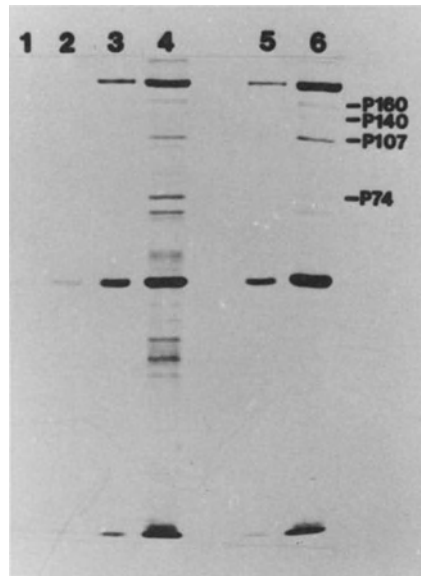

Fig. 7. Immunoprecipitation of virus-specific proteins of Yaba virus-transformed cells. Lanes 1, 3, and 5, normal, virus-infected, and cells transformed with virus that had been exposed to uv-irradiation for 1 min, immunoprecipitated with normal monkey serum. Lanes 2, 4, and 6 normal, virus-infected and transformed cells immunoprecipitated with serum from tumor-bearing monkey.

Yaba DNA cleaved with the same enzymes as above on a 0.7% agarose gel. Fig. 8 (C) shows the physical map locations for BamHI, EcoRI, and SalI determined using the information obtained from the hybridization of BamHI cloned fragments of EcoRI and SalI fragments Secondary digests of larger EcoRI and SalI fragments with BamHI were used to identify the positions for most of the BamHI fragments (42).

ACKNOWLEDGEMENT

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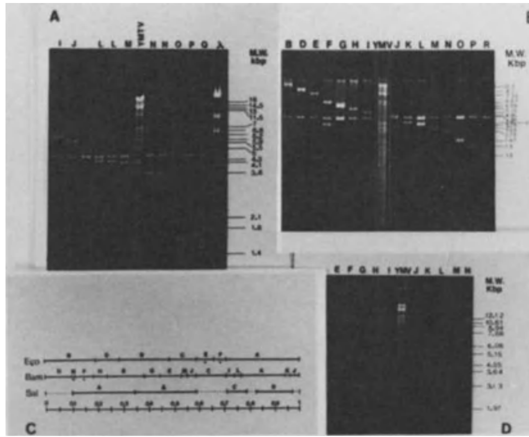

Fig. 8. Recombinant plasmid containing (A) PstI inserts, (B) HindIII inserts, (D) BamHI inserted were analyzed by cleaving the extracted plasmid DNA with respective restriction enzymes and running samples with Yaba DNA cleaved with the same enzymes on a 0.7% agarose gel, (C) Physical map locations for BamHI, EcoRI and SalI. Black dots indicate fragments whose position may change relative to one another. Broken lines indicate areas of the SalI map which could not be identified by hybridization.

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2

POXVIRUS INFECTIONS IN DOMESTIC ANIMALS

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ABSTRACT

Poxviruses are relatively unimportant causes of infection in domestic animals and progress in their study has been somewhat slow. Recent progress in the laboratory characterization of capripoxviruses and parapoxviruses, and the epidemiology and control of the infections they cause is reviewed. Attention is drawn to the uncertain epidemiology of cowpox and the recent recognition of infection in the domestic cat. The use of vaccinia virus as a vector for veterinary vaccines is also briefly discussed.

INTRODUCTION

Compared to other virus infections of domestic animals those caused by poxviruses are, with the exception of those caused by capripoxviruses, relatively unimportant. Attention has usually focussed on human smallpox, and vaccinia virus has usually been studied as a 'representative' poxvirus. In consequence smallpox has been eradicated and a vast amount of data has been collected on the structure and replication of vaccinia virus. In contrast progress on the pox diseases of veterinary importance, and in particular the development of safe, effective vaccines has been relatively slow (1). The techniques which have proved useful for the study of smallpox and vaccinia viruses are being applied to other animal poxviruses, but the fact that capripoxviruses and parapoxviruses do not infect small laboratory animals is a serious drawback.

Those poxviruses known to infect domestic animals are listed in Table 1; information on those which affect other animals will be found in Chapters 1, 2, 4-6. This chapter focusses attention on data collected during the last 10 years or so on infections caused by capripoxviruses, parapoxviruses and orthopoxviruses. Earlier work is not discussed but

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reference to it will be found in the papers cited. More general information on the replication and structure etc. of poxviruses can be found in recent reviews (2-4).

Poxvirus infections are characterized by the production of skin lesions. Some viruses, e.g. sheeppox, camelpox and swinepox produce generalized infection whereas others e.g. orf and pseudocowpox, produce localized infections. With some, e.g. cowpox virus, the infection may be localized in one host species (e.g. cattle) but generalized in another (e.g. domestic cats). Localized infections are usually transmitted by implantation of virus into the skin, either by virus entering broken or traumatized skin, or by inoculation of intact skin. Generalized infections may be transmitted by aerosol over short distances, by direct contact, or by fomites including insects.

Table 1. Poxviruses pathogenic for domestic animals.*

Genus	Virus	Animal hosts	Human infection
Orthopoxvirus	Buffalopox	Buffalo**	+
	Camelpox	Camel	-?
	Cowpox	Felines, cows**	+
Parapoxvirus	Orf	Sheep, goats	+
	Pseudocowpox	Cattle	+
	Papular stomatitis	Cattle	+
	Ausdyk	Camels	?
Capripoxvirus	Sheeppox	Sheep	-?
	Goatpox	Goats	-?
	Lumpy skin disease	Cattle**	-?
Avipoxvirus	Fowlpox	Fowls	-
Suipoxvirus	Swinepox	Pigs	-

*Poxviruses causing infection in other hosts are dealt with in other chapters.
**The reservoir hosts of these viruses are not known.

THE IMPORTANCE OF ANIMAL POXVIRUS INFECTIONS

Sheeppox, goatpox, lumpy skin disease, camelpox.

These infections are important in their own right, particularly in developing countries and communities whose agriculture is dependent on the particular animal. The importance of camelpox is difficult to judge

at present (see below 5, 6) but it has been suggested that sheeppox and goatpox are becoming more important as pressure on usable land increases in areas where the viruses are enzootic (1).

Parapoxvirus, Suipoxvirus, Avipoxvirus, buffalopox.

These infections are less serious, but nevertheless may cause economic losses when epizootics occur in local communities too dependent on susceptible species. In areas where different poxvirus infections occur in the same host e.g. sheeppox and orf, it may be important to establish a correct diagnosis quickly. Clinical diagnosis is not always certain and laboratory studies may be needed.

Unknown reservoir hosts.

Some poxvirus infections are of interest not just for their clinical or economic importance, but because information is lacking on the reservoir hosts of the viruses and on the way in which the viruses circulate in nature (7). This applies to cowpox, the cowpox-like viruses (Chapter 6), monkeypox (Chapter 1) and possibly buffalopox.

Possible confusion with other virus infections.

The clinical features of poxvirus infections in ungulates resemble those produced by more important virus infections such as foot-and-mouth disease, rinderpest and vesicular stomatitis. Consequently the possibility of poxvirus infection must be considered in the differential diagnosis of these more important infections, and vice versa. Fortunately the recognition of poxviruses by electron microscopy is a rapid and reliable method for confirming poxvirus infection (8) but the possibility of mixed infections should not be discounted.

Zoonotic poxviruses.

Some poxviruses are known to cause human infection and reliable information on some others is lacking (Table 1). Steps should be taken to minimize the risk of infection when infected animals are being handled. Human orf and pseudocowpox are occupational hazards, but cases of human cowpox may be missed if spread from hosts other than cattle is not considered (9). In particular the recognition of cowpox virus infection in domestic cats adds a new dimension to any discussion of poxvirus infection in domestic animals (see below).

CAPRIPOXVIRUS INFECTIONS

The viruses of sheeppox, goatpox and lumpy skin disease of cattle (LSD) have been placed in the genus Capripoxvirus (10). The viruses are very closely-related and there has been, and to some extent still is, uncertainty about their interrelationships; comprehensive reviews have recently discussed the early literature (1, 11, 12). Basically the viruses have been classified according to host specificity. However there is increasing evidence to suggest that the treatment of sheeppox and goatpox in this way is an oversimplification. There is also uncertainty about the antigenic relationships not just within the genus, but also between the capripoxviruses and other poxviruses.

Distribution.

Capripoxvirus infections of sheep and goats are endemic in the Indian sub-continent, the Middle East and Africa North of the Sahara. Europe and Australasia are free from infection, although there have been occasional importations of sheeppox into Mediterranean Europe (1, 12). A poxvirus infection of goats, distinct from orf, has been reported from the Western coast of the USA but so far has not been compared with other capripoxviruses (13). A variety of aetiologies have been considered as the cause of lumpy skin disease of African cattle, but a capripoxvirus is now accepted as being responsible for much of it (14).

Clinical features (12, 15).

Sheeppox and goatpox are malignant diseases characterized by the production of generalized skin lesions and pyrexia. Rhinitis and conjunctivitis usually precede the skin lesions, which are usually most common on the less hairy areas. Buccal lesions may occur and respiratory and pulmonary involvement is common in seriously affected animals. Animals of all ages may be affected but serious infection is most common in the young. Death in fatal cases is due to overwhelming virus infection of internal organs, particularly the lungs, often aggravated by secondary bacterial pneumonia. In general the infection tends to be a greater problem in sheep than goats.

Infection occurs in enzootic and epizootic forms and the differences in morbidity and mortality may reflect this (see below). It has also been suggested that strains of different virulence may exist, and there is evidence that not all breeds of animal are equally susceptible. Algerian sheep in particular are resistant to sheeppox (11).

Host range.

The traditional view of sheeppox and goatpox, discussed recently elsewhere (1, 12), is that they are epidemiologically distinct and that cross-species infection does not occur naturally and might not always occur experimentally. Recent experience with Nigerian isolates appears to confirm this (16). However in Kenya extensive outbreaks are caused by a capripoxvirus which naturally infects both sheep and goats (17).

Cross-species infection experiments with isolates from Nigeria, Kenya, Yemen, Turkey, India and Pakistan showed no absolute host specificity (15). Isolates from Yemen and Sudan were virulent in both sheep and goats, and the Kenyan isolate produced a mild infection in both species. However Nigerian sheeppox and Indian goatpox produced only mild infection in goats and sheep respectively (15). Strains with different host specificities circulate in Oman and Yemen; some cause infection in both sheep and goats, others in either sheep or goats in mixed flocks (18). The reasonable conclusion based on this and other evidence is that the taxonomic separation into sheeppox and goatpox species is untenable and that minor host range variants of an entity which could be called 'capripoxvirus' are in circulation (15).

Epidemiology and Management.

In fully susceptible flocks the morbidity may approach 100% with a mortality of 2-30% (12, 17, 18), although in outbreaks which coincide with peste des petits ruminants the mortality can be 50-90% (18). Enzootic infection in flocks previously exposed and so partially immune causes morbidity of 5-10% with negligible mortality (12, 17).

There has been speculation about the modes of transmission of capripoxvirus infection. Respiratory and pulmonary involvement suggested that aerosol spread may occur, and close contact of animals suggested that infection could be spread by direct contact. The involvement of arthropods has been suggested although transmission in insect-proof pens has occurred (17). Recent controlled experiments have shown that infection can be spread by each of these methods but their relative importance has yet to be determined (19, 20).

Field observations have shown striking differences in the speed and ease with which infection can spread. For example, in Oman the disease was seen to spread through flocks of 3000 animals in 1 month, yet to take three months to infect all animals in a flock of 12 (18).

Control and management of outbreaks in endemic areas without the use of effective vaccines is difficult (1, 14, see below). This has been emphasized by a recent analysis of animal husbandry in Yemen and Oman (18), but applies equally well to most areas in which capripoxvirus infection occurs. In particular the mixing of animals from different small, family flocks during the day, and the close confinement of each small flock in a corral or room of the owner's house at night, facilitate spread of infection. Control of infection in non-endemic areas ideally should be by quarantine to prevent entry of infected animals. Once introduced outbreaks should be controlled by slaughter. Vaccination in non-endemic areas, particularly with live vaccine, should be discouraged.

Early suggestions that the capripoxviruses of Africa and the Indian sub-continent were geographically isolated, and that this might result in the emergence of biologically and antigenically distinct viruses have recently been discounted; the well-established trekking routes along which animals may move between Africa and the Far East across the Middle East provide means by which the viruses could circulate (15, 18).

Epizootics of LSD occur in Africa and infection is thought to be spread by insects (14), but as respiratory infection occurs aerosol spread cannot be discounted. Clinical evidence of infection has been seen only in African cattle. The morbidity varies and survival and circulation of the virus between epizootics, when the morbidity is low, may be due to persistence in another host species. Recent studies have shown antibody to LSD in wild African buffalo (*Syncerus caffer*), particularly in areas where bovine LSD occurs (21).

Antigenic relationships and vaccination.

A variety of sheeppox vaccines has been used (1, 11). Inactivated vaccines induce only short-term immunity and live virulent vaccines must be used with care. Live attenuated vaccines have been used locally with some success (22, 23). However there is a great need for a 'universal vaccine' preferably attenuated, which could be used in any country where sheeppox and/or goatpox is a problem; effectiveness in controlling LSD would also be desirable. This goal necessitated the re-evaluation of the host specificity of viruses from different hosts and countries described above, and a similar assessment of their immunological relationships.

Early uncertainty about the antigenic relationships of the capripoxviruses (1, 11) has largely been resolved by recent work. Gel precipitation techniques have shown the complexity of capripoxvirus soluble antigens. Some workers have reported that more precipitin lines are produced by sheeppox-antisheeppox and goatpox-antigoatpox systems than by heterologous combinations (24). Others, using sensitive radio-labelled antigens, have found that the same number of lines were produced by sheep and goat isolates from various countries, and by LSD, with all antisera (25). Differences in the number of precipitin lines produced may be as much a reflection of the potency of the reagents as of differences between the viruses. Virus neutralization and immunofluorescence tests have failed to differentiate various capripoxviruses (15, 26). More significantly, cross-infection experiments in sheep and goats showed cross-immunity between viruses of ovine and caprine origin from various countries and the Kenyan strain of sheep and goatpox was suggested as a candidate vaccine (15). In fact other workers had attenuated this virus by passage in bovine cell cultures and used it successfully to control extensive natural outbreaks caused by the wild-type virus in Kenya (27). Interestingly these workers also showed its effectiveness in controlling LSD (27). More recently this vaccine has been used successfully in the Middle East (28). These trials indicate the short-term efficacy of the vaccine. If the immunity induced is of reasonable duration and if the vaccine is used properly there is a real prospect of eliminating capripoxvirus infections from areas in which they currently cause considerable economic loss.

Antigenic relationships between capripoxviruses and parapoxviruses.

Serological cross-reactions between different poxvirus genera can usually be demonstrated only with difficulty. However early reports of cross-reaction between capripoxvirus and parapoxviruses in gel precipitation tests have recently been confirmed (24, 25). The cross-reaction was sufficiently strong to be detectable with antisera from animals recovered from natural infections and could interfere with attempts to make a specific diagnosis by this method (25).

Genomic relationships and the status of capripoxviruses.

The very close relationship between capripoxviruses has been confirmed by restriction endonuclease analysis of their DNA (29, 30). Minor differences were detected in the genomes and some strains could be

assigned to ovine or caprine sources. This could not be done with some strains from Oman, and the interesting suggestion was made that these might have arisen by in vivo recombination. However all the viruses were very closely-related and homology was assessed at 80% or greater (29, 30).

In view of the very close relationship between all the capripoxviruses tested it has been suggested that the present separation into 3 species is not justified and that they should all be placed in a single species within the genus (15, 30).

PARAPOXVIRUS INFECTIONS.

Clinical and epidemiological features (8, 31).

Parapoxvirus infections affect mainly sheep and cattle but other species such as goats, camels, deer and musk ox may be affected; human infection is an occupational hazard (32, 33). In sheep lesions occur around the mouths of lambs (scabby mouth) and teats and udders of ewes (orf, contagious pustular dermatitis, contagious ecthyma of sheep). In cattle infection involves the teats of cows (pseudocowpox, paravaccinia, ring sores) and the mouths of calves (bovine papular stomatitis, [BPS]). Teat lesions are more common than oral lesions in cattle due, in part, to differences in husbandry; calves are usually separated from milch cows and infection is spread among the latter by milking.

Bovine and ovine parapoxvirus infections occur worldwide and the evidence available suggests that the former are more common and less important. Thus pseudocowpox is endemic in many herds but may go unnoticed or ignored, particularly where the standard of husbandry is poor (34). Attention may be drawn to it only when milk yields are affected. Mouth lesions in lambs have a more serious effect; they lead to poor weight gain, loss of condition and predispose to other conditions. Teat lesions in ewes may also prevent proper feeding of lambs. Because of this, particular attention has been paid to orf in countries such as New Zealand which have important lamb and wool industries (35).

Parapoxvirus infections are most conveniently diagnosed by electron microscopy on extracts of infected tissues. The characteristic appearance of the virions is unlike that of any other poxvirus (8, 34, 35).

Management and prevention.

The risk of infection can be minimized by improved husbandry and segregation of infected animals; any treatment required is supportive and symptomatic, e.g. careful feeding of infected lambs. It has been suggested that the persistence of virus in herds is due to survival of virus in scabs shed onto pasture and by latent and sub-clinical infection (8, 36). However virus can be isolated from chronic lesions for at least 6 months, and virus circulation may be maintained in this way (34). Immunity to natural infection is short-lived and reinfection occurs (34). An orf vaccine is available which contains live virulent virus and so must be used with care; vaccinated and non-vaccinated animals should be segregated to prevent cross-infection.

Immunity induced by vaccination is relatively short-lived and annual revaccination of adults may be needed to prevent accidental reinfection (14). Vaccination of ewes 6-8 weeks before lambing may prevent teat infection and thus prevent spread to lambs (37). However although antibody is transferred to lambs via colostrum it does not passively protect (38). This and the fact that at one time lambs were routinely vaccinated at 6 weeks of age meant that the newborn lambs were at risk. However successful vaccination of lambs aged 24-48hr is possible and this offers protection during the vital early weeks of development (37).

Human infection.

Human infection with parapoxviruses is an occupational hazard. Immunity is poor and reinfection occurs (33). Attention has been drawn to erythema multiforme as a complication of human orf (32). However most infections are mild and the majority are probably not reported; consequently the proportion of cases with this complication must be very low.

Interrelationships of parapoxviruses.

The traditional view has been to regard orf, BPS and pseudocowpox as separate species of the genus Parapoxvirus (10). However laboratory studies have not always separated them. The 3 viruses are extremely closely-related and their comparison is hindered by minor differences between isolates of any one species; these tend to obscure differences between the species. Recent studies still emphasize the close relationship but have produced some evidence for their separation.

Antigenic studies. Cross-neutralization tests on strains isolated from the 3 clinical entities and orf vaccine have shown considerable heterogeneity in their antigenic properties (39-41). Although neutralization by homologous antisera was often more efficient, sometimes markedly so, in some instances neutralization by heterologous antisera was more efficient. In some cases an orf isolate was neutralized more effectively by a BPS antiserum than the BPS isolate, which in turn was neutralized more effectively by orf antiserum (39). In this last survey a tentative suggestion of 2 grouping was made - each containing orf and BPS strains.

Immunofluorescence and the release of ^{51}Cr from infected cells by complement-dependent antibody have recently been used to study parapoxviruses (41). Potent antisera reacted with all the viruses, and homologous reactions were greater than heterologous. With less potent antisera apparently qualitative differences were obtained; e.g. a low titre antiserum to pseudocowpox reacted only with that virus. In addition antisera to BPS reacted with the outer envelope of BPS but not with that of pseudocowpox or orf viruses; unfortunately antisera to these last two viruses were not tested in this respect (41).

These results suggest that there are minor antigenic differences which need further clarification. Possibly studies with adsorbed or monoclonal antisera may provide useful information.

Genome analysis. Restriction endonuclease analysis of orthopoxvirus genomes has confirmed the validity of the traditional species, and different isolates of any species show considerable homology (42, 43). Similar studies have been made on parapoxvirus strains but with rather less success. Analysis of orf strains from Europe, the United States and New Zealand using a variety of endonucleases has shown considerable genome heterogeneity (39, 44-46). Little (39) or no (46) change in cleavage patterns was detected when virus was passaged through cell cultures and attempts to correlate the cleavage patterns with biological, immunological and epidemiological properties of orf strains may provide data of value (46). More recent analysis of orf strains has shown that the variation is restricted to the left half of the genome (47). Similar heterogeneity also occurs in pseudocowpox and BPS genomes and the classification of strains simply by comparison of cleavage

patterns is not possible (39, 44). However cross-hybridization studies with fragments of DNA from the ends of the genome showed hybridization only between members of the same virus species, although two strains originally classified as orf were reclassified as pseudocowpox as a result (44).

The status of parapoxviruses. The results recently obtained and discussed above tend to support the view that isolates of orf, BPS and pseudocowpox can be separated. However the 3 types of virus are clearly very closely-related, and taxonomists may wonder whether they all merit species status.

Contagious ecthyma of camels (Ausdyk).

There are reports that a parapoxvirus is responsible for some outbreaks of 'camelpox' (47, 48). At present there is not enough evidence to assess its importance as a pathogen (see below) nor has this virus been compared in the laboratory with other parapoxvirus isolates.

ORTHOPOXVIRUS INFECTIONS.

With the eradication of smallpox, camelpox is now the orthopoxvirus of greatest clinical importance. There is also interest in the epidemiology of monkeypox (Chapter 1) buffalopox and the epidemiology and interrelationships of cowpox virus, and closely-related viruses isolated from captive exotic species (7, Chapter 6). Of particular significance for veterinary infectious diseases in general is the suggestion that recombinant vaccinia strains should be used as vaccine vectors.

Cowpox.

Bovine cowpox is a relatively unimportant infection occasionally reported from Europe and the UK. Attention is drawn to it sometimes only when human cases occur. The traditional view was that cowpox virus circulated and was maintained in cattle, and that human infection was a consequent occupational hazard. However detailed surveys of bovine teat infections (34) and serological surveys of cattle in areas where human cases occurred (49) showed that bovine cowpox was very rare; human cases also occur in which no contact with cattle, infected or otherwise can be traced (49). The contrast with pseudocowpox is striking, and it was suggested that cowpox was not enzootic in cattle and that it circulated in a wild animal reservoir (49). This situation with cowpox is not

unique; it is supported by, and supports, similar conclusions on monkeypox (Chapter 1) and poxviruses closely-related to cowpox which have been isolated from captive exotic species (7, Chapter 6).

Feline cowpox. Evidence that Felidae were susceptible to orthopoxvirus infection was first obtained in 1973 (Chapter 6) and cowpox occurred in captive cheetahs in the UK in 1977 (50). The first reported case in the domestic cat occurred in 1978 (51). Increased attention has been paid to the topic since 1981 (Table 2) and details of further cases have been published (52-54). In particular clinical and epidemiological features of over 60 cases which occurred up to the end of 1985 have recently been reviewed, 47 of these in detail (55, 56).

Table 2. Cowpox virus infection recorded in the UK 1977-1986

	Cases recorded in each year 1977-1986.									
Host	'77	'78	'79	'80	'81	'82	'83	'84	'85	'86*
Cheetah	3	2	0	0	0	1	0	0	0	0
Cat	0	1	0	0	3	6	13	20	23	10
Human**	0	4	1	0	1	0	0	0	2	2
Bovine	0	0	0	0	0	0	0	0	0	0
Total	3	7	1	0	4	7	13	20	25	12

*Data for 1986 incomplete.
**Both cases in 1985 and 1 in 1986 had contact with infected cats.

Clinical features (55, 56). Only 3 of 47 cats did not develop multiple skin lesions although evidence suggests that most cases start with a single, primary lesion usually on the head or forelimbs. Often described as 'bite-like' the primary lesion may vary from a small (1 cm. diam.) ulcerated or granulomatous lesion to extensive cellulitis. Secondary skin lesions, small nodules (2-3 mm. diam.), usually appear about 11 days after the primary lesion. They increase in size over 2-3 days and form discrete ulcerated or scabbed lesions c. 2 cm. diam; in some animals they may be red hairless and moist. Secondary lesions may be widespread and buccal lesions occur.

Recovery is usually uneventful; scabs separate after 4-6 weeks, and the scars are eventually covered by new hair.

Most cats show no obvious signs apart from skin lesions and perhaps pruritis. Others may have vague signs such as inappetance, slight

pyrexia and respiratory signs. In some animals respiratory and pulmonary involvement may be more severe and may occasionally result in death from haemorrhagic pneumonia (51).

Pathogenesis (52, 55, 56). The presence of bite-like lesions suggests that infection occurs via the skin. However experimental infection can be established oro-nasally and may account for the occasional case in which no obvious primary lesion can be recognized. Virus spreads to and replicates in local lymph nodes and then spreads to other organs (e.g. lung, turbinates, spleen) and the skin via a white cell-associated viraemia. Experimental studies indicate that subclinical infection can occur.

Virus neutralizing and haemagglutinin-inhibiting antibodies persist for at least a year, but more information is required on their long term persistence and on the role of humoral and cell-mediated immunity in recovery from and resistance to infection.

Diagnosis (55-57). A clinical diagnosis of cowpox may be made in a cat with generalized lesions of the type described above, particularly if accompanied by some of the other signs described above, particularly if examination shows evidence of an older primary lesion. Primary lesions should be differentiated from simple trauma, tumours or leprosy and secondary lesions from those due to fleas, eczema, feline herpes or calicivirus infection.

Ideally the diagnosis should be confirmed by the laboratory. Electron microscopy of extracts of scab, biopsy or exudate is positive in about 70% of cases, and provides a rapid presumptive diagnosis. Virus isolation from similar specimens on chick chorioallantois and/or cell culture is slower, provides a more certain identification and is positive in about 90% of cases. Routine histology, which may show the conspicuous cytoplasmic A-type inclusions (8) may enable a tentative diagnosis to be made in cases where results from other tests are negative. Antibody tests are less reliable than detection of virus. Serum samples taken in the acute phase may be negative, although complement-enhanced neutralization may be demonstrated. Presence of antibody in a convalescent serum may simply indicate previous infection at some time. However evidence of healed skin lesions or a carefully taken history may support a diagnosis of cowpox here.

Management (52, 54, 55). Most animals recover although some may be ill for quite a long time; the prognosis is poor in animals with severe respiratory or pulmonary involvement. There is no specific therapy and supportive treatment should be provided where necessary. The limited evidence available suggests that corticosteroids may aggravate the condition and their use should be avoided. Little is known of the effect of underlying conditions on the prognosis of feline cowpox. However anything which depresses the immune response might be expected to exacerbate the condition and it is of interest that one of the few fatalities occurred in a cat which was positive for feline leukaemia virus antigen (55).

Human cowpox can be severe particularly in young children (49). Three instances of cat-to-man transmission have been reported (9), and veterinarians and owners of infected animals should be aware of the risks of infection. However that so few cases have resulted from contact with cats suggests that cowpox virus may not be very infective for humans.

Epidemiology (55, 56). Feline cowpox has been reported from widespread locations in the UK; both rural and urban animals were affected and no bovine cases were involved. Of 64 cases where the time of onset was known 59 began in August to December. The true incidence of feline cowpox is unknown, nor is it known how long it had occurred before the first case was recognized in 1978. Increased numbers of cases during 1981-1985 (Table 2) may reflect increased interest, and a decline in numbers reported during 1986 may reflect increased reliance on clinical diagnosis without laboratory aid being sought.

Only limited cat-to-cat spread occurs. This, and failure to detect cowpox antibody in sera from 285 cats suggests that cowpox virus is not maintained solely in cats. It seems probable that cats, like humans and cattle, are just indicator or accidental hosts.

The occurrence of feline cowpox is consistent with the view that the reservoir host is probably a small wild mammal. In particular the presence of bite-like wounds, and an autumn peak which coincides with increased rodent numbers and activity (58) supports this view. Also significant is the presence of orthopoxvirus antibody in small numbers of English bank voles and field mice (59). Cowpox and ectromelia (mouse-

pox) viruses are the only orthopoxviruses indigenous to the UK. Ectromelia is not thought to circulate in wildlife and the presumption that the antibody detected in wild rodents was in response to natural cowpox infection must be high. Further support for a wildlife reservoir comes from studies on a closely-related virus, isolated in Russia from captive species, which has been shown to circulate in wildlife (60, see Chapter 6).

Cowpox and 'cowpox-like' viruses.

Cowpox and ectromelia viruses are the only orthopoxviruses detected in the UK, and of these only cowpox has infected cats (55, 56). The situation in Europe is rather more complicated. Cowpox virus has been isolated there from bovine and human infections (61, 62) and recently in Holland from domestic cats (63). However increasing attention is being paid to orthopoxviruses isolated from captive exotic species particularly elephants (64). This topic is dealt with in detail in Chapter 6, but here it may be noted that these viruses are very closely related to cowpox and that their taxonomic position is uncertain. Minor differences have been detected between cowpox and these 'cowpox-like' viruses (65, 66), but more studies are needed. It is possible that these cowpox-like strains may be classified within the species cowpox, and that sub-species or variants may be designated. Whatever the final decision the existence of these strains means that more work is required to determine the range of indicator and reservoir hosts, laboratory properties and general biology of European orthopoxviruses.

Buffalopox.

Attention is occasionally drawn to outbreaks of poxvirus infection in buffaloes, particularly on the Indian sub-continent (67-69). The morbidity varies but fatalities are rare. Lesions are commonly seen on the face and teats and milk yield may be affected; human infection occasionally occurs. The virus responsible is very closely-related to vaccinia, and the persistence of buffalopox after the cessation of routine smallpox vaccination suggests that vaccinia, or perhaps variants of it, may have become established in nature (70). Detailed comparison of Indian vaccines and strains isolated from buffalopox outbreaks should resolve this question. It may also be necessary to examine the possibility that wildlife reservoirs may be involved in the circulation of these virus strains.

Camelpox.

Poxvirus infections in camels can be a serious problem in nomadic communities dependent on these animals. One complication, not always appreciated, is that at least 2 clinically similar conditions are caused by poxviruses - camelpox caused by an orthopoxvirus, and contagious ecthyma (Ausdyk) caused by a parapoxvirus (5-7, 48). A detailed survey showed that pox in Somali camels was a considerable problem. However it was not fully appreciated that 2 viruses were involved and it is not known whether this accounted for differences in morbidity and mortality seen in various herds (5). Evidence from Kenya suggests that camelpox is enzootic there (6). Although there are anecdotal accounts of human infection, camelpox virus is very host specific and detailed surveys suggest that human infection is very rare, if it occurs at all (5). Co-ordinated field and laboratory studies are needed in areas where pox in camels occurs to determine the distribution and relative importance of camelpox and Ausdyk.

The use of vaccinia virus as a vaccine vector.

One potentially valuable development resulting from molecular studies on vaccinia virus is the insertion of foreign genes into the vaccinia virus genome (71, 72). Such recombinant vaccinia strains are still infectious and animals infected with them develop antibodies and immunity to the foreign antigens. Consequently the insertion of genes which code for immunizing antigens of pathogens offers the prospect of using such strains as live vaccines for a variety of animal and human infections (73). Of veterinary interest are recombinant vaccinia strains which code for rabies (74), vesicular stomatitis (75) and porcine transmissable gastroenteritis (76) antigens.

The advantages and disadvantages of such an approach have been debated (70, 73, 77). The principal advantage is that of using the wide host range of, and prior experience with, vaccinia virus to construct vaccines which could be used in a variety of species to control infections not easily controlled by other means. However steps should be taken to ensure that the vaccine strains do not spread to other animals or species. The use of recombinants to control rabies in wildlife has been proposed (78) and possible genetic interaction between the vaccines and other orthopoxviruses which circulate in wildlife should be considered (70). Unwanted spread could be minimized by using

suitably attenuated recombinants. There is evidence that attenuation for some species may be achieved by insertion of the foreign gene into the vaccinia thymidine kinase sequence (79). An alternative approach may be to use as a vector, a parapoxvirus which has a more restricted host range (47).

CONCLUSIONS.

This review has shown how recent laboratory and field work has increased our knowledge of the basic properties of certain poxviruses and of the epidemiology and control of the infections they cause in domestic animals. At the same time areas have been indicated where future collaboration between laboratory and field workers should improve our understanding of these topics.

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3

POXVIRUSES OF RABBITS

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ABSTRACT

The biology of poxviruses which infect domestic rabbits (*Oryctolagus cuniculus*) is reviewed. Special emphasis is placed on one member of the *Orthopoxvirus* genus, rabbitpox virus, and three members of the *Leporipoxvirus* genus, Shope fibroma virus, myxoma virus and malignant rabbit fibroma virus. Recent advances in the molecular biology of these viruses and their interactions with target cells *in vivo* and *in vitro* are highlighted.

INTRODUCTION

Rabbits, like all vertebrate animals, are susceptible to a broad spectrum of viral infections (for review, see 1). For reasons that are in part historical, far more is known about diseases of rabbits caused by poxviruses than by any other virus group (2). For example, one particular poxvirus, myxoma, was the first virus to be used in a deliberate eradication programme of an endemic pest, the feral European rabbit in Australia (reviewed in 2-5). Another reason for the attention that has been paid to poxvirus infections of rabbits is that the first DNA tumor virus discovered was the rabbit fibroma virus, isolated and described by R. Shope in 1932 (6), which was studied extensively in the early days of tumor virology as a model for viral tumorigenesis (reviewed in 7).

In this chapter attention will be focused on members of the poxvirus family which can, either in the wild or in the laboratory, cause defined lesions or characteristic disease profiles in the European rabbit (*Oryctolagus cuniculus*: see reference 8 for review of rabbit taxonomy and genetics). The various symptomologies of these poxviruses in their natural hosts in the wild, including cottontail, brush or forest rabbits (*Sylvilagus* sp), squirrels (*Sciurus* sp) and hares (*Lepus* sp)

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have been described in detail elsewhere (9). Furthermore, the gross pathologies and histopathological profiles of poxvirus diseases in the laboratory rabbit have all been reviewed extensively (see individual virus sections for references) and will be given only minimal treatment here. Instead, special attention will be paid to the recent advances in the molecular biology of poxviruses that have impacted on the study of poxvirus diseases in rabbits. In particular, emphasis will be placed on the use of recombinant DNA technologies, such as cloning and sequencing, which have revolutionized our concepts of poxvirus replication and virus/cell interactions. These technologies have also provided powerful tools with which to assess new members of the different poxvirus genera for purposes of rapid and accurate diagnosis of viral infections, determining epidemiological profiles and assessing the structures and origins of novel variants or recombinants. Many of the "classic" parameters that have been used to classify and diagnose poxvirus isolates are, of course, still of great utility (9-11). However, now that such criteria as host ranges, disease symptomologies, morphologies of lesions in tissue culture and on chorioallantoic membranes (CAMs), serology, etc., can be used in combination with the powerful and sensitive techniques of restriction enzyme mapping, Southern blotting, and DNA sequencing, poxvirus isolates can be classified with greater precision than ever before. Furthermore, details for at least some of the features of poxviral pathogenesis and disease characteristics in rabbits can now be addressed for the first time at the molecular level.

GENERAL CHARACTERISTICS OF POXVIRUSES

The family of poxviruses share a number of characteristic features which render them unique among animal viruses: (1) The virions possess an assymmetrical brick-shaped morphology, with a defined internal "core" that contains a variety of endogenous enzymes required for viral RNA and DNA synthesis plus the linear double-stranded DNA genome, and (2) all stages of viral replication occurs outside the cellular nucleus in cytoplasmic structures referred to variously as "virosomes", "factories", or even "micronuclei". Because of its large genomic size and autonomous replication cycle, poxviruses are perhaps the least dependent upon cellular functions of all the animal viruses, and the typical poxviral DNA genome probably encodes up to several hundred gene products. The biology

Table 1. Poxviruses which can infect domestic rabbits (Oryctolagus cuniculus).

	Synonym	Natural host	Disease
Orthopoxviruses:			
Vaccinia[1]		Lab virus	Localized skin lesions[2]
Rabbitpox	rabbit plague	?	Lethal generalized infection
Leporipoxviruses:			
Shope fibroma virus	rabbit fibroma	Sylvilagus floridanus	Localized benign fibroma
Myxoma (California)	Marshall-Regnery fibroma	Sylvilagus bachmani	Lethal myxomatosis with internal lesions
Myxoma (S. America)	Aragao's (or Brazilian) fibroma	Sylvilagus brasiliensis	Lethal myxomatosis with internal & external lesions
Squirrel Fibroma	-	Sciurus carolinensis	Multiple benign fibromas[2]
Western squirrel fibroma	-	Scirus griseus	Localized benign fibromas[2]
Hare fibroma	-	Lepus capensis	Localized benign fibromas[2]
Malignant rabbit fibroma	Malignant rabbit	Lab recombinant[3]	Lethal invasive fibromatosis

[1] Other related orthopoxviruses (except variola) can also induce similar skin lesions after intradermal injection.

[2] The disease symptomology has only been observed in experimentally infected rabbits

[3] Generated by a spontaneous recombination between Shope fibroma virus and a still unidentified strain of myxoma virus (see text).

of poxviruses has been reviewed extensively elsewhere and is only briefly summarized in this chapter (10,12-14). The current system of nomenclature groups the poxviruses into six genera (15), of which only two, *Orthopoxvirus* and *Leporipoxvirus*, have relevance to infections of rabbits. As can be seen in Table 1, some of the disease syndromes have been observed only in experimentally infected lab rabbits, but are included here to provide a comparison with those poxvirus diseases for which domestic rabbits are known to be at risk.

Since much of the current research on many of these different viruses has focused on the structure and expression of the viral DNA, an abbreviated description of the overall poxviral genomic organization will be given. More comprehensive treatment of the structure and replication of poxvirus DNA can be found in references 16-18. Until recently, the majority of molecular studies on poxviral DNA have used vaccinia virus as the prototype, and so many of our current concepts of the DNA structure and regulation of viral gene expression has come from work on this virus. However, most of the following molecular details are believed to be common to all poxviruses as well:

(1) The poxviral DNA genome consists of a single non-segmented double stranded DNA molecule 150-250 kilobases in size.

(2) The ends of the packaged viral DNA are hairpin structures which covalently "cross-link" the termini.

(3) Also packaged with the viral DNA are specific viral enzymes such as RNA polymerase, poly (A) polymerase, RNA capping and processing enzymes, deoxyribonucleases, topoisomerase, protein kinase and nucleoside triphosphatase activities.

(4) The terminal regions at each end of the viral DNA are identical to each other for a distance varying from a few kilobases up to 15 kilobases, depending on the virus. These regions are of opposite orientation to each other and are referred to as terminal inverted repeat (TIR) sequences.

(5) Certain regions of the viral DNA, especially in the area of the TIR, are often hypervariable and subject to extremely rapid genetic drift, especially in the region of short tandemly-repeated sequences frequently encountered near the termini of *Orthopoxvirus* genomes.

The degree of cytopathology inflicted upon the target cell subsequent to poxviral infection varies enormously from poxvirus to poxvirus and is also modulated by the target cell. The most cytolytic poxviruses, such as vaccinia, drastically inhibit all macromolecular synthesis of the host cell shortly after the initiation of the infection and viral multiplication occurs with the concomitant death of the infected cell. The infectious progeny virus particles may be transmitted to neighbouring cells either by cell-cell contact or by free liberated virions. On the other hand, some poxviruses (such as Shope fibroma virus) cause a relatively benign infection which does not necessarily abbrogate cellular functions but instead can initiate a persistent or "carrier" state in which cellular and viral macromolecular synthesis co-exist. The overall subject of poxviral pathology and dissemination through host tissues has been reviewed extensively by Dales and Pogo (10).

In Table 2 is shown a partial list of restriction endonucleases which have proven to be useful in the past decade for classifying and mapping poxviral genomes. Reference 19 provides an introduction to these enzymes and the physical techniques used to analyze their DNA digestion products

Table 2. Restriction Enzymes of Particular Utility for Classifying Rabbit Poxvirus Isolates

Vaccinia[1]	Rabbitpox	SFV	Myxoma	MRV
HindIII[2-8,13,17]	HindIII[3,5,9-13]	EcoRI[14]	XhoI[14,19]	BamHI[18]
SalI[4,7,8,]	XhoI[5,6,9,11,12]	KpnI[14]	SalI[17]	PstI[18]
HpaI[4]	SmaI[5]	XhoI[14,19]	HindIII[17,20]	SstI[18]
EcoRI[4,6]	SacI[9,11]	HindIII[14-17]	BamHI[16-18]	XhoI[19]
BamHI[4,7,13,17]	KpnI[6,9,12]	BamHI[15-17]	PstI[18]	HindIII[20]
XhoI[5,6,8,14,19]	EcoRI[6,10,12]	BglI[15,16]	SstI[18]	
SmaI[5,8]	SstI[3,12]	PstI[15,16]		
KpnI[6,8]	BamHI[13]	PvuII[15,16]		
BglI[8]		SstI[15,16]		
SstI[3]		SalI[17]		
SacI[8]				

1 And other related orthopoxviruses
2 Ref. 20
3 Ref. 21
4 Ref. 22
5 Ref. 23
6 Ref. 24
7 Ref. 25
8 Ref. 26
9 Ref. 27
10 Ref. 28
11 Ref. 29
12 Ref. 30
13 Ref. 31
14 Ref. 32
15 Ref. 33
16 Ref. 34
17 Ref. 35
18 Ref. 36
19 Ref. 37
20 Ref. 38

and the particular utility of these methods to the analysis of poxvirus genomes has been reviewed by Holowczak (16). The individúal gel profiles and the collated fragment sizes are too voluminous for inclusion here but can be found in the individual references indicated. In addition to these profiles, considerable information can be gleaned from hybridization data derived from Southern blots, using either total or cloned poxviral DNA probes. For example, in Fig. 1 the BamHI profiles of vaccinia, SFV and myxoma are compared in terms of ethidium bromide staining and after blotting and hybridization with either SFV DNA probe or vaccinia DNA probe. Note that the use of such viral-specific DNA probes allows for precise distinctions between the DNA genomes of *Orthopoxviruses* (such as vaccinia) and *Leporipoxviruses* (such as SFV and myxoma). Under these "moderate" conditions of hybridization, the SFV and myxoma DNAs cross-hybridize but the stringency conditions can be varied so as to distinguish these viral genomes as well.

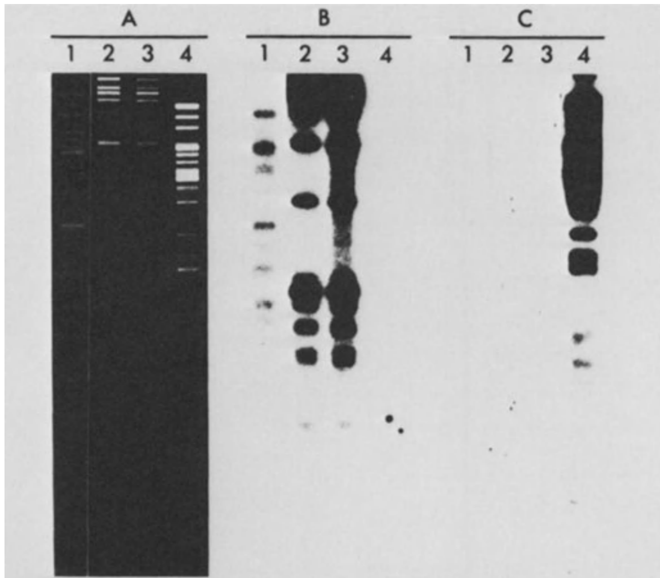

Fig. 1 Comparison of poxviral genomes by agarose gel electrophoresis of BamHI-digested viral DNA (Panel A), and by Southern blotting, using [32P]-SFV DNA probe (Panel B) and [32P]-vaccinia DNA probe (Panel C), under conditions of moderate stringency. Lanes: 1, myxoma (strain Lausanne); 2, SFV (strain Kasza); 3, SFV (strain Boerlage); 4, vaccinia (strain WR). (From reference 34, with permission).

ORTHOPOXVIRUS INFECTIONS OF RABBITS

All members of the orthopoxvirus genus except variola can grow in rabbit skin but the only member of this poxvirus group to cause a disease of veterinary significance in rabbits is rabbitpox virus (RPV). Recent evidence indicates that the DNA genomes of all the orthopoxviruses are closely related to each other in terms of the organization of their unique internal sequences but diverge considerably in the structures of their TIRs (e.g. see references 16,21,23-25,29,31). The molecular basis for the wide range of orthopoxvirus pathogenesis in rabbits, from the relatively benign dermal lesions induced by vaccinia to the systemic lethal infection of RPV, is still unknown.

Orthopoxvirus infections in rabbits following respiratory innoculation proceeds similarly to that described for extromelia in mice (reviewed by Dales and Pogo in reference 10). The virus first multiplies in the bronchiolar epithelium and alveoli, spreads through the lymphatic pathways to a variety of internal organs (such as liver and spleen) where multiplication occurs to varying degrees, depending on the virus and rabbit strain. If second site replication is sufficiently vigorous to cause a secondary viremia, the infection subsequently spreads to secondary target organs such as intestine and skin (39, 40). In the case of vaccinia the principle gross signs of infection in rabbits are slightly elevated temperature and a mild papular rash. The infection is usually resolved by the immune system, but can be severe and systemic in the immune compromised host (41). The relatively benign pathogenicity of vaccinia in rabbits is reflected in the rather low levels of extra-pulmonary replication and is in marked contrast to the high secondary organ levels of multiplication observed for RPV (next section). In fact, high intravenous doses of vaccinia will also precipitate internal organ viral replication and cause extensive viremia and death in rabbits. The pathogenicity of vaccinia in rabbits has been observed to increase in some recombinants between vaccinia and ectromelia (42), the latter of which is nonpathogenic in rabbits, but the reasons for this is not yet established. There is some evidence for an orthopoxvirus-specific antigen that is pathogenic to rabbits and induced by wild-type rabbitpox, cowpox, and neurovirulent strains of vaccinia but not by white pock variants of cowpox and dermotrophic strains of vaccinia (43, 44). Very little is known about the mechanisms of orthopoxvirus pathogenicity in rabbits, but pretreatment of rabbit skin with interferon inducers, or antiviral agents such as arabinosyladenine or phosphonoacetic acid is known to reduce the severity of vaccinia lesions (45-47).

An interesting exception to the rule that all members of the *Orthopoxvirus* genus except RPV are relatively non-pathogenic in rabbits is the case of buffalopox virus (9,48). Rabbits injected intradermally with buffalopox frequently succumbed to a systemic infection characterized by generalized poxviral lesions in a variety of internal organs and considerable viremia that is not well controlled by the immune system (49). On the other hand, in those rabbits that recover from buffalopox infection humoral and cell-mediated immune mechanisms completely clear the

virus from the infected rabbit tissues.

Recently it has been shown that certain classes of vaccinia mutants, such as the thymidine kinase-negative variants, are less pathogenic in test animals (50). It is anticipated that as more mutants of vaccinia and other *Orthopoxviruses* are created by recombination or site specific genomic manipulations, it will be possible to better evaluate the number and distribution of poxviral gene products which mediate viral pathogenesis and host range.

Rabbitpox virus.

Rabbitpox virus (RPV) induces a systemic, frequently lethal infection in rabbits that is the equivalent of ectromelia in mice, monkeypox in monkeys or smallpox in humans (1). Although never observed in the wild, at least half a dozen epidemics of the disease (sometimes called "rabbit plague" or "rabbit peste") have been reported in rabbit colonies. The first documented outbreak was at Rockefeller University in 1930-32 and the most recent in 1967 (51, and references therein). The disease profile and pathogenesis of RPV in rabbits has been comprehensively reviewed elsewhere (1,9,11), and will only be briefly summarized here.

The outbreaks in the United States and Europe have manifested in two forms: the exanthematous form, characterized by cutaneous eruptions similar to smallpox lesions in humans (exemplified by the Rockefeller strain of RPV) and the non-exanthematous or "pockless" form, characterized by minimal external lesions (exemplified by the Utrecht strain). The two forms can be distinguished *in vitro* by virtue of the fact that the former strain induces hemagglutinin activity (HA^+) while the latter is HA^-. The virus is transmitted from rabbit to rabbit by inhalation/ingestion of aerosols or droplets from infected nasal and eye discharges. The disease syndrome of the exanthematous form is characterized by fever, respiratory problems, nasal and conjuctival discharges, swollen lymph nodes and cutaneous lesions. Depending on the RPV strain and the genetic disposition of the rabbit, the skin lesions can vary from a macular rash and papules in the dermis and oral/nasal cavities, to severe skin hemorrhages and necrosis over the entire body, including the gums, palate and genitals. The non-exanthematous ("pockless") syndrome has few external gross lesions, but is frequently associated with conjuctivitus, diarrhea and, occasionally, pneumonitis. Internally, however, the rabbits infected with either type show evidence for pleuritus, pericarditus and possess focal, sometimes necrotic,

lesions on a variety of internal organs. The mortality rates vary from 10-20% for healthy adults to over 70% for the young. When recovery occurs it is usually complete and the rabbits do not maintain the virus in a carrier state.

The virus itself is closely related to vaccinia, especially certain strains of "neurovaccinia" but can be distinguished by pock morphology on chick CAM and rabbit skin (11). The origin and animal reservoir of RPV is completely unknown, but it has been suggested that RPV might be an escaped isolate of vaccinia. However, the marked differences in the pathology of RPV and vaccinia in the rabbit suggest caution, and the similarity of RPV lesions to those induced in rabbits experimentally infected with buffalopox (49) indicate that further work on *Orthopoxvirus* isolates are required to resolve this issue.

RPV grows in a variety of cultured cells and produces characteristic red, ulcerated pocks of chick CAM. Approximately 1% of all pocks are observed to produce an altered "white" pock morphology, and because of this RPV was one of the first animal viruses to be analyzed for genetic linkage by recombination (52,53). About 30% of the white pock mutants have a restricted host range in tissue culture and can be identified by failure to grow in pig kidney cells (54-58). RPV pock and host range mutants have been extensively analyzed recently to decipher the molecular mechanism(s) for their defects (59-64). RPV was also one of the first animal viruses to be used for the construction of temperature sensitive (ts) mutations for genetic analysis (57,65,66). Mutants of RPV that are resistant to the anti-poxviral agent thiosemicarbazone have also been described (67).

The close relationship at the DNA genome level between vaccinia and RPV has been investigated by DNA restriction enzyme mapping (see Table 2 for references). Briefly, the genomic organizations of the two viruses are very similar except in the region of the viral TIR (21,23,24,27-31). In the case of vaccinia, the length of the TIR is 11.8 kilobases (strain Elstree) and for RPV (strain Utrecht) it is 5.3 kilobases, or about one-half the size (21,24,30). Nevertheless, when TIR mRNA is hybrid-selected and translated *in vitro*, both vaccinia and RPV appear to express proteins of very similar size and distribution (68), indicating that the viral genes of RPV and vaccinia which map in the TIR are closely related. It is probable that a full appreciation of the differences

between RPV and vaccinia will require more extensive DNA sequencing analysis within these TIR regions.

DNA restriction mapping experiments on RPV white pock and host range mutants have revealed several intriguing features about spontaneous poxviral genomic rearrangements and genetic drift. Initial analysis of several such mutants indicated major deletions of sequences near the terminal regions of the viral DNA (63,64). However, closer examinations have revealed that many of these mutants have been subjected to substantial deletions and insertions coupled with transpositions between the left and right termini (61). Based on these mappings it has been found that the size of the RPV genome has a surprising degree of variability, with mutant genomes varying in size from a low of 148 kilobases (63) up to 210 kilobases (61), indicating that up to 25% of the viral genome is dispensable for viability, at least in terms of growth in culture.

LEPORIPOXVIRUS INFECTIONS OF RABBITS

All members of the *Leporipoxvirus* genus can infect domestic rabbits (see Table 1) but two of these (squirrel fibroma virus and hare fibroma virus) are never found naturally in rabbits except by experimental injection. Since the pathologies in rabbits of these latter two viruses has been presented elsewhere (1,9) and very little research has been conducted on their molecular structures, they will not be considered further here. *Leporipoxviruses* are of particular interest because they possess a number of biological features not to be found among members of the *Orthopoxvirus* genus. For example, despite the fact that all members of the poxvirus family do not physically enter the cell nucleus during their replicative cycle (for example, see discussion in reference 69), a few have been recognized for many years to be the causative agents for a number of proliferative diseases. Three notable examples of such "tumorigenic" poxviruses are:

(1) Shope fibroma virus (SFV), which induces benign fibromas in the adult rabbit (6) and invasive atypical fibrosarcomas in newborn (70-72) and immunosuppressed adult rabbits (71,73).

(2) Yaba tumor virus, an unclassified poxvirus found to cause subcutaneous histiocytomas in monkeys and man (reviewed by Rouhandeh, this volume).

(3) Molluscum contagiosum, also unclassified, the etiological agent for benign tumor-like epidermal lesions in man (74,75).

Recent research on the molecular basis for *Leporipoxvirus*-induced cellular proliferation has come focused on two members of the genus, SFV and malignant rabbit fibroma virus. The latter is a recombinant poxvirus derived from SFV and myxoma (discussed later) and has been particularly useful in assessing the poxviral gene products implicated in fibromatogenesis.

Shope fibroma virus.

First isolated by R. Shope in 1932 (6) from a subcutaneous fibroma derived from a wild cottontail rabbit (S. *floridanus*) caught in the Eastern United States, the rabbit fibroma virus was shown to be capable of inducing extensive localized dermal proliferation in the cottontail and domestic rabbit (reviewed in 1,3,7 and 9). Unlike the more extensively studied DNA tumor viruses (such as SV40/polyoma, papilloma virus, and certain adeno- and herpes-viruses), for which infectious virus is rarely detected in tumors, SFV-induced cellular proliferations are accompanied by viral replication and production of infectious progeny (7, 76). This distinction is also reflected in the responses of mammalian cells *in vitro* to these different viruses. Whereas the phenomenon of transformation of cultured cells is now well established for many DNA tumor viruses (eg. see 77), SFV does not enter the cellular nucleus (78) and does not permanently transform target cells *in vitro* into the immortalized phenotype. Instead, SFV induces a variety of different responses in cultured cells depending on the virus strain (79,80) and the cell type. For example, SFV can propagate on chick CAMs and rabbit embryo fibroblasts with a moderate level of cytopathology, while primary rabbit kidney cells, rabbit cell lines (such as the SIRC cornea cell line), and certain monkey cell lines (such as BSC-1, Vero and BGMK) aggregate into discrete clumps, sometimes referred to as foci, that consist of aggregated infected cells (78-82). Under the appropriate conditions of multiplicity of infection and passaging a "carrier-culture" state can be established *in vitro*, in which viral growth is sufficiently sublytic that the culture continues to proliferate while producing reduced amounts of viral antigen (86-88). With this mode of "semi-transformation" by SFV, novel phenotypic properties of the infected cells can be demonstrated, such as growth in soft agar. This balance *in vitro* between virus growth and

cell viability in culture is the closest model described to date which mimics the *in vivo* situation of fibroma cell proliferation in infected rabbits. Rabbit kidney cells persistently infected with SFV also become susceptible to vesicular stomatitus virus (VSV), to which they are normally resistant (88-91). In addition, monkey cells co-infected with SFV and VSV produce substantially reduced amounts of VSV defective interfering particles (92) and rabbits co-infected with SFV and VSV show reduced tumor formation but increased levels of VSV replication (93). The replication of SFV *in vivo* and *in vitro* can be inhibited by a number of antiviral agents, such as phosphonoacetic acid (46), arabinosyl-nucleoside analogues (47, 94), rifampicin (95), fluorodeoxyuridine (94), and the antibiotics congocidine and distamycin A (96).

The disease profile, gross pathogenesis and histopathology of SFV lesions in rabbits has been extensively reviewed elsewhere (1,3,4,7,9,97) and is only briefly summarized here. Unlike RPV, which is spread by aerosols and close contact, the primary mode of *Leporipoxvirus* transmission is by arthropod vectors and the major site of virus entry is by dermal innoculation. The host range in the wild is limited to the cottontail rabbit (*Sylvilagus floridanus*) in which the gross pathology is described as a slowly developing proliferant fibroma. Histologically, the tumors resemble molluscum contagiosum lesions in humans, and consist of undifferentiated mesenchymal cells and hyperplastic vascular endothelial cells, and often are infiltrated with mononuclear and polymorphonuclear leukocytes. The SFV tumors continually shed live virus which serve as the source of transmission by vectors, especially the mosquito. The viral proteins are excellent antigens (98) and in immunocompetent hosts the tumors regress spontaneously by virtue of a vigorous cell-mediated immunity (70,71,99-104), possibly abetted by circulating cytotoxic antibody (100,105,106) and interferon induction (107,108). When chemical co-carcinogens or gamma-irradiation is applied concurrently with viral innoculation, invasive sarcomas are elicited: in contrast, no such synergistic effects can be observed with other poxviruses, such as vaccinia or myxoma (7,109). Of particular note is that different strains of SFV vary in tumorigenicity (110-113) and isolates can spontaneously lose their oncogenic potential but remain infectious (7), implying that the viral genetic information governing

cellular proliferation is variable. In fact, many facets of the biology of tumorigenic poxviruses such as SFV can be rationalized by postulating that the virus either induces or encodes, as a function nonessential for viability, a growth factor or related mitogenic effector which stimulates target fibroblasts to proliferate.

One of the characteristic features of other DNA tumor viruses is their capacity to induce host nuclear DNA synthesis in response to specific viral gene products (77). The situation after SFV infection *in vitro* is more complex, and the cellular response appears to be a consequence of several competing viral activities which can either stimulate or inhibit host nuclear DNA synthesis, depending on infection conditions (86,88,114-116). At least some of the inhibitory activity detected under conditions of high multiplicity infections is related to the presence of endogenous DNAase activities which reside within SFV virions (117), but the nature of the stimulatory functions has not yet been elucidated.

In terms of SFV-induced proteins, it has been shown by polyacrylamide gel electrophoresis that both structural proteins in virus particles and nonvirion proteins induced in the cytoplasms of virus-infected cells show considerable variation form the profiles observed in parallel preparations from *Orthopoxvirus*-infected cells (118). Immunoprecipitation with heterologous antisera showed partial, albeit unquantitated, cross-reactivity between a few of the antigens induced by SFV and those of cowpox and vaccinia (119,120). The nature of these cross-reactive antigens is still not understood, but they do not induce neutralizing antibodies and infection of rabbits with vaccinia does not engender immunity to subsequent infection by SFV, and vice versa.

The DNA genome of SFV has been investigated by the techniques of recombinant DNA technology. The restriction enzyme profiles for a variety of SFV strains have been determined (see Table 2) and the BamHI, HindIII and XhoI restriction fragments have been cloned in bacterial plasmid vectors (32,34). The SFV DNA genome is 160 kilobases in length and shows a highly conserved arrangement between the different isolates of SFV. The viral DNA contains covalently closed hairpin termini (121) and possesses TIRs of 12.4 kilobases (32-34). Restriction maps of SFV DNA have been determined for a variety of restriction enzymes, some of which are illustrated in Fig. 2. As previously described (see Fig. 1), no demonstrable homology between SFV and vaccinia has yet been detected at

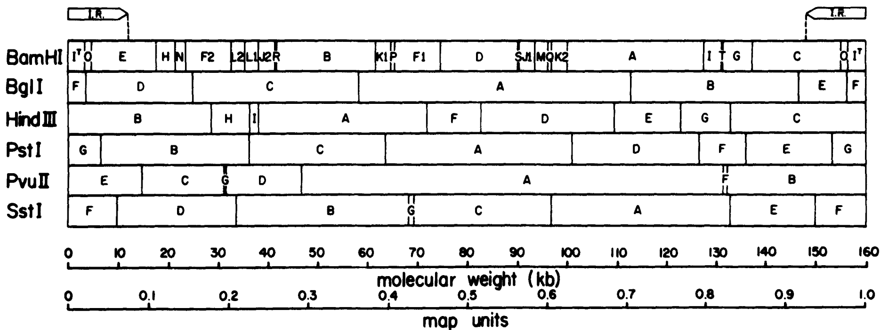

Fig. 2. Restriction enzyme map of the SFV (strain Kasza) DNA genome. The letters of each restriction fragment are in descending order, A being the largest. IR = Inverted Repeat. (From reference 33, with permission).

the level of DNA/DNA hybridizations, but there is some evidence that the two viruses induce several analogous gene products, such as RNA polymerase (122), thymidine kinase (TK) (123), and DNA polymerase (124,125). The SFV TK gene has been identified by hybridization with synthetic degenerate oligonucleotide probes and the nucleotide sequence compared to the vaccinia virus TK gene (126). As illustrated in Fig. 3, despite the fact that the DNAs encoding the vaccinia and SFV TK genes do not cross-hybridize under standard stringencies, they nevertheless possess extensive homology at the deduced DNA sequence level. Homology matrix

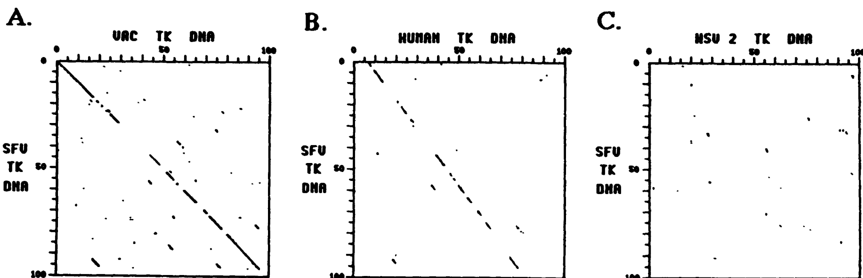

Fig. 3. DNA sequence homology matrix analysis of the TK genes derived from SFV, vaccinia virus, human and herpes simplex virus (HSV) type 2. The computer search program scored for string length homologies of 18 nucleotides, allowing for 6 possible mismatches. The axis of numbers refer to % full length for each TK gene. Nucleotide lengths: SFV = 528; vaccinia = 531; human = 702; HSV-2 = 1,128. (From reference 126, with permission).

analysis also suggests a close evolutionary relationship between the poxviral TK genes and a variety of eukaryotic cellular TKs, but not with the TK of herpes simplex virus (Fig. 3). Limited DNA sequence analysis of the SFV genome in the region neighboring the viral TK gene (126) has suggested that there is also a similarity between the organization of other viral genes of SFV and vaccinia in the middle of the two viral genomes, suggesting that the *Ortho-* and *Lepori-poxviruses* have descended from a common ancestral virus. On the other hand, the SFV TIR region has been completely sequenced at the DNA level (127-129) but no similarity can be detected with the TIR regions of vaccinia at the structural level or with respect to the encoded gene products (12,14, 16-18), suggesting that the viral TIRs of the two genera have evolved independently.

Several lines of enquiry have suggested that SFV genes which lie within or near the viral TIR are important for the biology of SFV-induced tumors. For example, recombination of only 7-8 kilobases of SFV sequences from this region into a myxoma genetic background resulted in the generation of novel tumorigenic *Leporipoxvirus*, malignant rabbit fibroma virus (MRV) (see last section). Furthermore, a subset of the SFV TIR DNA sequences are closely related to a small circular DNA species found in uninfected rabbit cells (127), suggesting that at least some of the SFV TIR may itself have been originally acquired by genetic recombination. The identity of this small circular DNA species remains to be determined but one plausible explanation is that it is a novel latent rabbit virus. However, since knowledge of rabbit viruses outside the poxvirus family is still very scanty (1), further analysis will be necessary in order to clarify the origin of this DNA species.

The expression of SFV genes encoded in the viral TIR has been analyzed by transcriptional mapping studies (130,131). Three of the expressed SFV TIR genes, designated T6, T8 and T9, are highly homologous to each other at the amino acid level and were probably created by a gene triplication event during the evolution of the SFV genome (128).

In terms of possible involvement in SFV fibromatogenesis, perhaps the most intriguing viral gene to date analyzed by DNA sequencing is designated the Shope fibroma growth factor (SFGF) gene, which maps just outside the right hand viral TIR in the unique internal sequences (132). As shown in Fig. 4, the SFGF amino acid sequence bears significant

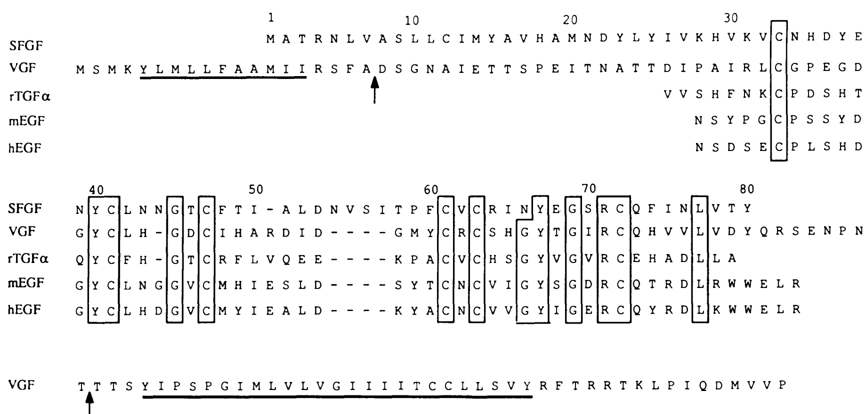

Fig. 4. Comparison of the amino acid sequence of the SFV growth factor (SFGF) gene with other members of the EGF/TGF-alpha family. The deduced precursor sequence of SFGF is compared with the precursor of vaccinia growth factor (VGF) and the secreted peptides for rat TGF-alpha (rTGF-alpha), mouse EGF (mEGF) and human EGF (hEGF). Identical amino acid residues are indicated in blocks. The proposed N-terminal signal sequence and hydrophobic C-terminal membrane spanning site of the VGF precursor are underlined (133) and the deduced cleavage sites for the generation of the secreted VGF polypeptide (135) are indicated by arrows. (From reference 132, with permission).

homology with a family of secreted mammalian peptide growth factors, including epidermal growth factor (EGF) and transforming growth factor-alpha. Vaccinia also contains a related gene, designated vaccinia growth factor (VGF), which was originally detected by homology searches in the published sequence databases (133,134). The role(s) of peptide growth factors such as members of the EGF family in normal cellular development and oncogenesis are complex, and is the subject of numerous recent reviews (136-142). The intact SFGF gene is also found in the MRV genome as well (last section), and its precise role in the tumorigenic phenotype of both SFV and MRV is a subject of current investigation.

Myxoma virus

The biology of myxoma virus and its interaction with the feral populations of *O. cuniculus* is a complex and fascinating topic and has been the subject of numerous comprehensive treatises (1-5,9,143). Unfortunately, there has been relatively little information gathered on the molecular biology of this virus and to date little is known concerning the molecular aspects of myxoma virus/cell interactions.

In its natural host (in S. America, *Sylvilagus brasiliensis*, the

tropical forest rabbit or "tapeti"; in N. America, *Sylvilagus bachmani*, the brush rabbit) myxoma virus causes very minor and benign lesions and would have probably never been systematically studied were it not for the fact that in *O. cuniculus*, the domestic rabbit, it causes a profoundly lethal disease, referred to as myxomatosis. Myxomatosis was first recognized as an infectious viral disease of imported rabbits in Uruguay at the turn of the century. Since then, the virus has spread, both by natural means and deliberate dissemination, to the point where it is now enzootic in wild populations of *Oryctolagus* in South America, Australia and Europe and in several species of *Sylvilagus* in North and South America. The virus produces disease only in leporids but there is a wide range of pathologies, varying from mild, self-limiting cutaneous lesions to full blown myxomatosis with > 99% mortality (reviewed extensively by Fenner and colleagues in references 3-5). Although the S. American form of myxomatosis tends to display more dramatic manifestations at the gross level (including extensive skin lesions and hemorrhages) than the California strain, both show characteristic internal features, including substantial proliferation of undifferentiated mesenchymal cells with large stellate morphology, often embedded within a matrix of secreted seromucinous material. In severe cases, the endothelial proliferation results in lumen narrowing of the local capillaries and necrosis of the myxomatous lesions is common.

The myxoma virus displays a close antigenic relationship to SFV and in fact the latter agent has been used as a vaccine against myxomatosis. The disease is spread by direct contact via discharges from lesions or conjuctival exudates and also by arthropod vectors, especially mosquitoes and fleas. The myxoma virus genetic information is subject to rapid variation and the mortality levels in infected feral rabbit populations has tended to moderate to much lower levels than when the first exposure was initiated. In addition, more resistant strains of rabbits tend to arise and fill ecological niches created by the first wave of infections (3).

The myxoma virus grows well in chick CAMs and a variety of cultured animal cells, including those derived from chicken, rabbit, squirrel, hamster, monkey and guinea pig. The replication *in vitro* of myxoma virus tends to be more vigorous than SFV and the cytopathologies somewhat more severe, depending on the conditions of infection (3, 144). Both

viruses are inhibited by the antiviral agent phosphonoacetic acid (145). Infected cells secrete into the medium a variety of viral specific polypeptides, some of which are sulphated and glycosylated, and unrelated antigenically to polypeptides secreted from vaccinia-infected cells (146). On the other hand, soluble antigens found in the serum of myxoma-infected rabbits appear to have a low but detectable, cross-reactivity with those induced by vaccinia (147), although the significance of this is unclear.

The viral DNA of myxoma virus has only been examined to date at the level of agarose gel electrophoresis after digestion with a relatively few restriction endonucleases (see Table 2). The myxoma DNA genome is approximately the same size as that of SFV, 160 kilobases, and the two show considerable cross-hybridization under conditions of moderate stringencies (34,36), indicating a close evolutionary relationship (see Fig. 1). Detailed restriction enzyme mapping studies remain to be done on the myxoma genome, and defined cloned DNA probes are needed before the extent of divergence between SFV and myxoma can be ascertained. Numerous isolates of myxoma possessing various degrees of attenuation, classified from level I to V (3), are available, but detailed mapping information on the myxoma genome will be required before the viral genetic elements governing pathogenesis, host range and virulence can be analyzed at the molecular level.

Malignant rabbit fibroma virus

In 1983 a novel tumorigenic poxvirus of rabbits was discovered in a laboratory rabbit colony which, at the time, was being used in a study of SFV (37,38). This new isolate, designated malignant rabbit fibroma virus (MRV, sometimes also abbreviated as MV or MRFV) causes fibromas in infected rabbits, which, at early times of infection, are histologically related, but not identical, to those induced by SFV (see Fig. 5). However, instead of regressing as in the case of SFV, these MRV-induced tumors were found to rapidly invade into multiple secondary sites of the rabbit in a systemic fashion. MRV infection is extremely lethal to the infected rabbit due to this disseminated malignancy as well as concomitant respiratory problems and purulent conjuctivitius brought on by supervening Gram-negative bacterial infections, particularly *Pasteurella multocida* and *Bordetella bronchoseptica* (37,38). MRV was shown to profoundly immunosuppress adult rabbits and this disruption of the immune system

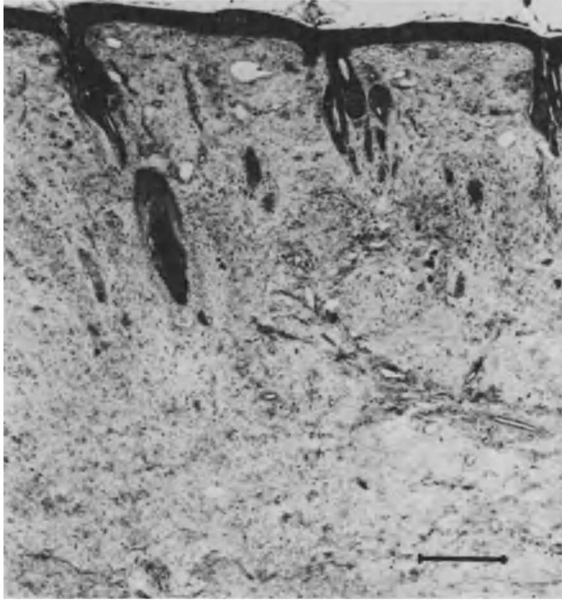

Fig. 5. Photomicrograph of primary malignant fibroma in rabbit dermis 6 days after intradermal inoculation with 100 infectious units of malignant rabbit fibroma virus, using hematoxylin and eosin staining (photo kindly provided by D. Strayer). The proliferating fibroma cells show high mitotic rate and appear either as dense clusters or scattered in mucinous ground substance. Bar = 1 mm. (From reference 37, with permission).

presumably plays a major role in allowing the tumors to progress from the benign localized tumors associated with SFV infection to the more invasive MRV-induced malignancy. The group which isolated and plaque-purified MRV ascertained that it was a contaminant of an SFV stock (strain Patuxent) they were using and, in fact, possessed many biological characteristics associated with both SFV and myxoma virus (37,38,148-155).

The pathology of MRV and the effect of this virus on rabbit lymphocytes during the progressive immunosuppression has been reviewed elsewhere by Strayer (156) and will be considered only briefly here. To date the MRV syndrome has only been observed in the original rabbit colony in Southern California from which it was isolated and in experimentally infected rabbits, but its extreme virulence in *O. cuniculus* suggests that wide dissemination could be possible if it were to gain access to the wild rabbit population. The MRV tumors (37,148) can be described at early times as myxosarcoma-like and are characterized by local raised tumor

masses quite unlike the diffuse flat lesions of myxomatosis, and yet distinct from the homogeneous fibroblastic proliferations in SFV tumors. As MRV infection proceeds, the infected rabbit rapidly loses the ability to mount an effective immune response to heterologous antigens as well as to the infecting virus and both B and T cell functions become profoundly compromised. The progressive metastasis of the MRV syndrome occurs via the spread of infected lymphocytes and, possibly, liberated infectious virus, via the reticuloendothelial system. The MRV infection is almost uniformly fatal to adult rabbits, but complete protection can be effected by prior immunization with SFV. A comprehensive comparison of the histopathology of SFV, myxoma and MRV lesions can be found elsewhere (148).

In culture, MRV replicates in a variety of cell types, including splenic lymphocytes, which are normally resistant to SFV. Interestingly, MRV is capable of propagating in resting T cells, although the extent of replication can be increased with mitogenic stimulation (153). In this respect, MRV is similar to HTLV III, the agent of human acquired immunodeficiency syndrome (AIDS), in that both viruses are capable of infecting quiescent T cells (152). The kinetics of growth of MRV in fibroblasts *in vitro* is somewhat more vigorous than SFV, and very comparable to that of myxoma virus. Both myxoma and MRV are rather cytotoxic to cultured cells and can prevent subsequent proliferation of rabbit kidney cells *in vitro*.

The organization of the DNA genome of MRV has been investigated by restriction enzyme digestions (36-38) and Southern blotting (36). As shown in the left panel of Fig. 6, the BamHI profiles of MRV DNA are closely related, but not identical, to that of myxoma (strain Lausanne) and quite distinct from SFV. Although moderate stringencies of hybridization permit cross-hybridization between the DNAs of SFV and myxoma (see Fig. 1), under highly stringent conditions (36) SFV DNA probes do not hybridize with myxoma DNA and under such conditions SFV sequences in MRV can be specifically distinguished. By virtue of mapping studies such as these, the origin of MRV can be confirmed to be associated with a recombination event that occurred at some point between myxoma and SFV. Based on the map positions of the SFV-derived sequences in MRV it has been possible to postulate a model to explain the structure of the MRV genome (Fig. 6, right panel). The original recombination event appears to have

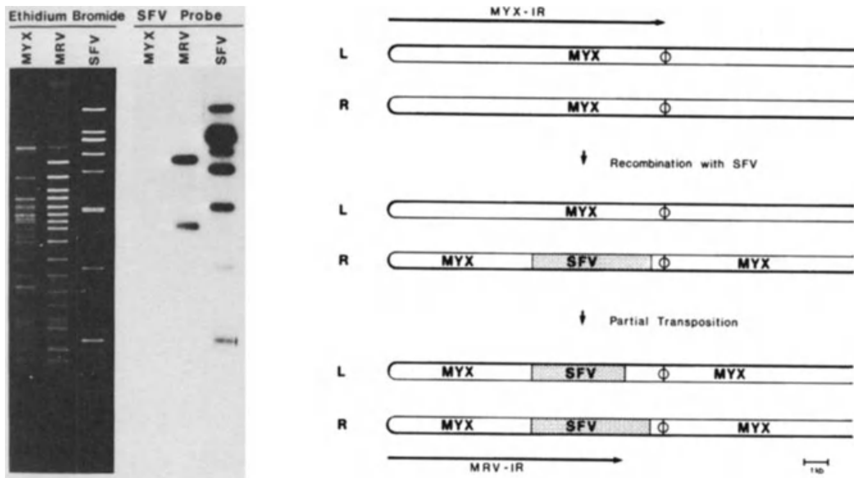

Fig. 6. The DNA genome of Malignant rabbit fibroma virus (MRV) was derived by recombination between Shope fibroma virus (SFV) and myxoma virus (MYX). In the left panel, the BamHI-digested viral DNAs were hybridized with (^{32}P) SFV DNA probe under conditions of high stringency such that the SFV and MYX genomes do not cross hybridize. Two BamHI fragments of MRV (A and H) contain SFV DNA sequences within them. In the right panel is presented a model to rationalize the origin of MRV by recombination between SFV and myxoma and to explain why more SFV DNA sequence information were transferred to the right MRV terminus than to the left. See text for details. (From reference 36, with permission).

been the genetic transfer of 7-8 kilobases from the right-end region of the SFV TIR to the right-end region of an unknown strain of myxoma virus DNA, with the concomitant loss of 7-8 kilobases of myxoma virus DNA sequences. Subsequent to this a still poorly understood process of terminal sequence transposition (reviewed in 14,16,17) resulted in the copying of 4-5 kilobases from the right MRV terminus to the left terminus (36). In this process approximately one-half dozen genes from SFV were transferred into myxoma (128), including an intact copy of the SFGF gene (Fig. 4).

At the present time it is not possible to say whether the recombination event which created MRV occurred during the passage of SFV through a rabbit concomitantly infected with myxoma, which can be found in a latent fashion in some rabbits (157), or was a result of an accidental laboratory contamination of myxoma virus during passage of SFV in culture. Perhaps when more information is obtained on the extent of myxoma DNA

genomic variations in the multiple strains of myxoma virus in the wild it will be possible to more explicitly ascertain the origin of the myxoma genetic background which forms the bulk of the MRV DNA genome.

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ECTROMELIA (MOUSEPOX) VIRUS

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ABSTRACT

Ectromelia virus, an orthopoxvirus, is a natural pathogen of colonized mice, and is the causative agent of mousepox. This disease follows three basic courses in mice susceptible to infection: 1) in the C57BL strains which express an innate resistance gene(s), and have a H-2^b MHC complex, the disease is inapparent; 2) in the A strains of mice which lack the innate resistance gene, a fulminating, acute disease with 100% mortality is observed; and 3) in strains such as BALB/c and DBA, an intermediate disease course is noted which may be acute or chronic. Severe disease and death is associated with necrotic lesions in the liver and the reticulo-endothelial system, with the spleen and the lymph nodes draining the initial site of infection being most affected. The major route of infection in nature is via abrasions on the surface of the skin which come in contact with infectious bedding, and there is no convincing evidence of aerosol transmission of the virus between cages and rooms. Mice infected either naturally or experimentally transmit the virus for a specific period of time depending on a number of factors, and surviving mice are then resistant to severe disease on reinfection, in certain cases, for a lifetime. Since virus transmission is through contact, epizootics may effectively be dealt with by localized quarantine and serologic monitoring, with the removal of cages which contain an animal which undergoes sero-conversion. The wholesale slaughter of mice exposed to this agent is not an acceptable control measure based on the known biology of the virus.

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INTRODUCTION

Since its discovery in colonized mice in 1930 (1), mousepox has been a scourge to many scientists using mice in research because of the high mortality associated with disease in susceptible strains of mice. Yet, it has served well as a model for the studies of exanthematous diseases of man (2), of immunologic responses to viral disease (3-8), and of innate resistance genes (9-12). Nevertheless, the fear of mousepox, due in part to an erroneous conception of its transmission and epizootiology (13, 14), has resulted in drastic control measures, namely the destruction of entire colonies of mice (14, 15). Considering current knowledge of the disease, there are reasonable alternatives to this control measure.

Mousepox has been considered enzootic in some mouse colonies in Asia and Europe, with only epizootic occurrences reported on the North American continent (16). Epizootics were recognized in several U.S. biomedical research institutions in 1979 and 1980, resulting in serious disruption to research and considerable expense for surveillance and control (17); however, important epizootiologic information was obtained (17, 18), and the outbreaks stimulated research on the biology of ectromelia virus in inbred strains of mice (19) as well as the development of a sensitive and specific serologic assay (20). Related research revealed that the common practice of immunizing mice against mousepox with vaccinia virus, effective in preventing disease, does not prevent transmission of ectromelia virus (21, 22).

Current knowledge of the biology and epizootiology of mousepox and its etiologic agent, ectromelia virus, along with a sensitive and specific serologic assay, provide rational and reasonable basis for prevention and control measures. Nevertheless, a need remains for a more effective vaccine.

PROPERTIES OF THE VIRUS

Classification

Ectromelia virus is a member of the genus *Orthopoxvirus* , one of six genera in the subfamily *Chordopoxviridae* of the family *Poxviridae*. Like other poxviruses of the vertebrates, members of this genus are distinguished by a large complex virion structure, a genome of double-stranded

DNA and a cytoplasmic site of replication. Allocation of virus strains to the genus is based on cross-protection experiments in animals and cross-reactivity in virus infectivity neutralization tests. Relationships among genus members have been further demonstrated by chemical and immunological analysis of virus-coded proteins (23-27), by nucleic acid hybridization studies (28), and through restriction endonuclease digestion of genomic DNA (29-32). This last analytical approach has revealed DNA sequence homology among the central region of the genomic DNA of a number of Orthopoxviruses (31), and divergence in sequence near the termini. It has been suggested that these conserved DNA sequences code for functions which are necessary for the direct production of progeny virus, and that the terminal regions code for virus functions that are tailored specifically to the individual virus-host relationship (31, 33). A prediction of this hypothesis would be that replication of these viruses in selected tissue culture cell lines would not require functions coded in the terminal regions of the virus genome. This appears to be the case, as naturally occurring mutants which have large DNA deletions in the terminal regions of the genome have been isolated in tissue culture from a number of Orthopoxvirus species (33-37). Furthermore, as would be expected of virus-coded functions which are not required for virus replication in tissue culture, no conditional lethal temperature sensitive mutants have been isolated in vitro which map to the terminal regions of the genome, although a large number of such mutants have been localized to the central, conserved region of the genome (38-40). It is of interest to note that where rabbitpox mutants (with deletions near the end of the genome) have been analyzed in animals, a number have been shown to be attenuated by the intracerebral and intradermal routes of inoculation in mice and rabbits, respectively (41).

Ectromelia virus variation

A cohesive study of the biological and biochemical properties of ectromelia virus isolates has not been carried out; however, a limited number of comparisons of isolates in the laboratory have shown distinctive properties (42, 43). The Hampstead strain was the original ectromelia virus isolate obtained by Marchal (1). It was maintained by passage both in the mouse and through eggs (44). Egg passaged virus was shown to lead to a substantial reduction in virulence for mice by the footpad route of infection (42). The Moscow strain of virus was isolated by Professor

V.D. Soloviev in Moscow (44), and has been made the benchmark for comparison of ectromelia virus virulence.

When the virulence of the Moscow strain in BALB/cByJ mice was compared by Lethal Dose$_{50}$ (LD_{50}) with a number of isolates from widely separated geographical locations, a tentative pattern emerged (45). Strains (Beijing 70, China; Washington University, USA; St. Louis 69, France) that had little or no tissue culture passage history, scored LD_{50} indices of a similar order of magnitude as that of the Moscow strain ($LD_{50} = 3.9 \times 10^1$); however, strains such as Ishibashi I-III and Hampstead (egg adapted) which had a number of _in vitro_ passages in egg or tissue culture showed a significantly higher LD_{50} ($LD_{50} > 10^4$). Japanese workers have previously shown that these two strains differed both in the character of the A type inclusion formed, and the plaque morphology on chicken embryo fibroblasts, where the Ishibashi strain produced larger plaques than Hampstead (43).

Of all of the ectromelia virus isolates available for analysis, the Moscow and Hampstead isolates have received the most attention by a succession of Australian researchers that has spanned five decades. Recently, an ectromelia virus isolate (strain NIH 79)* was cultured from an infected mouse in the 1979/1980 ectromelia virus epizootic at the National Institutes of Health (46). This isolate has been used in a number of studies examining the basic biology of ectromelia virus replication in inbred mouse strains (12, 19, 22).

Restriction endonuclease analysis of virion DNA has been employed successfully to analyse the similarities and differences among the _Orthopoxvirus_ species; however, it has only been used to a limited extent to compare the various isolates of ectromelia virus. Distinct differences were reported among the restriction endonuclease patterns of the genomic DNA of Hampstead, Moscow and NIH-79 strains of ectromelia virus (31, 45); however, independent attempts by Andrewes and Elford (47) and by Fenner (42) could not detect antigenic difference (haemagglutinin inhibition assay) between Hampstead and Moscow virus strains in mice, rabbits, guinea pigs or rats by single or repeated inoculations of virus protein.

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* A morbid C3H/HeN mouse from the 1979 ectromelia virus epizootic at NIH was sacrificed, and the virus from infected spleen/lymph node was passaged consecutively in a pathogen-free BALB/c mouse, a primary chick embryo culture, a BALB/cByJ mouse (twice) prior to virus cloning by three limiting dilution plaque purifications, and two passages in BS-C-1 cells prior to production of a working virus stock.

PATHOGENESIS

Clinical Disease

Two types of clinical (in addition to nonclinical or inapparent) responses in naturally and experimentally infected mice are evident (16, 46, 48). Highly susceptible mice such as A and C3H/HeJ strains usually die within 8 - 12 days after footpad inoculation with little or no clinical evidence of infection (19). This has been referred to as the acute versus the chronic form of the disease (16). DBA/2 and BALB/c strains can give either an acute or chronic response to infection. With the chronic form, depression, "hunching," ruffled coat, conjunctivitis, severe skin lesions and necrosis leading to amputation of limbs, ears and tail may be seen (Fig. 1). The chronic form may or may not terminate fatally. Typical signs are most obvious in the chronic form.

Fig. 1. A wild mouse (M. musculus domesticus, eastern shore) inoculated in the laboratory with approximately 1000 PFU of NIH79 strain of ectromelia virus by the footpad route of inoculation. This photograph was prepared 24 days following infection.

Fenner (16) describes the course of events, as observed in outbred mice experimentally infected with the virulent Moscow strain of virus by the footpad route, as follows: days one and two, local multiplication and spread to regional lymph nodes either as free virus or in circulating leukocytes(49); day two, primary viremia; day three, replication, with

necrosis, in the spleen and liver; day four, secondary viremia; days five and six, focal infection and replication in the skin; followed by a primary lesion at the site of inoculation on day seven, then a rash appearing on day seven, becoming severe and ulcerating on day eight. The severity of the rash depends on the degree of viremia, and may be absent or inapparent in resistant mice (19). In acutely fatal cases, the mice may die before enough time has elapsed for skin lesions to develop.

Factors Affecting Clinical Disease

The outcome of infection of the mouse with ectromelia virus is dependent on strain of virus and the genotype, age, and sex of the mouse. The strain of virus appears to be the least important of these factors, since strains isolated from naturally occurring outbreaks seem to be uniformly virulent prior to serial passage in eggs or cell culture (44), although careful studies by Fenner (42) showed distinct differences between the mouse-passaged Moscow and Hampstead strains in the ability to infect cagemates. While both strains were highly virulent, the Hampstead strain was not highly infectious.

The genotype of the mouse, on the other hand, has a dramatic effect on the course of the disease. Schell was first to demonstrate definitely that the C57BL strain showed greater resistance to disease than outbred strains and CBA and Bagg inbred strains (9). This difference was most dramatic when the footpad or intravenous routes of inoculation were employed, and was attributed to the development of a more effective immune response in the C57BL strain. Others similarly found that related strains C57BL/6 and C57BL/10 (B10) also demonstrated little or no clinical evidence of disease, whereas infections of A, BALB/c and DBA/2 strains yielded a highly fulminant disease course associated with high mortality (11, 12, 19, 48, 50). A more recent demonstration of the importance of genotype on the severity of the disease can be found in the work of O'Neill and co-workers (50). This group showed that a congenic strain B10A (5R) which differed from its parent B10 ($H\text{-}2^b$) by having H-2 alleles J^k, E^k, C^d, S^d and D^d, yielded over 100-fold more virus infectivity in spleen and liver, and a considerably higher mortality rate than the B10 mouse when infected by Moscow strain of ectromelia virus. The contribution of the $H\text{-}2^b$ genes to recovery from mousepox is only observed in mouse strains such as C57BL/6 or C57BL/10, which also express an innate resistance gene(s) of unknown function. The A strain, which lacks this resistance

gene (11, 12), is uniformly susceptible to ectromelia virus-induced death even in the case of strain A.By/SnJ which is $H\text{-}2^b$ (19).

Where examined (11, 51), it has been found that the cells cultured *in vitro* from both resistant and susceptible mice are equally permissive for ectromelia virus replication; this is consistent with the idea that resistance to clinical disease is not being expressed at the cellular level in non-lymphoid cells.

Fenner showed that both the Moscow and Hampstead (egg adapted) strains of ectromelia virus caused a more severe clinical disease in outbred, suckling mice and year old mice, than in eight-week-old mice (52). Using only Moscow strain of virus, Schell found a similar result in the resistant strain C57Bl (9).

A sexual dimorphism to disease has been observed in BALB/cJ and A/J mouse strains, and appears to have at least in part a hormonal basis (53). This sex-related difference in severity of disease although evident in the parental strains, was much more apparent in back-crossed populations (12). In all cases the female mice appeared more resistant to disease than males.

Pathology and Histopathology

Ectromelia virus replication in the cytoplasm of infected cells is associated with the appearance of two virus-induced intracytoplasmic inclusion bodies which are of diagnostic importance: type A (Marchal's bodies; 1) and type B (Guarnieri's bodies; 54, 55). The former are eosinophilic staining structures that are prominent in epidermal cells (Fig. 2), but not in the liver. This inclusion body has been classified as V+ or V- depending on whether virus is found in association. This V+ or V- character is a strain-specific property (43). Ectromelia, racoonpox, and cowpox viruses are the only *Orthopoxviruses* that regularly produce this inclusion body (55, 56). The latter (type B) are basophilic, present in all infected cells (Fig. 3), and may be easily overlooked.

Skin Lesions. The surface of the epidermis through which the virus first enters the mouse develops into the primary lesion. It is characterized by localized edema of the surrounding tissue, necrosis of both the epidermal and dermal cells, and the expected infiltration of inflammatory cells. With time, a hard, adherent scab forms, falls off, and leaves a deep hairless scar.

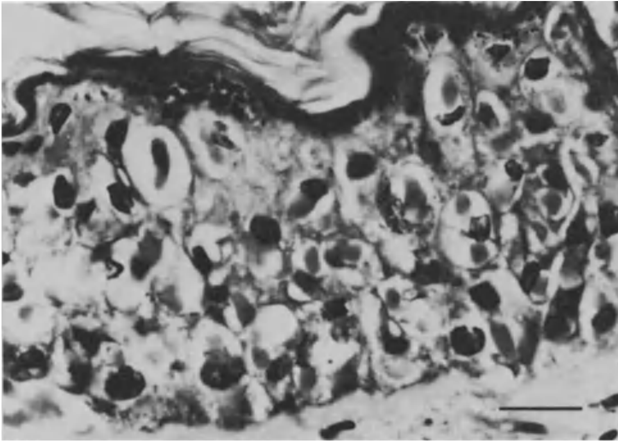

Fig. 2. Hyperplasia and hypertrophy of epidermal cells containing acidophilic cytoplasmic inclusions (Marchal's bodies). Hematoxylin and eosin stain. Line = 15 μm. (reprinted with permission from Ref. 46).

In some strains of mice, but not all, a secondary rash is observed. The first histologic changes observed are characterized by a few focal areas of epidermal hyperplasia with hypertrophy and ballooning of epithelial cells. Some cells may contain strongly staining eosinophilic cytoplasmic inclusions (type A). These secondary lesions can be found on the tongue, buccal mucous membranes, conjunctival epithelium, and vaginal mucosa (16).

Lesions of the Reticulo-endothelial System. The lymph nodes draining the primary lesion become enlarged and show partial to confluent necrosis spreading from the subcapsular sinus. In the most severe cases, the architecture of the lymph node is replaced by pyknotic nuclear debris and numerous inclusion bodies in a "featureless background" (57). In later stages of disease, the majority of lymph nodes (and Peyer's Patches) show lymphoblastic hyperplasia with or without focal necrosis. A remarkable feature of this disease in mice which recover is the rapidity with which the structure of the lymph node returns to normal. Grossly the spleen is engorged or pale, with isolated or confluent areas of necrosis (Fig. 4). In severe attacks, fibrous tissue can completely replace the necrotic spleen tissue, resulting in a scar which, on necropsy, is reliable evidence that the mouse has recovered from mousepox (Fig. 5) (57).

In the least severe form of the disease, histologic examination of spleens from infected animals reveals lymphoblastic hyperplasia of follicles and congestion of the sinuses of the red pulp. In severe cases,

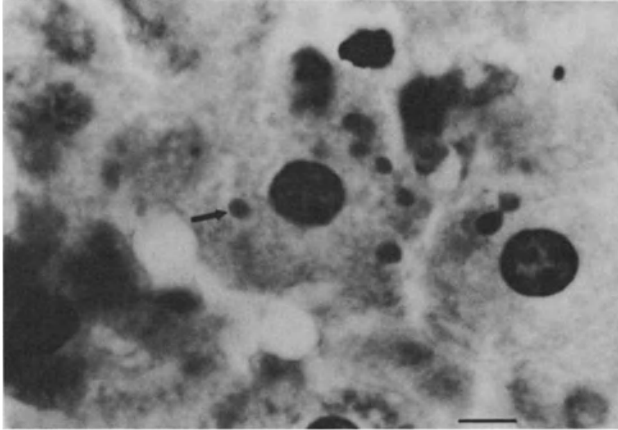

Fig. 3. Basophilic inclusions (Guarnieri's bodies) in hepatic cells. One cell contains six inclusions (arrow), several of which are surrounded by a faint halo. Hematoxylin and eosin stain. Line = 7 μm. (reprinted with permission from Ref. 46).

localized necrosis with fragmentation of the lymph follicles is observed, the extent of which depends on the mouse strain and age. Allen and co-workers found splenic lesions in 95% and 100% of naturally occurring and experimentally infected mice, respectively (46). Focal to confluent necrosis of the spleen is a consistent finding in mice that die with acute disease.

Liver Lesions. Livers may be swollen, friable, and mottled throughout with a delicate network of necrotic streaks producing a reticulated pattern. Allen and co-workers observed visible hepatic lesions in 15% of naturally infected mice which were sacrificed prior to death (46). Fenner observed hepatic lesions as the most dominant histologic finding in livers of fatal cases of mousepox, but that these lesions often occurred in the last 24 hours prior to death (57, 58). The major histologic finding in the liver is hepato-cellular necrosis which is usually focal, random in distribution, and showed no regular relationship to the normal tissue architecture. Curiously, in the acute disease, the inflammatory response in the liver is minimal (46).

Fig. 4. Section of spleen showing extensive areas of necrosis. Hematoxylin and eosin stain. Line = 300 μm. (reprinted with permission from Ref. 46).

Routes of Infection

Susceptible mice can be infected by all of the common routes of inoculation (57, 58). Some strains such as C57BL and C57BL/6 show a markedly different disease pattern depending on the inoculation route. By a peripheral route of inoculation such as the footpad, these strains show a subclinical disease picture (11, 12, 19), whereas the same dose of virus by the intraperitoneal route results in acute mousepox with high rates of mortality (10). Infection of mice by scarification, footpad inoculation or by instillation of the virus into the cornea result in a disease which is similar to that seen in naturally acquired mousepox (57, 59). The major route of infection in nature is via abrasions on the surfaces of the skin which come in contact with infected bedding. In 80% of naturally occurring cases, a primary lesion is detected and presumably represents the site of virus entry into the animal (58). Schell showed that between 1-2 particles of ectromelia virus were sufficient to infect a mouse ($1ID_{50}$) as demonstrated by a footpad inflammatory response (9), whereas $1LD_{50}$ required approximately 25 particles.

Immune Response

Both the humoral and cell-mediated arms of the immune system have been examined for their relative importance in recovery from disease. Pioneering research by Fenner showed that systemic administration of anti-ectromelia virus antibody reduced the clinical severity of the disease,

but had no effect upon the multiplication of virus in the inoculated foot (60). Immunization of the mouse with an attenuated Hampstead strain of ectromelia virus 4 weeks prior to footpad challenge with virulent Moscow strain was shown to be even more efficacious, as virus replication was severely limited, even in the foot. This work was extended by Blanden, who used cell transfer and graft vs. host experiments to provide compelling evidence that mononuclear phagocytes and cytotoxic T cells were most important in recovery from disease (3-5). Similar results were obtained by Tsuru and co-workers (61).

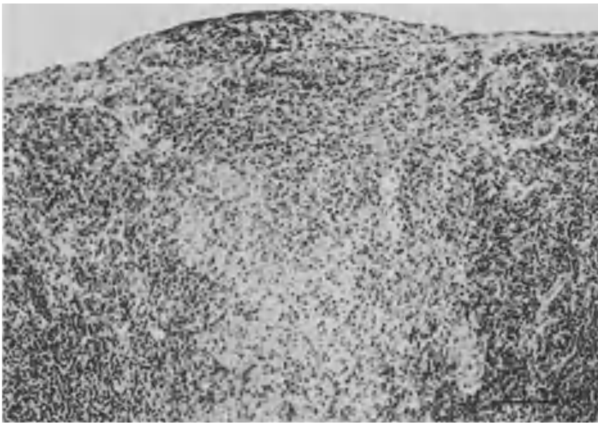

Fig. 5. Splenic scar with mound of fibroblasts and serosal cells on adjacent capsule. Hematoxylin and eosin stain. Line = 100 µm. (reprinted with permission from Ref. 46).

The apparent minor role of specific antibody in recovery from mousepox in resistant mice, which was first described by Blanden (3, 4), was supported by two independent experimental approaches which involved either infection with an immunosuppressive retrovirus (62) or depletion of $L3T4^+$ T helper cell population (63). Both approaches resulted in mice which were unable to mount a significant anti-ectromelia virus neutralizing antibody response, but nevertheless recovered from infection with similar kinetics as untreated controls.

EPIZOOTIOLOGY

Host Range

Chick Embryo and Tissue Culture Systems. The earliest studies involving the measurement of ectromelia virus infectivity were carried out on

the chorio-allantoic membrane (CAM) of the chick embryo. Burnet and Lush and Paschen independently showed that discrete, separate foci of infection (pocks) could be achieved by inoculating a dilute suspension of virus on the CAM (64, 65). A major deficiency with the use of chick embryos to measure virus infectivity resided in the variation from egg-to-egg of virus-induced pock formation. With the advent of tissue culture systems, an alternative method for measuring virus infectivity was available which was inherently more reproducible. The most commonly used cell lines for measuring ectromelia virus infectivity include L cells (3), BS-C-1 cells (20), chick embryo fibroblasts (9, 41), and mouse embryo fibroblasts (9). Schell showed that it took 19 and 20 virus particles to yield 1 plaque on mouse embryo fibroblasts and chick embryo fibroblasts, respectively (9). The CAM was slightly more sensitive with 12 particles needed to obtain one pock.

Ectromelia virus can replicate, to a limited extent, in some mouse hybridoma cultures although no obvious cytopathic effect was observed (66). It has also been shown that hybridoma cell lines which were able to support replication of the virus *in vitro*, could be infected during an *in vivo* passage in a diseased mouse.

Animals. Ectromelia virus has a very narrow host range - replicating well only in mice, and only in species from subgenus *Mus* and *Nannomys*, and not in prototype species from subgenus *Coelomys* or *Pyromys* (67). Syrian hamsters appear not to be susceptible to infection with ectromelia virus (68), whereas large doses of virus inoculated intranasally in the rat (69) or intradermally in the rabbit (70) resulted in at least limited, localized virus replication with the subsequent detection of circulating antibody. Paschen found that egg-passaged but not mouse-passaged ectromelia virus would replicate in the plantar surface of the guinea pig foot as well as the cornea (65). Intradermal inoculations of egg-passage virus gave a local indurated lesion and HI antibodies after 2 weeks.

Prevalence and Distribution

Mousepox is considered to be enzootic in many colonies of laboratory mice in Europe and Asia. North America has been free of disease except for epizootic occurrences (see 13, 16 for current review of prevalence). Documented introductions of ectromelia virus into mouse colonies in the United States have been traced to the inadvertent introduction of mice from unrecognized infected colonies (13, 17), or the inoculation of material

unknowingly containing ectromelia virus into susceptible mice (71). Experimental infection of four genera (seven species) of colonized wild Mus from North America, Europe, Asia and Africa demonstrated a wide range of susceptibility to disease -- from complete resistance to infection, to high mortality (67). These data suggest that populations of wild mice biologically capable of supporting enzootic mousepox exist in Europe and North America. Although little research has focused on the question of a virus reservoir in wild rodents in Europe, there are unconfirmed studies by Gröppel, who observed clinical signs (consistent with mousepox) in common voles and wood mice captured in the wild in Germany (72); however, no other diagnostic tools were used to confirm the clinical results. A second study in Britain by Kaplan et al. detected antibodies specific for Orthopoxviruses in sera from trapped skomer and short-tailed voles and wood mice (73).

Epizootic Mousepox

Detailed long term observations of experimental epizootics instituted with the virulent Moscow strain of virus in a highly susceptible outbred strain of laboratory mouse by Fenner supported the conclusion (59) that transmission occurred naturally in caged mice through contamination of skin abrasions with virus shed by infected cagemates (74). He has reported that bedding contaminated by infected mice is infectious for at least 24 hours (75). Contrary to reports in the literature (76), there is no convincing evidence of aerosol transmission of ectromelia virus between cages or rooms. Distant transmission probably occurs by the relocation of inapparently infected mice, or possibly by contaminated hands of careless animal caretakers and investigators. The inadvertent introduction of virus by the inoculation of mice with infected or contaminated biologic materials such as hybridoma cell lines is also potentially important (66).

The best information on the epizootiology of mousepox came from Fenner's observations of experimental epizootics (74). He found that "naturally" infected mice did not transmit to susceptible cagemates beyond 21 days, and virus could not be detected beyond 30 days, except in rare instances. Most importantly, he found that recovered mice were immune to reinfection, usually within two to three weeks. The immunity was solid for at least a year, then only limited replication of virus occurred in some of the challenged mice. Infant mice born to immune mothers were protected from fatal infections for several weeks by maternal antibody in the

milk; however, they could transmit virus. Since the protective effect of maternal antibody is lost within 4 weeks, mice not infected as weanlings would constitute a renewable, highly susceptible population with which to maintain the disease in a breeding colony (77).

In more recent times, with the growing use of inbred strains of mice, it has been important to learn if patterns of transmission and immunity in inbred strains were similar to outbred strains. It was recognized several years ago that at least one inbred strain (C57BL) was innately resistant to disease (9); however, observations of naturally occurring epizootics led Briody to conclude that C57BL mice could act as "immune carriers" of virus (76), implying long term, persistent infection and transmission of virus by this mouse strain. Furthermore, he reported that aerosol transmission was the most important mode of transmission and spread of virus.

Experimental and epizootiologic studies by Wallace and associates did not support Briody's conclusions that, in addition to C57BL strains, several other inbred strains were resistant to ectromelia virus (12, 19). Of seven inbred strains, C57BL/6J, AKR/J, C57LJ, BALB/cByJ, DBA/2J, A.By/SNJ and C3H/HeJ, experimentally tested by Wallace and Buller (19), the only strains that usually survived footpad inoculation with a virulent strain of virus (NIH-79) were the C57s and AKR mouse strains. Similar results for some of the strains have been reported by others (78). Furthermore, Wallace and Buller demonstrated that following footpad inoculation, C57BL/6 mice routinely transmitted the infection to cagemates, but for no longer than 17 days. Also, footpad-infected BALB/c mice regularly transmitted to cagemates before death at 10 - 12 days. When infected orally (intragastric inoculation), C57BL/6 mice shed virus in feces for up to 46 days, but were not infectious to cagemates beyond 36 days. Mortality in BALB/c mice infected orally was considerably less than by footpad infection (70% vs. close to 100%). Survivors usually developed the chronic form of disease. Small quantities of virus could be isolated from feces for as long as 29 days after oral infection and from skin up to 60 days; nevertheless, transmission could not be demonstrated beyond 30 days. Similar results were reported by Gledhill (79).

The first published systematic epizootiologic investigation of a natural outbreak of mousepox that included estimates of populations at risk (denominators) for calculations of prevalences of infection was done at the National Institutes of Health (NIH) in 1980 (18). Late in 1979,

mousepox was definitively diagnosed in three rooms on the NIH campus where mice on experiment were being held. In retrospect, it was likely that infected mice had been present in at least one of the rooms for several months. Prevalences of infection could be determined for two rooms on "site" and one room at an off-site "contract facility," also found to have housed infected mice. They were 3% of 939, 3% of 541, and 1% of 789, respectively. All rooms had housed susceptible strains of mice, usually four or five per cage. The salient feature of the epizootic was the slow and minimal spread of virus, which was later demonstrated to be highly virulent (19, 46, 78). Infected animals were found in only a few cages, which were located close to each other.

In summary, observations of well designed and controlled experimental epizootics in outbred mice along with more current detailed and systematic studies on experimentally and naturally infected inbred strains, has revealed the following important epizootiological characteristics of mousepox: 1) surviving mice develop immunity that limits the duration that virus is transmitted; 2) laboratory mice vary in resistance to disease, not infection; 3) all infected mice are capable of time-limited transmission of virus to non-immune cagemates; 4) spread of virus between cages and beyond probably occurs by one or more of the following methods: transfer of inapparently infected mice to cages of non-immune mice, inoculation of infected biological material into non-immune mice, or improper animal care techniques such as restraining mice with contaminated hands or instruments. Therefore, considering modern laboratory-animal husbandry practices (80), "explosive outbreaks" should be unusual today.

DIAGNOSIS

As indicated above, clinical signs such as skin lesions and amputations are suggestive of mousepox; however, suspicious skin lesions may also ensue from fighting, particularly in male mice. Since no other natural disease of mice is associated with the severe necrosis of lymph nodes, spleen and Peyer's patches, and on occasion intestinal engorgement which typifies acute mousepox in susceptible mouse strains, a provisional diagnosis can be made on the basis of gross and microscopic pathology (Fig. 6). For example, ectromelia virus infection cannot be differentiated from mouse hepatitis virus infection, a common mouse colony pathogen,

based solely on liver pathology, but examination of the reticulo-endothelial system reveals a striking pathological difference between the viruses. In a mouse hepatitis virus infection, necrosis is limited to specific regions of the spleen and lymph node follicles and the structure of the tissue is maintained. An ectromelia virus infection, on the other hand, causes total destruction of the lymph node architecture and severely affects the white pulp of the spleen. A provisional diagnosis can become definitive if distinctive type A or B cytoplasmic inclusion bodies are observed by hematoxylin and eosin staining, typical poxvirus virions are demonstrated by electron microscopy (46), or the virus is isolated from infected tissues, such as liver and spleen, by inoculation into cell cultures, onto the chorioallantoic membranes of eggs and/or into mice (16). In non-vaccinated mice, a positive ELISA, IFA, or virus neutralization assay employing vaccinia virus antigen, is diagnostic of past infection with an Orthopoxvirus, presumably ectromelia virus (20, 22). Antibody is usually detectable at ten days and may remain detectable for life. Antibody to ectromelia virus may be differentiated from anti-vaccinia virus antibody by employing both ectromelia and vaccinia virus antigen in the hemagglutinin-inhibition test (81) or ELISA (20).

CONTROL AND PREVENTION

The prevention of epizootics in a mouse colony require that 1) there is controlled access into the facility of all personnel, mice, and mouse products; 2) the facility has an adequate serologic screening program. The facility is the first line of defense against the accidental introduction of a pathogen such as ectromelia virus. All animals should be obtained from a source documented to be free of adventitious pathogens such as ectromelia, mouse hepatitis, Sendai viruses, etc. When this is not possible, introduced mice should be previously quarantined in direct contact with susceptible mice such as strain A, and serologically monitored for Orthopoxvirus antibody. Antibody specific for poxvirus is detectable 10 - 12 days after infection; therefore, testing serum collected from each mouse two or three times at biweekly intervals has a high probability of detecting the presence of ectromelia virus.

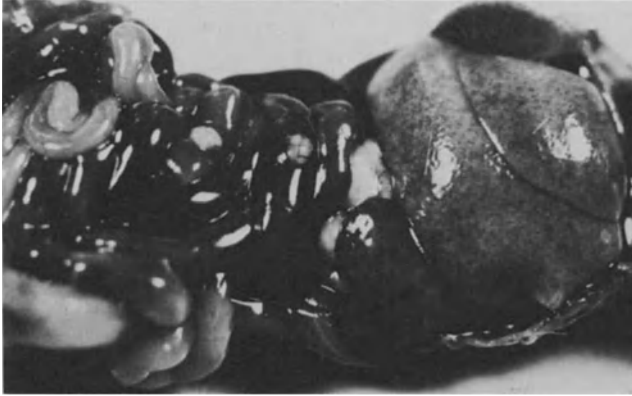

Fig. 6. Swelling and necrosis of the spleen and liver, enlarged Peyer's patches, and hemorrhagic small intestine in BALB/c mouse with mousepox. (reprinted with permission from Ref. 46).

It has been demonstrated that certain cell lines and cultures, including hybridomas, can support inapparent ectromelia virus replication (66). Consequently, biological materials of mouse origin should be tested for virus in cell culture or non-immune susceptible mice prior to introduction into the mouse facility.

If an infection with ectromelia virus is found within a colony, it is likely that it will be localized if all animal care technicians practice proper husbandry procedures (80). In this event, a localized quarantine followed by biweekly serological testing with removal of cages containing reactors until the remaining mice are negative for two to three screenings should resolve the problem. This is a much more satisfactory solution than destroying all mice presumed to be exposed, as has been practiced.

All materials exposed to infected mice should be autoclaved and incinerated where practical. Ectromelia virus has a limited survival time on fomites which is affected by the association of cellular debris, humidity, and temperature (57, 82); however, this environmental stability is of no consequence should traditional disinfectants (final concentrations of the following chemicals: paracresol 1%, phenol 2%, Mercury bichloride 0.1%, or alcohol 40%, (56)) be used in routine cleaning of contact surfaces.

The common practice of immunizing mice against mousepox with vaccinia virus should be exercised with caution. Although it has been demonstrated that such vaccination may prevent morbidity and mortality, it does not necessarily prevent the spread of virus, thus having the potential of creating a "silent" reservoir of virus. Furthermore, serum from

vaccinated mice will react in ELISA and IFA diagnostic serological assays, when, as normally is the case, vaccinia virus antigen is the basis of the serological assays.

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5

POXVIRUS INFECTION IN ZOO-KEPT MAMMALS

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ABSTRACT

Between 1960 and 1986 at least 22 outbreaks of pox disease have been observed in elephants (Elephas maximus, Loxodonta africana), rhinoceroses (Ceratotherium simum, Diceros bicornis), okapis (Okapia johnstoni) and other mammals (family Felidae, ordo Edentata) in European zoological gardens. 21 outbreaks lie within a circle with a diameter of 1070 km around a taken center near Magdeburg. Only the Moscow outbreak occurred outside of this circle. Since in some zoological gardens children are allowed to ride on elephants, it had been assumed that the virus had been introduced into the zoo by persons recently vaccinated against smallpox. In 10 cases orthopoxvirus strains were isolated which were similar but not identical in their biological properties. But in contrast to vaccinia virus these virus strains produce small (1 - 2 mm in diameter) lesions with a haemorrhagic center on the chorioallantoic membrane, intracytoplasmic inclusions of type A V +, and characteristic skin lesions in laboratory mice. Since they resemble cowpox virus they were called cowpoxlike virus strains. The coincidental occurrence of some outbreaks and the restriction to a limited region within Europe support the hypothesis that zoo-kept mammals are only indicators of a certain hidden virus cycle. DNA analysis with restriction enzymes revealed a certain heterogeneity of the genome outside the conservative region characteristic for the orthopoxvirus genus. Recent virus isolations from a cat and humans living in close contact with domestic cats in the Netherlands and Germany revealed a close relatedness between these strains and the cowpoxlike virus strains

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isolated from zoo-kept mammals. These findings support the hypothesis that a small wild-living mammal may be the primary reservoir for cowpox or cowpoxlike viruses.

INTRODUCTION

In 1960 the first outbreak of pox disease occurred in zoo-kept elephants (5 Elephas maximus, 2 Loxodonta africana) in Leipzig. Four of five Asian Elephants in the zoo showed severe illness, while all the African Elephants recovered (1). In the following years similar outbreaks were observed in mammals of zoological gardens and circus enterprises in Germany, the Netherlands, Czechoslovakia, Austria, Poland, and the Soviet Union (Table 1; Fig. 1; 29, 32, 33). Since isolated virus strains resembled vaccinia virus in some biological properties, it was assumed that the virus originated from humans vaccinated against smallpox (4, 13).

VIRUS ISOLATION AND CHARACTERIZATION

Material and methods

The isolation and identification of pox virus strains followed standard procedures described in detail by LENNETTE & SCHMIDT (34). Infected egg membranes were fixed in 3 % glutaraldehyde and embedded in Durcopan (Fluka AG, Buchs, Switzerland). Contrastation in a 7 % aqueous solution of uranylacetate and plumbium hydroxide followed the procedure of MILLONIG (35).

Monkey kidney cells (RC-37) were grown and propagated as described previously (36). The origin and history of the virus isolates used for comparative studies is summarized in Table 2 (see also 37 - 39).

For ^{32}P-labelling of the viral genomes individual isolates were grown on RC-37 cell cultures, the viral DNA was labelled with ^{32}P-orthophosphate (carrier-free, in HCl-free aqueous solution, New England Nuclear) in vivo and analyzed as described elsewhere (38).

Table 1: Outbreaks of cowpoxlike disease in zoo-kept mammals in Europe

Locality	Geograph. co-ordin.	Time of outbreak	Mammal species	Deaths	Transfer to humans	Isolation and characterization of virus	References
Leipzig (zool. garden	51.19 N 12.20 E	2.5.60	5 Elephas maxim. 2 Loxodonta afr.	2	several cases, 2 with eye-disease	positive, poxvirus	1,2
Berlin-Friedrichsfelde (zool. garden)	52.20 N 13.25 E	a) 17.9.60	3 Elephas maxim.	O	2 cases, 1 with erosion of lip	positive, poxvirus	3
		b) 20.9.61	1 Elephas maxim.	O	O	negative	
Erfurt (Thüringer Zoopark)	50.58 N 11.01 E	1962	1 Loxodonta afr.	O		positive, "Vakzinevirus"	4
Copenhagen (zool. garden)	55.40 N 12.35 E	2.8.63	4 Okapia johnst.	1 calf	1 case: local infection of hand	positive, not vaccinia virus	5
Rotterdam (zool. garden)	51.55 N 04.28 E	23.11.68	5 Okapia johnst.	1 calf	O	positive, cowpoxvirus	6,7
Magdeburg (zool. garden)	52.07 N 11.38 E	3.7.71	2 Elephas maxim. 1 Loxodonta afr.	O	O	positive, cowpoxlike virus	8,9
Augsburg (circus C.A., frequent change of locality)	48.23 N 10.53 E (48.45-51.36 N 6.34-9.16 E)	7.-14.10.71	11 Elephas max. 4 Loxodonta afr.	2	2 cases: local infection of forearm and hand	positive, "Vakzinevirus"	10,11
German Democratic Republic (travelling circus)		20.3.72	12 Elephas maxim.	1	O	positive, "Vakzinevirus"	12,13
Liberec (zool. garden)	50.46 N 15.03 E	10.6.72	2 Elephas maxim.	O	O	positive	14

Table 1. (continued)

Locality	Geograph. co-ordin.	Time of outbreak	Mammal species	Deaths	Transfer to humans	Isolation and characterization of virus	References
Amsterdam (zool. garden)	52.22 N 04.54 E	31.8.73	2 Elephas maxim.	0	0	positive, cowpoxvirus	15
Moscow (zool. garden)	55.45 N 37.35 E	a) 13.11. 1973	about 19 cats (Felidae: Felis leo, F. pardus, Leopardus pardalis, Acinonyx jubatus), 2 Myrmecophaga jubata	nearly 100 % pulm. form	0	positive, cowpoxlike virus	16,17,18
		b) Oct. 1974	2 Felis concolor; F. bengalensis	killed	0		
Laxenburg near Vienna (circus)	48.13 N 16.20 E	early in 1974	1 Elephas maxim.	0	0	positive, cowpoxlike virus	19
Leutershausen near Ansbach	49.18 N 10.24 E	Febr. 1975	1 Elephas maxim.	1	0	positive, elephant poxvirus	20
Münster (Allwetter-Zoo)	50.57 N 07.37 E	5.9.77	2 Ceratotherium simum	0	0	positive, cowpoxlike virus	21, 22
Frankfurt/Main (zool. garden)	50.07 N 08.40 E	16.9.77	3 Elephas maxim.	1 after 10 mo	0	positive, cowpoxlike virus	21,23
			1 Loxodonta afr.	1 after 20 mo	0		
			1 Diceros bicorn.	1 after 4 mo	0		
Lodz/Poland (zool. garden)	51.46 N 19.30 E	end Dec. 1977	2 Elephas maxim.	0	0	not done	24

Table 1. (continued)

Locality	Geograph. co-ordin.	Time of outbreak	Mammal species	Deaths	Transfer to humans	Isolation and characterization of virus	References
Weingarten near Speyer (circus F.)	49.19 N 08.26 E	19.2.79	4 Loxodonta afr.	1	0	negative	25
Reutlingen (circus)	49.29 N 09.11.E	Febr. 1979?	2 Elephas maxim.	0	0	not done	26
East Berlin ? German Democrat. Republic		Nov. 1980	9 Elephas maxim.	0	0	positive, related to vaccinia virus	27,28
Hameln/Hannover (circus)	52.06 N 09.21 E	24.12.80	1 Elephas maxim.	0	0	positive, cowpoxlike virus	29
Hamburg (zool. garden)	53.33 N 09.59 E	13.3.84	8 Elephas maxim.	0	0	positive, cowpoxlike virus	30
Spandau/ West Berlin (circus)	52.33 N 13.12 E	22.3.86	2 Elephas maxim.	0	0	positive, cowpoxlike virus	31

(The following outbreaks are mapped in Fig. 7: 1) Leipzig 1960; 2) Berlin-Friedrichsfelde 1960; 3) Erfurt 1962; 4) Copenhagen 1963; 5) Rotterdam 1968; 6) Magdeburg 1971; 7) Augsburg 1971; 8) Liberec 1972; 9) Amsterdam 1973; 10) Moscow 1973,1974; 11) Vienna 1974; 12) Ansbach 1975; 13) Münster 1977; 14) Frankfurt/Main 1977; 15) Lodz 1977; 16) Speyer 1979; 17) Reutlingen 1979/80?; 18) Hameln/Hannover 1980; 19) Hamburg 1984.)

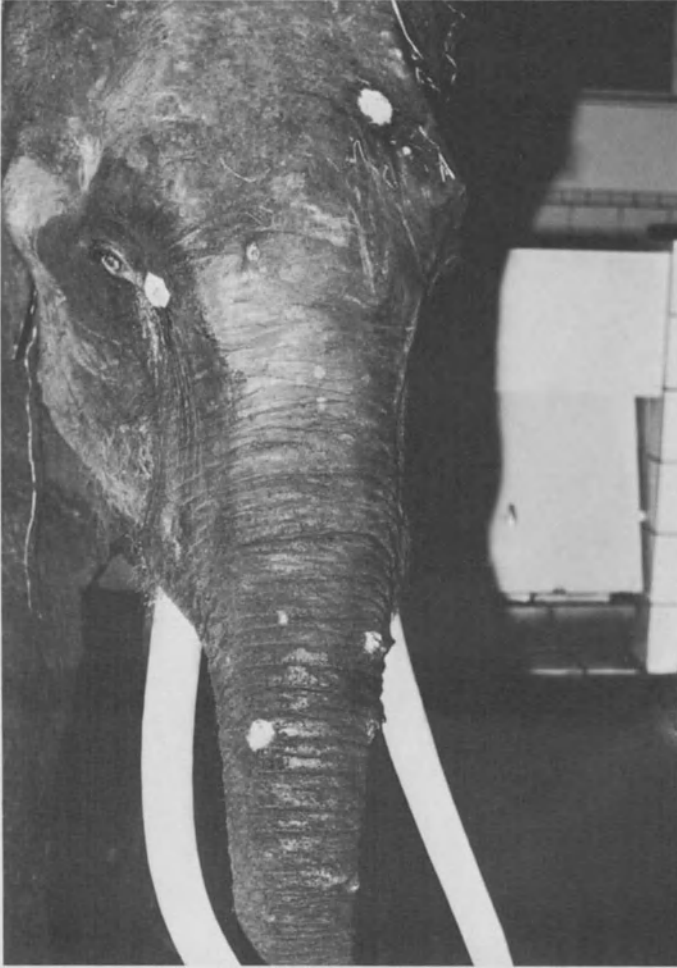

Fig. 1. Skin lesions at trunk and head of an Asian elephant: outbreak Hameln/Hannover 1980.

The following restriction enzymes were used: BamHI, HindIII, and SmaI. These enzymes were purchased from Biolabs (Beverly, Mass./USA; BRL Neu-Isenburg, Germany). Incubations were carried out according to a standard procedure for each enzyme, and the resulting DNA fragments were separated on 0.5,

Table 2. Origin and history of orthopoxvirus strains analyzed by DNA cleavage.

Virus	Strain	Species	Locality of Outbreak	Year	Origin of Virus Strain and (reference)
EP-1			Augsburg	1971	MAHNEL, Munich (11,40)
EP-2			Ansbach	1975	MAHNEL, Munich (20)
EP-267			Frankfurt	1977	MAHNEL, Munich
EP-4	elephant	Asian	Hannover	1980	MAHNEL, Munich (29)
EP-5	pox	Elephant	Vienna	1974	KUBIN, Vienna (19)
EP-6			Amsterdam	1973	HEKKER, Utrecht (29)
EP-7			Amsterdam	1973	HEKKER, Utrecht (29)
EP-8			Hamburg	1984	PILASKI, Düsseld. (30)
OP-1	okapi	okapi	Rotterdam	1968	HEKKER, Utrecht (7)
OP-2	pox		Copenhag.	1963	FREUNDT, Aarhus (5)
RP-1	rhinoceros pox	White Rhinocer.	Münster	1977	PILASKI, Düsseld. (22)
CPX	cowpox "Brighton"	cow			BAXBY, Liverpool
Cat-P	carnivore pox	anteater felidae	Moscow	1973	MARENNIKOVA, Moscow (16,17)
H-CP-LSax	human poxvirus isolate	human	Lüneburg	1985	NASEMANN, Hamburg (50)
Rab-P	rabbit pox	rabbit			HEKKER, Utrecht
BP-1	buffalo pox	buffalo			BAXBY, Liverpool
ECT	ectromelia mouse pox	mouse			MAHNEL, Munich
CP-1	camel pox	camel			RAMYAR, Teheran (41)
VAC-LS	vaccinia "Elstree"				HEKKER, Utrecht (42)

0.8 or 1.0 % slab gels (Seakem Biomedical, Rockland, Me.). Electrophoresis was performed at 4°C in vertical gels (35 x 20 x 0.3 cm) at 75 V (constant voltage). The gels were dried and autora-

diography was performed using Kodak XAR-5 films.

Biological and physicochemical characterization of virus isolates

The virus isolates obtained from zoo-kept mammals produce small efflorescences (diameter 0.8 to 1.5 mm) with haemorrhagic center at the chorioallantoic membrane of the hen's egg (Fig.2),

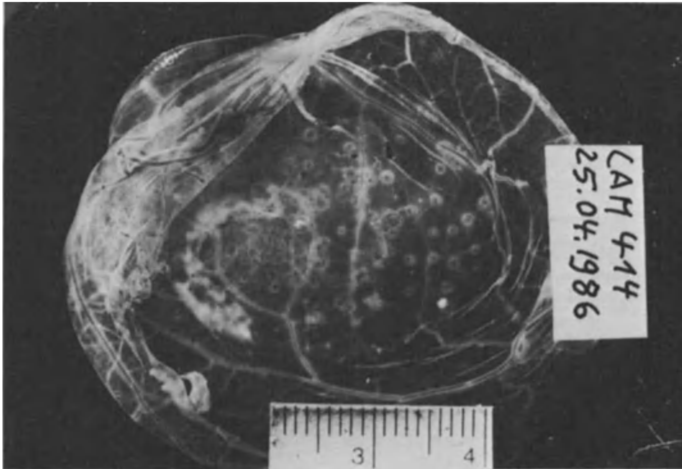

Fig. 2. Egg membrane 48 h after an infection with the isolate EP-9 (Berlin 1986): one white pock variant and over 50 typical cowpoxlike efflorescences with haemorrhagic center.

containing cells with inclusion bodies of type A V + besides B-type inclusions. BAXBY & GHABOOSI (45) had demonstrated in two other isolates from German elephants (EP-1 = Augsburg 71; EP-2 = Ansbach 75; Table 1,2) that these virus strains resemble cowpox virus in their ability to produce A-type inclusions. They should therefore be regarded as "cowpoxlike viruses". Also three other virus isolates from Asian Elephants (Amsterdam 73, Vienna 74, Hameln/Hannover 80) and one virus strain isolated from an Okapi (Okapia johnstoni, Rotterdam 68) produce these inclusion bodies which incorporate virions (i.e. A V +; Fig. 3). Only the A-type inclusions of the Moscow virus contain no virions and are therefore designated as A V -.

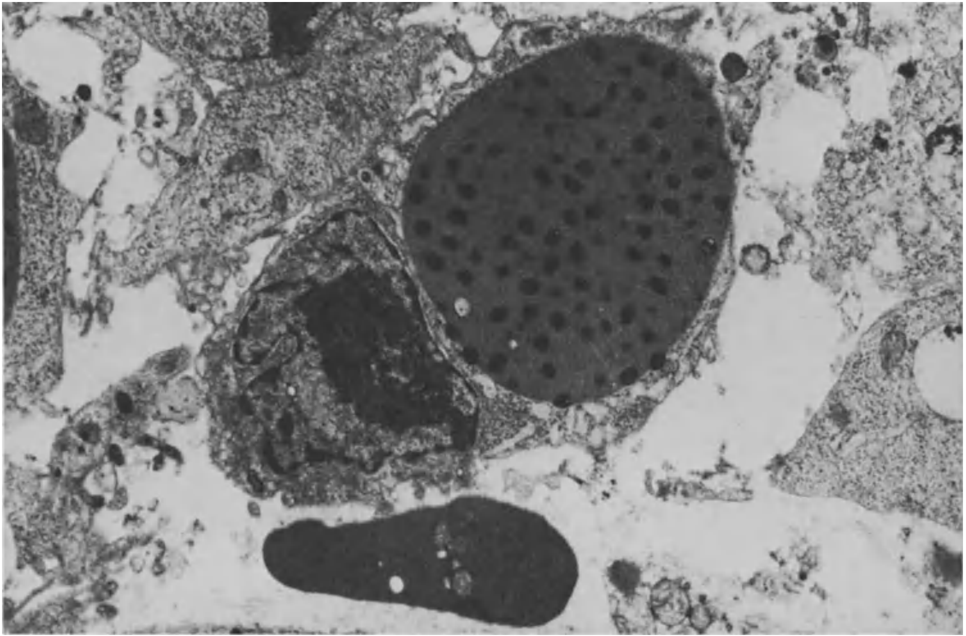

Fig. 3. Cytoplasmic inclusion of type A V + in cells of the chorioallantoic membrane 72 h after an infection with the isolate EP-8 (Hamburg 1984).

These cowpoxlike virus strains can also be distinguished from vaccinia virus (strains Elstree, Bern, MVA) by other biological markers like plaque morphology in sheep embryo fibroblasts and by their pathogenicity for the rabbit skin. Five strains were tested in 6-week-old NMRI mice by intracerebral inoculation. They produced characteristic skin lesions which resemble those caused by ectromelia virus (Fig. 4).

TURNER & BAXBY (46) have demonstrated by polypeptide analysis of orthopoxvirus strains that ectromelia virus, elephant

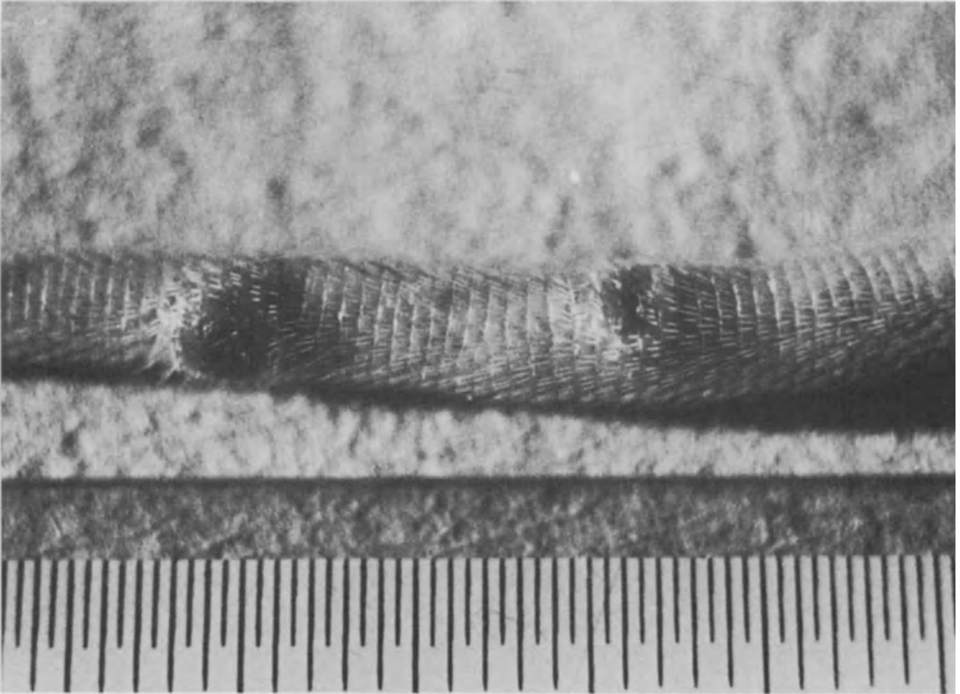

Fig. 4. Characteristic efflorescences at the tail of an adult NMRI mouse 19 days after an intracerebral infection with the isolate EP-267 (Frankfurt 1977).

virus, and Moscow virus produce the same polypeptide pattern and can be separated from the vaccinia group by the absence of a polypeptide of molecular weight 53,000 (i.e. p 53). Cowpox virus has a polypeptide of molecular weight 37,000 which is absent in Moscow virus and elephant poxvirus (4).

However, DNA cleavage with restriction enzymes is a better suited method for identification and characterization of pox-virus strains. As demonstrated in Fig. 5 the DNA cleavage patterns of the virus strains isolated from zoo-kept mammals

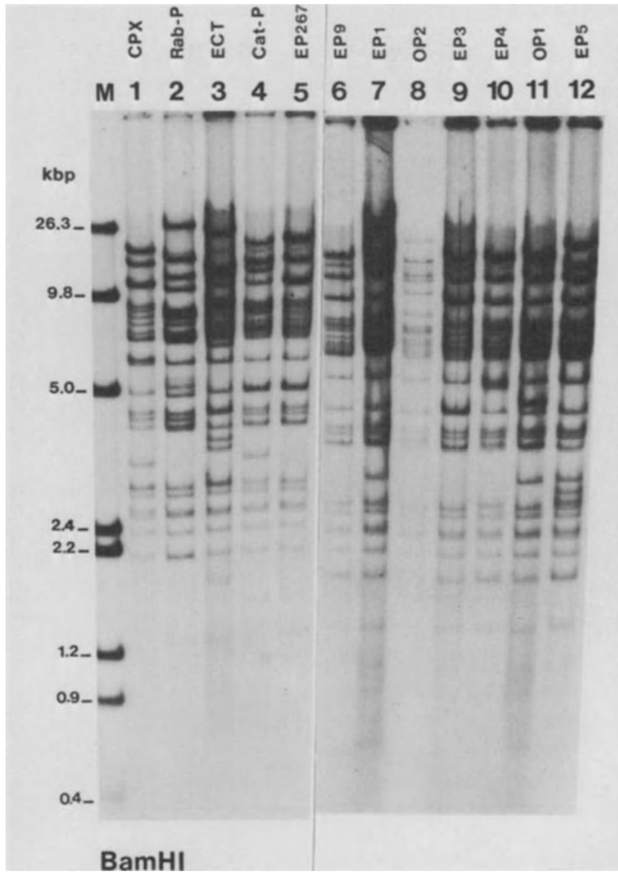

Fig. 5. Autoradiogram of the cleavage patterns of the DNAs of poxvirus strains isolated from elephants (EP-1, lane 7; EP-3, lane 9; EP-4, lane 10; EP-5, lane 12; EP-9, lane 6; EP-267, lane 5) and okapis (OP-1, lane 11; OP-2, lane 8) in comparison to the DNA of cowpox (CPX, lane 1), rabbitpox (Rab-P, lane 2), ectromelia (ECT, lane 3), and catpox (Cat-P, lane 4). The DNAs were cleaved with the restriction endonulease BamHI. The resulting DNA fragments were separated on 0.8 per cent slab gels. Phage Lambda DNA cleaved with MluI (M) served as molecular weight marker.

in Europe show a high degree of similarity and can be distinguished by this method from the patterns of cowpox and vaccinia virus.

Furthermore, it was found that the HindIII and SmaI cleavage patterns of a poxvirus isolated in 1985 from a 6-year-old child at Lüneburg in Lower Saxony (H-CP-LSax) are identical to the DNA fragmentation patterns of the elephant poxvirus strain EP-267 which was isolated in 1977 from an Asian Elephant at the Frankfurt zoo (39; Fig. 6). This finding allows the following

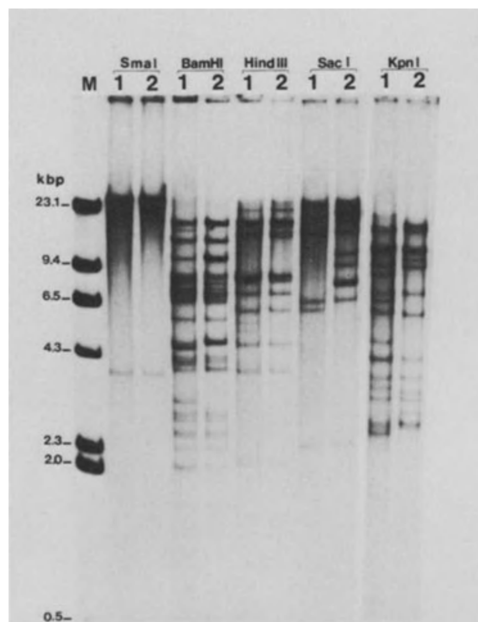

Fig. 6. Autoradiogram of the cleavage patterns of the DNA of a poxvirus isolated from man (H-CP-LSax, lanes 1) in comparison to the DNA of the elephant isolate EP-267 (lanes 2). The DNAs were cleaved with endonucleases SmaI, BamHI, HindIII, SacI,and KpnI. The DNA fragments were separated as described in Fig. 5.

interpretation: Firstly the H-CP-LSax isolate could be a variant of EP-267 virus in which the HindIII and SmaI sites are extremely conserved; secondly H-CP-LSax could be a recombinant virus of unknown origin.

EPIDEMIOLOGY

Geographical range of cowpoxlike disease outbreaks in zoo-kept mammals

To get an impression of the geographical range of the outbreaks the different places were mapped. Out of 22 localities observed in Europe between 1960 and 1986 21 lie within a circle with a radius of about 535 km around a taken center near Magdeburg (Fig. 7). Only the Moscow outbreak occurred outside of this

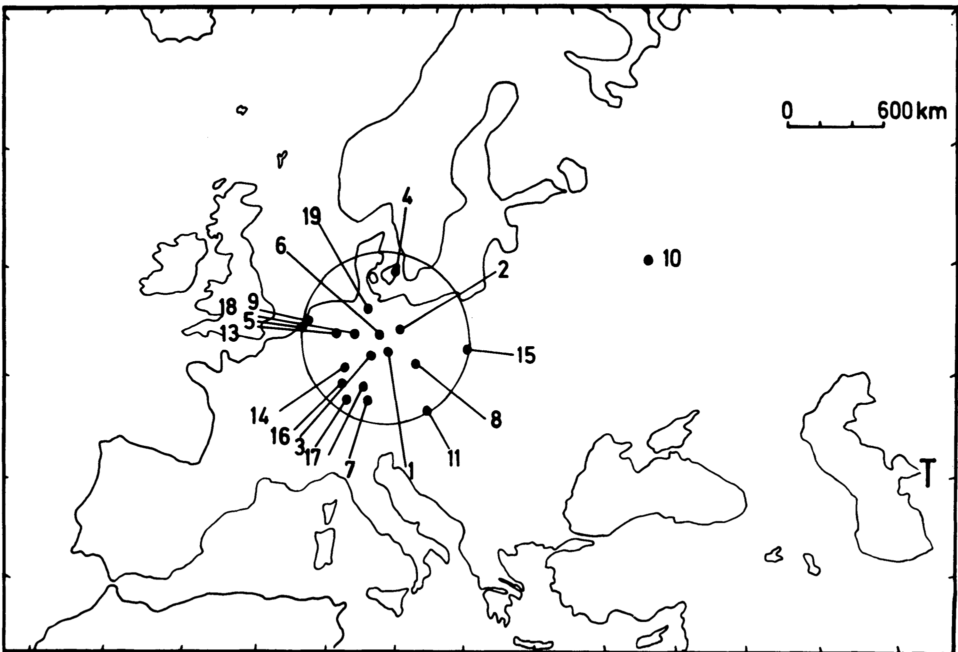

Fig. 7. Most of the localities of outbreaks of pox disease in zoo-kept mammals lie within a circle with a diameter of about 1070 km (explanation of figures in Table 1). A poxvirus strain was isolated in 1974 from wild-living rodents in Turkmenia (T) by MARENNIKOVA et al. (16).

circle. No outbreaks in zoo mammals except in cats have been reported from England, Scandinavia, and the Mediterranian countries. Our knowledge about outbreaks in the communist countries is fragmentary.

MC GAUGHEY (43) reported about several outbreaks of severe pox disease in elephants in India during the 19th century. One epidemic lasted with varying virulence for more than ten years killing more than 50 per cent of the animals in a herd. So far there exist no reports concerning pox virus isolation or serological examination of elephants in the Asian or African countries.

Since in the USA and Canada a lot of zoological gardens exist where elephants and other mammals are kept under conditions like in Europe one would expect similar outbreaks in these countries. Surprisingly so far no case of cowpoxlike disease in elephants or zoo-kept mammals has been reported from this region. The only case suspicious of pox disease in 1973 in a young Asian Elephant kept at the Woodland Park Zoological Garden in Seattle/USA could not be confirmed by virus isolation or serological examination (44).

Coincidental occurrence of outbreaks in Europe

In September 1977 an outbreak of pox disease occurred in White Rhinoceroses (Ceratotherium simum) in the Münster zoo. The first clinical signs suspicious of pox disease were seen at September 5th at the conjunctiva of the right eye of the female rhinoceros which had been imported from Africa in 1973. At September 16th her son, born June 16th 1977 in the Münster zoo, showed typical efflorescences at the forehead (22).

In the same month only 11 days later a similar outbreak was observed in elephants of the Frankfurt zoo. At September 16th a female Asian Elephant fell ill with pox disease. Skin alterations suspicious of pox disease were seen in the following time in a Black Rhinoceros (Diceros bicornis) on October

21st and in a female African Elephant (Loxodonta africana) on November 11th 1977. The airline distance between the two outbreaks is abaout 230 km. There was no transport of animals or material between the zoological gardens for several months before the outbreaks. In both cases virus strains could be isolated which could be distinguished from vaccinia virus by several biological and physicochemical markers. It was therefore postulated that the two outbreaks had occurred independently from each other (21, 32).

In December 1977 a third outbreak occurred in Asian Elephants at the zoological garden of Lodz in Poland. Even when no virus strain was isolated the clinical symptoms were typical for pox disease (24).

Artificial infection of zoo-kept elephants with vaccinia virus

On January 31st 1973 a 9-year-old Asian Elephant was vaccinated at the Woodland Park Zoological Gardens in Seattle/USA with vaccinia virus ("Wyase Dryvac", Wyeth Laboratories, Philadelphia) by making a superficial incision (about 2 cm in length) at the skin of the left external ear. 7 days later a "dime-sized" erythematous lesion was observed suggesting that a vaccination take had occurred. In a serum sample collected on January 26th 1976 a neutralizing antibody titre against vaccinia virus of 1 : 2,800 was found (9).

An Asian Elephant vaccinated in Germany (near Berlin) with vaccinia virus strain "Elstree" by subcutaneous inoculation developed a severe illness with clinical symptoms like foamy conjunctivitis and polyarthritis. Apparently no rash was observed (19).

We have seen no clinical symptoms in elephants and rhinoceroses after a subcutaneous vaccination with the MVA strain of vaccinia virus (developed by A. MAYR, Munich) using the same mode of application as described by GEHRING & MAYER (12). We have also vaccinated Asian Elephants in zoological gardens in

Frankfurt, Cologne, Münster, Hamburg, and Berlin with vaccinia virus strain Elstree employing the method of FOSTER (44). In all cases no clinical symptoms were observed.

Epidemiology of cowpoxlike virus infection in zoo-kept mammals

Since cowpoxlike viruses differ in their biological and physicochemical markers from vaccinia virus humans can not be the source of pox disease in these mammals. In 1973 an outbreak of pox disease occurred in Carnivora and members of the family Edentata at the Moscow zoo (No. 10 in Fig. 7) causing a fatal, fulminant pulmonary form of disease without skin lesions and a dermal form of rash. The biological markers of the isolated cowpoxlike virus strains were nearly identical with those of a virus isolated in 1974 from the kidney of a wild Big Gerbil (Rhombomys opinus), a rodent caught in Turkmenia (16, Fig. 7).

If wild-living rodents are involved in a primary cycle of cowpoxlike viruses isolations from rodent predators and also other findings concerning cowpoxlike skin disease in Carnivora would be of interest. SCHÖNBAUER et al. (47) found hairless skin lesions, 6 - 8 mm in size, in a domestic cat from Vienna, Austria. These efflorescences contained typical inclusion bodies of type A V + which were indistinguishable from inclusions we observed in cells of the CAM after an infection with cowpoxlike virus strains.

Until recently it had been assumed that cowpox infections in domestic cats are confined to England. The isolation of a cowpoxvirus from a domestic cat and the human owner near Utrecht in the Netherlands has shown that these cases can also occur at the continent (48). The girl at Lüneburg/Lower Saxony from whom the cowpoxlike virus strain (H-CP-LSax, Fig. 6) was isolated had no contact with zoo-kept mammals but lived in close contact with some pet animals like cats, a rabbit, a guinea pig, and a dog. Since the DNA cleavage pattern of this virus is similar to the pattern of a cowpoxlike virus strain (EP-267)

isolated in 1977 from an Asian Elephant the source of infection must have been one of these pet animals most likely a cat. It could be well documented that the domestic cats belonging to the family of this girl outside of Lüneburg were free to hunt outside in the field. These findings lead us to the conclusion that the reservoir of cowpox-related viruses may be a small mammal hunted by domestic cats, as already assumed by BAXBY (49).

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6

HERPESVIRUS INFECTION IN OLD AND NEW WORLD MONKEYS

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INTRODUCTION

Of the viruses affecting human and nonhuman primates, the herpesviruses are probably of greatest interest and concern. As a family, the Herpesviridae are one of the most universally distributed of the mammalian viruses, found in both warm and cold blooded animals as well as in plants and invertebrates. Clinically, the herpesviruses are responsible for a diversity of host responses including common cold sores (gingivostomatitis), neurologic disease, exanthemata such as varicella -- chicken pox/zoster (shingles) -- and atopic eczema, cytomegalovirus disease of the newborn, oncogenic disease in poultry and primates, keratoconjunctivitis, genital herpes, hepatoadrenal necrosis, hepatitis, respiratory disease, and various nonspecific syndromes. Latent infection may be one of the most important attributes of the herpesviruses.

Historically, herpesvirus infections have been recognized since the early days of medicine. Mettler (1) cites the Roman physician Herodotus, in the year 100 A.D., who described "herpetic eruptions which appeared about the mouth at the crisis of simple fevers." The presence of a virus was observed in ocular and labial lesions by Lowenstein in 1919 (2). "Herpes" is derived from the Greek "to creep."

Recognition of herpesviruses in nonhuman primates is more recent and originated with the studies of Sabin and Wright in 1934 (3) and the isolation of a herpesvirus from a fatal human case of B virus (Herpesvirus simiae) infection following a monkey bite. A similar virus had previously been reported, from a human infection, but was considered to be a neurotropic form of herpes simplex (4). Successful isolation of B virus from the central nervous system of a rhesus monkey (Macaca mulatta) and from rhesus monkey kidney cell cultures was reported in 1954 (5,6). Clinical disease in rhesus monkeys was first described in 1958 (7) and

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further studies indicated that the presence of herpesviruses in nonhuman primates was not an isolated occurrence.

New herpesviruses SA8 and SA15 were isolated from the African vervet monkey (*Cercopithecus aethiops*) and baboon (*Papio* spp.) (8-11). The vervet monkey also harbors another distinct herpesvirus, a cytomegalovirus (African green monkey cytomegalovirus-AGM-CMV) (12).

Herpesviruses in New World monkeys, different from those previously isolated from Old World monkeys, were reported by several investigators (13-15). A herpesvirus (*H. saimiri*-1) recovered from tissues of dead marmosets (*Saguinus nigricollis, S. oedipus*) and designated *H. tamarinus* or Herpes *T.* Herpes M, *H. platyrrhinae*, was subsequently shown to be indigenous to the squirrel monkey (*Saimiri sciureus*) rather than the marmoset (13-18).

These findings were significant as they demonstrated that nonhuman primates, like their human counterparts, responded to a primary herpesvirus infection in a similar clinical fashion. More important, again as in the human, a latent infection resulted. It was also emphasized that herpesviruses could cause a more virulent form of clinical disease in an alien host compared with that seen in the natural host. Of some consequence was the finding that the squirrel monkey had another virus (*H. saimiri*-2), also latent, that had the ability to produce a malignant disease when inoculated into the marmoset (19-21).

These discoveries resulted in a new perspective in simian virus research: that viral oncology was not restricted to lower forms of animal life, but could involve primates (human and nonhuman) as well. The presence of viruses in these animals also suggested another and perhaps a new area for concern associated with the use of nonhuman primates in research, the aspect of biohazards. Accordingly, monkeys and apes in the laboratory setting, rather than being considered merely as test tubes or another medium for the cultivation of an infectious agent, had to be considered as biologic entities with a considerable and distinct microflora, viruses being only one of the many components. The need for caution in the handling of these animals is, therefore, explicit!

Recognition of the existence of oncogenic (19-24) viruses in primates was not only a hallmark in primate virology, but resulted in expanded exploration of simian viruses. As a consequence, another oncogenic herpesvirus (*H. ateles*-2) present in the black spider monkey

(Ateles geoffroyii) was demonstrated. This virus when inoculated into cotton-top marmosets caused a malignant lymphoma with leukemia (24). Several other herpesviruses have been isolated from squirrel, spider, and owl monkeys. Not all of these are oncogenic; the neurotropic (cytocidal) viruses behave much like human herpesvirus (herpes simplex virus, HSV) in their infectivity. In addition, a large number of lymphotropic viruses have been isolated from Old World monkeys and apes; their oncogenic potential is to be determined (Table 1). A number of excellent reviews provide details regarding these viruses (58-73).

Table 1. Herpesvirus infections of nonhuman primates

Virus* (common name)	References
Subfamily: **Alphaherpesvirinae**	
Herpes simplex-1 (HSV-1)	Lowenstein, 1919 (2)
HSV -2 (HSV-2)	Schneweis, 1962 (25)
B virus (H. simiae)	Sabin & Wright, 1934 (3)
	Gay & Holden, 1933 (4)
	Melnick & Banker, 1954 (5)
	Keeble et al., 1958 (7)
Chickenpox (varicella-Zoster)	Weller, 1953 (26)
LVV (Liverpool vervet virus)	Clarkson et al., 1967 (27)
HPV (PMH)	McCarthy et al., 1968 (28)
Delta patas herpesvirus	Ayres, 1971 (29)
Medical Lake macaque (MLMV)	Lourie et al., 1971 (30)
Rhesus CMV	Asher et al., 1969 (36)
SA8	Malherbe et al., 1963 (10)
H. tamarinus (herpes T, marmoset herpes, herpes M, H. platyrrhinae)	Holmes et al., 1963 (13,15)
	Melnick et al., 1964 (14)
H. saimiri-1 (HVS-1)	Melendez et al., 1966 (17)
	King et al., 1967 (18)
H. aotus	Sheldon & Ross, 1966 (40)
Chimpanzee herpes	McClure & Keeling, 1971 (41)
H. ateles-1 (HVA-1)	Hull et al., 1972 (43)
Subfamily: **Betaherpesvirinae**	
Chimpanzee CMV (cytomegalovirus)	Vogel & Pinkerton, 1955 (31)
CMV-human	Rowe et al., 1956; (32); Smith, 1956 (33)
SA6	Malherbe & Harwin, 1957 (8)
SA15	Malherbe & Harwin, 1957 (8)

Table 1 (continued)

Virus* (common name)	References
Subfamily: Betaherpesvirinae (continued)	
Vervet CMV (AGM-CMV)	Black et al., 1963 (9)
	Dreesman & Benyesh-Melnick, 1967 (34)
	Smith et al., 1969 (35)
Aotus CMV (Owl monkey CMV)	Ablashi et al., 1972 (37)
Marmoset CMV (SSG)	Nigida et al., 1979 (38)
H. aotus-1	Daniel et al., 1971 (42)
H. aotus-3	Daniel et al., 1973 (48)
Subfamily: Gammaherpesvirinae	
EBV (Epstein-Barr virus)	Epstein et al., 1964 (39)
H. ateles-2 (HVA-2) (strain 810)	Melendez et al., 1972 (24)
H. ateles-3 (HVA-3) (strain 73)	Deinhardt et al., 1973 (45)
H. saimiri-2 (HVS-2)	Melendez et al., 1968 (19)
H. aotus-2	Barahona et al., 1973 (47)
Rhesus leucocyte-associated herpesvirus-1 (LAHV, HVM), herpesvirus-2	Frank et al., 1973 (55)
H. papio - H. hamadryas (HVP)	Lapin et al., 1975 (49)
	Deinhardt et al., 1978 (44)
HVP - H. anubis	Falk et al., 1976 (50)
H. pan (chimpanzee)	Landon et al., 1968 (51)
	Gerber et al., 1976 (52)
H. pongo (orangutan)	Rasheed et al., 1977 (53)
H. gorilla	Neubauer et al., 1979 (54)
African green monkey EBV-like virus (AGM-EBV)	Bocker et al., 1980 (56)
Subfamily: Not characterized	
H. papio	Kalter & Heberling, 1972 (46)
Herpesvirus (M. fascicularis)	Heberling et al., 1981 (57)
Herpesvirus M. (HVMA)	Lapin et al., 1985 (140)

*A number of other herpesviruses have been recovered from several different Old and New World nonhuman primates. These have been either not described in sufficient detail to warrant inclusion at this time or have been indicated in reports as "unpublished data."

See Roizman et al., 1981 for the International Committee on Taxonomy of Viruses (ICTV), Herpesvirus Study Group for recommended provisional nomenclature and taxonomy.

TAXOMONY AND NOMENCLATURE

The large number of herpesviruses isolated from animals and plants has led to confusion and difficulty in establishing an orderly system of nomenclature as well as in classification. This problem is particularly true among the primate viruses because the natural host is often not known and passage of these viruses from one species to another, particularly the human, is a frequent occurrence. In addition, the use of vernacular or common names has become so ingrained that change is not only difficult but is resisted. Much of the problem in appropriate classification is the lack of detailed study on the many isolates that have been recovered and reported in the literature. Thus, although several attempts have been made to provide an appropriate taxonomic schema for the herpesviruses in general and the simian viruses in particular, final acceptance remains in limbo (58,68,73).

Over 35 herpesviruses recovered from nonhuman primates may be found listed in the literature (61,62,68). The precise number of additional herpesviruses is unknown, but more than likely is an extensive number. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses has provided a most detailed attempt at answering the question of nomenclature and taxonomy of the herpesviruses (68). Another group, concerned only with viruses of nonhuman primates, has provided a simpler simian virus nomenclature in an attempt to avoid various problems associated with the more formal approach (74). Most investigators, however, continue to use the vernacular or common nomenclature (Table 1).

The family Herpesviridae is divided into three subfamilies, principally on the basis of biological characteristics: **Alphaherpesvirinae** (Herpes simplex virus group), **Betaherpesvirinae** (cytomegalovirus group), and the **Gammaherpesvirinae** (lymphoproliferative virus group) (68). Simian herpesviruses are found in all three subfamilies. In this chapter the disease capabilities of the varicella-zoster viruses (**Alphaherpesvirinae**) are discussed as a separate group: "Exanthematous Disease."

BIOLOGICAL CHARACTERISTICS

All Herpesviridae share the same biological properties and details are provided elsewhere (58,63,64,66,68,70-73). In common with other herpesviruses, the simian herpesviruses have double stranded, linear DNA;

32-75 G/C moles %; and a molecular weight of 80-150 x 10^6. Proteins consist of more than 20 structural polypeptides with molecular weights of 12,000 to 200,000 including a number of major glycoproteins. Both lipid and carbohydrate are present although the total weight of each is unknown. The ether sensitive lipid present in the virion envelope and carbohydrate is covalently linked to the envelope proteins. Morphologically, all the viruses appear the same, with the virion approximately 120-200 nm in diameter. Although the virion and the icosahedral nucleocapsid differ in their immunological specificity, it is the envelope of the virion that contains the host determinant antigens and the viral antigens. The envelope glycoproteins induce neutralizing antibody and are generally responsible for detection in viral diagnosis. Obviously, variations in biologic, biochemical and infectious properties exist. Although routine differentiation is generally dependent upon the antigenic properties of the virion, these properties overlap and antigenic relatedness is common to the family. Thus, the biologic and biochemical properties, along with the composition, size, and arrangement of the deoxynucleotide sequences, provide the basis for differentiating the family Herpesviridae.

A number of herpesvirus properties are important for understanding the pathogenesis of this virus family. The host range of herpesviruses is extremely variable both in nature and as a result of laboratory manipulation, frequently crossing species barriers. An extreme example of this is infection of humans with B virus. In the natural host, the rhesus monkey (*M. mulatta*), infection resembles that seen in the human following natural infection with herpes simplex, and is characteristic of herpesvirus infections in their original hosts. Infection of humans with this virus frequently results in death. Herpes simplex infection of monkeys also frequently results in a clinical form of disease dissimilar to that seen in the human.

Alterations in infectivity occur in cell culture where certain of the herpesviruses have the capacity for cellular transformation, producing continuous cell lines that may result in tumor formation when inoculated into appropriate animal hosts. This variability is important in attempting to differentiate the herpesviruses.

One of the most important biological properties of the herpesviruses is that of latency. Following primary infection, herpesviruses become

latent in sensory ganglia (**Alphaherpesvirinae**), various glands and tissues (**Betaherpesvirinae**), and lymphoid tissues and cells (**Gammaherpesvirinae**) within the host. Activation of this latent infection occurs at intervals in spite of the presence of neutralizing antibody. The precise triggering mechanism is not known although various stimuli are known to induce recurrence. This capability is of greatest concern in B virus infections of *Macaca* spp. The site of HSV latency is in neurons of the sensory ganglia, but virus isolation from these sites is difficult and requires careful laboratory manipulation (prolonged coculture of ganglion explants with susceptible cells). One may speculate that the viral DNA is integrated into the cell chromosomes, making recovery difficult. Reactivation may be symptomless (virus shedding in saliva) or result in typical herpetic disease either localized ("cold" sore, genital lesion) or generalized with or without neurologic disease.

Transmission of herpesviruses is probably the same among all species that carry the virus. Most infections are the result of contact between moist mucous surfaces, either open lesions or saliva. Other avenues of infection are airborne, transplacental, transfusions, intrapartum, by breast milk, and via water. This latter mechanism is unusual, but has been noted.

DISEASES

Confusion and lack of information exists regarding the diseases each of these viruses is capable of inciting, their epidemiology, susceptible hosts, clinical features in these various hosts, pathology (if any), diagnosis, prevention and control, as well as the current thoughts on therapy, particularly in the various species of nonhuman primates. Herpesviruses are complex and their host relationships are not well understood. It is generally not clear how this biological entity functions in causing disease. For example, numerous glycoproteins are present in the envelope of each virus. Which of these, if any, are responsible for the relatedness among several of the herpesviruses -- herpes simplex, B virus, SA8, and others? What are the initial events leading to infection? Following infection, why is it possible in one instance to develop a productive infection with the biosynthesis of infectious progeny and cell death or, conversely, to develop a nonproductive infection with the perpetuation of viral genome and survival of the host

cell. This latter aspect that leads to latency is undoubtedly one of the most important concerns in understanding herpesvirus infections. Thus, to understand infection and disease due to the herpesviruses, or for that matter any infectious agent, one must recognize not only the biology of the infecting agent, but comprehend the interplay between agent and host. Accordingly, the various exogenous and endogenous factors that govern the susceptibility of each particular host animal must be considered.

Infection and disease due to the herpesviruses may be separated into several clinical groups according to the host response. McCarthy and Tosolini (62) suggested that herpesvirus infection of nonhuman primates results in: generalized and neurological disease, exanthematous disease, lymphoproliferative or oncogenic disease and cytomegalovirus (cytomegalovirus-like) disease. These groupings are not helpful in specifying the causative agent as one or several of the herpesviruses may simulate any of these clinical entities.

Generalized and Neurological Disease

Neurotropic herpesviruses capable of causing generalized infection and disease frequently followed by neurological sequelae include: *H. simplex* (HSV) 1 and 2, *H. simiae* (B virus), SA8, *H. tamarinus* (herpes-T, marmoset virus HMV, *H. saimiri-1*), *H. ateles-1*, *H. aotus-1* and several non-primate herpesviruses. These viruses in culture are cell-free and cytocidal.

Although the nonhuman primate is not the natural host, the relative frequency with which HSV is isolated from nonhuman primates, as well as the close biological relationship of all these viruses, necessitates inclusion of HSV in any discussion of herpesvirus infections of New and Old World monkeys.

Epidemiology. These neurotropic viruses represent a wide range of Old and New World monkey isolates. Although they may have a number of characteristics in common, they may be subdivided on the basis of their antigenicity, which parallels in part their geographic origins. The vast number of strains and serotypes suggest that a spectrum of interrelationships exists.

Two distinct epidemiological patterns emerge, providing a mechanism for transfer of each of these viruses to their natural or alien host. Both vertical and horizontal transmission maintains the virus within its specific or natural host. Virus may be passed to the fetus during

pregnancy, to the offspring at the time of birth, or shortly thereafter. Probably the major source of infection is from bites and scratches although aerosols are known to be responsible for infection in captivity. Contaminated food and water supplies may account for some transmission, but are probably not a major source. Animals are infected in nature but the exact mechanism of virus transfer is not known (75). Experimental genital infection has been reported and natural genital infection (chimpanzees), although rare, has been recognized (76). Regardless of the pattern of transfer, once infected the animal remains infected for life (latent infection). This latency permits continuous spreading of virus as shedding occurs periodically. The exact reason for virus shedding is undetermined. These herpesviruses reside in the ganglia from which they may be recovered by appropriate laboratory procedures. Activation and virus shedding is probably similar in all species.

Herpesvirus infections in an alien host follow the same transmission pattern, but the end result is most often not the innocuous disease seen in the natural host, but rather a highly virulent, oftentimes fatal, generalized form of the disease. Many epidemiological aspects of cross infections are unknown. HSV infection of nonhuman primates is a common occurrence and generally involves contact with a human who is shedding the virus. Aerosols are also probably associated with transmission from human to animal and spread of HSV from one animal to another is recognized.

As seen in Table 2, antibody to these herpesviruses varies from colony to colony. Populations of young animals will have less antibody than colonies of older animals. What accounts for these colony differences is unknown, but if there are positive animals in a colony, the prevalence will increase in time. Although both *H. saimiri* and *H. tamarinus* are indigenous to the squirrel monkey, why differences in infectivity exist within the same colony is speculative.

Clinical. The usual clinical manifestation of viruses within this group in the natural host is either an inapparent infection or a mild form of herpetic disease after a short incubation period of a few days or several weeks. Most characteristic is the presence of typical herpetic ulcers or vesicles at the mucocutaneous junction principally on the lips, tongue, oral mucosa, or on the genitalia. The most extreme manifestation

Table 2. Herpesvirus antibody in colony animals

Colony No.	H. simiae (M. mulatta)	H. saimiri (S. sciureus)*	H. tamarinus
1	10/12 (83.3%)	0/10 (0%)	0/10 (0%)
2	27/54 (50.0%)	1/7 (14%)	1/7 (14%)
3	0/10 (0%)	60/66 (91%)	37/70 (53%)
4	15/25 (60.6%)	25/25 (100%)	0/25 (0%)
5	0/18 (0%)	6/6 (100%)	2/6 (33%)
6	23/42 (54.8%)	8/8 (100%)	4/4 (100%)
7	15/42 (35.7%)	--	--

*The same colony of S. sciureus was tested against H. saimiri and H. tamarinus.

of herpes virus infection is that associated with involvement of the central nervous system.

If the disease is more severe than a localized lesion, fever and lymphadenopathy develop along with a generalized ulcerative dermatitis, conjunctivitis, anorexia, irritability, and weakness. Neurological disease may follow the generalized symptoms either immediately or after a lapse of time (which may even be years). Symptoms of encephalitis include: lethargy, twitching, convulsions, hemiplegia, difficulty in swallowing, progressive paralysis, coma and death.

Pathology. Herpesvirus infections are characterized by a number of features that, if not pathognomonic, are at least quite distinctive of these viruses. Information on the pathology in the various infected species is extensive and will not be repeated here (7,59,80-85). Essential pathologic features following infection with one of the cytocidal herpesviruses are: 1) the development of characteristic intranuclear inclusion bodies, 2) multinucleated giant cell formation, and 3) cell necrosis. All of these features may be observed in any one or all tissues or organs during one clinical episode.

Although the nonhuman primate is not the natural host for HSV, the lesions that occur are similar to those seen in the human.

Central nervous system involvment is usually manifested by an encephalitis or with indications of a meningitis. As in the human, lesions are most frequent in the cerebral cortex, but may occur in other lobes as well as in the thymus and other nuclei.

Typically, reactions to HSV infection are host dependent. Marmosets do not develop the above clinical picture. Death generally results with a minimum of clinical findings: anorexia, diarrhea, dehydration, hypothermia, and death. The histopathology, however, is typical of herpesvirus infection. Infection of tree shrews apparently is even more variable (77).

Infection with herpes-T in monkeys is also typically herpetic. In the squirrel monkey, its natural host, lesions are confined to the lips and oral mucosa. No significant lesions are found in any other tissues. In the marmoset, where deaths have occurred, there is evidence of a generalized infection.

SA8 pathology data are limited to that obtained from experimental studies (78,79). In the baboon, pathology in the spleen and adrenals resembles that seen following infection with other herpesviruses.

H. ateles-1 and *H. aotus*-1 are nononcogenic and typical of the neurotropic herpesviruses in their pathology. The original *H. ateles* infected spider monkey had characteristic herpetic oral lesions prior to death (43).

Diagnosis. The vast number of herpesviruses (Table 1) responsible for infection/disease, the ubiquity of this virus family and its antigenic interrelatedness, much of which is not understood, can make differential diagnosis difficult (86). Probably the most complicating factor is the antigenic input of an unknown infecting herpesvirus from foreign sources. Thus, the daily contact of an individual with herpesviruses from diverse sources probably has some effect on the antibody response of each individual. Current molecular studies appear to have provided mechanisms capable of overcoming this difficulty.

Within the herpesvirus family, there are groups of viruses that are more closely related and these continue to pose diagnostic difficulties. For example, in determining HSV infection, diagnosis consists of differentiating among HSV-1, HSV-2, B virus, and SA8.

At times the clinical evidence may offer a presumptive herpesvirus diagnosis; however, the specific etiologic diagnosis can rarely be made on clinical findings alone. To ascertain the specific cause of the infection, the following approaches, either singularly or preferably in combination, are necessary: 1) virus isolation, 2) detection of virus in the tissue, and 3) serologic methods able to distinguish the various herpesviruses and determine a significant antibody change.

1. Virus isolation: virus may be recovered from overt lesions by inoculation directly onto various cell cultures, chorioallantoic membrane of developing chick embryos, and into rabbit brain. The virus may then be typed by one or another of the available serologic procedures. Most important is the availability of reference reagents and appropriate controls. Monoclonal antibodies reacting with specific viral antigens will do much to develop specific and rapid diagnostic procedures.

2. Detection of virus _in situ_: Herpesviruses may be recognized in tissues by appropriate staining and electron microscopy. However, while this may be satisfactory for demonstrating the presence of a herpesvirus, these methods do not differentiate the various herpesviruses. Specific identification may be accomplished by use of enzyme-immuno cytology, immunofluorescent procedures, or immunoelectronmicroscopy with appropriate antisera. Hybridization with radiolabeled viral DNA probes and detection by autoradiography is also available.

3. Serology: serologic procedures are now available for both the identification of virus in specimens and the detection of antibody. Several procedures are currently in use, as well as under investigation, for the rapid, specific and sensitive identification of virus antigen or antibody. Classical procedures include the serum neutralization test, complement fixation, immunofluorescence, radioimmunoassay, immunoperoxidase (ELISA), immunoelectrophoresis (Western blot) and immunoblot assay. All of these methods have their advocates.

Recently, a dot-immunobinding assay (DIA) was described (87,88) and has shown great promise. The procedure is simple, highly specific and sensitive, requires little in the way of laboratory equipment and is inexpensive. Inactivated (psoralen) B virus antigen has been used, eliminating the need for rigid safety requirements. Utilizing the combining power of nitrocellulose for protein, detection of antibody or antigen may be accomplished in 3-5 hr. Differentiation between HSV-1,

HSV-2, and B virus is possible. Katz et al. (89) have similarly described an ELISA for detection of group-common and virus-specific antibodies in sera induced by herpesviruses.

Interpretation of laboratory results requires caution and experience. This is particularly true in the interpretation of herpesvirus infections. The ubiquity of this virus group and their presence in the mouth, body tissues and fluids, as well as the presence of antibody, frequently leads to confusion. The site of virus isolation and the clinical picture must be viewed critically. Likewise, antibody without a significant rise in titer during convalescence is inconsequential. Here, too, one must consider the clinical aspects since certain physiological changes in an individual may result in the recurrence of a latent herpesvirus infection. Furthermore, herpesvirus recurrences may not be associated with antibody increases and so limit the value of serologic testing in laboratory diagnosis.

The appearance of lesions on the lips or tongue of an animal would suggest a herpesvirus infection. Suspicion regarding the specific agent would rest upon the species involved. However, confirmation by appropriate laboratory tests is necessary as infection by other herpesviruses, as emphasized above, readily occurs.

Difficulties in providing a definitive diagnosis by serologic procedures frequently necessitates isolation and identification of the virus. As indicated, isolation is not difficult, but specific identification of the isolate may be a problem. Although the availability of monoclonal antibodies may be helpful in distinguishing between HSV-1 and -2 as well as SA8, such sera are not readily available to the routine diagnostic laboratory. The classical procedures may not distinguish between these viruses.

All **Alphaherpesvirnae** may be isolated with relative ease in a variety of cell systems. Both human and nonhuman cells are susceptible. Hela, LLCMK2, kidney cells of various primates, etc., are all satisfactory. Other procedures are satisfactory, but offer no particular advantages over cell culture.

Hilliard et al. (90) have recently adapted restriction endonuclease analysis, which has been shown to be of value for the precise identification of the herpesviruses, for the identification and differentiation of B virus.

Prevention, Control, Therapy. The most practical precaution for preventing outbreaks of herpesviruses in a colony of nonhuman primates involves good husbandry and the institution of rigid public health measures. Inasmuch as HSV is not a natural disease of these animals, control of the human population is imperative. The studies of Daniel et al. (91) suggest that a vaccine against HSV is practical and effective. More recently, Skinner et al. (92) vaccinated juvenile and adult rhesus monkeys with a subunit formaldehyde inactivated vaccine Ac NFU(S^-)MRC against HSV with no local or systemic side effects. Antibody to both HSV-1 and -2 developed. However, herpesvirus vaccination must be viewed in terms of host-virus relationships. It is well known that recurrences occur in spite of the presence of antibody. Constant infections persist in the presence of humoral and cell-mediated immunity. Whether or not more effective vaccines utilizing subunit glycoproteins or recombinant attenuated strains may induce higher levels of immunity is not known (93,94). Effective herpesvirus vaccines have been demonstrated in animals, for example Marek's disease of chickens.

In addition to vaccination, therapeutic measures are also available and effective. HSV specifies and induces the enzymes required to synthesize its own DNA. Two enzymes are apparently most susceptible to alteration: the viral DNA polymerase and the viral thymidine kinase. Pyrimidine and purine analogues such as phosphonoacetate and phosphonoformate are strong inhibitors of several of the herpesviruses. Acycloguanosine (acyclovir) which is selectively phosphorylated by thymidine kinase (viral) is also highly effective in inhibiting certain of the herpesviruses and has recently been licensed for I.V. and topical use. Interferon is under investigation in several laboratories and has shown some effectiveness against HSV infections. Topically applied interferon has been found to be effective against HSV-1 induced epithelial keratitis in nonhuman primates (95-97). The effectiveness of interferon for other infections is still in need of study.

Discussion: Current studies with molecular techniques would indicate that approaches to the control of neurotropic herpesvirus infections are possible. The often times expressed fear of oncogenicity associated with the use of live herpesvirus vaccines is diminishing. Genetic engineering procedures now make it possible to remove the genomic region responsible for cell transformation.

Epidemiological evidence for the association of HSV with human cancer has resulted in a number of experimental attempts to demonstrate this in nonhuman primates (98-102). In general, these studies all failed to show any direct relationship. Marmoset cell lines infected with HSV did not develop any indications of malignant transformation (103). Perhaps of some relevance was the observation of activation of endogenous mouse type C virus by U.V.-irradiated HSV-1 and -2 (104). Whether or not this activation results in tumorigenesis was not demonstrated. However, inasmuch as C-type viruses or their genomic materials are present in most vertebrates (105), it is of interest to speculate on such a possibility.

Exanthematous Disease

The herpesviruses thus far isolated from nonhuman primates and considered capable of causing a chickenpox-like disease include: *H. varicellae* (varicella-zoster, V-Z), Liverpool vervet virus (LVV), Patas monkey herpesvirus (HPV, PMH), Delta herpesvirus (DHV) and macaque vesicular disease virus (Medical Lake Macaque Virus, MLMV). In addition, chickenpox-like disease has been observed in chimpanzees and other apes. The viruses isolated have typical V-Z like characteristics. These viruses in culture grow with difficulty and are cell associated. Extracellular virus is usually defective.

The V-Z viruses are all antigenically related, but the simian isolates appear to be more closely related to each other than to the human virus, V-Z (106) and to some extent HSV (107). White et al. (108) indicated that after contact with humans, an 8-month-old gorilla developed a disease clinically resembling chickenpox. A similar finding was reported by Marennikova et al. (109) in a gorilla with skin lesions resembling smallpox. It is not clear whether human V-Z infects nonhuman primates with disease production or not. Recently, Padovan and Cantrell (110) provided an overview of infection in nonhuman primates by this group of viruses.

Epidemiology. Clarkson et al. (27) described an outbreak of a fatal exanthematous disease in vervet monkeys following the introduction of young vervets into the Liverpool School of Tropical Medicine. These young vervets were imported from Nairobi and placed in a room with other vervets that had been in residence for over a year. All animals appeared normal; however, some 12 days following arrival, one of the newly imported animals died. At necropsy, a papular rash covering the entire

body was noted. Seventeen days later, one of the original resident animals died with similar skin lesions. The diagnosis of B virus infection was made. The disease continued through the colony, but did not spread to rhesus or mona monkeys nor to baboons. The possibility that this outbreak was due to V-Z was suggested. Another outbreak was observed in a group of patas monkeys (Erythrocebus patas) imported from Chad and Nigeria via a dealer and undergoing quarantine in one room (28). A similar disease to that in the Liverpool vervets occurred in patas monkeys at the Delta Regional Primate Research Center and hence the name Delta herpesvirus (29). It was suggested that this virus may be the simian counterpart of human chickenpox.

Outbreaks of vesicular disease similar to zoster occurred in a variety of monkeys (M. nemestrina, M. fuscata, and M. fascicularis) at the Medical Lake Field Station of the Washington Regional Primate Research Center (30). Deaths occurred in a small number of cases. Although the origin of these animals was not described, it was noted that a survey of monkey sera from Malaysia indicated that the disease was common in captive animals but not in animals in the wild.

An orangutan living under the same conditions with a gorilla that developed "chickenpox" did not develop the disease, but developed antibodies (108). The gorilla case resulted from contact with a human and occurred 15 days after contact. Another human living in the same household became ill at the same time as the gorilla. Virus was isolated from the vesicles followed by development of antibody. In the Moscow zoo, a case of smallpox-like generalized infection developed in a 2 1/2-yr-old gorilla (109). Although the appearance of the rash did not resemble varicella, the virus isolated had the properties of V-Z. The epidemiology of this case is not clear. The animal, upon arrival in Moscow from West Germany, had signs of an upper respiratory disease, followed by a cough. A diagnosis of bronchopneumonia was made and the animal treated. The rash developed 14 days after arrival.

Clinical. The incubation period for simian varicella varies from 7-15 days. The clinical picture of both human and nonhuman primate disease is similar, except the disease in simians is more severe. The cutaneous, oral, and visceral lesions are similar to those seen in fatal human varicella. Lesions appear abruptly as small papules (1-3 mm) which rapidly progress to vesicle formation covering, at times, the entire

body. Ulceration follows with a dried crust covering the coalescing ulcers. Depending upon the severity of the illness, fever, anorexia, lethargy, and lymphadenopathy are noted. Deaths occur in a large number of animals following evidence of clinical symptoms, but not always. Deaths have been observed in the absence of clinical findings. Marked variation in the response of different simian species is observed.

A pneumonia may develop, but has not been reported in most instances. Deaths, when they occur, are within 48 hr after the development of the rash. CNS involvement has been observed only following intracerebral inoculation.

Pathology. Although some variation in histopathology is apparent in the different species as a result of infection with the different viruses, the general histopathology is that of a herpesvirus infection. Upon necropsy, most tissues show some degree of involvement. Hemorrhage, focal or diffuse necrosis, and inclusion bodies are seen in involved tissues: liver, spleen, adrenals, lymph nodes, pancreas, etc.

The skin lesions consist of vesicles containing a clear serous fluid or cellular material. Virus may be found in these fluids. Vacuolation and hemorrhage is observed in the dermis underlying epidermal lesions. Hyperplastic areas all contain inclusion bodies (27-30).

Diagnosis. Serologic diagnosis is readily accomplished by one or another of the standard diagnostic methods. As all the viruses in this group are antigenically related to each other, human V-Z and HSV, specific determination of the agent requires careful use of test procedures and reagents (106). LVV, HPV, DHV, and MLMV are immunologically identical. CZHV is antigenically distinct but does have some relationship to LVV, PHV, and MLMV. DHV however does not cross relate to CZHV and is probably different (106,107). LVV, PHV, and MLMV appear to be one group whereas DHV and CZHV are each members of distinct virus groups (107).

All of these viruses grow well on one or another of simian kidney cell cultures, Vero cells, human diploid cells, but all do not grow on the same cell systems. Likewise, human cells are not as sensitive as nonhuman cells to the simian virus, particularly for primary isolation. Other host systems, suckling mice, chick embryos, rabbits, are not susceptible. Direct visualization by staining smears of cellular material, immunofluorescence or electron microscopy are valuable for presumptive diagnosis, but do not distinguish from other herpesviruses.

Prevention, Control, Therapy. Outbreaks are either sporadic or occur shortly after the arrival of new animals into a colony. Thus, the usual husbandry procedures for minimizing colony outbreaks are not applicable. The DHV outbreaks in *C. aethiops* and *E. patas* apparently were caused by activation of latent infections resulting from stress of other spontaneous diseases or experimental manipulation. The relationship to V-Z makes one suspect that somewhere the human virus may have been involved, although there is certainly no evidence to support this suggestion.

The resemblance of this disease in nonhuman primates to that seen in the human has suggested that studies on simian varicella-like disease may be used to understand the human disease as well as to evaluate chemotherapeutic and immunological therapy. Felsenfeld and Schmidt (111) showed that V-Z protected patas monkeys from DHV. Similarly, attenuated human strains are protective in rhesus monkeys as well as in humans (112). Also DHV infection of African green monkeys could be inhibited by (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU). Viremia, appearance of rash, and other clinical symptoms were reduced or eliminated (113). This same group of investigators (114) were able to demonstrate the prophylactic and therapeutic effects of recombinant type a interferon A (rIFN-a-A) in DHV infections of African green monkeys. Antiviral effects could be shown when rIFN-a-A was administered 4 hr prior to virus inoculation or when deferred until 44 hr post virus inoculation. Interferon was effective in the prophylaxis of "simian varicella" (undesignated strain) in an epizootic outbreak (115).

Discussion. Simian varicella does not occur with any frequency. Although the source of infection is not understood and fatality may be high, the disease is not a major cause for concern.

The similarity to the human disease, however, offers a model for studies of its human counterpart and, as such, has usefulness in biomedical research. Successful use of varicella vaccines in humans suggests its equal value in the nonhuman primate population; however, the low incidence of disease in these animals makes the value of such an undertaking questionable.

Cytomegalovirus (CMV) Infection

Several cytomegaloviruses have been observed or isolated from a variety of nonhuman primates: chimpanzee, African green monkey, rhesus

monkey, baboon, owl monkey, marmoset, squirrel monkey, and gorilla (8,9,31,34-38,116-120). The interrelationships of these viruses to each other or to the human cytomegaloviruses are not clear.

Epidemiology. The origin and epidemiology of cytomegalovirus (CMV) infection in nonhuman primates is unclear. Serological surveys do show the presence of antibody to one or another CMV in most nonhuman primates examined. Because these viruses may be isolated with relative frequency from various tissues, latent infection undoubtedly plays an important role in maintaining the virus in the natural host as well as providing a mechanism for virus spread. Persistent shedding of CMV in the urine of a healthy rhesus monkey has been reported (121). It is felt that CMV is host specific with little if any crossing to other species. However, Muchmore (122) reported a possible human CMV infection following a chimpanzee bite.

Rhesus monkeys were found with antibody to the African green monkey CMV (123). Similarly, the African green, Java, and rhesus monkeys as well as baboons were reported to be susceptible to African green monkey CMV (120). It was further demonstrated that during captivity rhesus and African green monkeys became infected with both the rhesus and African green monkey CMV strains. These strains are immunologically distinct.

Although there are no data to suggest that the same concern shown for human infection with CMV, i.e. congenital infection (which occurs) and immunosuppression (SAIDS) needs to be extended to nonhuman primates, it is highly probable that the pathogenesis of the human and simian CMV's is similar.

Clinical. There is no evidence of overt disease occurring in nonhuman primates as a result of CMV infection, with the possible exception of CMV in chimpanzees (31). Experimental infection of marmosets with a human strain of CMV (125) or rhesus monkeys with African green monkey CMV (129) failed to induce overt disease. Ventricular dilatation and leptomeningitis in rhesus monkeys following intrauterine infection with a rhesus CMV as well as CNS lesions in infected fetuses is recognized (126). Neurological disease was reported in squirrel monkeys as a consequence of CMV congenital infection (127).

Pathology. Cytological evidence of CMV infection is characterized by the presence of intranuclear and intracytoplasmic inclusion bodies in the salivary glands or other involved tissues. In chimpanzees with

disseminated CMV disease, intranuclear inclusions were seen in the cortex of the adrenals. Where disease was extensive, involvement was characterized by areas of dense leucocytic infiltration and necrosis that in some places extended through the entire width of the cortex. A myocarditis was also seen which was characterized by numerous focal collections of lymphocytes and plasma cells along with areas of necrosis (31).

Spontaneous cytopathology seen in cells under cultivation generally appears as scattered round cells which are lysed in 1-3 days leaving large holes surrounded by dark cells. The time of appearance of cytopathology varies, but is generally slow in developing.

Diagnosis. The diagnosis of CMV infection is based upon observation of typical CMV histopathology, isolating virus, and serologic demonstration of antibody. Histologic evidence of infection is characterized by typical intranuclear and intracytoplasmic inclusions that are pathognomonic for CMV disease. For virus isolation, several cell lines, usually host related, are preferable. Fibroblasts of embryonic origin are preferred although adapted CMV strains show a wider ability to proliferate. Tissue or specimens for virus isolation should be used directly rather than after freezing. If freezing is unavoidable, the storage medium should contain sorbitol (equal volumes of 70%). Inoculated cells can be kept at least 3-6 weeks.

The complement fixation test is the test of choice for most laboratories. However, newer methodologies (FA, ELISA) suggest possible substitution. Recent infections may be best determined using indirect immunofluorescence for detection of virus specific IgM.

Prevention, Control, Therapy. In the human, concern over CMV infections involves pregnant women and immunosuppressed patients. Pregnant and immunosuppressed nonhuman primates are generally not monitored for CMV infections. Further, inasmuch as the epidemiology of CMV infections is not clearly understood, mechanisms for prevention, control, or therapy would be difficult to apply. Vaccines as recommended for the human have not been attempted in nonhuman primates but probably would be effective. Obviously, removal of known shedders or those who are serologically positive from a colony in need of being kept free of CMV would help minimize the problem.

Discussion. The existence of a nonhuman primate counterpart of CMV suggests that these species should be used for models of the human

disease. Various monkeys and apes have been successfully infected with the human strain of CMV. SA6 isolated from baboons is identical to the vervet monkey isolate (8). Their occurrence is so infrequent, however, that little is known about them.

Lymphoproliferative Disease

The ability of herpesviruses to induce malignant disease is a well established phenomenon: Lucke virus causing adenocarcinoma in the leopard frog kidney (128), avian neurolymphomatosis due to Marek's disease virus (129), and cottontail rabbit lymphoma due to H. sylvilagus (130). In the human, the relationship of herpesviruses to oncogenesis, while not totally resolved, is highly suspect: Epstein-Barr virus (EBV) was isolated from cell cultures derived from Burkitt's lymphoma and is suspected of being etiologically related to certain human lymphomas (nasopharyngeal cancer, Hodgkin's lymphoma). EBV is known to be the cause of infectious mononucleosis (IM) (39,131-133). More tentative is the relationship of HSV-2 to human cervical cancer (73).

In the nonhuman primate, the relationship of herpesviruses to oncogenesis is unequivocal (45,58,65,134,135). Although all the isolated lymphotropic herpesviruses have not as yet been clearly identified with disease, a sufficient number have been to establish the relationship. H. saimiri-2 isolated from primary squirrel monkey kidney cell culture was shown to cause malignant lymphoma and leukemia in unrelated simians such as the owl monkey, marmoset, spider monkey, cinnamon ringtail monkey, and African green monkey (19,21,22,24). No disease is induced in the natural host nor in a number of other primate species. This same group of investigators shortly thereafter isolated another herpesvirus, H. ateles-2, from a spider monkey kidney cell culture that was also able to induce malignancies in cotton top marmosets (24).

Although it is a human agent, EBV is antigenically related to the EBV-like simian viruses and has produced tumors in nonhuman primates after experimental inoculation (136-138).

Epidemiology. Simian oncogenic herpesviruses have been recovered from both Old and New World nonhuman primates. EBV is a human pathogen and does not cause disease in nature in nonhuman primates. The natural hosts for HVS and HVA are the squirrel monkey and spider monkey, respectively, in which they do not cause overt disease, nor do they share DNA homology with EBV or EBV-like viruses. The EBV-like herpesviruses have

all been isolated from Old World simians and all share some homology with EBV-DNA. With the possible exception of the baboon EBV-like virus (*H. papio*-HVP) (49), the other isolates, chimpanzee herpesvirus (*P. troglodytes*-CHV) (51), orangutan herpesvirus (*P. pygmaeus*-HVO), gorilla herpesvirus (*G. gorilla*-HVG) (54), African green monkey (AGM-EBV) (56), are not known to cause disease in their natural hosts nor in other primates. Two lymphotropic EBV-like viruses had been isolated from *Macaca* sp. and these like the other isolates were not associated with disease (55,57). More recently Lapin and his associates (140) recovered a lymphotropic virus from the stumptail monkey (*M. arctoides*). This virus also does not appear to cause disease in its natural host nor has there been any indication of a relationship to other macaque lymphotropic viruses (55). The EBV-like herpesvirus isolated by Heberling et al. (57) was recovered from cynomolgus monkeys with a high incidence of lymphoma although a causal relationship was not demonstrated. All of these viruses are of interest inasmuch as they have striking similarities with EBV. Their epidemiology resembles that seen for EBV in the human. Major differences, however, exist between the Old and New World viruses.

Virus transmission is horizontal by means of oral secretions and natural infection is high. Latency results as these oncogenic herpesviruses will reside in leucocytes for the life of the animal (71).

Clincial. With the exception of HVP which has been associated with a continuing outbreak of lymphoma at the Institute of Experimental Pathology and Therapy (Sukhumi, USSR), the other viruses are not known to cause clinical disease in their natural hosts.

Lapin and his associates (44,49,50,139) in a series of studies have described a wide variety of lymphomas in the baboon colony: non-Hodgkins lymphoma of the lymphoid type (the predominate disease), lymphosarcomas, prolymphocytic lymphosarcomas, reticulosarcoma, lymphoplasmacytic and immunoblastic lymphoma, and lymphogranulomatosis. Advanced disease is frequently accompanied by immunosuppression (141).

Experimentally, it is evident that these lymphotropic viruses have the capacity to induce infection in animals; however, disease is more limited in occurrence. Early attempts to transmit infectious mononucleosis to monkeys were essentially negative or undefined (142-144). Gibbons were also inoculated with EBV containing material with development of transitory clinical disease and evidence of antibody (145). The presence

of antibody in Old World monkeys and apes, however, had been previously demonstrated (146-149), but was absent or infrequently found in New World monkeys (150,151). In retrospect, it is highly probable that these findings were reflections of antibody-crossing with the various EBV-like viruses isolated from Old World, but not New World, primates.

Using EBV-transformed squirrel monkey cells, IM heterophile antigen was demonstrated on the cell membrane of transformed squirrel monkey cells (152,153). None of the animals developed palpable tumors or hematological abnormalities, but three of four animals developed heterophile agglutinins and EBV-specific antibodies. In another study (136), these investigators were able to induce neoplasia resembling human malignant lymphoma (reticulum cell sarcoma) in cottontop marmosets following inoculation with cell free virus or autologous cells transformed by EBV. Detectable tumors were noted in 31-46 days. Epstein et al. (137) were able to demonstrate that EBV-containing cultured lymphoblasts induced in owl monkeys a reticuloproliferative disease characteristic of malignant lymphoma.

Further evidence for the oncogenicity of EBV for experimental animals (marmoset) was clearly demonstrated when it was shown that nuclear antigen EBNA was present in the cells of the experimental marmoset tumor (154). In order to produce lymphoproliferative disease, Falk et al. (155) demonstrated that 10^4 transforming units were necessary.

The clinical disease induced by *H. ateles*-2 is similar to that of *H. saimiri*-2; however, HVA disease is more uniform than that due to HVS. HVA in cottontop marmosets produces an acute lymphocytic leukemia or a poorly differentiated malignant lymphoma. Tumor development is 100% and death occurs in 2-5 weeks. Also the HVA leukemic reaction has a lower cell count and multiple infarctions due to thrombosis are rare. In the owl monkey, HVA infection results in a lymphoblastic or stem cell lymphoma; however, HVA infected owl monkeys do not all develop disease, but survivors develop a long lasting chronic infection (67,134).

Pathology. The Old World monkey lymphotropic viruses are B-cell tropic whereas the New World monkey viruses are T-cell tropic. EBV, like its Old World monkey virus counterparts, is also B-cell tropic. The EBV-like viruses all have the ability to transform or immortalize primate B lymphocytes in culture (50,156). The cell range of this capability varies with the virus. Although, these viruses are considered B-cell

tropic, they do produce tumors of the T-cell type as well as non-T, non-B cell types. According to Markaryan (157) examination of lymphomatous tissues will show numerous chromosome breaks and structural rearrangements. Advanced disease in baboons shows that the lymphocytes are suppressed and the animals are hyporesponsive. Virus genome is present in tumor tissue or transformed cell lines as a result of infection with all these viruses (141).

The T-cell tropic viruses also differ from their Old World monkey counterparts in that they are not only transforming but are cytolytic as well. HVS and HVA are able to induce tumors in rabbits, a capability not shown for the Old World monkey viruses. These viruses also produce a widespread reticulum invasion of the major organs: lymph nodes, spleen, liver, etc., with replacement of the normal cellular structure. In the tamarin, the HVA neoplastic cell type is a poorly differentiated lymphoblast but more uniform than those in HVS tumors. In owl monkeys again the HVA histpathology is clearly differentiated from that of HVS: the tumors are lymphoblastic or stem cell lymphomas, invasion of kidneys is extensive where the cells form expanding nodules in the cortex and medulla; the adrenals, lungs, and liver are generally free of pathology (134).

Diagnosis. With the exception of HVP infection in the baboon, the host animal does not develop disease. Accordingly detection of infection/disease is not a diagnostic problem, but rather a matter of determining that the clinical disease was due to the introduced agent.

Identification is based upon characterization of recovered virus and ascertaining its true nature by various immunological and hybridization studies. The B-cell tropic viruses have four major groups of antigens as determined by immunofluorescence: virus capsid antigen (VCA), early antigen (EA), membrane antigen (MA), and nuclear antigen (EBNA). DNA homology is shared by all EBV and EBV-like viruses; HVS and HVA do not hybridize with these viruses but do share some homology with each other. These two viruses share a common antigen which may be detected by immunofluorescence.

Serological studies detect these various antigens, but interpretation may be difficult. Using crude antigens, antibody may be detected to each of the viruses. The B-cell tropic viruses will all show some degree of cross reactivity with each other and to HSV, but not with the T-cell

tropic viruses and, conversely, the T-cell tropic viruses cross react but not with the B-cell tropic viruses. Finding of antibody in normal animal populations also complicates interpretation. Antibody to the various groups of antigens varies among the different primate species. As disease is not a factor except in the case of HVP infected baboons, the significance of the various antibodies is speculative. Detection of these various antigens in New World monkeys and in cell cultures has been reported (67), but the pattern of development is not as clearly defined as in EBV and EBV-like infections. EA, MA, and LA (late antigens) are detected following infection and tumor development; but apparently NA (nuclear antigens) do not appear after HVS infection. NA has been reported in HVA infected cells (158).

The B-cell tropic viruses are readily recovered by cultivating peripheral lymphocytes or from lymphoid cell lines obtained from tumors. HVP has been recovered from the throats of colony animals where there is a high incidence of disease but not from the colony maintained in the forest surrounding Sukhumi (159). HVS may be isolated from squirrel monkey degenerating kidney cells or from circulating lymphocytes and tumor tissue of infected animals. HVA may also be recovered from kidney cells as well as by cocultivation of peripheral lymphocytes with permissive cells.

Prevention, Control, Therapy. Infection with the lymphotropic herpesviruses does not result in typical herpesvirus disease. As a consequence, with the exception of what has been shown in Sukhumi, these are all experimental situations and result from experimentally inoculating susceptible animals. HVP has been shown to be controlled by segregating the animals (159). It should be noted that the original outbreak in the Sukhumi colony followed attempts to induce tumors in baboons by inoculation of human leukemic materials (49). The possibility of vaccinating against these malignancies was suggested by the use of an inactivated HVS vaccine in marmosets (160) and EBV in the cotton-top marmoset (161).

Discussion. A number of other herpesviruses have been isolated from peripheral leucocytes of nonhuman primates (Table 1). These leucocyte-associated viruses have thus far shown no capacity to induce disease in either their natural host or when experimentally inoculated into other species. Serologic evidence of infection may be detected in various surveyed Old World monkeys (162). LAHV (HVM) (163) and HVMA (140) are

antigenically distinct although HVMA shows some homology with EBV and HVP. The isolate reported by Heberling et al. (57) has not been studied in sufficient detail. Like other herpesviruses in this group, these viruses persist in the host animals' lymphocytes and may be isolated from oropharyngeal swabs as well as from lymphoid tissue (162). At least two strains of LAHV exist, but more study is required for full evaluation. Complement fixing antibody, but not neutralizing antibody to LAHV has been found in cynomolgus monkeys, African green monkeys, patas monkeys, and chimpanzees. As indicated in Table 1, several distinct isolates have been made from the squirrel and spider monkeys. Certain of these are neurotropic and not associated with tumorigenesis; these should not be confused.

SUMMARY AND CONCLUSIONS

The Herpesviridae consist of a large group of viruses with diverse disease-producing capabilities. In addition, their biological properties are such that it is difficult to minimize these capabilities. Confusion results from the many different isolates obtained from the same species. These viruses are antigenically distinct and have different invasive capacities. However, therapeutic agents useful against HSV and other herpesviruses with the same characteristics have been shown to be inhibitory. The question of whether such therapy might produce mutants has not been resolved. Vaccines have been developed, but again their efficacy is questioned principally because the herpesviruses _in vivo_ are not accessible to antibody.

Another major problem with herpesviruses is their latency and recrudescence. This ability is probably basic to the character of this virus group and as a result is a major factor in maintaining this virus as a principal cause of disease.

Probably of greatest concern are those herpesviruses with a predilection for lymphocytes (**Gammaherpesvirinae**) and their close association with cancer. Although the role of these viruses in oncogenesis has not been fully established, this relationship, particularly in animals other then humans, is well recognized. The ability of herpesviruses to become integrated into the host's nucleic acid and remain undetected is of considerable concern when attempting to understand their association with oncogenesis. What, then, is the precise role that the herpesviruses

play in oncogenesis? Are they activators, cocarcinogens, inhibitors of mechanisms of host functions? The possible association of retroviruses and herpesviruses in malignancies needs further study.

Our understanding of the infectious process of the herpesviruses, or of any viruses for that matter, is woefully lacking. Here we have a group of viruses capable of at least two different mechanisms of infectivity within the same host: a cytocidal capability and persistence. Persistence may be subdivided into a number of different pathways depending upon the cell system involved. Here the interplay between virus and cell is even less clear because again the end result is extremely variable: latency, immunosuppression, tumorigenesis, polyclonal activation, genetic involvement and many more. It is apparent that the herpesviruses deserve the attention they receive in our attempt to further our knowledge of disease and disease processes.

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7

BABOON LYMPHOMA VIRUSES

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ABSTRACT

Enzootic outbreak of lymphoma has occured at the Sukhumi monkey colony beginning from 1967 to nowadays. This enzootic has resulted in over 280 fatal cases among baboons. Lymphoma-associated B-lymphotropic herpesvirus (HVP) antigenically related but not identical to the Epstein-Barr virus (EBV) and sharing with EBV upto 40% DNA homology, as well as T-lymphotropic C-type retrovirus H(S)TLV-I have been isolated from lymphomatous animals. The above viruses are released into the environment and spread horizontally. The majority of the disease cases among baboons represented different morphological variants of non-Hodgkin's B- and T-cell lymphomas. No correlation between immunological type of lymphoma and the level of antibodies to HVP and H(S)TLV-I has been found. The majority of monkeys showed an increase of antibody titers to both viruses in prelymphoma period. Integrated H(S)TLV-I-like provirus was determined in baboon lymphoma DNA. HVP-specific DNA was found in tumour and some normal tissues of monkeys from the high-risk lymphoma stock.

INTRODUCTION

Lymphomas (leukemias) are the most frequent neoplasms in different monkey species. In principle, these tumours resemble all clinical and morphological variants of human haemoblastoses. However, an overwhelming majority of them belong to various types of non-Hodgkin's B- and T-cell lymphomas.

A small number of lymphomas (leukemias) described in

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different nonhuman primate species, mainly in macaques and gibbons, is associated with viruses (GALV, H(S)TLV-I, H(S)TLV-III, D-type). The greatest number of haemoblastoses (lymphomas) are described in hamadryas baboons of the Sukhumi monkey colony where the disease started in 1967 among the animals injected parenterally with human leukemic blood as enzootic and has resulted by now in over 280 fatal cases.

Table 1. Mortality as a result of lymphoma in hamadryas baboons of the Sukhumi monkey colony.

Y e a r	Number of adult baboons in the stock	M o r t a l i t y Number	%
1966	346	0	0
1967	365	1	0.27
1968-1980		179	1.98
1981	1126	16	1.42
1982	1041	18	1.73
1983	1254	13	1.04
1984	1140	14	1.22
1985	1248	18	1.44
1986 (1 Nov.)	1281	23	1.79
	Total number of baboons died of haemoblastoses - 282		

The disease is associated with two viruses: DNA-containing B-lymphotropic herpesvirus HVP, related but not identical to EBV, and C-type retrovirus, also related to but not identical to H(S)TLV-I (see Fig. 1 and Fig. 2 below).

Regular isolation of both viruses mentioned above from lymphomatous hamadryas baboons as well as from animals in the prelymphoma period or those in the high-risk stock was performed, stereotype immunological shifts in animals with initial signs of the disease and in sick animals was observed. The dynamics of antibody titers to herpesvirus HVP and retrovirus H(S)TLV-I are not only indicative of probable association of lymphoma with HVP and H(S)TLV-I viruses but also of the participation of these viruses in the development of neoplasms. The question about the character of interrelation between EBV-like herpesvirus and C-type retrovirus H(S)TLV-I needs additional and special investigation. However, the viral nature

of the baboon lymphoma is beyond any doubt due to the above facts and enzootic character of the disease.

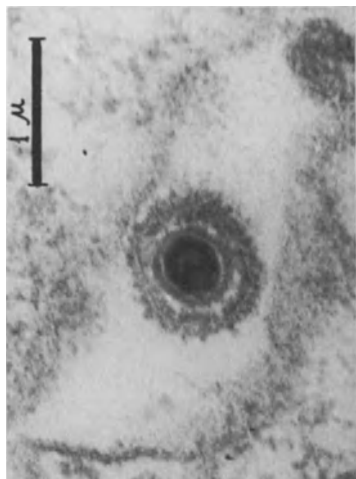

Fig. 1. B-lymphotropic herpesvirus HVP.

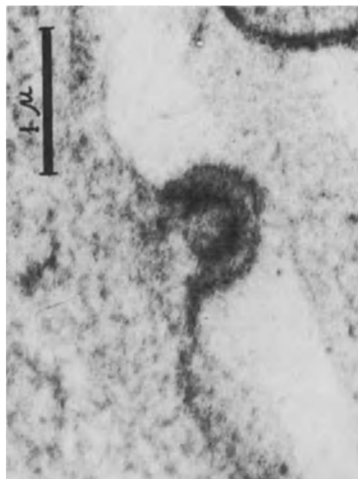

Fig. 2. C-type retrovirus.

Baboon lymphomas belong to the B-cell type in approximately 50% of the cases and of about 50% to the T-cell type. Only a few cases belong to null-cell variants. It is of interest that no parallelism between the prevalence of either B- or T-lymphotropic viruses and the titers of antibodies to them and the cell variant of lymphomas has been noted (1).

Isolation of EBV-like herpesviruses, first from hamadryas baboons, and then from other monkey species including apes, was preceded by revealing antibodies to viral capsid antigen (VCA) of EBV (2-8) in sera of animals in places of their natural habitat. There was an impression that EBV was widely spread among Old World monkeys presenting a reservoir of the virus for human populations. However, the isolation and molecular-biological characterization of B-lymphotropic herpesvirus from lymphomatous hamadryas baboons (and later on also from healthy animals of this species) have allowed the conclusion that in this case we are dealing with related virus similar antigenically, but nonetheless having certain

antigenic differences and bearing about 40% DNA homology with EBV virus (9-16).

The fact that the isolated baboon herpesvirus designated as HVP (Herpesvirus Papio) is closely related but different from EBV has allowed the hypothetical suggestion that a subfamily of B-lymphotropic EBV-like herpesviruses of primate order exists. The latter was confirmed by subsequent isolations of the related viruses from various primate species (herpesviruses of gorillas, orang-utans, chimpanzees, African green monkeys and macaques) (17-21) /Fig. 3).

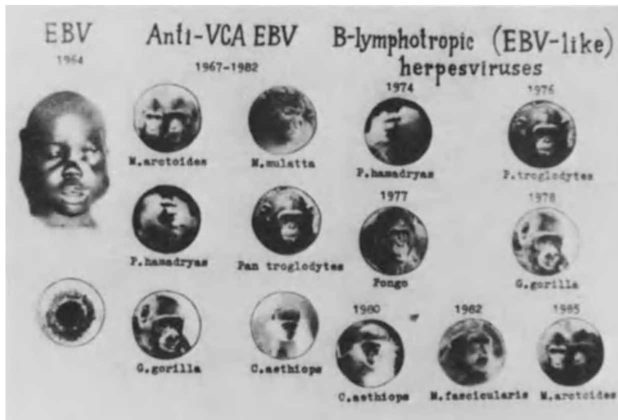

Fig. 3.

The situation with the C-type retrovirus seems to be similar to that with HVP. Isolation of HTLV-I from patients with T-cell lymphoma (originally misdiagnosed as Mycosis fungoides and Cesary syndrome) by R. Gallo's group (41) and ATLV virus by the Japanese investigators (15,42) (in further research both groups of investigators agreed that the viruses were identical) has shown the relation of these viruses to human lymphomas/leukemias. In 1982-1983 I. Miyoshi et al., K. Yamamoto et al., B. Lapin and A. Voevodin et al. described antibodies to H(S)TLV-I in Japanese macaques, African green monkeys and finally in hamadryas baboons (22-27). These investigations, having gone through almost the same stages as in the case of HVP studies, have led to the conclusion that monkeys are the carriers of HTLV-I-like viruses closely related

to HTLV-I, but clearly different from it. These viruses were designated in literature as STLV-I.

MATERIALS AND METHODS

Investigation of lymphomatous viruses associated with hamadryas baboon lymphomas was carried out in the "high-risk" stock of baboons of the Sukhumi monkey colony numbering over 2000 animals, mainly of the 9th, 10th and 11th generations born in this colony. Annual mortality in connection with different cytological types of B- and T-cell non-Hodgkin's lymphomas was 1.22 to 2.5% among the animals of "susceptible" age, i.e. over 3 years (43,44).

Frequently occurring variants of the non-Hodgkin's baboon malignant lymphomas are shown in Figs. 4 - 7.

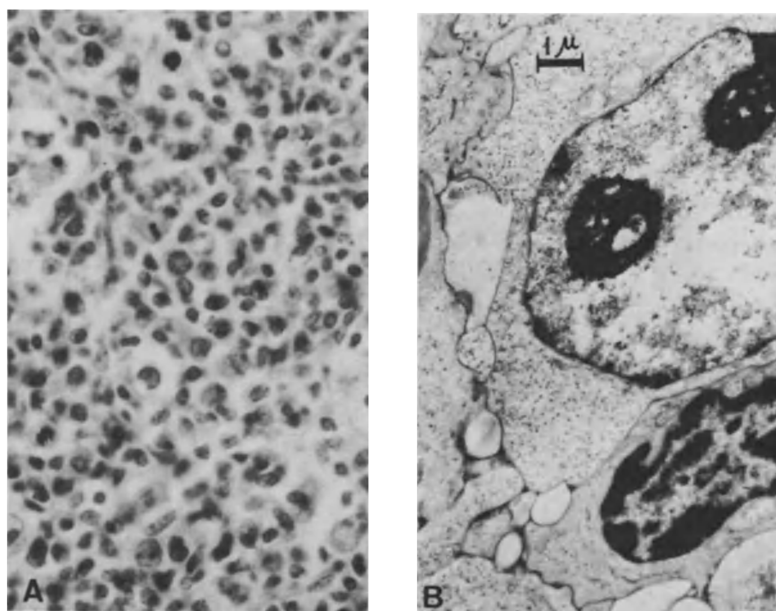

Fig. 4 B-cell baboon malignant lymphomas. Centroblastic-centrocytic lymphoma. a) Histostructure (H. & E. staining). X 500. b) Ultrastructure.

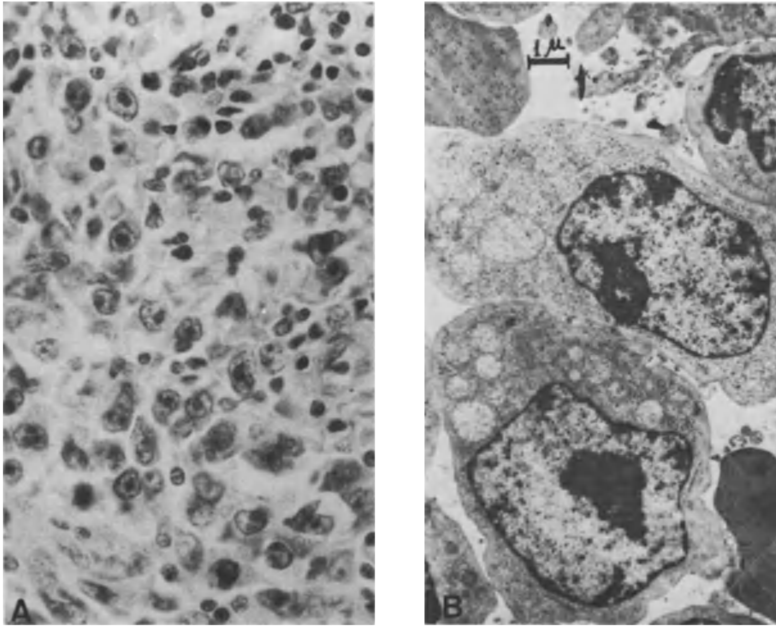

Fig. 5. B-cell type immunoblastic lymphoma. a) Histostructure (H. & E. staining). X 700. b) Ultrastructure.

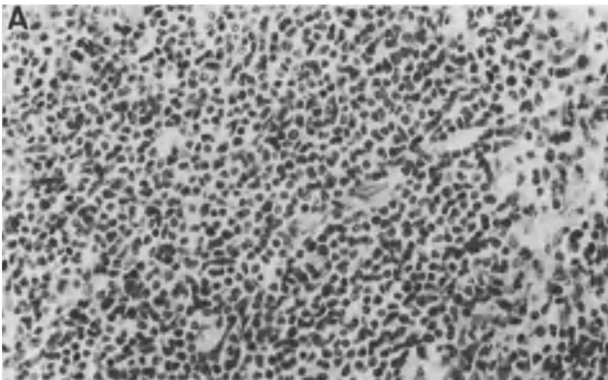

Fig. 6. T-cell baboon non-Hodgkin's malignant lymphomas. Prolymphocytic lymphoma. a) Histostructure (H. & E. staining). X 300. b) Ultrastructure.

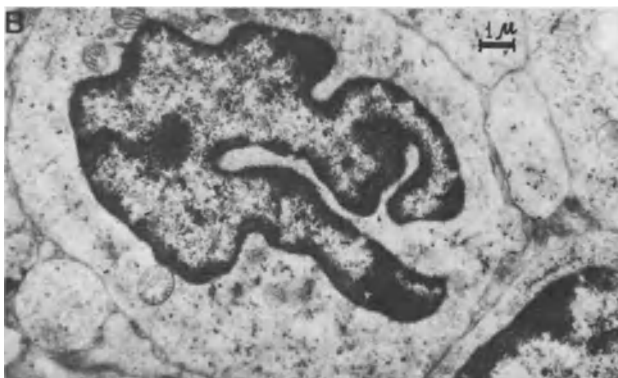

Fig. 6b.

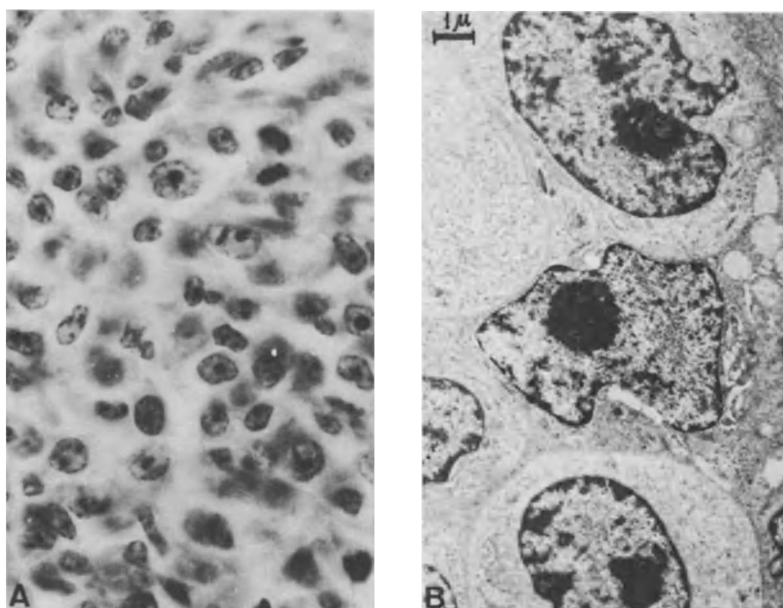

Fig. 7. T-cell immunoblastic lymphoma. a) Histostructure (H. & E. staining). X 700. b) Ultrastructure.

Control group for the "high-risk" stock consists of 600 monkeys kept in game reserve belonging to the Sukhumi monkey colony of the Institute of Experimental Pathology and Therapy. These monkeys have never been in contact with the "high-

-risk" animals of the main stock. The animals are regularly subjected to veterinary examinations with special attention to spleen and lymph nodes (mainly to their size and solidity as well as biopsy of lymph nodes). Peripheral blood, cell immunity status, and the presence of antibodies to HVP, H(S)TLV-I, H(S)TLV-III and type-D retrovirus are also investigated. Antibodies were revealed by indirect immunofluorescence test and immunoenzyme assay (ELISA).

Virus-producing cell lines 594S-F9, P3HR-I (for HVP study) as well as HUT-102, C91PL, C10MJ-2 lines (for H(S)TLV-I study) were used as antigens. As reference sera to study HVP we used HVP- and EBV-positive and negative sera of monkeys and men, and to study H(S)TLV-I - human sera from HTLV-I-positive healthy donors and monoclonal antibodies GIN-14 against p19 and p28 HTLV-I kindly provided by Dr. Y. Hinuma (25,39).

The absence of H(S)TLV-III virus-carriers and D-type retroviruses was documented by the absence of antibodies to the latter ones, that was established in indirect fluorescence tests using H9 cells infected with HTLV-III and Fcf_2Th cells infected with SAIDS D-Washington virus (the latter was kindly provided by R. Benveniste).

Integration of H(S)TLV-I-like provirus in baboon lymphoma DNA was investigated using Southern blotting analysis of high molecular weight lymphoma DNA digested by restriction enzymes PstI, BamHI, EcoRI and SstI. Full genome HTLV-I DNA cloned in SstI site of pSP-65 vector was used as a molecular probe (28).

HVP DNA in lymphoma tissue was revealed by reassociation kinetics (16). Cell immunity was studied by testing the response of peripheral blood lymphocytes to polyclonal mytogens (Con A, PHA, PWM). Correlation between populations and subpopulations of B- and T-lymphocytes in peripheral blood and lymphoid organs was also investigated. Immunological typing of malignant lymphoma was performed using immunological and cytochemical tests. Lymphomas were also typed morphologically.

RESULTS

Many lymphoid cell lines have been established both at the laboratory of Experimental Oncology of the Institute and at collaborating laboratories (9-12) from lymphomatous and healthy animals of the Sukhumi colony. These lines produce herpesvirus (HVP) and some of them produce simultaneously HVP and C-type retrovirus H(S)TLV-I. As it was started above, HVP has antigens typical for EB virus - viral capsid antigen (VCA), early antigen (EA), membrane antigen (MA), and nuclear antigen (NA), althogh it also has antigenic determinants specific for HVP. In this respect, VCA, being practically undistinguishable from that of EBV, is an exception. The DNA buoyant density values of both EBV and HVP equal to 1.717-1.718 g/cm^3, sedimentation coefficient being 55S (13). The greatest part of the viral DNA in the infected cells is present as covalently linked circles. The structural organization of the genome of both Epstein-Barr virus and baboon herpesvirus is very similar, whereas the general homology of their DNAs does not exceed 35 - 40% (13,29,30).

An important biological peculiarity of the hamadryas baboon herpesvirus is its ability to transform lymphocytes of homologous animals as well as monkeys of other species. There are also rather few reports about the possibility to transform human lymphocytes by HVP (31), but these data need additional confirmation.

Of indisputable interest are data on the induction of fatal lymphoproliferative disease in New World monkeys as a result of injection with HVP-producing culture established from a lymphomatous hamadryas baboon (11). HVP is released into the environment through nasopharyngeal mucosa and lacrimal gland, which seems to be the main route of horizontal transmission of the virus. Based on the data about the presence of antibodies to VCA-HVP (as an indicator of the virus infection) in different groups of baboons imported from Ethiopia we have come to the conclusion that the prevalence of this infection can fluctuate in a wide range. According to our data it varied from 35 to 90%, however, it is indisputable

that this range can be much higher. The prevalence of infection among hamadryas baboons living in conditions of the Sukhumi colony increases with time reaching practically 100%. The animals born in the colony have maternal antibodies to VCA eliminating in the course of the first year of life (32). However, due to the horizontal transmission of the virus the number of infectious animals increases reaching maximum approximately by the age of 5 years (33 / Fig.8).

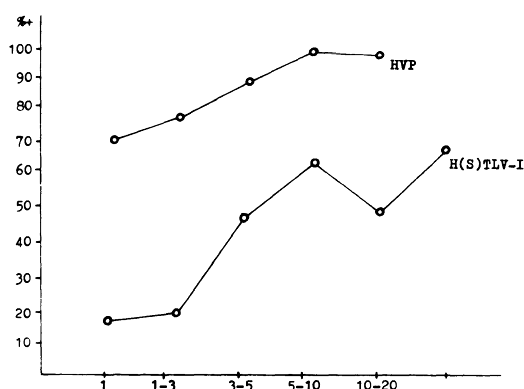

Fig. 8. Age-dependence of HVP and H(S)TLV-I seropositivity.

Laboratory isolation of the virus is made from nasopharyngeal smears, the filtrate of which is able to transform lymphocytes of young homologous (or other susceptible) animals forming continuous virus-producing lymphoid lines. The release of the virus into the environment has a seasonal dependence, the peak being observed in autumn and spring (34).

At the beginning of the 70s, when studying the lymphoma outbreaks by electron microscopy of haemopoietic cells, kidneys, lacrimal and submaxillary glands, we revealed virus-like particles resembling C-type retroviruses. Simultaneously, similar particles were found in plasma sediments in ultracentrifugation as well as in ultracentrifugation of the latter in

sucrose and caesium chloride gradients. Possessing physical, chemical and morphological characteristics of "oncornaviruses" these particles had no antigenic relationship with the retroviruses known at that time (35). C-type retroviruses were also revealed in the HVP-producing cell cultures. They were identified only after the American and Japanese investigators isolated the C-type retrovirus which is known now as H(S)TLV-I. As it was noted above, beginning from 1982 there appeared some publications about the revealing antibodies to H(S)TLV-I virus in some monkey species (Macaca fuscata, Cercopithecus aethiops, Papio hamadryas). This was followed by the isolation from monkeys of the viruses related to but at the same time different from HTLV-I. These data suggested the existence of a group of simian HTLV-like viruses. When comparing nucleotide sequences and restriction maps of these viruses it has been established that those sequences differed from one another and also from HTLV-I (25-27, 36-38).

Investigating sera of considerably large groups of healthy baboons of the "high-risk" stock (177), healthy monkeys of the game reserve located in the forest near Sukhumi (118), baboons imported from Ethiopia during quarantine isolation (195), and monkeys that died of lymphoma as well as sera obtained in prelymphoma period, we have found that practically all but one lymphomatous monkeys have antibodies reacting with H(S)TLV-I antigens (Table 1). These antibodies were detected in almost half (45.2%) of the "high-risk" stock baboon sera. As far as the baboons from our game reserve and those newly imported to the colony are concerned, the per cent of sera positively reacting to HTLV-I antigens was much lower: 7.6% for the forest baboons and 5.6% for those brought from Ethiopia. These data convincingly showed the difference in in the prevalence of infection in different populations and also suggested possible horizontal virus transmission being most clearly pronounced in the Sukhumi monkey colony. It was probably due to more overcrowded conditions in the colony as compared to the conditions in nature or forest reserves. This is also proved by clear increase in the number of seroposi-

Table 2. Prevalence of antibodies reacting with HTLV-I in lymphomatous and healthy baboons of different populations.

Group	Number tested	Number positive	Percentage positive
Lymphomatous baboons	58	57	98.3
Sukhumi main stock baboons	177	80	45.2
Control animals from Sukhumi game reserve	118	9	7.6
Control animals imported from Ethiopia	195	11	5.6

tive baboons with age.

Significant infection prevalence among the "high risk" stock baboons as well as the elevation of antibody titers to HTLV-I antigens in prelymphoma period have posed a question about possible involvement of H(S)TLV-I in the baboon lymphoma development. To solve this question, the Southern blotting analysis was used to investigate lymphomatous baboon DNA for the presence of integrated H(S)TLV-I-like provirus. High molecular weight lymphoma DNA was digested by restriction enzymes mentioned above. As a molecular probe, full genome HTLV-I DNA was used (it was kindly provided by Dr. R. Gallo). All the 10 samples of Pst-I digested lymphoma DNA investigated have been found positive. The spectrum of the fragment proved to be similar, but clearly different from that of HTLV-I. Apart from the three fragments found in all specimens that, probably, were internal fragments, each sample revealed also individual fragments (Fig. 9). These findings proved monoclonal integration of H(S)TLV-I-like provirus in different sites of the baboon lymphoma DNA. These data were confirmed by the analysis of lymphoma DNA digests by the restriction enzymes BamHI and EcoRI (the restriction sites for which were absent in H(S)TLV-I

provirus). In a number of cases, some fragments were smaller in size than the expected one of STLV-I provirus indicating that in some of the baboon lymphomas there was an integration of the defective proviruses (28).

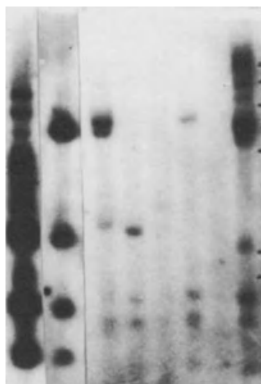

Fig. 9. Southern blot analysis of PstI digests of baboon lymphoma DNA (probe: full-length HTLV-I provirus). 1 - human HTLV-I-producing cells (C91PL); 2 - pSP-65 plasmid with full-length HTLV-I provirus insert; 3-6 - baboon lymphomatous lymph nodes; 7 - HVP/STLV-I infected baboon lymphoid cell line.

Southern blotting analysis of SstI digests of the baboon lymphoma DNAs reveals 8.5 kb fragment that corresponds well with the data by H. Guo et al. (38) who investigated the DNA of lymphoid H(S)TLV-I-producing cell line. The latter has been established from a baboon - the recipient of blood from lymphomatous Sukhumi colony baboon after seroconversion for anti-H(S)TLV-I. Thus, the findings of the present research are indicative of a possible involvement of H(S)TLV-I virus in the malignant transformation of lymphocytes and correspondingly in the lymphoma development. On the other hand, it showes the similarity (and simultaneously the difference) of the virus isolated from lymphomatous baboons with the human virus (HTLV-I).

As we mentioned above briefly, there is an increase in the titers of antibodies to HVP- and H(S)TLV-I-antigens in the prelymphoma period. We have succeeded in performing this by retrospective analysis of sera from normal baboons that died of lymphoma. The sera were obtained at the prelymphoma period, stored frozen and then examined (after confirmation of lymphoma diagnosis). It was found that the monkeys that

seemed clinically healthy showed a clear elevation of antibody titers to HVP-VCA and HVP-EA and simultaneously to H(S)TLV-I antigens (27, 39). Till now we have not succeeded in establishing the relationship between peculiarities of morphological and immunological variants of lymphomas and the level of titers or viruses HVP and H(S)TLV-I.

The role of both viruses described earlier (HVP and H(S)TLV-I) (40) as well as the question of their interaction in the process of the lymphoma development and progression needs special study. However, the peculiar dynamics of antibodies to both viruses with characteristic and clear elevation of antibody titers in the prelymphoma period indicates an association between baboon lymphoma and these viruses.

The decrease of antibody titers observed subsequently in many lymphomatous animals (in some of them they remain on the initial level or even increase) can be explained by the immunosuppressive action of the virus(es) or immunodepression due to lymphoma development and progression. This decrease of the titers to HVP and H(S)TLV-I is accompanied also by changes in cell immunity, i.e. by a decrease in the response to polyclonal mytogens as well as by disbalance of T-lymphocyte subpopulations (that can be caused by the same factors).

The suggestion that there is an association of B- and T-lymphotropic viruses with baboon lymphoma can be subjected to criticism based on the lack of correlation between the number of diseased baboons and the number of the virus-carriers. However, such a situation may happen with all (or almost all) tumour viruses, since in fact we do not know the viruses that are able to induce tumours in 100% of infected animals. This situation is also well known in infectious pathology where infection of an individual even with a rather virulent agent does not mean the development of the disease.

Returning to the baboon lymphoma we could once more stress that extensive physical contacts of monkeys in the Sukhumi colony (such as grooming, fighting with bites, contacts with excrements, heterosexual and bisexual contacts, etc.) can play a significant role in the realization of the oncogenic potential

of the association of HVP and H(S)TLV-I.

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8

B VIRUS (CERCOPITHECINE HERPESVIRUS 1) INFECTIONS IN MONKEYS AND MAN

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ABSTRACT

B virus can be grouped together with the human herpes simplex viruses (HSV) and the bovine herpes virus 2 (BHV-2) to the genus Simplexvirus, subfamily Alphaherpesvirinae, family Herpesviridae. B virus is indigenous to rhesus monkeys (Macaca species) and most likely represents the simian counterpart of HSV. Virus can be recovered from herpetic lesions and at the beginning of the infection from oral and genital swabs. Latently infected animals have virus in the ganglia subserving the genital tract and the oral cavity. B virus is hazardous to man for its ability to cause a fatal encephalomyelitis. HSV immunity may be cross-protective.

INTRODUCTION

One of the most frightening zoonoses is the infection of man with the cercopithecine herpesvirus 1, which is indigenous to macaque monkeys (Macaca spp.). Several synonyms are used for this virus: herpesvirus simiae, simian herpesvirus, herpesvirus B or B virus. The latter name is generally accepted (1). Biological and molecular biological investigations imply that B virus should be grouped to the genus Simplexvirus, subfamily Alphaherpesvirinae in the family Herpesviridae (2,3).

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The human herpesvirus 1 and 2 (herpes simplex virus types 1 and 2; HSV-1, -2) and the bovine herpesvirus 2 (BHV-2; bovine mammillitis virus) are recognized members of this genus (3). A further herpesvirus, the cercopithecine herpesvirus 2 or SA 8, isolated from African green monkeys (vervet monkey, Cercopithecus aethiops) and baboons (Papio ursius) shows serological crossreactivity with HSV, B virus and BHV-2 and therefore should also be grouped to the genus Simplexvirus (3a, Pauli and Ludwig, unpublished).

HISTORY

The first case of an acute ascending myelitis in man with fatal outcome, following a bite of an apparently normal rhesus monkey, was observed in 1932. Two research groups reported on the isolation of virus from brain and cord (4,5). The inoculation of these isolates into rabbits reproduced a disease similar to that in man, supporting the neurotropism of the virus. The agent described by Sabin and Wright (5) was called B virus, whereas Gay and Holden (4) named their isolate W virus (initials of the patient: Dr. W. B.). These authors regarded their virus as an atypical variant strain of the human herpes virus. Sabin (6-8), however, concluded from cross-neutralization experiments in rabbits and from pathogenicity studies that the B virus and the human herpes virus represented two distinct entities. Comparing the pathological and immunological data obtained after infection of animals with other herpesviruses, he suggested that B virus was intermediate to herpes and pseudorabies virus (8). In a more detailed study, using the chorioallantoic membrane technique, Burnet and co-workers (9) indicated that these three viruses

spring from a common ancestor and were specific for their respective hosts: herpesvirus for man, B virus for monkeys, and pseudorabies virus for pigs.

Although the early investigations (8-10) already implied that B virus is indigenous to rhesus monkeys, it was not until 1949 that a second B virus isolate, again originating from a fatal case of encephalomyelitis in man was described (11). After several unsuccessful attempts to isolate B virus directly from monkeys, a few years later virus strains were obtained from a rhesus monkey used in a poliomyelitis virus study (12) and from tissue cultures of normal rhesus and cynomolgus monkey kidneys (13). These results together with the serological investigations supported the hypothesis that B virus can be latent in Macaca species. The increasing use of rhesus (Macaca mulatta) and cynomolgus monkeys (Macaca philippinensis) in research and for biological production underlined the importance of B virus infections for man, not because of the frequency of the infection, but because of the seriousness and fatality of the disease.

DISEASE IN MACAQUES

Although B virus was first described half a century ago, little is known about the disease in the natural host, the macaque monkeys. The disease in general resembles the infection with herpes simplex virus in man showing the picture of a primary stomatitis (2,14). The disease is usually benign and can be recognized from the herpetic ulcers on the tongue, in the buccal cavity and on the muco-epithelial border of the lips. Lesions heal spontaneously in about a week. Only a few reports on the involvement of B virus in fatal infections of rhesus monkeys (Macaca mulatta; 15,16) or

bonnet monkeys (Macaca radiata; 17) are known. Inoculation of the virus into antibody-negative monkeys resulted in infection as shown by seroconversion, virus isolation and sometimes mild clinical signs (ulcers at the site of inoculation; conjunctivitis). Reproduction of the fatal disease in monkeys, however, was never observed (16-18). These findings support the idea that additional factors contribute to the outcome of B virus infection.

Based on the close serological relationship of the simian with the human herpes virus (HSV) the suggestion arose that B Virus is the simian counterpart of HSV. The known oral lesions and the demonstration of latency in sensory ganglia subserving the oral region supported this idea (19-21). The mouth as the site of infection has always been emphasized, because monkey bites had caused human infections. Recent reports on the isolation of B virus from naturally and experimentally infected animals, on the contrary, stress that B virus infections are not only restricted to the oral cavity, but are localized even more frequently on and in the genital tract and involve the ganglia subserving it (18,22).

LATENCY AND REACTIVATION

It is known that reactivation of latent herpesviruses in different species, including man, can be observed as a response to a variety of stimuli (2,23-27). Furthermore excretion of infectious virus can occur in the absence of clinical symptoms. Attempts to isolate B virus from monkeys proved to be difficult. This was only possible over a short period during a primary infection either with or without clinical signs. Induction of virus excretion in antibody positive animals was unsuccessful in most

of the investigations, although all kinds of stress factors known to induce virus replication and excretion in latently infected man or vertebrates were applied (21,28).

There is only one report on B virus isolation from latently infected monkeys. Seropositive females (M. fascicularis) were treated with anti-human-lymphocyte globulin over a period of 15 days. From one of these animals B virus could be isolated from vaginal swabs over a period of 12 days, from day 6 after the beginning of treatment. Oral swabs from that animal were positive only once on day 17. The mouth and vagina were free from herpetic lesions. All efforts to isolate B virus from the other treated animals remained unsucessful.

Isolation of B virus from monkeys suffering from herpetic lesions or dying due to infection has been reported (16,17,29-31). In well controlled investigations the excretion of B virus during epizootics in breeding colonies was followed. From six monkeys 8 isolates were obtained, three originating from the mouth, three from the vagina and one from the penis. All samples contained high titers of infectious virus. It is noteworthy to mention that no lesions were obvious in regard to the isolations from the oral cavity or genital tract. This allows the conclusion that most of the B virus infections in macaques are not associated with clinical symptoms.

Virus isolations appear to be likely shortly after infection before neutralizing antibodies arise. In the same series of investigations it could be shown that in vitro reactivation of B virus was possible. Trigeminal and dorsal root ganglia harboured latent virus. From two additional monkeys virus was reactivated from the lumbar-sacral ganglia (18,22).

TRANSMISSION OF VIRUS

Transmission of B virus from monkey to monkey is likely when animals have primary infections. Under experimental conditions the virus could be isolated only from newly infected animals. Latently infected animals seem to play a minor role in transmission (22). The infection of man can occur through bites or by secretions (saliva or vaginal excretions). The virus invades the body through scratches, lesions or exanthemas. A further danger certainly comes from infected tissue culture cells. Latent virus can be reactivated in vitro from nerve cells as well as from kidney cell cultures (13,19,20,32). Therefore animals harbouring latent virus should be considered as being infectious. Screening for antibodies is the easiest way to identify such animals.

Infection of macaques in the wild depends on age and population density. About 15% of freshly captured animals - most of them are usually two-year-old juveniles and younger ones - have antibodies (31,33,34). On Santiago Island with its high population density, controlled studies showed that more than 80% of the monkeys at the age of 3-4 years harbour specific antibodies (35).

After capture during transportation and housing in cages the number of antibody-positive animals increases, indicating that the infection spreads easily (34,36). Seroconversion in breading colonies has recently been followed. B virus transmission from monkey to monkey even occurred when the animals were housed in gang cages (18,22). Careful examination revealed that the mouth and vagina were free from lesions. This leads to the conclusion that virus excretion occurs without clinical signs in infected monkeys.

As already mentioned, juvenile animals have a low prevalence for infections with B virus if conditions are normal. The rate of infection increases with age, also pointing to sexual transmission. The analagous situation to HSV-2 infections in man with an increase from puberty onwards is obvious (37).

Birth of antibody-negative siblings from positive mothers and lack of infection during birth and nursing supports the hypothesis that latent B virus is not shed during pregnancy or after delivery.

DISEASE IN MAN

Relative few cases of B virus infections in man have been reported since the first description in 1933/34 (for review: 2). Most of the infections were fatal and characterized by an ascending encephalomyelitis. The duration of the clinical disease varied from 3 to 21 days. All the infections could be traced back to contacts with macaques. Most interesting in this respect are reports concerning the source of infection or the duration of time between a putative infection and the outcome of clinical signs (1,38). The authors even reported on the isolation of B virus from a patient who had not been in contact with monkeys for at least 10 years. The history of the infection and the clinical signs point to an activation of latent B virus. The patient survived the infection probably due to intense medical treatment. Most of the reported cases in man are comparable to the fatal systemic HSV infection of infants. From the 25 recorded B virus infections 20 were fatal.

DIAGNOSTICS

Most investigations concerning the diagnostics of B virus infection are hampered by the close serological relationship to HSV. Sera from monkeys positive for B virus antibodies are able to neutralize HSV. The titer against the heterologous virus is sometimes even higher than against B virus itself (2). For reasons of hazard some investigators even preferred to test monkey sera against HSV, keeping in mind that both viruses are closely related and that macaques captured in the wild would only have contact with B virus. Unequivocal results by serological testings would be helpful in establishing breeding colonies free of B virus. It is necessary to evaluate human sera for the presence of anti-B virus antibodies, tracing a possible infection with this virus. Until now several attempts have been made to establish adequate test systems. In our hands radioimmunprecipitation in combination with competition experiments followed by polyacrylamide gel electrophoresis proved to differentiate between antibodies directed against HSV-1 and B virus (2). Such tests, however, cannot be performed under routine laboratory conditions.

Rapid identification of virus isolated from man or monkeys is necessary. Restriction enzyme analysis revealed that the differentiation of closely related viruses or of variants of a virus is possible. As recent reports have shown, B virus can clearly be differentiated from the human herpesviruses and pseudorabies virus (2) and from the SA 8 virus (39). This information should lead to the development of tests for rapid routine diagnostics.

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9

SIMIAN VARICELLA VIRUS

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ABSTRACT

Several outbreaks of a varicella-like exanthematous disease have occurred in captive nonhuman primate populations. In each of these epizootics, the causative agent isolated from infected monkeys was determined to be a simian herpesvirus with properties resembling human varicella-zoster virus [VZV].

Furthermore, many of the clinical and pathological signs of simian varicella virus [SVV] infection in monkeys, including the vesicular skin rash and visceral lesions, are also characteristic of human varicella infection. It has also been found that the genome of SVV shares considerable homology with the genome of VZV. Therefore, in addition to causing sporadic epizootics in primate colonies, SVV infection of nonhuman primates also serves as a valuable laboratory model for human varicella infection, and is an important tool for virologists interested in investigating the pathogenesis, immunology and antiviral therapy of varicella-zoster.

INTRODUCTION

In the past 20 years, several outbreaks of a varicella-like exanthematous disease have occurred in captive nonhuman primate populations (1-7). In each of these epizootics, the causative agent was determined to be a simian herpesvirus with properties resembling human varicella-zoster virus [VZV]. Since the disease caused by simian herpesvirus was so similar to varicella in humans, the virus was called simian varicella virus [SVV]. Whenever spontaneous epizootics of SVV occur in primate colonies among Cercopithecus aethiops, Erythrocebus patas or Macaca species, high morbidity or mortality rates can occur. For example, 9 C. aethiops were involved in an outbreak of SVV at the Liverpool School of Tropical Medicine in 1966 (6). Nearly 56% of the infected animals died within 48 hours after appearance of an exanthematous rash. Another devastating outbreak of SVV occurred in a

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colony of 95 patas monkeys at the Glaxo Laboratories in Great Britain in 1967 (7). During a span of three weeks, 27 animals died. Spread of the disease was halted only after the remaining animals housed with the infected monkeys were killed. Two epizootics of SVV similar to that observed in patas monkeys in Great Britain struck patas monkeys housed at the Delta Regional Primate Research Center, Covington, Louisiana in 1968 and 1973 (1-4). Both of these outbreaks resulted in mortality rates exceeding 50%. Three outbreaks of Medical Lake Macaques Virus (MLM) in macaques (*Macaca nemestrina*, *M. fuscats*, *M. fascicularis*) occurred at the Medical Lake Field Station of the Washington Primate Center between 1969 and 1970 (5). In these outbreaks approximately 71 animals developed an exanthematous rash, concentrated mainly in the inguinal and axillary regions, but unlike the epizootics in vervets and patas monkeys discussed above, only 6% of infected animals died from the disease.

Individual strains of SVV have been named according to the location of their isolation and/or the species of monkey from which they were isolated. For example, in the SVV outbreaks in Great Britian, the SVV isolates obtained from vervets in Liverpool, England were called Liverpool Vervet Virus [LVV] while those isolated from patas monkeys at the Glaxo Laboratories were named Herpes Patas Virus [HPV]. On the other hand, viruses isolated from SVV epizootics in patas monkeys at the Delta Primate Center have been named Delta Herpes Virus [DHV]. Finally, those isolates of SVV which originated in macaques at the Medical Lake Field Station have been named Medical Lake Macaque Virus [MLM].

ETIOLOGY

SVV can be isolated from infected monkeys by inoculating cultures of simian cells with the fluid of newly appearing vesicles or with whole blood, serum, or extracts from spleen, lungs or kidneys (8). Alterations of infected cell monolayers is usually observed in three to four days and consists of round, swollen, refractile cells which later sloughed as the area of infectivity enlarges. Typical type A inclusion bodies can be seen in infected cells which are appropriately stained. Unfortunately, it is very difficult to obtain large amounts of cell-free virus from infected cell monolayers. SVV is highly cell-associated *in vitro*. Thus, little infectious virus is released by infected cells into the culture medium. Even freeze-thawing of infected monolayers fails to release cell-free virus in titers

greater than 10^6 plaque forming units per milliliter of medium (9). Serial subculture is accomplished, therefore, by the transfer of infected cells onto monolayers of non-infected cells. The difficulty in preparing stocks of cell-free virus with high titers has made it impossible to infect monolayers of susceptible cells at a multiplicity of infection high enough to deliver one virus particle to each cell. The inability to synchronize the growth of SVV *in vitro* has hindered studies on the biochemistry and molecular biology of this virus.

Electron microscopy of SVV infected cells reveal spherical particles approximately 180-200 nm in diameter consisting of an electron dense core surrounded by an icosahedral capsid (10) (see Fig. 1). The icosahedral capsid in turn is surrounded by an envelope. The genome of SVV has been shown to be DNA (11). On the basis of its morphological, genetic and biological properties, therefore, SVV has been classified within the family Herpesviridae.

Several laboratories have attempted to determine if viruses recovered from different outbreaks of simian varicella represent a single strain of SVV. Felsenfeld and Schmidt found that DHV, HPV, LVV, and MLM were indistinguishable by both cross-neutralization and complement fixation tests (12). Harbour and Cant also found it difficult to differentiation between DHV, LVV, HPV and MLM by a number of serological tests (13). However, they did find that antiserum against chimpanzee herepsvirus reacted with MLM, LVV, and HPV isolates but not with the DHV isolate. On the basis of these results, these authors have suggested that there could be two distinct groups of simian varicella viruses, a MLM/LVV/HPV group and a DHV group. Restriction endonuclease analysis of viral DNA has been used by a number of workers to compare the genomes of different isolates of herpesviruses. Gray and Oakes were able to study the restriction endonuclease digests of SVV DNA by analyzing DNA extracted from the cytoplasm of SVV infected cells (14). Upon comparing the electrophoretic mobilities of HindIII digestion products of MLM DNA with Hind III digestion products of DHV DNA, it was found that the cleavage products of the two DNAs were indistinguishable from each other. This study therefore, supports those serological studies which have suggested that all epizootics of SVV have been caused by a single virus strain.

The clinical similarities between simian varicella infection of monkeys and VZV infection of humans has raised the possibility that SVV might be genetically related to VZV. Although the initial study by Ayres using cross-

neutralization assays did not detect a serological relationship between DHV

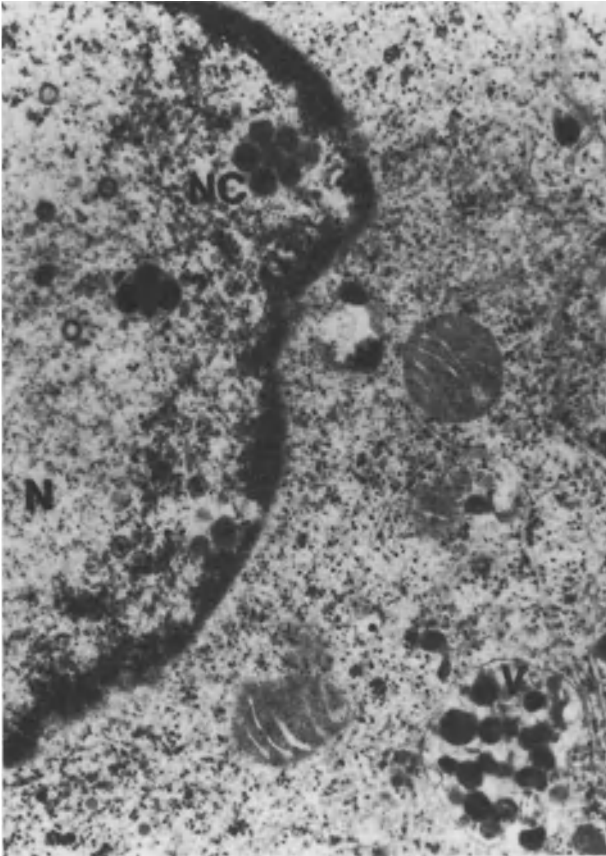

Fig. 1. Electron microscopy of a Delta herpesvirus infected Vero cell. N = cell nucleus. NC = viral nucleocapsid. V = cytoplasmic vacuole containing degraded DHV particles.

and VZV (15), several subsequent studies have suggested that SVV and VZV are antigenically related. Blakely et al. reported that macaque monkeys infected with SVV synthesized complement fixing antibodies to VZV (5). Immunodiffusion studies utilizing sera from the SVV infected macaques indicated

reactions of identity between SVV and VZV. A series of studies by Felsenfeld and Schmidt have further defined the serological relationship between SVV and VZV (16-18). These investigators reported that rhesus monkeys immunized with SVV generated both neutralizing and complement-fixation antibodies which cross-reacted with VZV. Conversely, rhesus monkeys immunized with VZV produced antibodies which cross-reacted with SVV. In addition, patas monkeys immunized with VZV did not develop any clinical signs of disease when they were challenged with SVV.

The detection of extensive serological cross-reactivity between SVV and VZV strongly suggests that the genomes of the two viruses might be genetically similar. Gray _et al._ have directly determined the amount of nucleotide sequence homology between SVV and VZV by hybridizing VZV DNA to SVV DNA immobilized on nitrocellulose paper (14). Southern blot hybridizations performed under stringent hybridization conditions did not detect DNA homology between VZV DNA and simian varicella virus DNA. However, hybridizations performed under conditions of lower stringency revealed that VZV and SVV genomes possess regions of conserved nucleotide sequences sharing 70% to 75% nucleotide sequence homology. These findings indicate therefore that while SVV and VZV are not genetically identical, they do share a close evolutionary relationship.

CLINICAL FINDINGS

The incubation period of simian varicella in experimentally inoculated monkeys ranges from 5-15 days (5,19,20). However, in most reported natural outbreaks of SVV infection, initial appearance of papular rash and deaths were seen 12-28 days following introduction of newly acquired monkeys into established primate colonies (5,6,7). Although the mode of transmission is not known, it is reasonable to suspect aerosol or contact transmission, particularly contact with contaminated fomites (21,22).

Clinical signs of disease may vary in severity depending on the species of monkey infected. In general, SVV tends to cause a milder infection in macaques than in African green and patas monkeys. Also, inoculation of the virus attenuated through cell culture passage resulted in a milder infection in patas monkeys (23). Typically, the disease is characterized by an extensive maculo-vesicular rash involving all skin surfaces except for the palms and soles (see Fig. 2). Fever, lethargy and anorexia sometimes preceeded appearance of these lesions by 1-2 days (22). The rash progresses

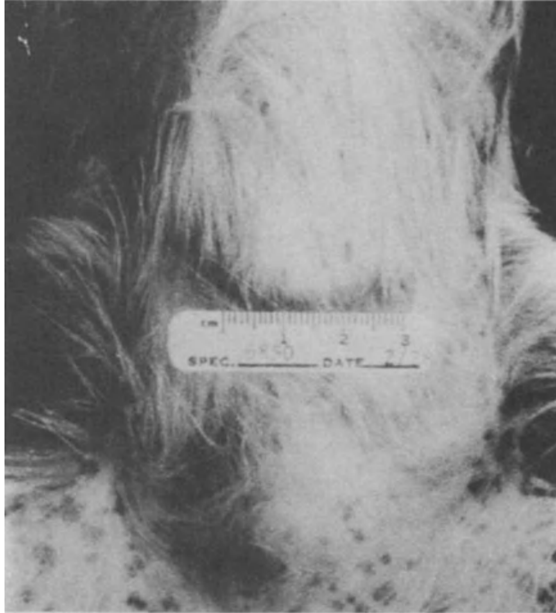

Fig. 2. Severe macular rash on the skin of a *C. aethiops* experimentally infected with Delta herpesvirus. (Reprinted with permission from ref. 37)

from macules (2-4 mm) to shallow vesicles to light encrustations and individual lesions often times coalesce to form larger lesions (21,22). Vesicles contain clear serous or cloudy fluid and upon rupturing leave a brownish-red crust. Oral ulcerations and facial edema were reported concomitant with the rash in some cases. Most deaths occur within 72 hours after appearance of the varicelliform eruptions (6,7,21). Morbidity and case fatality rates of 93% and 58%, respectively, were reported in an outbreak of the disease in patas monkeys (22), while respective figures in macaques tended to be generally lower (5).

PATHOLOGY

Although severity and distribution of the lesions varied, pathological changes were essentially similar in all reported cases of SVV infections in monkeys. Characteristic lesions included a generalized epidermal rash, necrosis and hemorrhage of various visceral organs, and characteristic intranuclear eosinophilic inclusions in a variety of cells (6,7,19,20,22,23).

The rash consisted of focal epidermal hyperplasia and vesiculation, with intranuclear inclusions in cells in the hyperplastic areas and margins of vesicles. Mucosal ulceration and hemorrhage are variable and can occur at all levels of the digestive tract from mouth to colon. In fatal cases, multifocal necrosis and hemorrhage were reported in most internal organs including the lungs, liver, urinary bladder, adrenal cortex, spleen, pancreas, lymph nodes and gonads. Generalized vascular involvement was reported in most cases and is believed to be the underlying cause of the generalized hemorrhage (10). Vascular changes, including necrosis with intranuclear inclusions in endothelial and smooth muscle cells of blood vessels were reported in various organs but particularly in the lungs (10,19,22)

Affected livers were described grossly as being coursely granular, friable and mottled with hemorrhage, while pulmonary lesions consisted of reddened firm raised areas which varied in distribution from focal involvement to the entire lobe surface. Microscopically, multifocal coagulative necrosis and hemorrhage with minimal inflammatory cell infiltration were reported in livers, with intranuclear inclusions in hepatocytes at the periphery of lesions and occasionally in Kupffer cells. Respiratory tract changes included necrosis of the bronchial epithelium and large areas of necrosis and hemorrhage, fibrin and neutrophil and macrophage infiltration in bronchiolar and alveolar areas. Intranuclear inclusions were noted in epithelial and endothelial cells in the affected areas (19).

EPIZOOTIOLOGY

The source for SVV outbreaks in primate colonies has not been identified. As noted above, SVV causes a much more severe infection in _C. aethiops_ and _E. patas_ than it does in macaques. Furthermore, it has been found that macaques living in the wild have antibodies to SVV (5). This raises the possibility that macaques might serve as a reservoir for SVV; a hypothesis supported by the observation that in several outbreaks of SVV, _C. aethiops_ and _E. patas_ species had been exposed to macaques shortly before the

onset of disease. For example, in the SVV outbreak at the Bowman Gray School of Medicine, newly arrived African green monkeys broke out with the disease shortly after they had shared recirculated air with a colony of macaques housed in a nearby room (21). And in a SVV epizootic at the Delta Regional Primate Research Center, all of the afflicted patas monkeys had been housed in animal quarters together with macaque species. It is known that macaques seroconvert following exposure to SVV (8) but the frequency of latent infections in survivors of epizootics is unknown. However, reactivation of latent SVV infection in monkeys is thought to occur and in at least one outbreak in an established colony of *E. patas* at the Delta Regional Primate Research Center, the source of the infection was believed to have been the result of reactivation of a latent infection (22). In that particular outbreak, all animals had been housed in an isolation unit for at least 6 months with two animals which had been infected with the virus in an epizootic 4 years previously. If it is indeed proven that macaques are the reservoir for outbreaks of SVV in primate colonies, serologically testing of macaques may aid in identifying individual macaques that have the potential to infect other species.

In the many laboratories involved in the isolation and characterization of SVV, many professional and staff personnel have come in contact with infected animals and/or their tissues. However, there have been no reports of transmission of SVV to humans. Even though there is no indication that SVV is infectious for humans, one should nevertheless take the same precautions in handling SVV as one would any infectious agent.

IMMUNE RESPONSES TO SIMIAN VARICELLA VIRUS INFECTION

Cell-mediated responses to SVV have not been investigated. However, both RIA and ELISA have been very useful in determining the kinetics of virus-specific IgG and IgM responses to SVV in experimentally infected monkeys (24-26). The findings of Anchilli *et al.* are typical (24). In these studies, IgM antibodies were detected as early as 8 days after SVV inoculation. Peak titers of IgM were reached 12 to 13 days later and remained stable for about 7 days. At this time, IgM antibody titers started to drop until they were no longer detectable 40 days after infection. IgG antibody on the other hand, did not appear until five days after the appearance of IgM. Levels of IgG antibody reached a plateau about 20 days after infection where it remained stable for approximately 2 months before

slowly declining to non-detectable levels (see Fig. 3).

The role of antibody in host resistance to SVV infection is not known. However, it has been observed that passively administered zoster immune globulin can modify the severity of VZV infections in children with cancer (27). Whether this occurs by virus neutralization or by some other mechanism such as antibody-dependent cellular cytotoxicity is not clear. However, these results suggest that the anti-viral antibody which appears in SVV infected animals is playing an important role in containing the infection.

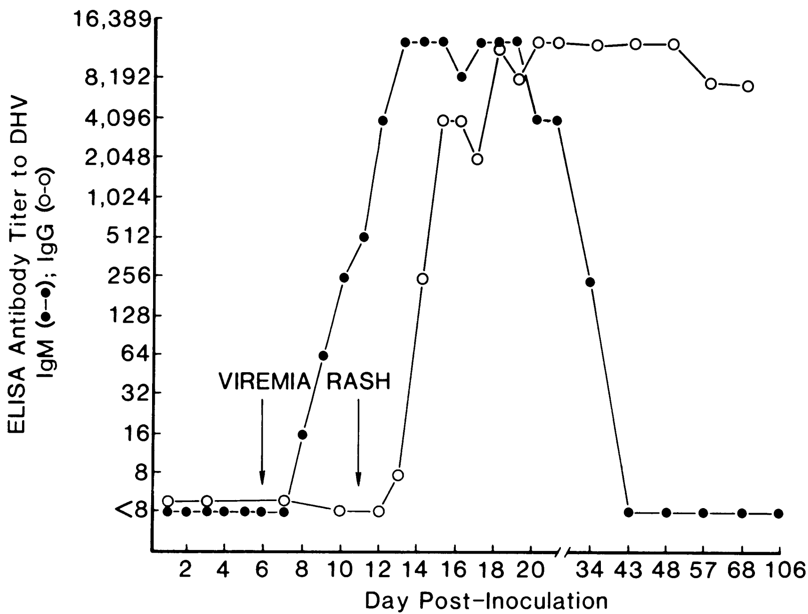

Fig. 3. Time course of IgM and IgG production following experimental DHV infection in the patas monkey. Double indirect and indirect ELISA tests were used to measure IgM and IgG antibody to DHV, respectively. (Reprinted with permission from ref. 25).

SIMIAN VARICELLA VIRUS AS A MODEL FOR VARICELLA ZOSTER VIRUS INFECTION

Animal models are commonly used in medical research to investigate the pathogenesis of viral diseases. Unfortunately, studies on the pathogenesis of VZV in humans have been hampered by the lack of satisfactory animal models. Myers et al. were able to establish an experimental infection of weanling guinea pigs using VZV adapted for growth in fetal guinea pig cultures (28). Guinea pigs inoculated intranasally or subcutaneously shed virus from the nasopharynx and viremia was detected in some animals. In addition, animal to animal transmission of VZV and production of a specific antibody response to VZV was reported. However, no varicella-like clinical illness such as vesicles or exanthema were observed in infected animals. Therefore, this model may be of limited value for study of the pathogenesis of human VZV infection.

The large degree of homology between the genomes of SVV and VZV and the clinical similarities between simian and human varicella infections suggest that SVV infection of non-human primates can be used as a model for human VZV infections. In fact, it has been found that SVV infection of non-human primates can be used to model both the mild type of VZV infection which is commonly associated with chickenpox in young children and the more severe disseminating type of VZV infection commonly seen in immunocompromised individuals. Whether a particular SVV infection resembles chickenpox or disseminating varicella depends upon which species of monkey is used in an individual experiment. In general, SVV infection of cynomolgus monkeys and African green monkeys are benign and involve only the skin, liver and a few lymphnodes (5,22). Thus, these species can be used as a model to investigate the pathogenesis and immunology of chickenpox. Infections of patas monkeys however, often leads to severe complications including the development of pneumonia and encephalitis (29). Thus, patas monkeys are better used to study the pathogenesis and host responses to the progressive form of varicella often seen in adults and immunosuppressed patients. Experimental models of SVV infection have recently been used to study the efficacy of antiviral agents in varicella infections. Acyclovir, bromovinyl deoxyuridine, phosphonoacetic acid, AraT, AraU and interferon have all been shown to be effective in promoting recovery from SVV infections (30-35). Recently, a live-virus vaccine has been developed in Japan (36). Since VZV can be used to immunize patas monkeys against SVV infection, SVV infection of non-human primates might be useful in the testing of VZV vaccines.

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10

HERPESVIRUS INFECTIONS OF EQUINE ANIMALS

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ABSTRACT

The clinical signs, significance and relevance of EHV infections are reviewed, and the research of recent developments on their molecular biology are presented. Data on restriction enzyme analysis and blot hybridization support the separation of EHV-4 from EHV-1. Both viruses share common cross-reacting glycoproteins significant for diagnostics and treatment. Molecular biological data show clearly that EHV-1 can cross the species barrier, causing disease in ruminants. Despite considerable DNA heterogeneities amongst EHV-2 strains, hybridization studies with various strains showed a high degree of homology. Application of rapid screening methods for the differentiation of field isolates and suggestions on vaccination programs are discussed briefly.

INTRODUCTION

Horses are domestic animals with great economic importance and of ethical value for man. They carry a variety of viral pathogens, but only a few (especially alpha-viruses) can be hazardous for man, and cause zoonoses. Besides the influenza viruses the species-specific herpesviruses are still the most important and critical agents influencing performance and health of racing and riding horses, as well as animals used for experimental purposes and vaccine production. This applies also to horses in the wild and the historically old races kept in zoos. Based on recent work which covers different aspects and types of equine herpesviruses (1,14,39)

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this review summarizes new data on molecular epizootiology, immunogenic components, molecular structure of the genomes as well as diagnosis and prevention.

DEFINITION AND SYNONYMS

The viruses are going to be classified into four distinct types: equine herpesvirus 1 (EHV-1), also known as equine abortion virus (EAV). This virus represents the former subtype 1 of EHV-1 (45). EHV-2 is the equine cytomegalovirus (ECM) (46). EHV-3 is known as equine coital exanthema (ECE) virus (36a,43). The recently separated EHV-4 stands for the rhinopneumonitis virus which was previously named subtype 2 of EHV-1 (2,15).

HISTORICAL BACKGROUND

Virus-induced abortion of the fetus was first described and reproduced in the horse by Dimmock and Edwards (19). From then on the disease became known in different parts of the world (49,53). A respiratory syndrome associated with EHV-1 was studied by Doll et al. (24,25). Based on the pathology in respiratory tissues, the virus was named rhinopneumonitis virus. Due to neurological syndromes the epidemiology of EHV-1 infections became more complex (6,13,15,20,49,57). Although it was supposed that pregnancy is a prerequisite for neurological disease (31,32,35), other research groups demonstrated that neurological complications occurred also in non-pregnant mares, stallions, geldings and foals (20,57,58).

EHV-2 was recognized to be clearly different from the other types and its role in disease conditions was and is poorly understood, since this virus could be isolated from diseased and healthy animals as well as from normal tissue culture probes (5,33,53,56). Because of slow growth, the cell

association and ability to form intranuclear inclusions, this group of agents was called equine cytomegaloviruses (62).

The equine coital exanthema virus (ECE virus), typed EHV-3, was isolated and characterized in only four countries: in Germany by Petzoldt (42) and Thein (57a), in the USA by Bryans (8) and by Ludwig et al. (36a), in Canada by Girard et al. (27) and in Australia by Pascoe et al.(41). The problems associated with the few EHV-3 cases most probably contribute to the lower incidence of the virus.

The respiratory isolates of the EHV-1 group were recently separated and form the EHV-4 group. Shimizu et al. (50) were the first to report antigenic differences between EHV-1 strains, suggesting two subtypes. The differentiation of such isolates was supported by comparing European strains with the Kentucky D strains (37). DNA analysis showing 20% genetic homology between the two previous subtypes of EHV-1 now justifies classifying EHV-4 as another type (2,15,55).

CLINICAL SYMPTOMS

Infection of horses with EHV-1 is associated with clinically distinguishable entities: 1) respiratory disease, 2) abortion and perinatal disease and 3) the neurological syndrome.

Respiratory disease

Acute EHV-1 respiratory disease occurs mainly in foals, weanlings and yearlings and is characterized by fever, anorexia and profuse serous nasal discharge, which later becomes mucopurulent. Extensive necrosis of the epithelial cells of the upper respiratory tract, especially within the nasal cavity, is accompanied by an acute inflammatory response. Virus may reach

the lungs, and especially in young horses causes bronchopneumonia with damage from secondary bacterial infections (53). In horses with antibodies to EHV-1 a milder infection is common.

Abortion and perinatal disease

Pregnant mares may abort 14 to 120 days after exposure without any clinical signs of disease (22). Most mares abort at 6-11 months of gestation. EHV-1 has rarely been isolated from natural and experimental infection of the genital tract (43,59). The condition is milder than that caused by EHV-3, and unlike EHV-3 it does not involve the perineal skin.

Birth of weak and dying foals in conjunction with EHV-1 abortion has been reported (38). More recently a neonatal foal disease associated with perinatal infection by EHV-1 without concurrent abortion or respiratory disease was described (21). The syndrome involved still births, the birth of weak, depressed foals that died within 24 hours and foals apparently normal at birth, which developed severe respiratory distress within 18-24 hours and died within 24-72 hours after birth.

Encephalomyelitis

Natural outbreaks of EHV-1 induced nervous disease were reported both in association with abortion or respiratory disease and without concurrent abortion or respiratory disease (15,31,33a,36b,57). The clinical signs vary from mild ataxia to complete recumbancy with fore- and hindlimb paralysis. An incubation period of about 7 days was recorded for both natural and experimental cases (28,32).

EHV-2

Clinical significance of EHV-2 is not known with certainty, since viruses have been isolated from apparently healthy as well as clinically ill horses (5,53), e.g. acute respiratory disease and keratoconjunctivitis have

been associated with this virus (53,56). The agent is widely spread in young animals and infection occurs probably by inhalation of contagious material coming from the respiratory tract of other horses. The high incidence of infection is associated with long-lasting virus persistence and continuous viral shedding.

EHV-3

ECE virus is the primary causal agent of equine coital exanthema. After an incubation period of a week or less, early vesicular or pustular lesions are formed on the penis and preputial mucosa or in the vulva and perineum. When uncomplicated by secondary bacterial invaders, eroded areas usually resolve within a fortnight, followed by local depigmentation. Infection does not impair subsequent fertility. Besides the lesion in the genital tract, lesions may appear on the conjunctiva as well as on the lips, and external nares and nasal mucosa (34).

EHV-4

Members of this virus group (previously subtype 2 of EHV-1) are associated with respiratory syndromes mentioned for EHV-1, although the infections are usually milder and restricted to the upper respiratory tract. The infection is not invasive, not accompanied by systemic disease and does not cause viremia (1). Experimental infection of pregnant mares did not cause abortion. Based on DNA restriction analysis, apart from a single isolation (4) obtained from an aborted fetus and a most interesting isolate recovered from the brain of a horse with encephalomyelitis (57b) this virus is mostly a respiratory isolate from mild disease.

SEROLOGY

EHV-1 is immunologically distinct from EHV-2, EHV-3 and EHV-4 (14,27,40,44). Serologically the different strains of EHV-1 are in general

uniform as based on neutralization tests, although some respiratory isolates of EHV-1 (EHV-1 DNA fingerprint type) of lower virulence (non-abortigenic) have slightly different neutralization properties; e.g. the strains NM-3 and SL-D (7,9,14). The serological separation of EHV-1 and -4 was proposed by Shimizu et al. (50) because there was some evidence that the two subtypes were neutralized with different strength. A lot of uncertainties arose when horse sera were used for differentiation. Our conclusion is that monospecific rabbit sera clearly show the subtype specificity in support of the new nomenclature and separation of the viruses into EHV-1 and -4 (14,15).

Another group of isolates classified as EHV-2 had no cross-neutralization with any of the equine herpesviruses. Among themselves they are very heterogenous as based on neutralization: in two instances the serological differences initiated discussions to divide them into different antigenic types (30,33).

The few EHV-3 isolates from different countries are serologically closely related but seem to differ from a donkey coital exanthema virus (39).

In agreement with our present knowledge on the molecular composition of equine herpesviruses, other serological test methods (ELISA, fluorescence antibody test, complement fixation test) detect major group-specific antigens which most probably are located on the nucleocapsid or in the core (39).

GENOME STRUCTURES

The genome of the equine herpesviruses consists of the covalently linked L and S segments. The unique short segment (U_S) is flanked by

inversely oriented repetitive sequences allowing for two isomeric forms. Viruses with this kind of structure have been grouped as D-type herpesviruses, e.g. EHVs, BHV-1, PsR virus, VZV (47). As shown in Fig. 1, EHV-1, -3 and -4 have this kind of genome structure (48,60,61). Electron microscopic studies with the EHV-2 DNA indicate that the genome exists in more than one isomeric form (39). Recent studies on the sequence of the left genomic terminus in EHV-1 showed that tandemly repeated sequences of about 450 bp are flanked by inversely oriented identical 100 bp long sequences (14). These flip-flop structures are so far unknown for other herpesviruses, but an AT rich sequence which follows a palindrome structure is found to be incorporated into the 100 bp region. Similar findings have been reported for BHV-1 (29) and VZV (18). These consensus sequences in three D-type herpesviruses might play a major functional role in replication.

The genetic relationship of EHV-1 and -4 covers a total of 20% of their genomes (2,14), whereas EHV-1 and -3 are only 10% homologous. Similar studies revealed that EHV-2 shares only little homology with other members of EHV (1-3%) (2,14,51).

Based on the DNA restriction profile the equine herpesviruses show a considerable amount of variability which is by far more prominent in EHV-2 than in EHV-1, -3 or -4 and on the other hand, more obvious in EHV-4 strains than in EHV-1 strains (4,14,39).

Using the fingerprinting technique EHV-1 isolates from abortions and neurological disease could not be differentiated (15) although intra-strain variations were evident.

The variability in EHV-1 is found to be located at the genome termini and the junction (U_L/I_R) fragment (16). Analysis of the DNA of vaccine strains revealed the absence of certain fragments present in wild-type

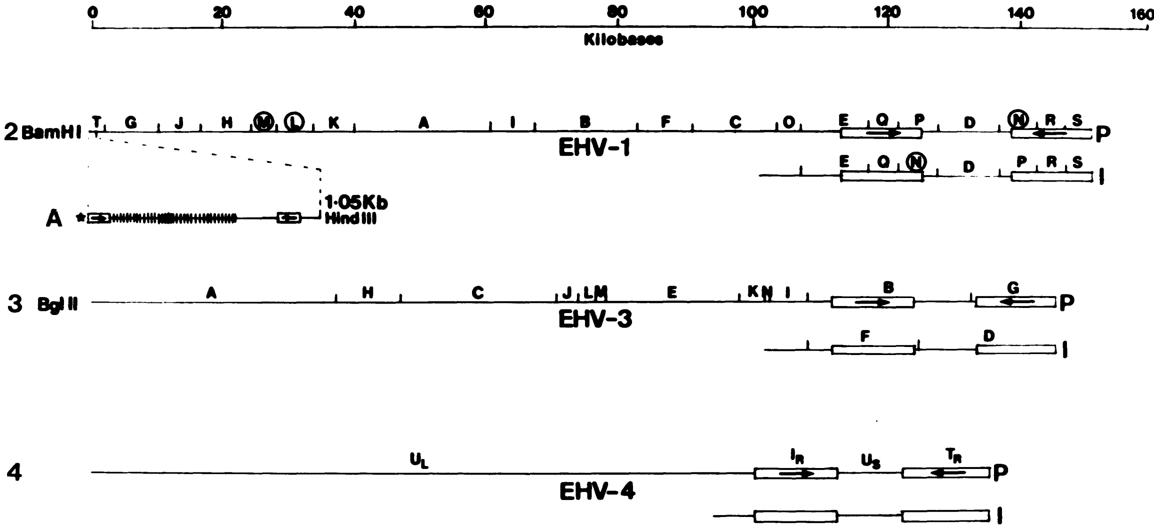

Fig. 1: Genomic arrangement and physical map of EHV-1, EHV-3 and EHV-4:
Line 2, Bam HI restriction map (60). Changes in the vaccine strain Prevaccinol and respiratory isolates NM3 and SL-D are marked by enclosed letters. 2.A, represents the flip flop structure in the left terminal fragment (as revealed by analysis of the sequence from a plasmid clone containing the BamHI (*), Hind III subfragment. * represents the modified end by the BamHI linker. Slashed lines are tandemly repeated sequences.The boxes with arrows represent inversely repeated sequences (100 bp). Line 3: BglII restriction map of EHV-3 (48); Line 4: Genomic arrangement of EHV-4 DNA drawn from the abstract presented at the 10th Herpesvirus Workshop 1985 in Ann Arbor, USA, by Whalley et al., (61).

viruses (15). It is of profound interest that two respiratory isolates of reduced virulence (e.g. failure to cause abortion after experimental infection) and having fingerprints of EHV-1, lack identical bands also absent in the vaccine strain Prevaccinol. (Fig. 1) (15).

Despite the heterogeneity of the EHV-2 isolates in their DNA restriction profiles, they show an almost total homology in DNA hybridization (Fig. 2) (14).

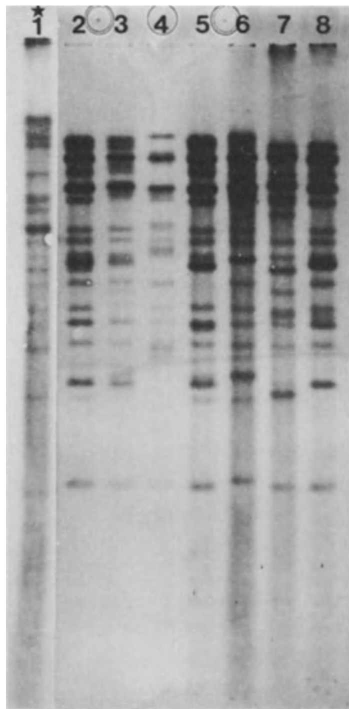

Fig. 2: Autoradiograph of the Southern blot showing BamHI restriction profiles of different EHV-2 isolates after hybridzation with a labelled EHV-2 DNA probe. Lanes 1 & 6 are respiratory isolates: strain stanion (15) and strain LK (46), respectively. Lanes 2 & 4, strains T16 and T366 isolated from cases of conjunctivitis. Lanes 3, 5, 7 & 8 are strain T432, strain NHV (testicle of a stallion), strain Karpas (33) and strain cytomegalo (30), respectively. The whole virus DNA probe (lane 1, strain stanion) is radioactively labelled and marked with a star.

ANTIGENS AND PROTEINS

Structural proteins of EHV-1, -2, -3 and -4 have been characterized by PAGE (1,3,12,39). In respect to strain variation, immunity and protection the glycoproteins in the envelope play the major role. Out of 28-30 structural proteins reported for EHV-1 and -4 at least 12-14 glycoproteins (25-260 K) exist in EHV-1 and EHV-4. Of these, in the case of EHV-1 8 glycoproteins were found to be major ones (approx. mol. w. 240 K, 190 K, 140 K, 120 K, 110 K, 96 K, 63 K and 45 K) and four to six were minor glycoproteins (260 K, 90 K, 74 K, 61 K, 38 K) (1,14). Interestingly enough, at least 8-9 glycoproteins with molecular weights of 240 K, 190 K, 140 K, 120 K, 96 K, 61 K, 41 K, 38 K and an additional protein of 33 K were also present with slight mobility differences in case of EHV-4. From the preliminary data with immunoblotting studies using immune rabbit serum the proteins 240 K, 140 K, 120 K, 90 K, 74 K, 41 K and 38 K were found to be common immunogenic components in these viruses. Besides the proteins 90 K and 74 K, which are cross-precipitated by heterologous sera of EHV-1 and -4, the proteins 140 K, 41 K and 38 K are the prominent ones which react immunologically (14).

In the case of EHV-2 at least 7 glycoproteins have been reported, out of which three (83 K, 78 K, 73.5 K) and four (111 K, 68 K, 61 K, 41 K) represent major or minor proteins, respectively (12). EHV-4 and EHV-3 share approximately 20% and 10% DNA homology with EHV-1, respectively. It is likely that several viral proteins are closely related in these species. This has already been established for EHV-1 and EHV-4 (1, 14). However, except for the major capsid protein, no reports are available for EHV-1 and -3. In contrast the protein profiles of EHV-2 vary extensively as compared to other members of the group.This is also in accordance with DNA homology studies (e.g. less than 3% homology with other EHVs) (51).

RELEVANCE OF EHV INFECTIONS

Of the four types, EHV-1 infections are most important with regard to clinical disease and other parameters. This virus is responsible for "abortion storms", for neurological complications and death of foals. Based on neutralization tests, DNA restriction profiles and blot hybridization studies (Fig. 3) we have recently found that EHV-1 crosses the species barrier to bovine animals. Isolates from non-equine hosts show clear markers in their genomes indicating that the adaptation in other hosts is able to alter some viral DNA restriction sites (17). The serological relationship of EHV-1 and -4 leads to vagaries in epidemiological situations. There is an indication that the horse populations, which have been experiencing EHV-4 infection show less severity in symptoms and fewer abortions; this points to a kind of protective effect. Although EHV-4 cannot easily be isolated, its importance in death of foals and in respiratory diseases of racing horses has always been postulated. The significance of EHV-2 infections is often uncertain. This virus seems to be an opportunistic agent and is widely spread already in young animals. Therefore a synergistic effect with other virus and/or bacterial infections may be assumed. The equine coital exanthema virus is certainly of less importance.

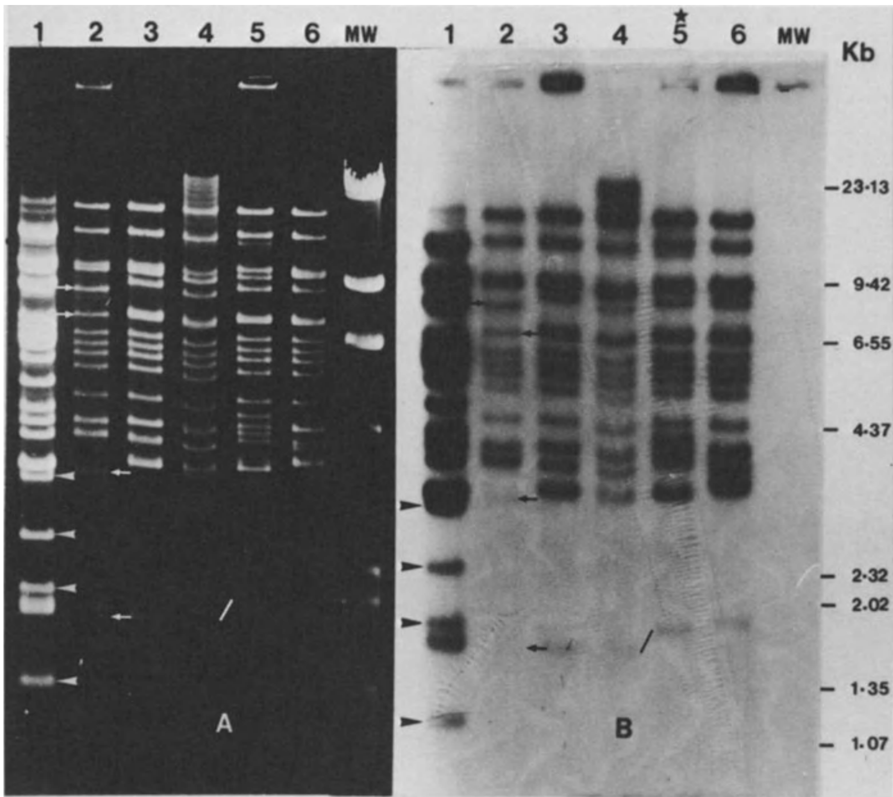

Fig. 3:

A: BamHI restriction profiles of the DNA of different EHV-1 isolates from non-equine hosts (ruminants). Lane 1, strain Ro-1 (isolated from the brain of an antilope) (15); Lane 2, 3 & 4 are bovine fetal isolates from abortions: the strains 136/B, ERV/B_1 and VD-122, respectively. Lanes 5 & 6 are reference strains, Aust. IV and Army 183 (15).

B: Autoradiograph of the Southern blot from the same gel (A) after hybridization with a ^{35}S labelled whole EHV-1 DNA probe (marked with a star). Extra bands present in case of strain Ro-1 and the absence or the mobility differences of different fragments in respective isolates are marked.

ONCOGENECITY AND LATENCY OF EHVs

Recently, it has been shown that three equine herpesviruses EHV-1, EHV-2 and EHV-3 have oncogenic potential (39), however, no reports concerning EHV-4 exist. EHV-1 transformed and tumor cells have a specific viral DNA fragment integrated within the host DNA (39). Cell cultures infected with ECMV (EHV-2) at high multiplicity may establish persistent infection and oncogenic transformation. "Dot hybridization assays" confirmed the presence of virus-specific DNA sequences in such cell cultures and in the animal tumor tissues (39). Cell lines harbouring virus specific DNA sequences have been shown to express EHV-3 specific proteins by indirect immunofluorescence tests. EHV-1 and EHV-3 transformed cells express one or more proteins that react with antisera to HSV-1 proteins encoded by specific DNA sequences and known to represent the transforming region (39). DNA sequences harboured in case of equine herpesviruses particularly in case of EHV-1 have been shown to map in a similar region of the genome (approx. 0.32-0.38) (39). Furthermore, recent hybridization studies in our laboratory revealed that map units approx. 0.3-0.45 are colinear in the genomes of EHV-1, BHV-1 and PsR virus (14). The conservation of the transforming gene sheds an interesting light on the evolutionary relationship of these viruses.

The role of latency in epizootiology of EHV-1 and EHV-4 infections has recently been reviewed (1). No concrete data on EHV latency is known so far. Infectious EHV-1 can only be isolated after cocultivation of intact buffy coat cells with virus susceptible cells.

In view of the ability of other herpesviruses, both of man and animals, to establish latency in white blood cells (47), it is prudent to believe that latent EHV-1 is harboured and persists in leukocytes. A similar situation could be true for EHV-2 as it is often isolated from buffy coat cells and

leukocytes. On the other hand, apart from a single isolation (out of 22 attempts) of a EHV-4 strain (previous subtype 2 of EHV-1) by co-cultivation of trigeminal ganglion tissue no further evidence for the existence of EHV-4 latency has been reported. Isolation of EHV-4 from the brain tissue of a horse which had succumbed after an encephalitis (Thein, personal communication) may actually represent reactivated latent virus.

DIAGNOSTICS, PREVENTION AND IMMUNIZATION

In general the isolation of equine herpesviruses is mostly bound to equine cells, although EHV-1 has a broader cell spectrum. EHV-2, -3 and -4 with some exceptions depend solely on equine cells for their replication. In EHV-1 infections the virus can be recovered from nasal swabs and from organs or from brain which would correlate with a systemic spreading. EHV-4 is mainly located in the respiratory tract, which is also true for EHV-2. The latter virus has often be found as a contaminant in equine cell lines and can be isolated from buffy coat and leukocytes. Diagnostic procedures and characterization of equine herpesviruses have improved considerably by the application of DNA fingerprinting, which avoids the uncertainties of serological methods. This method in combination with Southern blot hybridization can even be used for rapid and efficient screening of large numbers of field isolates (16).

Equine herpesvirus infections have not been eradicated due to the latency of these viruses. Preventive methods are concerned with hygienic management and treatment, with isolation of the infected animal groups and separating them into quarantine. Vaccination is mainly considered as a preventive tool. Several types of vaccines have been used, but the live attenuated vaccines are not free from complications. Reports show that

EHV-1 vaccines led to abortion and even to paralysis in horses (53). There is evidence that a certain DNA fingerprint type which has become prominent in larger horse populations during the last few years in the USA can be associated with fingerprints of vaccine strains (4). This complicates vaccination programs with the live vaccines and needs further evaluation and safer vaccines. Progress in mapping the major immunogenic components of all these equine herpesviruses, preferentially of EHV-1 and -4 will certainly lead to recombinant vaccines. EHV-2 may be a good vector for incorporation of genetic material, since this virus is the most ubiquitous one among the four types.

Until an effective recombinant vaccine has been developed and proved to be protective, a possible suggestion would be to use inactivated EHV-1 vaccines combined with live attenuated EHV-4, since the latter virus is less pathogenic and is known to induce some cross-protection.

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11

HERPESVIRUS INFECTIONS OF BOVIDAE

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ABSTRACT

Herpesviruses of bovine animals restrict the economy of animal-breeding. Some of them cause severe diseases like infectious bovine rhinotracheitis/infectious pustular vulvovaginitis and malignant catarrharal fever, some are involved in immunodepression, and all of them are able to establish latency. Recent molecular biological findings may pave the way to a better understanding of the pathogenesis of bovine herpesvirus infections and lead to effective eradication programs.

INTRODUCTION

The importance of virus infections of captive animals lies mainly in their hazardousness for larger animal populations and in some cases for man. Virus infections of bovidae - this family includes a variety of domestic animals - are of interest because of economic reasons. Therefore all infections influencing their meat, food and wool production can be of considerable relevance. Herpesvirus infections of bovidae are known to cause great financial losses and may have consequences on human nutrition.

Based on our present knowledge one can expect that each subfamily of bovidae has its indigenous herpesvirus. Most information exists about the bovine and the caprine herpesviruses due to their economic importance

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(1-3). This short review covers herpesvirus infections of bovidae emphasizing recent progress in the molecular biological characterization of those agents.

CLASSIFICATION

Only a few of the numerous herpesviruses isolated from bovidae, including pseudorabies virus (4), equine herpesvirus 1 (5) etc. have been characterized and classified:

- bovine herpesvirus 1 (BHV-1) is the causative agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (IBR/IPV).
- BHV-2 comprises strains of bovine herpesmammillitis, but also viruses responsible for Allerton disease.
- BHV-3 represents viruses causing the African malignant catarrhal fever. Another suggested name for this virus is alcephaline herpesvirus 1.
- BHV-4 covers a group of isolates which represent the "Movar-type" herpesviruses (6-8).
- The classification of BHV-5 is uncertain. This virus is definitely not the causative agent of "Jaagsiekte".
- The goat herpesvirus has been named BHV-6. Since this virus is indigenous in goats, it may be reclassified as caprine herpesvirus in the near future.

DISEASES AND LATENT INFECTIONS

BHV-1 is known to be the causative agent of several clinical syndromes in cattle. The most prominent ones are IBR and IPV. Distinct strains are regarded to be responsible for the different clinical entities, most of them

can be differentiated according to the DNA restriction enzyme patterns (9). The strains of the IBR-type mainly cause infections of the upper respiratory tract, which are sometimes associated with febrile systemic infections. Often co-infections with other viruses or secondary bacterial infections complicate the clinical picture (10). Less frequently, conjunctivitis, orchitis, endometritis, enteritis and mastitis have been reported. The virus is also known to be abortigenic. The IBR-type of BHV-1 has shown to be latently present in normal fetuses.

The IPV-like strains clearly differ in their organ tropism and can be separated by DNA analysis. They are responsible for recurrent inflammations of the mucous membranes of the vulva and vagina or the preputium. None of the IPV-like strains could be associated with abortions.

Recently another group of BHV-1 viruses, responsible for meningo-encephalitis in calves, has been described (11).

BHV-2 has mainly been isolated from two well described clinical entities. Firstly, the classical herpesmammillitis which is mainly observed in European countries (e. g. Scotland, Eire) and the USA. This form is characterized by ulcerative alterations at the teats accompanied by swelling and severe oedema (12). Sometimes ulcera in the vulvovaginal mucosa and the mouth were reported (13). The second form, a generalized infection of the skin, has been reported from cattle in Africa, but is also known in the USA. Skin nodules, which undergo necrosis, are prominent. Since only superficial layers of the epidermis are affected, the lesions usually heal within a few weeks without scar formation.

The causative agent of African malignant catarrhal fever has been classified as BHV-3. Under natural conditions this virus is present in alcephaline animals, suggesting the name: alcephaline herpesvirus 1. In Africa it is responsible for severe infections in zebous and cattle. These agents may be different from the virus involved in the clinical entity known as "bösartiges Katarrhalfieber" in Europe. This disease, described first by Goetze and Liess (14), can be observed sporadically in cattle that were in contact with sheep or buffaloes. Infected animals show a febrile systemic infection characterized by mucosal lesions mainly at the head and in the intestine, severe rhinitis and diarrhea accompanied by central nervous system disorders. The disease is always fatal.

In European countries another virus has several times been isolated from cases of "bösartiges Katarrhalfieber" (15). This virus is now grouped to BHV-4.

It remains to be clarified whether BHV-4 is responsible for any disease in cattle. Only a few isolates are able to induce mild febrile infections with conjunctivitis and tracheitis when administered experimentally. Others, which have been isolated from aborted fetuses, were associated with metritis or could be isolated from cases of "bösartiges Katarrhalfieber" (15,16).

Recent studies, however, suggest that BHV-4 might be an opportunistic virus, which can induce immunosuppression, rather than the causative agent of defined clinical entities (6,16,17).

The caprine herpesvirus 1, also named BHV-6, was isolated from goats (mainly young animals) which showed conjunctivitis and rhinitis.

Severely affected goat kids died of the disease. Pathological exploration revealed ulcerations and necroses in the mucosae of their gastro-intestinal tracts (18).

It is generally accepted that bovine herpesviruses like other herpesviruses are able to establish latency after primary infection. Latently persisting viruses can be reactivated to recurrencies by immunodepression and other stimuli (19- 22).

The location of latent virus in general is still unknown, but for BHV-1 predominatly the cervical and sacral ganglia have been discussed as the site of latency. Other reports claim that the virus may be latent in lymphocytes (23,24). The ability of these viruses to hide in a latent stage promotes the wide spread of BHV-1, BHV-4 and BHV-6 and, in particular, protects the viruses from eradication. It is of special interest that BHV-6 appears to be latent in a high percentage of animals in countries where goats are a major economic factor (25).

GENETIC MATERIAL, ANTIGENS AND PROTEINS

Despite identical morphology and structural composition, considerable differences in genome organisation and antigenic properties exist among the various bovine herpesviruses.

According to its genome structure BHV-1 belongs to the D-type herpesviruses. Its DNA consists of a small segment (2.2×10^7), the unique short (U_S) region, which is bracketed by two inverted repeats, and a large segment (U_L) which is 6.6×10^7 in size (3, 26). Adjacent to the terminal

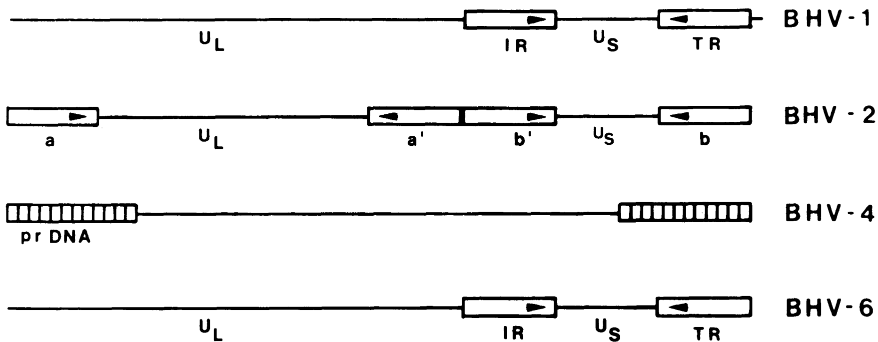

Fig. 1. Genome organisation of bovine herpesviruses
U_L: unique long; U_S: unique short; IR: internal repeat; TR: terminal repeat; pr DNA: polyrepetitive DNA.
(Redrawn with modifications from ref. 8,27,28,30)

repeat a short "DNA tail" has been found (27; Fig. 1). DNA fingerprinting allows the differentiation of IBR- and IPV-like virus strains as well as the separation of the encephalitic strains (10, 11).

The genome of BHV-2 represents a group E virus DNA similar to that of HSV. Single sets of DNA sequences are framed by reiterated inverted repeats at the termini and internally. The DNA of BHV-2 shows approximately 15% homology to the DNA of HSV and encodes at least one highly conserved glycoprotein homologous to glycoprotein gB of HSV. The molecular weight of the total DNA is 8.8×10^7 dal (28).

Only little information exists on the genetic material of BHV-3 , but its DNA can be differentiated from that of BHV-4 by restriction enzyme analysis (3). The genome arrangement has preliminarily been suggested to resemble that of other gamma-herpesvirinae (29).

The genome of BHV-4 has a size of 7.6 x 10^7 dal and according to its structure falls into the group of gamma-herpesvirinae. The genomic organisation clearly represents a group B herpesvirus with a single unique DNA segment framed by numerous reiterations at the termini. These terminal parts of the DNA contain polyrepetitive units of 1950-2750 bp (8).

The DNA of the caprine herpesvirus can clearly be differentiated from BHV-1 by restriction endonuclease analysis. The sequence arrangement of its genome is similar to that of BHV-1, falling also in the group D herpesviruses. BHV-6 is closely related to BHV-1 and shares approximately 60 - 80% DNA homology (30; S. I. Chowdhury, personal communication).

Usually herpesviruses specify 50-100 polpeptides; 30-50 of them represent structural proteins. About 5-15 are glycosylated and integrated in the viral envelope. These glycoproteins are most important inducers of immune defence mechanisms.

BHV-1 specifies at least 3 major glycoproteins (74 K, 91 K and 105 K; Fig. 2). Some of them are processed or dimerized (9,31). The 74 K protein induces the strongest neutralizing immune response and is considered to represent the major immunogenic component (9,32,33).

Purified BHV-2 contains 12-15 glycosylated proteins ranging from 25-150 K in size (34). A glycoprotein of 130 K is responsible for eliciting neutralizing antibodies. This protein shares common epitopes with glycoprotein B of HSV (35). Presently, no information exists on the polypetides of BHV-3 and BHV-4. Although antigens of BHV-4-infected

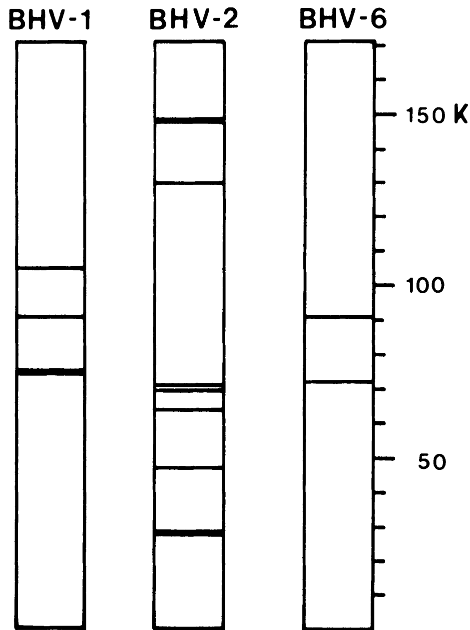

Fig. 2. Major glycoproteins of bovine herpesviruses
The scale gives the relative molecular weight, schematic view. (Redrawn from ref. 10, 31, 34, 36.)

cells could be differentiated by immunological techniques into nuclear bound antigens and others present in the whole cell (17). The polypeptides specified by goat herpesvirus (BHV-6) have not yet been reported. Several of its proteins, however, cross-react with BHV-1, and two are glycoproteins of 74 K and 91 K (36).

RELEVANCE OF INFECTION

BHV-1, -4 and -6 are known to be distributed worldwide, but BHV-2 and -3 are prevalent only in a few countries (BHV-2: Great Britain, North

America, Southern Africa; BHV-3: Africa, sporadic cases in the USA and Europe).

The most relevant virus is certainly BHV-1 because of its potential to cause overt disease with loss of animals. Furthermore it induces immunosuppression and thereby contributes to complex disease forms in cattle. Clinical outbreaks due to goat herpesvirus infections have only been reported from Switzerland and California. Our own studies revealed that the virus is widely spread in a latent stage in countries, where the goat is an economic factor for nutrition, like Greece, Turkey, North Africa etc. (25, 37).

African malignant catarrhal fever (BHV-3) is of certain relevance for cattle and zebous in endemically infected areas, because of the lethality of the disease.

The importance of BHV-2 appears to be a minor one, since only localized outbreaks are known, which may temporarily influence the milk production of infected animals.

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12

AUJESZKY'S DISEASE (PSEUDORABIES) IN LABORATORY AND CAPTIVE ANIMALS

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ABSTRACT

Aujeszky's disease (AD) is an economically important ailment of the pig. The etiological agent is a herpesvirus. Its primary host and reservoir is the pig, in which the disease is seldom fatal over a certain age, while it is a fatal disease in many animal species coming into direct or indirect contact with the pig. Under natural conditions AD rarely occurs in captive animals (foxes, minks, dogs in kennel and zoo animals) but never in laboratory animals. However, these species are susceptible to the virus by artificial infection. Therefore these species are used as an aid in the diagnosis, vaccine development and investigation of the biological properties of the virus.

INTRODUCTION

Aujeszky's disease virus (ADV) or pseudorabies virus is a member of the family Herpesviridae (1). It is an enveloped virus of 120-150 nm and it has a linear double-stranded DNA genome with molecular weight of 90×10^6 (2).

ADV causes an acute, most often nervous and fatal disease in domestic animals (3). The natural reservoir of the virus is the pig in which it behaves as a contagious disease, while all other species contract the virus from the pig, directly or indirectly. Indeed, as to the main characteristics of the disease in primary (pig) and secondary (other species) hosts, AD behaves as if it were two different infectious diseases.

Main features of AD in pigs. 1. Susceptibility to ADV is age dependent: it may cause up to 100% mortality in piglets under

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two weeks of age, showing signs of nervous symptoms (only paralytic). In growing and adult pigs the respiratory form is more common with a few nervous cases and with abortion in pregnant sows. Subclinical cases have also been described. 2. In pigs recovered from acute infection the virus can maintain an infection in the tonsils resulting in virus shedding and persistent infection in herds. 3. The virus may colonize ganglia (latent infection) from where it can be reactivated by stress, and virus is excreted again in the nasal secretions. 4. As a result of the above, apparently the pig is the only species where a continuous chain of animal-to-animal or herd-to-herd infection (spread) is maintained under natural conditions. 5. The source of virus is the pig, or meat (offal) derived from infected pigs.

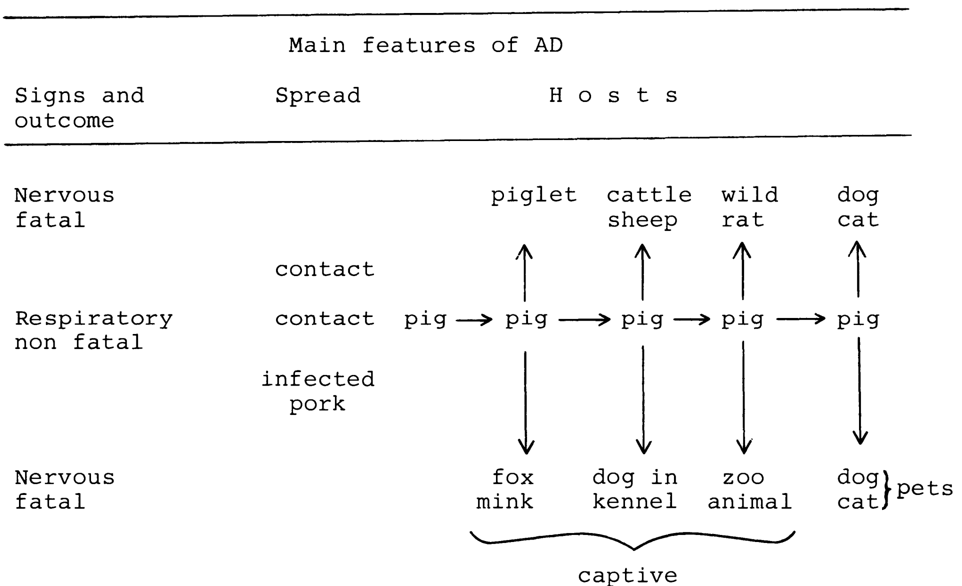

Fig.1. The "chain of being" of ADV in nature

Main features of AD in other species. 1. Under natural conditions ruminants and carnivores are highly susceptible, while rodents to a lesser degree. Clinical disease is always rapid (few days), accompanied by nervous signs (hyperaesthesis resulting in pruritus).

2. These species do not excrete virus upon infection thus - with exceptional cases - lateral spread (animal-to-animal or herd-to-herd) does not occur, therefore AD is not a contagious disease amongst these species. Mortality in a group of animals is related to the number of animals initially infected by pigs. 3. When acute disease has subsided in herds, surviving animals remain susceptible and harmless to each other concerning virus transmission. Latency does not develop in these species. 4. The main modes of infection in ruminants are airborne transmission or contact by oral and nasal secretions of pigs. Carnivores (pets or captive) and fur animals are infected with uncooked pork or abattoir offal (Fig.1).

AD does not occur in laboratory animals under natural conditions (that is unaided by man), though they are invariably susceptible to artificial (parenteral) infection. Accordingly, laboratory animals play a significant role in the diagnosis of AD, innocuity test of vaccines and investigation into the biological properties (e.g. virulence) of ADV strains. A particular form of artificial infection is by accidental infection of foreign hosts with not fully attenuated live pig vaccines.

NATURAL AD IN CAPTIVE ANIMALS

AD outbreaks may occur in mink, fox and nutria, in dogs kept in kennels and zoo animals.

AD in fur animals was first observed on a mink and a fox farm near Leningrad in 1956 (4). Similar cases were reported in Czechoslovakia (5) and in other countries where fur animals are kept on farms (6-10). Clinical signs were: lethargy, diarrhoea, abdominal pain, paralysis of mandibula, dispnoe, hyperaesthesis. Foxes showed intense pruritus of the mouth and head, while it is rare in the mink. Most of the foxes died in a week, while disease may last longer in minks. Mortality ranged from 50 to 80%. A typical post-mortem finding was lung oedema. After boiling pork, the disease ceased abruptly. Experiments suggest that lesions in the mouth are indispensable for oral infection (11).

Both live (12) and inactivated (13) vaccines are available for fur animals. Žuffa's attenuated strain is combined with butulism

vaccine (12).

Dogs kept in kennels can also be infected with pork containing ADV. Clinical signs: salivation, dispnoe, diarrhoea, hyperaesthesis, oedema of the head and pruritus (not always). Animals die in 2 to 3 days. Post mortem findings are variable, often with lung oedema. Sick animals do not respond to treatment (14-17).

There is one report on AD in a zoo; pumas fed cattle heads died (18).

Specific virus identification is necessary for diagnosis. Immunization of dogs is ineffective (19).

AD INFECTION DUE TO ACCIDENTS

AD was induced in sheep and lambs after inoculation with automatic syringe previously used for vaccinating pigs against AD (20-21). Virus survived in the syringe for 3 days. In a case reported from the USA, restriction enzyme pattern of the DNA of virus reisolated from dead lambs was identical with that of strain Norden that is used as a pig vaccine in the United States and is a derivative of strain Buk (21).

Another interesting case involves the killing of some ten thousand one-day-old chicks in Holland that were vaccinated intramuscularly (i.m.) with Marek's disease virus vaccine at the hatchery (22). Symptoms started in the rearing house at day 2 to 4 after delivery. They were lying on one side with one leg paralysed, stretched backward before dying shortly thereafter. They showed excitation upon touching. Virus was isolated from the brains of dead birds in eggs and identified as herpesvirus by electronmicroscope. On i.m. or subcutaneous (s.c.) inoculations the isolated virus produced the same symptoms in day-old chicks. Since no avian herpesvirus elicits similar clinical picture, AD was suspected. The virus was neutralized by ADV-specific serum and intranuclear inclusion bodies were found in neurocytes. The virus which contaminated the Marek's disease vaccine was most likely the Dutch AD vaccine for pigs that derived from strain Buk. This strain had been passaged some 650 times in chicken-embryo fibroblast culture resulting in both adaptation to this

species and retaining virulence to very young chicks.

SUSCEPTIBILITY OF LABORATORY ANIMALS TO DIFFERENT ADV STRAINS

Susceptibility to virulent strains.

Rabbit. In the early period of AD research when the means of exact titrations were not available, investigators observed that rabbits were the most sensitive to ADV (23). Therefore this animal was used most often for diagnosis. Up to the present many findings have attested that LD_{50} of virulent strains is <10 pfu (e.g. 24) or even equals 1 pfu (25).

Rat and mouse. The question of the susceptibility of these species has arisen because of the suggestion that they might transmit virus between swine herds. It has been shown, however, that rats are fairly resistant: successful peroral infection requires some 10^6 pfu and the presence of lesions of the mucosa of the mouth is a precondition. Rats or mice fed carcasses died of AD may contract the disease but they do not transmit infection through contact with diseased or dead animals (26). They are most susceptible to intranasal infection but even so 10^4 pfu is the required dose (27). Rats may die in a few hours showing no symptoms at all, or may display pruritus. In the light of the above findings it is not likely that the rat either acts as virus reservoir or plays a role in the herd-to-herd spread of AD.

Susceptibility of the adult mouse is similar to the rat. Young mice (less than one-week-old) are hundredfold more susceptible than older ones ($LD_{50}<100$ pfu on i.m. infection). Symptoms are similar to that of rats (28).

Chicken. There are some reports from the early period on the susceptibility of poultry (23). The first systematic study was carried out by Ivánovics at al. in 1954 (29). They found that chickens were susceptible up to two weeks of age on intracerebral (i.c.) and s.c. injection. However, the virus strain they used had undergone hundred passages in chicken cells thus it is hard to assess the role of adaptation played in the virulence of their strain for chickens. Further studies revealed that one-day-old chickens are highly susceptible to a virulent

field isolate by the i.c., i.m. and s.c. route of infection. LD_{50} is about 100 pfu by the i.c. infection, while it is 1000 pfu by i.m. or s.c. inoculation. Susceptibility greatly decreases with age: LD_{50} by i.c. infection increased about hundred-fold per week, while chicks older than 2 days were resistant to both i.m. and s.c. infection (30).

Susceptibility to attenuated ADV strains.

Interest in this problem can be attributed to the following: a/ When attenuated vaccines are prepared it is desirable to be able to assess the degree of attenuation of a strain; b/ It would be useful to find correlation between the susceptibility of any laboratory animal and that of the pig, to be able to use the former as a model in vaccine trials; c/ It was hoped that identification of vaccine strains would be possible by virulence test in laboratory animals (31,32). This has, however, been made obsolete by the restriction enzyme analysis of the DNA of ADV strains.

Ivánovics at al. (33) were the first to show that a high number of passages (over 300) of a virulent strain in chick-embryo cells caused a change in biological properties, namely occurrence of pruritus in mice decreased considerably.

Attenuated ADV strains are numerous and varied as to their origin: most of them (25, 34) were obtained by serial passages in chicken-embryo fibroblasts (CEF), by passages in the presence of a mutagene (35) and by the isolation of naturally occurring avirulent mutants (36).

Susceptibility of laboratory animals to some attenuated and vaccine strains are summarized in Table 1. The general conclusion can be drawn that there is a tendency of parallel decrease of virulence in the different species, including the pig.

Rabbit. Since the rabbit is regarded the most susceptible to parenteral inoculation this was the preferred species to check the degree of attenuation. Bartha was the first who showed that there was a correlation among the cytopathic form (rounding up), the small plaque in primary pig kidney cells and the biological properties in animals (36). His attenuated mutant

Table 1. Virulence of avirulent and vaccine strains of ADV in different species

Investigator Strain	Species				
	Pig	Sheep	Chick* (i.c.) LD_{50} (pfu)	Mouse	Rabbit
Suhaci et al (38)					
Buk 98	++		$<10^2$	+++	+++
Žuffa (12, 39)					
Buk-TK-200	-(p+)		$<10^2$		(+++)
Buk-TK-900/IV.	-(f-)	-	10^5	(++)*	(++)
Buk-TK-300/9,2**		-	$>10^5$	(+) *	-
Buk-TK-900/6,2**			$>10^5$	- *	
Škoda (25, 40)					
Buk-624	-(f+)	+++	10^3		(+++)
Norden	-	+++	10^3	(+++)	(+++)
PrV-X (46)			$>10^5$	(+)	-
Bartha (36)					
K/61	-	-	$>10^5$	(+)*	(++)
Tatarov (35)					
MK-25	-	-	10^5	-	-
Virulent strains	+++	+++	$<10^2$	+++	+++

p+ = may be pathogenic for piglet
f+ = pathogenic for swine foetus (40)
+++ = virulent, with pruritus in laboratory animals
(+++) = kills rabbit and mouse without pruritus
(+) = marked increase of LD_{50} and mean death time, no pruritus
* = data on chick and mouse (48)
** = mink vaccines

(K/61; K for small, in Hungarian, 61 for the year of isolation) was clearly less virulent for rabbits of more than 2 kg (20 to 60% mortality, 5 to 6 days of mean death time, lack of pruritus) than the virulent parent strain. K/61 is also avirulent for piglets of any age and for sheep, however, it is not for dogs, cats and minks (37).

Strain Buk(arest) that had originally been passaged 98 times on chorionallantoic membrane of chicken embryo (38) in Roumania, was further passaged in CEF in Bratislava, Czechoslovakia and various vaccines were obtained (25, 34, 39, 40).

Škoda's line of passage resulted in some degree of attenuation by the 115th passage: although it still killed rabbits, this was done without pruritus (25). This line behaved similarly even after the 1000th passage though less virulent plaques could be isolated (41). Passage 624 became avirulent for piglets (even after i.c. infection) and for 2-month-old calves (i.m. inoculation). Probably this latter one examined by other workers showed 90% mortality (without pruritus) and a mean death time of 4 days (31) thus occupying a position of intermediate virulence between strain Bartha and virulent isolates.

The Buk strain was passaged independently by Žuffa (34, 39) in CEF resulting in a number of attenuated strains (Table 1). Passage 900 (designated TK 900/IV; TK for tissue culture in the Slovak) lost its virulence for piglets (39), swine foetus (42) and sheep but retained 100% virulence (without pruritus) for rabbits (43). However, a plaque isolated from the 900th passage and plaque-purified six times showed a similarly decreased virulence for rabbits like strain Bartha (39). An avirulent virus for the rabbit can arise at a much lower passage level because a totally avirulent clone (TK 300/9,2) was selected by him from the 300th passage of Buk. This is now used both for sheep and mink immunization (12).

Another avirulent strain (MK-25) was induced by Tatarov in Bulgaria by passaging a virulent strain in the presence of iododeoxyuridine (IUDR). His IUDR resistant mutant is avirulent for sheep, rabbits and mice (35). It has been revealed that this strain is a thymidine-kinase negative (TK^-) mutant (44).

Persistent infection by Buk 624 (25, 45) in calf kidney culture resulted in marked attenuation of this strain and it became harmless for rabbits (46).

Mouse. Although generally mice are somewhat more resistant to parenteral inoculation than rabbits, their lesser susceptibility to attenuated strains are comparable to that of rabbits (Table 1). Vaccine strains do not cause pruritus in mice, it takes 5 to 8 days for the K/61 (Bartha's strain), 3 to 4 days for the Buk and 2 to 3 days for virulent strains to kill them, at the level of 10^4 pfu administered by the s.c. route (32).

Chicks. These can be used to differentiate some avirulent strains (e.g. K/61 and Buk 624) from virulent ones (47). Chicks inoculated i.c. with $>10^5$ pfu of strain Bartha show no symptoms at all, while in most cases <100 pfu of a virulent strain kills them in 4 to 6 days. Buk 624 again occupied an intermediate position (47). Studies on other attenuated strains, on the one hand, revealed that day-old chicks are less sensitive than rabbits or mice. As to their susceptibility, they resemble sheep (Table 1, 48). On the other hand, to some of the Buk strains highly adapted to CEF, chicks are as sensitive by i.m. as by i.c. inoculations, while to field isolates they are less sensitive by the i.m. route than by i.c. injection (48).

One can conclude that not only the course of attenuation of a virulent ADV can be checked easily in laboratory animals but they are reliable indicators of the safety of the vaccine strains.

LABORATORY ANIMALS IN DIAGNOSIS

Up to the early sixties when the use of tissue culture became widespread, laboratory animals had to be used in virological diagnosis from specimens and as indicators in virus neutralization assays. (A detailed treatment of that period is found in ref. 3 and 23).

Aujeszky (49) in his classical experiment in 1902 inoculated rabbits, guinea pigs and dogs, and isolated an agent from rabies that caused nervous symptoms in cattle, dogs and cats. The characteristic and unique signs displayed by the

rabbit (high susceptibility and sure reproducibility, intense local reaction: pruritus at the site of s.c. inoculation, short course of the disease, lack of progressive paralysis) convinced him and us that he was dealing with a disease - as now called Aujeszky's disease (in Europe) or pseudorabies (in USA) - basically different from rabies.

As early as in the thirties neutralization assays were carried out in laboratory animals (e.g. in guinea pigs) in surveys to estimate the spread of infection of pig herds in the USA (50) and in England (51).

Since many investigators have reported since the late fifties that easily and cheaply prepared tissue cultures, such as CEF (52), rabbit kidney (53), primary pig kidney (54), PK15 (28), calf testis (55) etc. are very sensitive to field isolates of ADV, for humanitarian reasons there is little justification to use experimental animals for diagnosis, where tissue cultures are available, as was pointed out by others as well (3). I can also suggest that when animals have to be used, they are to be killed after the onset of the first typical signs as there is no recovery anyway. The short course of the disease requires at least two daily inspections. That restrain can be exercised in the use of animals is further supported by the fact, that sensitivity of tissue cultures to ADV is equal or surpasses that of the rabbit (24, 25). Some reports reveal an unnecessary overexamination of samples resulting in already known knowledge and many dead animals: e.g. strains isolated from two cats died of AD were inoculated into rabbits whose virus containing organs were fed to cats to see if they died. They did. Also experimental inoculations were performed with virus isolated in tissue culture, in dogs, cats, ferrets, rabbits and mice by i.c., s.c. an i.ocular route (56).

ROLE OF LABORATORY ANIMALS IN VACCINE TRIALS

They can be used to test the innocuity of vaccines for pigs or, in theory, to test the immunogenicity of vaccines. However, since most of the vaccines (Table 1) have retained some residual virulence for cheap laboratory animals this

notion must have only a very limited application. Mice were used to assess the efficacy of an inactivated subunit vaccine against AD (57).

USE OF LABORATORY ANIMALS IN MARKER RESCUE EXPERIMENTS

In the early eighties several research groups published data on the structure of the DNAs of some vaccine strains, that suggested that part of the genome became deleted (58-61). It has been shown that strain Bartha and Norden (derived from Buk 624) both have a deletion of 2,7 million daltons on the small unique (Us) region of their genomes (62). These strains derived independently, therefore it was of interest to determine if the deletion had any causal relationship to the decrease of virulence of the vaccine strains. Marker rescue experiments were performed by recombining a specific fragment derived from a virulent strain, into the deleted region of the attenuated strains to see if virulent recombinants could be generated. Also, virulent recombinants were made between two avirulent strains (strain Bartha and a TK^- mutant). The virulence of ADV recombinants was measured either by i.c. inoculation of one-day-old chicks (63), or in mice (64) by others.

One-day-old chicks are especially suitable for marker rescue experiments. Avirulent parent strains that are inoculated i.c. in large doses ($>10^5$ pfu) are not only innocuous but are cleared from the brain in 2 to 3 days. Accordingly, virulent recombinants generated even in small proportion (a few %) are able to multiply in the presence of nonmultiplying parents present in excess. Thus chick brain serves as a selection system for the virulent portion of a mixed virus population. It is interesting to note that strain Bartha rescued only at Us although is not virulent for chicks, attained an increased growth capacity as compared to the original avirulent strain. This still avirulent strain that gained increased growth capacity could be enriched in the chick brain and it became possible to select rescued recombinants by analysing only a relatively small number of plaques (63). A second rescue of strain Bartha (rescued already at the Us region) by Bam HI fragment 4 of a virulent strain

made this vaccine strain virulent for chicks by i.c. inoculation. The usefulness of chicks for this type of experiment lies in the fact that, on the one hand, the virulence of strain Bartha is virtually nil in chicks thus any slight increase of virulence could easily be detected. On the other hand, the double rescued strain Bartha which was selected by the chick system turned out be virulent for pigs as well (65).

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HERPESVIRUS OF CATS

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ABSTRACT

Feline viral rhinotracheitis is a major respiratory disease of cats. It is caused by a herpesvirus, designated feline herpesvirus I. This chapter reviews the present state of knowledge of the virus and the disease it produces. The epizootiology of the disease is discussed with particular reference to the latent carrier state. Finally measures for prevention and control of the disease are briefly reviewed.

INTRODUCTION

Feline herpesvirus I (FHV 1) is clinically the most significant respiratory pathogen of cats. It was first isolated in 1957 in the U.S.A. by Crandell and Maurer (1), and the disease, an acute, febrile syndrome characterised by copious ocular and nasal discharges, was called feline viral rhinotracheitis (FVR) (2). The other major respiratory pathogen of cats is feline calicivirus. Although both of these viruses are equally widespread in the cat population throughout the world, the disease caused by feline calicivirus is generally much milder (3). Feline calicivirus and other, less common causes of respiratory disease in cats have been reviewed elsewhere (3,4).

Other herpesviruses besides FHV 1 may also infect the cat. A second feline herpesvirus has been described by one group of workers (5). This virus is serologically distinct from FHV 1 and other mammalian herpesviruses, highly cell-associated, and apparently associated with the feline urolithiasis syndrome (5,6). In addition, Aujeszky's disease virus (suid herpesvirus 1) of pigs may infect cats, producing an intense pruritis

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which progresses rapidly to terminal coma and death; epidemiologically however this is of no significance, since cat to cat spread does not seem to occur (7,8,9).

THE VIRUS

FHV 1 has been classified as an alphaherpesvirus by the International Committee on Taxonomy of Viruses, along with such herpesviruses as herpes simplex virus, infectious bovine rhinotracheitis virus, and Aujeszky's disease virus (10).

Morphologically FHV 1 appears to be a typical herpesvirus (11,12). In negatively stained preparations both mature enveloped and non-enveloped particles may be seen with cubic symmetry. The pleomorphic envelope has an average diameter of 178 nm; the nucleocapsid has an average diameter of 108 nm and is presumed to have 162 capsomeres (reviewed by Povey (13)). Limited thin-section morphogenesis studies have demonstrated nucleocapsids in the nucleus of infected pneumocytes by 12 hours post-infection, acquiring envelopes as they pass through the inner nuclear membrane, with mature enveloped cytoplasmic particles being present by 24 hours (14).

Information on physico-chemical properties of FHV1 is limited. The molecular weight of FHV 1 DNA has been estimated to be approximately $80x\ 10^{6}$, with a G + C content of approximately 46-50 moles % (10,15). The genome structure of FHV 1 has recently been described by Rota and Maes (16,17): FHV 1 DNA is 133 kilobase (kb) in size and composed of a 103 kb unique long segment covalently bound to a 30 kb short segment. The short segment contains an 8 kb unique region flanked by 11 kb repeats. This structure is consistent with the structure of herpesvirus genomes which have two isomeric forms. The locations of some immediate - early and late genes were also described.

There are two reports of SDS-polyacrylamide gel electrophoresis of FHV 1 structural polypeptides. Fargeaud et al (18) reported 23 virion polypeptides, six of which were thought to be glycoproteins. Maes et al (19) demonstrated at least 17 virus-specific polypeptides, three of which were shown to be glycosylated.

The stability of FHV 1 to various chemicals, heat, and pH has been reviewed in detail by Crandell (20) and Povey (13). Briefly, FHV 1 is

sensitive to the action of ether and chloroform (21,22); formalin (21,22) sodium deoxycholate (23); and β-propiolactone (20). The virus is sensitive to trypsin (22,24) and is acid-labile (21,22). Virus stored in cell culture fluid is inactivated in 154 days at 4°C, 33 days at 25°C, 36 hours at 37°C, and 4-5 minutes at 56°C (21). It loses all viability after six months at -22°C, and drops significantly in titre after six months at -50°C (22,25). The optimum temperature for storage is -70°C or below.

FHV 1 is a comparatively labile virus in the environment, surviving for only up to 18 hours in a moist external environment at 15°C, and less than 12 hours in a similar but dry environment (26). As an aerosol, it is relatively unstable at midrange and higher relative humidities (27). The virus is sensitive to a number of common disinfectants, including cationic and aniomic detergents, and hypochlorite (28).

Haemagglutination of feline erythrocytes by FHV 1 has been demonstrated but guinea-pig, dog and chicken red blood cells are not haemagglutinated (29,30,31). Feline red cells are also haemadsorped by infected cultures at 4°C (29).

Until recently it was considered that the natural and experimental host cell range of FHV 1 was highly restricted in contrast to some other herpesviruses such as Aujeszky's disease virus or herpes simplex virus. Despite attempts to culture it in a number of laboratory animals including dogs, and in cell lines from various species (1,13,32,33), *in vivo* it appeared only to infect members of the Felidae, and *in vitro*, apart from one unconfirmed report of adaptation to a rabbit kidney cell line (11), and one report of abortive infection in human cells pre-treated with inactivated Sendai virus (24), its replication is confined to cells of feline origin. Recently, however, herpesviruses indistinguishable antigenically from FHV 1 have been isolated from dogs with diarrhoea (34). The pathogenic and epidemiological significance of this is unclear, but the viruses isolated appear to have similar DNA profiles on restriction enzyme analysis, and similar polypeptide patterns to field and standard strains of FHV 1 (35).

All FHV 1 isolates so far examined appear to be closely related antigenically on the basis of conventional serological cross-neutralisation tests (36,37,38); more refined serological techniques such as neutralisation kinetics or plaque reduction assays have not been used.

Recent work using restriction enzyme analysis of the viral DNA has confirmed this high degree of similarity between strains isolated from various parts of the world (15,35), and which in general is reflected in the relatively uniform biological behaviour of isolates. Nevertheless strains of modified virulence do exist, having been produced in recent years for use in vaccines (39,40,41). When one of these, a ts mutant (39), was examined it also showed a similar DNA cleavage pattern to the other isolates when the major DNA fragments were compared (15). More extensive work is needed to confirm this apparent lack of heterogeneity in FHV 1.

PATHOGENESIS AND PATHOLOGY

FHV 1 is highly infectious to susceptible cats and generally produces a reasonably uniform upper respiratory tract syndrome. The natural route of infection is almost certainly intranasal, oral or conjunctival. Experimentally, the intranasal route is most commonly used, but several other routes have also been investigated (reviewed by Povey (13)). Because of the affinity of some other herpesviruses for both respiratory and genital tracts, some attention has been given to a possible genital tract tropism for FHV 1. Bittle and Peckham (42) showed that vaginal instillation of virus resulted in congenitally infected kittens. Transplacental infection and abortion has been demonstrated following intravenous inoculation of virus, but although abortions also occurred following the more natural intranasal route of inoculation, no virus was recovered from aborted material (43). Thus abortion was attributed to non-specific effects of the severe debilitating upper respiratory disease and not to the effects of the virus itself.

In the typical, respiratory experimental infection, replication of FHV 1 as assessed by (1) pathological findings together with the presence of intranuclear inclusion bodies and (2) the occurrence of maximal virus titres in tissues, takes place predominantly in the mucosae of the nasal septum, turbinates, nasopharynx and tonsils; other tissues including conjunctivae, mandibular lymph nodes and upper trachea are also often involved (44,45). A viraemia has only rarely been reported (45,46,47).

Pathological lesions consist of multifocal areas of epithelial necrosis with neutrophilic infiltration and exudation with fibrin (44,47). Necrosis

and resorption of the turbinate bone may also be seen, and indeed an apparent predilection of FHV 1 for growing bone has been noted epxerimentally following intravenous inoculation of young kittens (48). Resolution is generally slow, but by two to three weeks, epithelial regeneration with some squamous cell metaplasia, and sometimes hypertrophy, may be seen. The disease is apparently not dependant upon the presence of microbial flora, for it has been reproduced experimentally in germ-free cats (47). Nevertheless it is likely that the effects of the disease may be enhanced by secondary invasion by bacteria.

THE CLINICAL SYNDROME

FHV 1 produces a characteristic syndrome in susceptible cats (44,46,49). The incubation period is usually 2-6 days, but may be longer. Experimentally it has been shown that increasing virus dosage is significantly correlated with a shortening of the incubation period and to some extent with the severity of clinical signs (49) but in general, the syndrome is reasonably uniform.

Early signs of the disease include depression, marked sneezing, clear ocular and nasal discharges, and sometimes hypersalivation. There is usually fever (39.5°C) and loss of appetite. As the disease progresses, the discharges gradually turn muco-purulent. Conjunctivitis and sometimes dyspneoa and coughing may develop, and there may be a recurrence of the pyrexia. A leucocytosis with a left shift is present throughout the course of the disease. The majority of clinical signs has usually resolved in 10-20 days but some animals may be left with chronic sequelae. Mortality may be high in very young or debilitated cats. Other signs seen less commonly include tongue ulcers (50), ulcerative and interstitial keratitis (51) and a primary viral pneumonia (52); generalised disease may also occasionally occur, particularly in younger animals (53,54). These and other rarer manifestations such as skin ulcers and nervous signs, have been reviewed by Gaskell and Wardley (4) together with a discussion of various factors which on some occasions may account for variations in the host's response.

MAINTENANCE OF THE VIRUSES IN THE POPULATION

FHV 1 is a highly successful virus in cats. It is worldwide in distribution (20) and together with feline calicivirus, accounts for the majority of cases of feline respiratory disease (4,55). Clinically, it is the most significant of the feline respiratory pathogens. Serological surveys prior to vaccination demonstrated serum neutralising antibody titres in 26-70% of cats, depending on the nature of the sample population (56,57,58): in general, infection is less common in isolated household pets than in colony animals. Thus in cats, FHV 1 has filled the respiratory ecological niche which in many species is filled by a number of other virus families.

FHV 1 is relatively fragile and short-lived in the external environment. Thus outside the cat it probably only persists long enough for indirect transmission to occur within the closed confines of a cattery. It has no known reservoir hosts, and vertical transmission does not naturally seem to occur. Therefore like many herpesviruses it must rely for its continued survival on its ability to persist in the host, such persistence being achieved firstly by continuous horizontal spread from the acute case to susceptible cat, and secondly, by means of carriers.

The FHV 1 carrier state is characterised by a latent phase with only intermittent episodes of virus shedding (59,60). In the latent phase, virus is undetectable by normal sampling techniques, but during re-excretion episodes, virus is present in oro-pharyngeal secretions and the cats are infectious to other cats (61). As with other herpesvirus carrier states there is no evidence that the carrier state is self-limiting (62).
Studies have shown that at least 80% of FHV 1 recovered cats are likely to be carriers, and that at least 45% of them are likely to be epidemiologically important, that is likely to shed virus under natural stress conditions (59,60). Cats may excrete virus spontaneously (approximately 1% of a group of carriers on any one day), but they may also be stimulated to shed as a result of various stresses (58,59,60). Thus experimentally it has been shown that both corticosteroid administration, and less consistently, the stress of moving animals into new quarters, may stimulate episodes of virus re-excretion in 69% and 18% of FHV 1-recovered cats, respectively. This has also been confirmed in the field situation to

a limited extent, where FHV 1 re-excretion was recorded in 3 of 75 cats 9-12 days after entering a boarding cattery (4). There is also some evidence that the stress of lactation may induce an episode of virus shedding from queens (59,60): the significance of this is discussed below.

With experimentally-induced episodes of virus shedding there is a delay from the first day of stress to the onset of re-excretion of 4-11 days (mean 7 days) (59,60). Animals then shed virus for 1-13 days, and in some cases this is accompanied by mild clinical signs. Occasionally though, such clinical signs may be seen in carriers unassociated with detectable episodes of virus shedding. There is some evidence of a refractory period after an episode of induced re-excretion during which animals are less likely to experience another episode (63).

The site or site of latency of FHV 1 is not as well established as in some other alphaherpesvirus infections. Recent work has demonstrated FHV 1 in trigeminal ganglia tissue fragment cultures from 18% of FHV 1-recovered cats (64): numerous previous attempts to demonstrate latent virus using coculture or explant culture techniques successful in some other herpesvirus infections were unsuccessful (45,65,66). Thus it appears that either additional or alternative sites for latency are important in FHV 1, or that there is less latent virus in the trigeminal ganglia, or it is under stricter control. It is also possible that virus isolation from ganglia might be enhanced by slight alterations in culture technique: minor differences have been shown to influence the recovery rate in other systems (67). In-situ DNA hybridisation studies should also be performed.

The practical implications of the carrier state are:

(1) carriers are difficult to identify because of their intermittent shedding pattern, though the chances of detecting virus shedding might be increased either following a stress or when clinical signs are present.

(2) Any animal with a known history of respiratory disease, or with persistent or recurrent signs of respiratory disease, should be suspected of being a carrier. Similarly any queen who repeatedly produces litters that develop respiratory disease is probably a carrier.

(3) Although FHV 1 carriers should always be regarded as potentially infectious as they may shed virus spontaneously at any time, they are much

more likely to be so in the three week period after a stress (eg any change of housing, or during lactation).

(4) Animals may become field virus carriers, without having shown any clinical signs, under protection from either passive immunity or from systemic vaccination (61,68). There is some evidence though that intranasal vaccination will protect against the subsequent development of the carrier state, at least in the short term (69). There is no evidence that vaccination will "cure" pre-existing carriers, although it is possible it might reduce detectable episodes of virulent virus shedding.

TRANSMISSION

The major method of spread of FHV 1 is by direct cat-to-cat contact. During the acute stage of the disease, virus is shed in high titre in oro-pharyngeal, nasal and conjunctival secretions for 1-3 weeks; during re-excretion episodes from carriers, levels shed are generally lower, though individual animals may shed similar amounts (63)

Transmission is achieved though infectious discharges and sneezed macrodroplets making contact with the mucosa of the upper respiratory tract. Cats have been infected by the aerosol route but this is probably not an important natural route for there is some evidence that the cat does not produce an infectious aerosol of FHV 1 during normal respiratory movements (61). The distance through which sneezed macrodroplets can be carried is not known but in relatively still air it appears they may reach a distance of 1.2 m (26).

Indirect or fomite transmission via a contaminated environment, personnel, or feeding and cleaning utensils may also occur and it is probably an important route of transmission where groups of cats are housed together. However, in view of the fragility of FHV 1 outside the cat, indirect sources of virus are unlikely to be of long term importance in the transmission of the disease. Other factors that influence the survival of FHV 1 in the external environment, and hence indirectly affect the efficacy of transmission include temperature, relative humidity and ventilation (4,26).

It is likely that under natural conditions the efficacy of cat-to-cat transmission of the virus will depend on both the amount of virus being

shed by the infecting animal and on the duration and intimacy of contact of the susceptible animal with the infected secretions. Therefore it might be expected that virus might be more readily transmitted by cats in the acute stage of the disease where the discharges are usually more copious and in slightly higher titre, than by shedding carriers. Thus it has been shown that although cross-infection from acutely infected to susceptible cats may be readily achieved (23,32,70) under experimental conditions, fairly intimate contact of several days duration appears to be necessary before successful transmisison may occur from a shedding carrier (61).

Under more natural conditions, however, it is likely that the greatest importance of the carrier lies in its ability to transmit the virus within the close confines of family groups, and particularly from carrier queens to kittens. A study on virus shedding patterns in queens and kittens in the 10 week post-partum period have demonstrated a marginally increased shedding rate at this time: four of ten queens re-excreted virus, and four kittens from three litters developed a contact infection (61). Furthermore, some kittens became infected subclinically under cover of passive immunity and became latent carriers: such a mechanism is an ideal way for the virus to perpetuate itself in the next generation since it achieves transmission without the hazards associated with the development of clinical disease.

IMMUNITY

Although a number of conventional serological tests have been developed for FHV 1 (reviewed by Povey (13) and Gaskell and Goddard (63)) most studies have concentrated on the detection of serum neutralising (SN) antibody. Following primary infection, SN antibody is slow to rise and even by 40 days may only be present in approximately 70% of cats (49). Following an initial episode of virus re-excretion, however, a significant rise in antibody levels occurs in most cats: titres then remain relatively stable regardless of subsequent episodes of virus shedding (62). Despite the relatively low, or in some cases non-detectable levels of SN antibody after acute disease, resistance to challenge has been demonstrated 21 days after experimental infection, and partial protection after five months (71). In vaccination trials, most studies have demonstrated reasonable protection at

three months but equivalent protection has been reported after one year (72). In studies on early protection with an intranasal vaccine, although apparently specific protection was operating by six days after vaccination, no detectable neutralising antibody was present in nasal washings or serum although there were low levels of IgA and IgM (73).

Thus it appears that SN titres, as in other herpesvirus infections, are not necessarily indicative of resistance to infection, and that other immune mechanisms, particularly cell-mediated responses, are undoubtedly of importance in determining the animals immune status. Little work has been done, however, on cell-mediated and other immune responses to FHV 1 infection. Antibody and complement-mediated lysis of FHV 1 infected cells has been demonstrated in vitro and has been shown to limit intracellular virus spread (74,75). In vivo, cytolytic antibody and lymphocyte transformation responses have been studied in both acute and recrudescent disease, and interestingly cats which experienced episodes of virus re-excretion had a lower resting cytolytic antibody capacity than those with did not (76). Wardley et al (74) also demonstrated antibody dependent cell-mediated cytotoxicity and possibly T-cell cytotoxicity of FHV-1 infected cells, though the phenomenon of MHC-restriction was not then addressed. More recently, Tham and Studdert (77) have recorded MHC-restricted cytotoxic activity of peripheral blood T cells from FHV 1 vaccinated cats in autologous target cells, and also delayed hypersensitivity skin reaction to FHV 1.

Available data suggests that maternally-derived (essentially colostral) antibody in kittens may persist for 2-10 weeks with mean levels falling below detectable levels by 6-9 weeks of age (61-78). However little work has been done on relating these antibody levels to actual protection against challenge: some kittens with no detectable FHV 1 antibody may still be protected against the disease (61).

PREVENTION AND CONTROL

A number of vaccines are available for use against feline respiratory disease. Generally these are combined FHV 1/feline calicivirus vaccines, and there are three basic types: modified live systemic vaccines, modified live intranasal, or inactivated adjuvanted systemic. An assessment of these

vaccines and their usage has been given elsewhere (79). In general, they are relatively successful in preventing disease in the majority of healthy, previously unexposed cats. However, because of the nature of the epidemiology of the disease, effective disease control, particularly in colonies with endemic problems, needs to be approached through a combination of vaccination and management. This has been described in detail elsewhere (3) but brief guidelines are given below:

A: In breeding colonies which are disease-free:

- All cats should be vaccinated routinely if there is any contact, direct or indirect with other cats.
- Inactivated vaccines are preferable, though with care, modified live should be satisfactory.
- Care should be taken to avoid buying in carriers i.e. any cat with a history of association with respiratory disease.
- All incoming cats should be quarantined for three weeks and ideally screened virologically, and also serologically if not previously vaccinated.

B: In breeding colonies with endemic disease:

In some circumstances, it may be feasible to restock the colony with specific pathogen-free cats and employ a barrier system to keep virus out. However, in many situations, the only reasonable course is to attempt disease control. This may be done by:

- Regular vaccination programmes
- Booster vaccinating queens either prior to mating, or during pregnancy if with a killed vaccine
- Keeping cats as stress-free as possible
- Avoiding the use of particular queens with a history of respiratory disease in their kittens.
- Moving queens into isolation at least three weeks before term so (i) kittens are not exposed to any carriers in the colony and (ii) any shedding episode from the queen as a result of the move will be over before kittening.
- Early-weaning kittens into isolation away from their mother if it is likely she herself is a carrier

- Vaccinating all kittens as soon as maternal antibodies are at a non-interfering level (normally 9-10 weeks) and certainly before exposure to any other cats
- Earlier vaccination schedules - e.g. starting at about six weeks with systemic vaccines, or possibly by using the intranasal route in very young kittens where maternal antibody is still present.
- Employment of good management practices to prevent spread of virus within a colony of cats. Such measures include solid partitions between adjacent pens, at least 1. 2 m between open frontages, and appropriate disinfection procedures (reviewed by Gaskell (3)).

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HERPESVIRUS SYLVILAGUS: LYMPHOPROLIFERATIVE AGENT OF COTTONTAIL RABBITS

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ABSTRACT

Herpesvirus sylvilagus is an indigenous virus of cottontail rabbits. Infection of rabbits results in a lymphoproliferative disorder characterized by spleen and lymph node hyperplasia, the appearance of atypical lymphocytes in the peripheral blood, and an intense T-cell proliferation. *In vivo*, the virus is present latently in B and T lymphocytes in both a covalently closed circular form and a linear duplex form. *In vitro*, the virus is able to bind to lymphocytes but a productive infection is not detected. In productively infected cultured rabbit kidney cells, the virus induces the synthesis of at least 45 virus-induced polypeptides of which at least 14 are glycosylated.

INTRODUCTION

The herpesvirus family is composed of a large number of complex viruses with diverse properties. Studies of the close association of several of these viruses with lymphoproliferative disease in man and lower animals have provided a great deal of information concerning the possible oncogenicity of these agents. *Herpesvirus sylvilagus* infection in cottontail rabbits (*Sylvilagus floridanus*) provides a useful model for studying the complex virus-host relationships of lymphotropic herpesviruses. Experimental infection of wild cottontail rabbits with *H. sylvilagus* produces a primary lymphoproliferative disease. The severity of this response varies among animals from a benign lymphoid hyperplasia to a severe lymphoma-like disease, thus resembling the syndromes of infectious mononucleosis and Burkitts lymphoma seen in humans infected with Epstein-Barr virus (EBV).

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Although the biological properties of *H. sylvilagus* are similar in many respects to those seen with EBV, the ability to grow *H. sylvilagus* lytically in cultured cottontail rabbit kidney (CRK) cells offers an advantage over EBV, for which no simple permissive system is known. Not only does this system facilitate the production of purified virions and virion DNA, but, in addition, events occurring during the full expression of the viral genome can be monitored and the viral gene products can be characterized. Furthermore, a nononcogenic variant has been obtained by serial passage in cultured cells. A study of this strain will be valuable in providing a means to understand the oncogenicity of this virus. The following report summarizes several aspects of the biology of *H. sylvilagus*.

NATURAL HISTORY

Herpesvirus sylvilagus was first isolated from primary cell cultures prepared from the kidney tissue of an apparently healthy cottontail rabbit (*Sylvilagus floridanus*) trapped in southern Wisconsin (1, 2). Numerous subsequent isolations of the virus as well as the presence of neutralizing antibody in wild-caught animals indicates that this virus is a natural pathogen of cottontail rabbits. As with EBV, *H. sylvilagus* has a very narrow host range; infection appears to be strictly limited to wild rabbits of the genus *Sylvilagus*. It is apparently unable to infect ordinary laboratory strains of rabbits of the genus *Oryctologus* or other commonly used laboratory animals (1).

In tissue culture, the virus grows readily in cells of either *Sylvilagus*, *Oryctologus* or *Lepus* origin (1). Here it undergoes a morphological development similar to that seen with other herpesviruses; immature virions accumulate in nuclear inclusion bodies and the primary envelopment occurs at the nuclear membrane (1, 3). As with other herpesviruses (4, 5), *H. sylvilagus* can also acquire its envelope at preformed cytoplasmic membranes. Mature virions of this group (Fig. 1), have a diameter of 200 nm and are comprised of an electron-dense core, a capsid of medium electron density and an outer envelope consisting of a unit membrane. Although virions of this group appear to have an electron-dense layer between the capsid and envelope, this same area is electron-lucent in the smaller virions (d = 130 nm) that are enveloped at nuclear membranes.

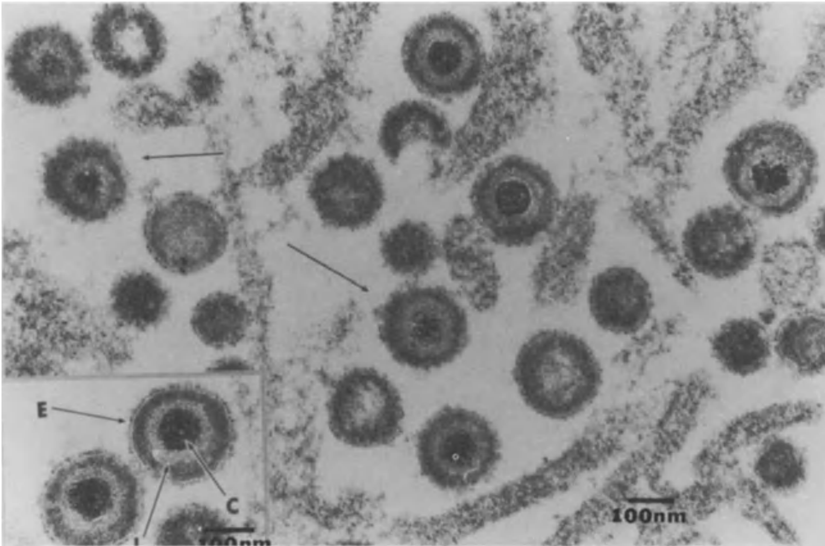

Fig. 1 and inset. Mature virions in the extracellular space. Electron-dense cores (C) slightly stained capsids (I) and the outer envelopes (E) are recognizable. The space between capsid and envelope is filled with a homogeneous, electron-dense matrix. A fuzzy coat is present on the surface of some of the virions (arrows). (Reprinted with permission from ref. 3).

In tissue culture, H. sylvilagus is primarily cell associated with less than 1% of the replicating virus released into the supernatant medium. The growth cycle is long; an eclipse period lasts 10 to 12 hours and peak virus titers and maximum cytopathic effects (CPE) are not reached until 40 to 50 hours later (6, 7).

PATHOGENESIS

Experimental infection of cottontail rabbits with H. sylvilagus is characterized by a chronic low-grade viremia that persists for the remainder of the animal's life (8). The pathological change seen in these animals is primarily a generalized lymphoproliferation. Within 2 to 3 weeks after inoculation the spleen and lymph nodes show a rapid enlargement due to an increase in the size and number of lymphatic

nodules. In most animals, such hyperplasia reaches a peak at 6 to 8 weeks and declines thereafter over the next 2 to 3 months. However, in approximately 15% of all inoculated animals, the changes in the lymph nodes progress to an obliteration of the follicular architecture as well as the cortical and medullary sinuses by large numbers of actively dividing lymphocytes (Fig. 2). Spleens of these animals also show a marked loss of normal architecture (8).

In most animals, other organs are also infiltrated with immature lymphocytes. Although the kidney, liver and myocardium are most frequently involved, invasion of the lungs, pancreas, submaxillary gland and intestinal wall is seen in more severely affected animals. In all instances, destruction of the parenchymal cells appears due to the crowding by the invading lymphocytes rather than infection by the virus (8).

The pathological changes in the tissues are also reflected in the peripheral blood. A leukocytosis with a relative lymphocytosis begins about 2 weeks after infection and is characterized by the appearance of large, abnormal lymphoid cells in the peripheral blood (7, 8). By fluorescent antibody studies, Kramp *et al*. (9), have shown that the intense mononuclear proliferation that is observed results from a large increase in the population of T lymphocytes. The subpopulation of T cells involved is not known.

VIRUS-LYMPHOCYTE RELATIONSHIP - *IN VIVO*

The predominant virus-lymphocyte relationship *in vivo* is non-permissive; the virus is present latently in both the B and T lymphocytes of the peripheral blood, lymph nodes and spleen and is readily recoverable by co-cultivating infected cells on permissive cell monolayers (9, 10). The number of cells capable of forming infectious centers is low and ranges from 1 per 10^4 to 1 per 10^6 cells (10).

As with other herpesviruses (11, 12), *H. sylvilagus* is shed from the mouth of infected animals as infectious, extracellular virus. This occurs despite the presence of significant levels of serum antibody (13). Interestingly, the virus that is shed in the saliva appears to be in the form of infectious virus-antibody complexes (J. Goodrich, unpublished data). These complexes are neutralized by the addition of goat antisera against cottontail gamma and alpha but not mu chains,

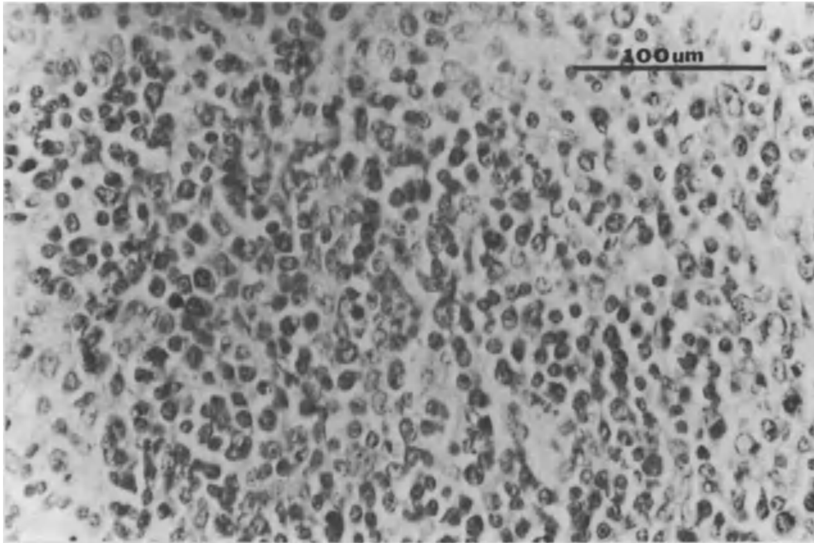

Fig. 2. Section of popliteal lymph-node from cottontail developing lymphoma-like disease 4 weeks after inoculation of H. sylvilagus. Immature lymphocytes obliterate normal follicular structure and fill cortical sinuses. Hematoxylin and eosin stain. (Reprinted with permission from ref. 8).

indicating that both IgG and IgA, but not IgM, are bound to the virions in the saliva of virus-shedding animals. Free anti-virus antibody is also found in the saliva of these animals and is capable of sensitizing additional virus but not neutralizing it. The significance of these 'subneutralizing' or 'nonneutralizing' levels of antibodies in the pathogenesis of H. sylvilagus infection is not clear.

In contrast to reports with Marek's Disease virus (MDV) (14) and EBV (15), the source of the oropharyngeal H. sylvilagus does not appear to be the result of a productive infection of non-lymphoid cells since viral antigen is not seen in cells other than lymphocytes in any tissues of the mouth, pharynx or adjacent structures. Instead, the presence of masses of antigen-positive lymphocytes migrating through and accumulating in the tonsillar crypt lumen provides evidence that

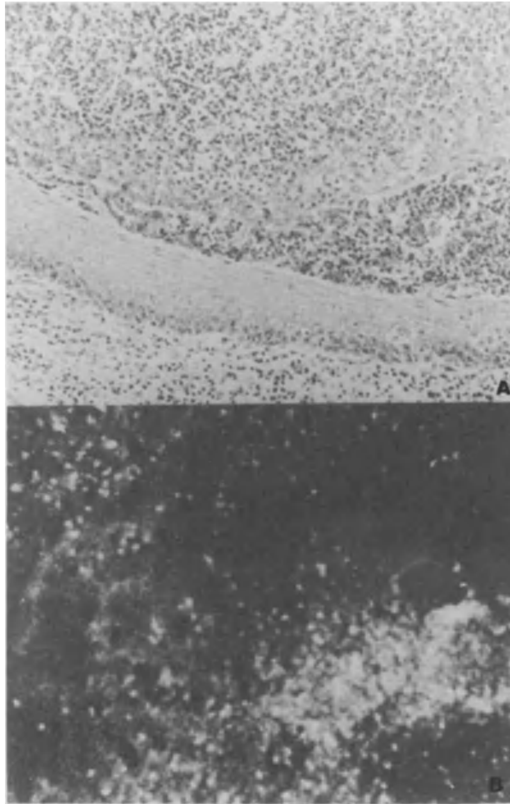

Fig. 3. (A) Palatine tonsil of a H. sylvilagus-infected rabbit showing migration of lymphocytes through wall of tonsillar crypt and accumulation in lumen; eosin and hematoxylin stain. (B) Fluorescent antibody stain of adjacent section showing migration of antigen-containing lymphocytes and accumulation of viral antigen in lumen. (Reprinted with permission from ref. 13).

this is the site of maturation and release into the oral cavity (Fig. 3) (13).

VIRUS-LYMPHOCYTE RELATIONSHIP - IN VITRO

Despite the well-known transformation potential of several members of the herpesvirus family, attempts to develop H. sylvilagus-transformed

lymphocyte lines by several methods have been unsuccessful (10). R. Cohrs (personal communication) has, however, obtained a morphologically transformed juvenile cottontail rabbit kidney (JCRK) cell line using UV-inactivated H. sylvilagus. Cells of this line have lost their contact inhibition and have become anchorage independent as judged by their ability to form colonies in soft agar.

The early interaction between H. sylvilagus and cottontail rabbit lymphocytes in vitro has recently been studied in some detail (A. Patick, unpublished data). Although both purified, mitogen-stimulated and non-stimulated mononuclear cells from uninfected rabbits were unable to support either a productive infection or form infectious centers when plated on permissive cells, a subpopulation of these cells was shown to be able to bind to H. sylvilagus as demonstrated by a membrane immunofluorescence assay. As shown in Table 1, binding of virus to a population of mononuclear cells occured readily at 37°C . At 30 min, 10% of lymphocytes appeared positive. By 2 hr this percentage increased to 30% but then decreased by 24 hr. H. sylvilagus was also able to bind to lymphocytes at 4° but this reaction appeared slower in that a longer incubation period was required to attain a level equal to that found when lymphocytes were incubated at 37°C.

Table 1. Adsorption of Herpesvirus sylvilagus to cottontail rabbit lymphocytes in vitro.[a]

			% Membrane Fluorescence (positive cells)[b]		
Culture and Treatment	Serum	Temp	30 min	2 hr	24 hr
H. sylvilagus infected	+	4°	0	6.5	25.1
H. sylvilagus infected	+	37°	1.2	30.0	14.0
H. sylvilagus infected	-	4°, 37°	0	0	0
Mock infected	+	4°, 37°	0	0	0

[a] Lymphocytes were purified from cottontail rabbit peripheral blood and incubated with H. sylvilagus at either 4° or 37°. At various times after infection, the inocula were removed, cells washed extensively and indirect membrane immunofluorescence assay was carried out using known positive (+) or negative (-) serum.

[b] 500 cells were scored to calculate % of membrane immunofluorescent cells. The percentages given here are mean values from 2 separate experiments.

MOLECULAR BIOLOGY

Virion DNA. Based on analyses performed by Medveczky, *et al*. (16), *H*. sylvilagus DNA has the following features: (i) by SmaI restriction endonuclease digestion, the size of *H. sylvilagus* DNA is estimated at 158 kb; (ii) the prescence of repetitive elements is suggested by the detection of a supermolar 0.55 kb SmaI fragment in *H. sylvilagus* DNA (iii) the presence of both 0.5 and 0.25M fragments in BAMHI-cleaved DNA suggests that *H. sylvilagus* genome may undergo isomerization as has been described for herpes simplex virus (17). These observations are similar to those seen with a herpesvirus isolated from a cottontail rabbit (CTHV) and are consistent with its proposed structure; CTHV DNA consists of two segments of unique segments flanked and joined by tandem repeats of different lengths (16).

Conflicting reports as to the size and structure of the *H. sylvilagus* genome arise from the analyses performed by R. Cohrs (personal communication). Digestion of *H. sylvilagus* DNA with the SmaI restriction endouclease results in 25 fragments including the super-molar fragment of .55 kb. Based on the sum of these fragments, the size of the DNA was estimated at 111.75 kb. In addition, although Cohrs (personal communication) also obtained sub-molar fragments upon EcoR1 digestion, these were shown to be the result of a heterogenous population of DNA molecules that differed by the number of repeated DNA segments and not due to inversion of the genome. From his analyses, he proposes that the structure of *H. sylvilagus* is similar to that of EBV and contains an internally located repeated DNA segment.

The state of the *H. sylvilagus* genome has been examined in spleen cells of infected cottontail rabbits (16). As with EBV, *H. sylvilagus* DNA is found in both a covalently closed circular form and a linear duplex form. Both viral DNA forms appear to be present in approximately 0.2 copies per cell.

NONONCOGENIC VARIANTS

Attenuation of *H. sylvilagus* has been achieved in this laboratory by long-term serial passage in New Zealand rabbit kidney cells. Cottontail rabbits infected with this strain do not develop lymphoproliferative disease. They do, however, acquire persistent infections as indicated by the isolation of small plaque virus variants

and by the detection of *H. sylvilagus* neutralizing antibodies. In addition, animals infected with this attenuated strain and subsequently challenged with the wild-type strain do not develop a lymphoma-like disease (H.C. Hinze, unpublished observations).

It is not known at present by what mechanisms the attenuated *H. sylvilagus* has lost its lymphoproliferative inducing capabilities. Medveczky *et al*. (18) have analyzed DNA from both attenuated and wild-type viruses. Cleavage comparisons of these DNA's have revealed a deletion of about 1 kb in the unique coding region of the DNA. This deletion may interfere with the transcription and translation of a putative transforming protein or may even modify the immunogenicity of a viral antigen, resulting in more effective elimination by the host immune system. Alternatively, this deletion may occur in a part of the genome that is indirectly involved in the transformation process. No difference is seen in the growth of infectious attenuated virus in lytically infected cells *in vitro*. Replication of attenuated virus does, however, appear more rapid, with maximum CPE and peak virus titers appearing 24h earlier than the wild-type virus (A. Patick, unpublished observations).

VIRUS-INDUCED POLYPEPTIDES

Mature virions are composed of 44 proteins ranging in molecular weight from 18 to 230 kilodaltons. Seventeen polypeptides, including a major protein of 150 kilodaltons, are found within the nucleocapsid (19). By one-dimensional gel electrophoresis, at least 4 major glycoproteins and four major phosphoproteins can be identified in the mature virus. The structural complexity of these modified proteins are evident in 2-dimensional electrophoretic profiles; at least 13 phosphoproteins, 9 glycoproteins, and 4 which are both glycosylated and phosphorylated have been identified (20).

We have recently identified and characterized the polypeptides that are induced during a productive infection (21). SDS-PAGE analysis of pulse-labelled, whole cell extracts resolved a minimum of 18 virus-induced polypeptides (VIP), including the major nucleocapsid protein of 150 kilodaltons (VIP 8) (Fig. 4). After infection, host synthesis is gradually inhibited; polypeptides characteristic of the uninfected cell (h_1, h_2) gradually disappear from the gel profiles

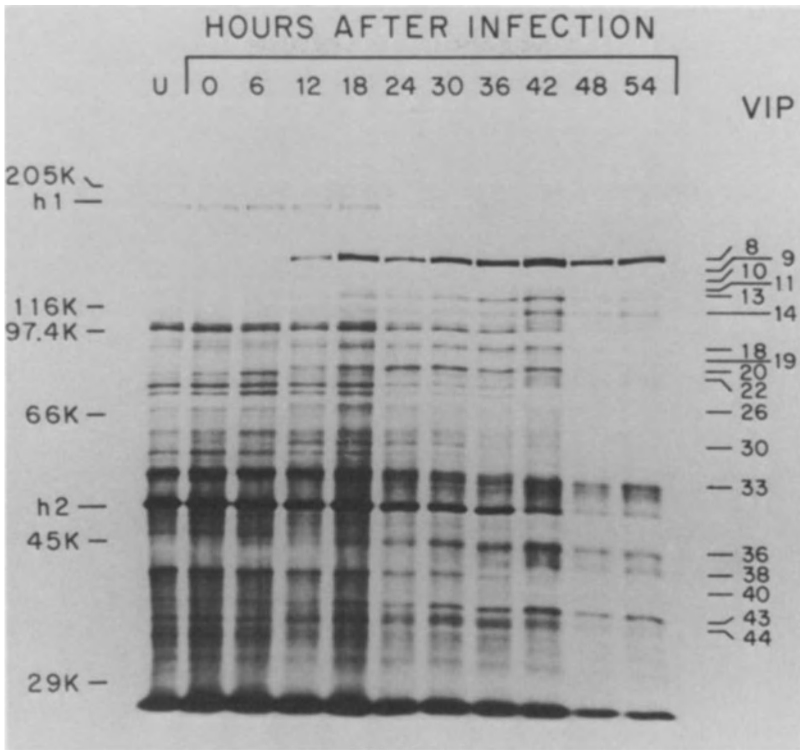

Fig. 4. Time course of appearance of polypeptides synthesized in _H. sylvilagus_-infected cells. Infected cells were pulse-labeled with [^{35}S]methionine (50 μCi/ml) for 6 hr periods at various times after infection as indicated. U, Uninfected sample. At the end of each labeling period, cell cultures were solubilized and equal amounts of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Numbers correspond to virus-induced polypeptides (VIP) designated in Table 2. h, Host polypeptides. Locations of molecular weight reference markers are shown on left. Time of exposure was 24 h. (Reprinted with permission from ref. 21).

while those unique to the infected cell gradually appear. In order to selectively enrich for virus-induced polypeptides, radiolabeled cell extracts were immunoprecipitated with _S. aureus_ protein A. By SDS-PAGE analysis (Fig. 5) at least 45 VIP were resolved, ranging in molecular weight from 230 to 27 kilodaltons. Furthermore, it appears

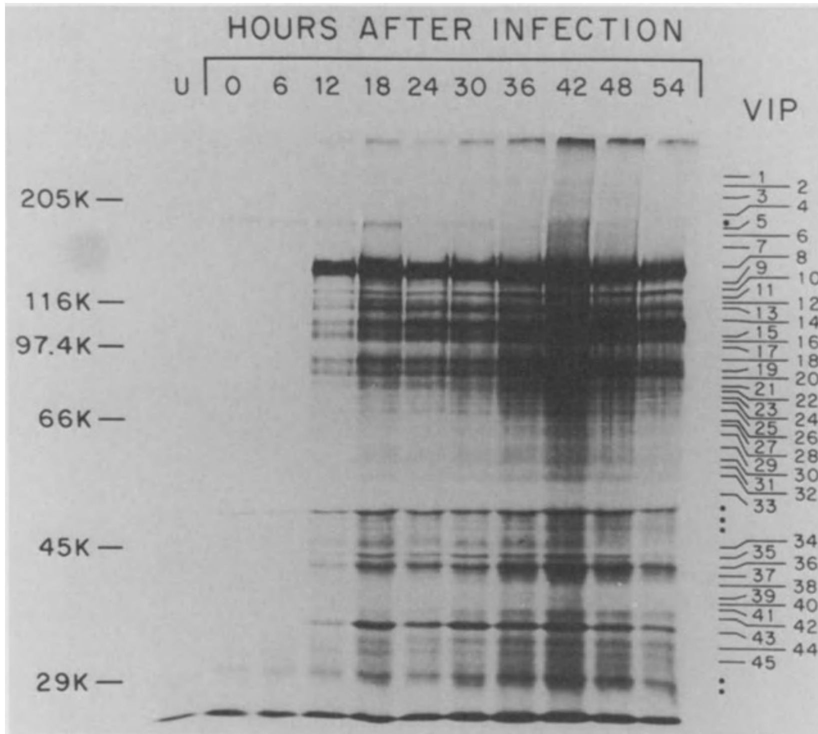

Fig. 5. Immunoprecipitation of [^{35}S]methionine-labeled polypeptides from H. sylvilagus-infected cells. Infected and uninfected (U) cell cultures were labeled with [^{35}S]methionine (50 µCi/ml) for 6 hr periods at the times indicated. Equal amounts of protein from extracts prepared from cell cultures were incubated overnight with 10µl of undiluted immune serum from a cottontail rabbit (titer, 1:8.000). Immune complexes precipitated by addition of S. aureus protein A were denatured and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Virus-induced polypeptides (VIP) are numbered in order of decreasing molecular weight (Table 2). Nonspecifically precipitated polypeptides are indicated by a dot in right margin, and molecular weight reference markers are shown on left. Time of exposure was 5 days. (Reprinted with permission from ref. 21).

that H. sylvilagus polypeptides were synthesized at different times after infection, suggesting the existence of complex controls

Table 2. Summary of <u>Herpesvirus</u> <u>sylvilagus</u>-induced polypeptides

Virus-induced Polypeptide	Apparent mol wt (x 10^3)	Time of Appearance/ Disappearance (hr p.i.)[a]	Time of Maximum Intensity (hr p.i.)[b]	Observed in direct extracts[c]
1	230	12-18		-
2	220	18-24		-
3	200	12-18		-
4	190	30-36		-
5	185	42-48		-
6	180	0-6		-
7	170	0-6/18-24		-
8[d]	150	6-12	45	+
9	140.5	12-18		+
10	140	0-6	45	+
11	135	12-18/42-48		+
12	130.5	12-18/42-48		-
13	130	12-18	45	+
14	120	6-12	45	+
15	110.5	6-12	45	-
16	110	0-6	45	-
17	105.5	30-36		-
18	100	6-12	45	+
19	93	6-12	45	+
20	92	6-12/42-48	39	+
21	89	6-12	45	-
22	88	30-36		+
23	86	0-6	45	-
24	83	0-6	45	-
25	78	0-6	45	-
26	77	12-18		+
27	75	0-6		-
28	71	0-6		-
29	68	6-12		-
30	64	6-12		+
31	62	6-12	45	-
32	59	6-12	45	-
33	54	12-18		+
34	43	12-18	45	-
35	40	0-6	45	-
36	39	0-6	45	+
37	38	12-18		-
38	37	12-18		+
39	36	30-36		-
40	33	30-36		+
41	32.5	30-36		-
42	32	0-6	45	-
43	31	6-12	45	+
44	29	0-6	45	+
45	27	0-6	45	-

regulating their synthesis and accumulation as has been described for other herpesviruses (22) (Table 2).

The modification of these polypeptides by glycosylation has recently been examined. In these studies, it appears that H. sylvilagus induces at least 14 glycoproteins in lytically infected cells. These range in molecular weight from 130 to 27 kilodaltons. Recently, the release of glycoproteins into the culture medium of several herpesviral infected cells has been reported (23, 24). Preliminary results here have also shown that at least 6 glycoproteins are released into the culture medium by H. sylvilagus infected cells. The most predominant glycoprotein of 54 kilodaltons (VIP 33) is found primarily in the culture medium of infected cells and less abundantly in cell extracts. Preliminary studies also indicate that VIP 33 is found on the surface of infected cells. The biological significance and immune reactivity of this glycoprotein as well as others are currently being examined.

Table 2 (Continued)

[a] Determined by direct visual inspection of autoradiogram (Fig. 5). p.i., Postinfection.

[b] The relative amount of (^{35}S)methionine located within major bands was measured with the aid of a reflectance fluorescence transmission scanning densitometer; the times of maximum intensities are listed at the midpoint of the pulse period.

[c] These polypeptides were observed in whole-cell extracts analyzed directly in sodium dodecyl sulfate-polyacrylamide gels (see Fig. 4).

[d] Major viral nucleocapsid polypeptide (reprinted with permission from ref. 21).

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THE PATHOGENICITY AND MOLECULAR BIOLOGY OF GUINEA PIG CYTOMEGALOVIRUS

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ABSTRACT

Infection of guinea pigs with guinea pig cytomegalovirus (GPCMV) results in an acute infection followed by chronic persistent infection. The severity of the acute phase of infection is dependent upon the strain of the host and whether the host is pregnant. During the persistent phase of infection, virus persists in the salivary glands, the pancreas, and lymphoid tissues. GPCMV infects the placenta of pregnant guinea pigs, crosses the placenta and infects the fetuses. Transplacental transmission of the virus can occur throughout the entire gestation period. The molecular cloning of approximately 97% of the GPCMV genome has made it possible to determine the DNA structure, generate restriction endonuclease maps of the DNA, identify regions of DNA sequence homology with human cytomegalovirus (HCMV), begin to analyze patterns of transcription, and detect GPCMV infection in cultured cells by _in situ_ hybridization.

INTRODUCTION

Guinea pigs, mice, rats, hamsters, and man become infected with their own species-specific cytomegaloviruses. This chapter will be devoted to GPCMV. No attempt will be made to compare GPCMV to cytomegaloviruses of other species except in the summary when the value of GPCMV as a model for HCMV infection will be discussed. Although GPCMV infection occurs naturally in guinea pigs with the percentage of antibody-positive animals from commercial distributors varying from 8 to 50% (1), most information about GPCMV has been obtained from experimental infection of guinea pigs _in vivo_ or infection of guinea pig cells in culture. The pathogenicity of GPCMV has been previously reviewed (1-4). The purpose of this chapter is to rereview and update what is known about the pathogenicity of GPCMV and to review the molecular biology of the virus for the first time. Pre-

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viously unpublished data concerning the sequence homology of GPCMV and HCMV DNAs and the temporal regulation of GPCMV gene expression are included.

PATHOGENICITY

Intranuclear GPCMV inclusions in the salivary gland duct cells of guinea pigs naturally infected with GPCMV were first observed in 1920 (5). The investigator thought the inclusions represented the vegetative cycle of a protozoan encysted in the salivary glands and kidneys. Six years later, Cole and Kuttner (6) demonstrated that the inclusions in guinea pig salivary gland duct cells were caused by a virus. Studies in the late 20's and early 30's showed that emulsions of infected salivary glands produced immunity in guinea pigs subsequently inoculated intracerebrally with the virus (7) and that intratracheal inoculation of the emulsion caused interstitial pneumonia (8).

Acute infection

Experimental infection of guinea pigs with GPCMV has been carried out using weanling or adult animals inoculated by subcutaneous, intracerebral or intraperitoneal routes. In addition, two different strains of guinea pigs have been used: the outbred Hartley strain and the inbred strain 2. Several problems with experimental infection of guinea pigs need to be discussed. First, since GPCMV can naturally infect guinea pigs and cause a self-limiting infection in which virus can persist, it is difficult to commercially obtain animals that do not have existing antibodies. Newborn and weanling animals can possess antibody passively transferred from their mothers. Second, the infectivity of GPCMV is markedly reduced by heparin; hence, blood samples taken for analysis of infectious virus have to be collected using anticoagulants such as Alsever's solution, sodium citrate, and EDTA (9). The inhibition of GPCMV infectivity increases as the concentration of heparin increases. In several early reports (10,11), the ability of the investigators to analyze viremia during acute infection was hampered by the fact that the blood drawn from the experimental animals was collected into heparin (12).

When guinea pigs are experimentally infected with GPCMV, acute infection occurs and the disease process and outcome depend upon several variables, including the strain of guinea pigs. When weanling Hartley guinea pigs were inoculated intracerebrally or subcutaneously with salivary gland-passaged virus or virus that had been passaged only briefly in tissue cul-

ture (10), the animals developed viremia and viruria, and infectious virus was isolated from numerous organs; all but one animal remained apparently healthy through 24 days pi. By cocultivating tissues from animals infected subcutaneously with guinea pig embryo cells, the authors detected infectious virus in the brain, kidney, lung, liver, pancreas, spleen, salivary gland and thymus by 9 days pi. GPCMV was isolated from the brain tissue of animals inoculated intracerebrally or subcutaneously, but only brain tissues from animals inoculated intracerebrally showed histologic changes including intranuclear inclusions in the cerebrum, the pons and the cerebellum. The titers of virus from salivary gland and thymus increased through at least 24 days pi and were at high enough levels that they could be measured directly from tissue homogenates and did not require cocultivation. Persistent infection also was observed (see below).

In 1976, studies on GPCMV infection of adult Hartley guinea pigs were published by Dr. G. D. Hsiung (11), initiating a long and productive literature on GPCMV from Dr. Hsiung's laboratories. When adult Hartley strain guinea pigs were infected, an acute self-limiting infection similar to that reported for weanling animals was observed. The acute infection lasted approximately 10 days. The pathogenesis was similar whether salivary gland-passaged virus was introduced intraperitoneally or subcutaneously (11). A transient mononucleosis syndrome, weight loss, lymphadenopathy, transient viremia, and viruria occurred during the first 2 weeks after inoculation (12). GPCMV was detected in the blood, lung, spleen and kidney by 2 days pi. By 12 to 14 days, virus was no longer detectable in the blood and was detected in the lung, spleen and kidney only occasionally. Infectious virus was first isolated from the salivary gland at 5 days pi, persisted in the salivary gland and increased until it reached maximal levels by 3 weeks pi. At 3 to 4 weeks pi, intranuclear and intracytoplasmic inclusions were found in the duct cells of the salivary glands, mature virus particles were seen in cytoplasmic vacuoles within the duct cells and infectious virus was recoverable from the salivary gland tissue. The infected Hartley guinea pigs usually recover from infection by 4 to 6 weeks pi. When pregnant Hartley guinea pigs were inoculated with GPCMV, the acute infection was more severe and interstitial pneumonia and pronounced splenomegaly were seen (13). The death rate was higher and in those animals that survived, virus was cleared less efficiently.

The mononucleosis syndrome of adult Hartley guinea pigs was further investigated (14,15). When inoculated subcutaneously with salivary gland-passaged virus, the animals developed a mononucleosis with splenomegaly, lymph node enlargement, anemia and circulating lymphocytosis with atypical lymphocytes. Infectious virus was isolated from the plasma, the granulocyte-erythrocyte and the mononuclear fractions of the peripheral blood at a time pi when infectious virus also was isolated from numerous body tissues. Infectious GPCMV was isolated from the spleen from 3 to 30 days pi but the highest titers were found on day 7. Equally high titers of GPCMV were found in the macrophage, B-cell and T-cell populations of the spleen. The spleens reached their maximum weight at approximately 11 days pi. The hematological changes were transient and blood counts, spleen size and histology returned to normal by 1 month after inoculation with GPCMV.

Detailed examination of the enlarged lymph nodes seen during GPCMV-induced mononucleosis was also carried out (15). Infectious GPCMV was isolated from mesenteric, axillary, and cervical lymph nodes at 1 and 2 weeks pi. Histological changes in the lymph nodes were observed. When the lymph node tissue was stained by immunohistochemistry using immune guinea pig anti-GPCMV sera and avidin biotin glucose-oxidase staining, many cells demonstrated nuclear staining but only a few of these antigen-containing cells had inclusions. This study showed that cells can be infected with GPCMV without having classical inclusions.

In contrast, when adult inbred strain 2 guinea pigs were inoculated with salivary gland-passaged GPCMV, disseminated disease occurred and a large percentage of the infected animals died. Many of the animals developed a severe bilateral interstitial pneumonia which was fatal within 3 weeks (16). During disseminated GPCMV infection, the virus was able to replicate in hepatocytes, macrophages, megakaryocytes, salivary gland duct cells, fibrocytes, myocardial fibers, pancreatic acinar cells, adrenal cells, and polymorphonuclear leukocytes. Many cells contained viral inclusions including dense bodies and enveloped dense virions in the cytoplasm as well as intranuclear inclusions (16). When strain 2 guinea pigs were inoculated with tissue culture-passaged GPCMV, no fatalities occurred; infectious virus was recovered from a wide variety of tissues including the lungs, but histopathologic changes were minimal and viral inclusions were not observed (17). When strain 2 guinea pigs were vaccinated with tissue culture-passaged GPCMV and then inoculated with salivary gland-passaged

virus, only 11% of the animals developed viremia and none of the animals died.

Persistent infection

GPCMV infection also has a chronic persistent phase. The ability of GPCMV to persist, particularly in salivary gland and thymus, was first observed in studies of weanling Hartley animals inoculated with GPCMV (10). When adult Hartley guinea pigs were inoculated with salivary gland-passaged virus, infectious GPCMV was isolated from the salivary glands and pancreas 4 to 10 weeks pi (12). Inclusion-bearing cells could not be detected beyond 6 weeks pi even though infectious virus could still be isolated at 30 weeks pi (18). Persistent GPCMV infection was enhanced during pregnancy, as demonstrated by the fact that salivary gland virus titers were significantly higher in pregnant than in nonpregnant animals (13).

The persistence of low levels of infectious virus in the blood of animals 30 to 90 days after GPCMV inoculation was not detected by cocultivation but was demonstrated by the ability to transmit virus from blood samples to young antibody-free guinea pigs (12). Blood taken from animals 30 to 60 days pi caused an infection in the salivary glands of healthy guinea pigs. Blood taken from animals 60 to 90 days pi did not cause salivary gland infection but did induce anti-GPCMV antibody in healthy antibody-free recipients. Although GPCMV was isolated routinely from the salivary glands and pancreas of persistently infected animals, virus also was isolated less frequently from the spleen, kidney and urine (19). GPCMV was cleared from the bone marrow by 2 weeks pi but persisted in the thymus, the macrophage and B-cell populations of the spleen, and the lymph nodes for at least 60 days (14). The lymph nodes in persistently infected animals remained larger than those in control animals for at least a year (15).

Establishing persistent GPCMV infection in strain 2 guinea pigs requires careful control of virus inoculum and yields animals that differ from persistently infected Hartley animals in their increased incidence of viruria. Persistent viruria was seen in 57% of strain 2 animals and in only one (5%) of the Hartley animals inoculated (19). It is interesting that (a) there was no correlation between the presence of virus in the urine or the isolation of virus from the renal tissue of these animals, and (b) virus was isolated more commonly from the urine of persistently infected strain 2 females than males.

Transplacental transmission to the fetus

It is important to realize two facts about pregnancy in guinea pigs. First, the anatomy of the guinea pig placenta is very similar to that of the human placenta in that there is a single trophoblast layer (20). Second, the gestation period in guinea pigs is 68 to 70 days and the trimesters have been arbitrarily broken down into 0 to 20 or 25, 21 or 26 to 40 or 49, and 41 or 50 days to delivery.

The susceptibility of guinea pig fetuses to GPCMV infection was demonstrated as early as 1936 (21,22) by inoculating fetuses in the fourth or fifth weeks of gestation by needle puncture through the uterine wall. The fetuses developed meningitis and generalized infection. More than 40 years later it was demonstrated that GPCMV infection of pregnant guinea pigs could lead to GPCMV infection of the fetuses (23-25). When pregnant Hartley guinea pigs were sacrificed at various times after GPCMV inoculation, virus was isolated from 9 out of 37 fetuses ranging in age from 27 to 60 days and the tissues in which virus was found varied from fetus to fetus (23). From days 5 to 9 after inoculation, GPCMV was found in the blood and salivary glands of the pregnant mothers but from days 15 to 24, GPCMV was found only in the salivary glands of the mothers. When female guinea pigs were inoculated on the day they were mated and the resulting 6 pregnant mothers sacrificed from 44 to 60 days pi, no virus-infected fetal tissues were found in the 16 fetuses examined. Similarly, when animals that were persistently infected with GPCMV were mated, transmission of GPCMV to the fetuses was not seen. GPCMV was isolated from the placentas of mothers inoculated with GPCMV during pregnancy but not from mothers inoculated on the day they were mated.

In a second study (24), mothers were allowed to go to term and transmission of the virus was measured by examination of the newborns. This study differed from that of Choi and Hsiung (23) in that mothers were inoculated during the second half of pregnancy. Only 3 of the 15 guinea pigs used did not have antibody to GPCMV prior to inoculation. All 3 nonimmune animals had litters containing at least one infected newborn. Three of the 12 immune mothers had infected litters indicating that the presence of pre-existing antibody did not prevent fetal infection. GPCMV was isolated from the lung, spleen or brain of newborns from the nonimmune mothers and was isolated only from the lung of the newborns from immune mothers.

No congenital abnormalities were found in any of the infected newborns in this study or in the infected fetuses in the studies by Choi and Hsiung (23).

The work of Johnson and Connor (25) also demonstrated that GPCMV is transmitted transplacentally but the evidence was not as strong as had been reported by others. When pregnant guinea pigs were inoculated subcutaneously at 14 to 64 days gestation and sacrificed at term, transplacental transmission of GPCMV occurred in only 3 animals and all 3 were inoculated during the second trimester of pregnancy. These data indicate that GPCMV transplacental infection was limited to the second trimester. It is interesting that several of the animals inoculated during the first and third trimester did not have antibodies to GPCMV prior to experimental infection and that 2 of the mothers that bore infected fetuses did have anti-GPCMV antibodies prior to infection. This latter observation confirms previous findings that pre-existing antibody does not prevent transmission of the virus to the fetus. As in the previous two studies, no congenital abnormalities were observed in the fetuses from which virus was isolated.

Subsequent detailed studies confirmed that fetal GPCMV infection occurred regardless of when in the gestation period the mothers were inocluated (26). However, the frequency of virus infection in the newborns and of stillbirths increased when experimental infection was initiated in late gestation (26,27). Because the optimum time for isolating GPCMV from infected fetuses was 11 to 15 days after the mother was inoculated regardless of when during gestation infection was initiated, failure to isolate virus from a fetus taken close to term from a mother inoculated 55 days earlier may not mean that transplacental transmission is restricted to the second trimester but may be due to the length of time between inoculation of the mother and examination of the fetus. The ability of mothers infected during the first trimester to develop antibody may help prevent their fetuses from becoming infected more than those of mothers infected late in gestation who do not develop antibody before delivery. In recent experiments, GPCMV was isolated from newborns (3 to 15 days of age) of mothers infected during the first, second or third trimesters (28) indicating GPCMV infection persisted in fetal tissues throughout the gestation period at least in some animals. GPCMV was isolated most frequently from the salivary glands of newborns from mothers infected during early stages of gestation, whereas virus was isolated from a variety of tissues in newborns from mothers infected later in pregnancy.

Placental infection may influence whether GPCMV infection of the fetus occurs. GPCMV isolation from the placentas of infected mothers was documented even in early studies. Recently, it was shown that (a) GPCMV can be isolated from the placenta long after virus is cleared from the maternal blood; (b) virus can be isolated from the placenta whenever fetal infection occurs; and (c) fetal infection does not always occur when there is placental infection (29). These findings indicate that the placenta can be a reservoir for GPCMV but may also limit transmission of the virus to the fetus.

The data accumulated on GPCMV infection of fetuses suggest that fetal infection mimics that of the adult. During the first 10 to 14 days after fetal infection, infection is acute and the virus can be isolated from a variety of organs and tissues. With time, virus persists only in the salivary gland and to a lesser extent in the spleen. The extent and severity of fetal infection is also influenced by the presence of circulating maternal antibody. Guinea pigs that have seroconverted prior to pregnancy are able to transfer immunity to their offspring but the antibody is short-lived (30). The most long lasting protection is seen in animals born to mothers that experienced infection during pregnancy, but it is not possible to determine whether these offspring obtained their neutralizing antibody passively from their mothers or developed their own antibody as a result of the intrauterine infection.

Strain 2 guinea pigs also can be used to study transplacental transmission of GPCMV (31), although to prevent maternal death, it was necessary to inoculate with low doses of virus. As had been previously demonstrated for Hartley guinea pigs, placental and fetal infection of strain 2 animals occurs regardless of the stage of pregnancy at the time of maternal inoculation. Since all mothers in the only study carried out to date were sacrificed by 4 weeks pi, future studies will have to be done to determine how long infection of the strain 2 fetuses persists, the eventual extent of infection, and whether the fetuses will survive to term.

When mothers vaccinated with either low passage tissue culture-passaged GPCMV or an envelope antigen vaccine prepared from virions and dense bodies were challenged with salivary gland-passaged GPCMV, GPCMV was isolated from the tissues of 27% of the fetuses of control nonimmune mothers

and from <1% of the vaccinated mothers (32). Vaccinated animals continued to shed GPCMV for long periods of time, as detected in throat cultures (33). In addition, when these animals became pregnant at 45 to 55 weeks after vaccination, 41% of the pregnant vaccinated animals had positive throat cultures for GPCMV compared with 28% of the nonpregnant vaccinated controls. When weanling animals were vaccinated with low doses of a high passage tissue culture-passaged GPCMV, virus was not shed and pregnancy did not reactivate the virus. GPCMV was not detected in fetuses or newborns of animals vaccinated with low or high passage tissue culture-passaged virus. In addition, both low and high passage vaccine protected fetuses from transplacental transmission of GPCMV following challenge of the pregnant animals with the more virulent salivary gland-passaged virus.

MOLECULAR BIOLOGY

Characterization of GPCMV DNA

Two different cell lines were used for preparation of GPCMV DNA (34). The first was guinea pig embryo fibroblast (GPEF) cells. The second was the 104Cl cell line, a benzo(a)pyrene-transformed and cloned line derived from strain 2 guinea pig embryo cells (35). Purification of GPCMV DNA yielded an average of 80 µg of GPCMV DNA/10^8 GPEF cells and 110 µg/10^8 104Cl cells. When confluent GPEF cell cultures were transfected with calcium phosphate-precipitated GPCMV DNA, typical GPCMV CPE became apparent 7 to 9 days after transfection indicating that there were at least some full-length GPCMV DNA molecules present in the purified GPCMV DNA samples used.

When the restriction endonuclease cleavage patterns were determined for GPCMV DNA and compared with those for HCMV DNA, it was apparent that each virus DNA had its own distinct electrophoretic profile (34). Cleavage of GPCMV DNA with *Hin*dIII generated at least 21 fragments ranging in size from 30.9×10^6 to 0.5×10^6 daltons; *Eco*RI cleavage generated at least 36 fragments ranging from 27.9×10^6 to 0.6×10^6 daltons; and *Xba*I cleavage generated at least 40 fragments ranging from 22.4×10^6 to 0.6×10^6 daltons (36). Further characterization showed that GPCMV DNA has a CsCl buoyant density of 1.713 g/cm^3 [guanosine plus cytosine (G + C) = 54.1%] which is slightly less than that of HCMV DNA (34).

Molecular cloning and mapping the genome

Sixteen of the 21 GPCMV *Hin*dIII fragments were cloned (36). Twenty-eight of the 36 GPCMV *Eco*RI fragments were individually cloned into the pACYC184 and pBR322 vectors. Failure to clone *Hin*dIII-A was compensated by the ability to clone *Eco*RI-D, -C, and -H, which are colinear with *Hin*dIII-A.

The *Hin*dIII, *Eco*RI, and *Xba*I restriction endonuclease cleavage sites were mapped to specific sites on the GPCMV genome by hybridizing ^{32}P-labeled fragments to Southern blot transfers of total GPCMV DNA cleaved with the three different enzymes. Each ^{32}P-labeled cloned *Hin*dIII or *Eco*RI fragment hybridized only to a band in its own digest identical to itself in electrophoretic mobility. No cross-hybridization between any internal fragments was seen. Cross-hybridization to multiple bands in each of the other two digests was observed and made it possible to position many of the fragments on the genome. Three terminal fragments were identified. It was concluded from studies carried out to identify the terminal fragments that two populations of GPCMV molecules exist. The predominant form (70% of the population) consists of molecules in which both terminal fragments contain repeat sequences of a maximum of 0.7×10^6 daltons (*Hin*dIII-R and -M, *Eco*RI Y and A, and *Xba*I-N and -F). The minor population (about 30%) consists of molecules in which one terminal fragment (*Hin*dIII-R, *Eco*RI Y, and *Xba*I-N) is identical to that in the predominant structural form, whereas the remaining terminal fragment (*Hin*dIII-O, *Eco*RI B, and *Xba*I-G) is identical except it is missing the 0.7×10^6-dalton repeat sequence.

The data obtained from hybridization with cloned, gel-isolated internal and terminal fragments and from double digestions allowed linear arrangement of all the GPCMV *Hin*dIII fragments. All but four of the *Eco*RI fragments were aligned. The *Xba*I restriction endonuclease cleavage map was also generated except for three regions of uncertainty. Two important conclusions were obtained from the molecular cloning and physical mapping of the GPCMV genome: (a) the size of GPCMV DNA was calculated to include 239 kilobases (Kb), corresponding to a MW of 158×10^6; and (b) the GPCMV genome consists of a long unique sequence with terminal repeat sequences but without internal repeat regions. The structural organization of GPCMV DNA is unique for a herpesvirus DNA; it does not contain the four isomer configurations and is more similar in its organization to the structure reported for murine CMV DNA (37).

Homology between GPCMV and HCMV DNA sequences

No detectable DNA sequence homology has been reported between DNA from HCMV isolates and simian or murine strains. When cloned HCMV Towne strain XbaI fragments (38) were reacted with GPCMV DNA, homology with GPCMV DNA was located between 0.5 and 0.77 map units on the Towne strain genome (34). When GPCMV DNA was reacted with total HCMV AD169 DNA cleaved with HindIII or XbaI, the sequences of HCMV DNA homologous with GPCMV DNA mapped between 0.06 and 0.33 units on the AD169 genome (approximately 25% of the genome). The differences in the map position between Towne and AD169 strain DNAs simply reflect the fact that the orientation for the long unique segment published for AD169 DNA is inverted relative to that for Towne DNA.

The locations of the homologous HCMV DNA sequences on the GPCMV genome also were mapped (Fig. 1). The regions of the GPCMV genome which share sequence homology with HCMV AD169 DNA are located at 0.05-0.09 and 0.52-0.73 map units and taken together they represent a size of about 58 Kb or approximately 25% of the GPCMV genome.

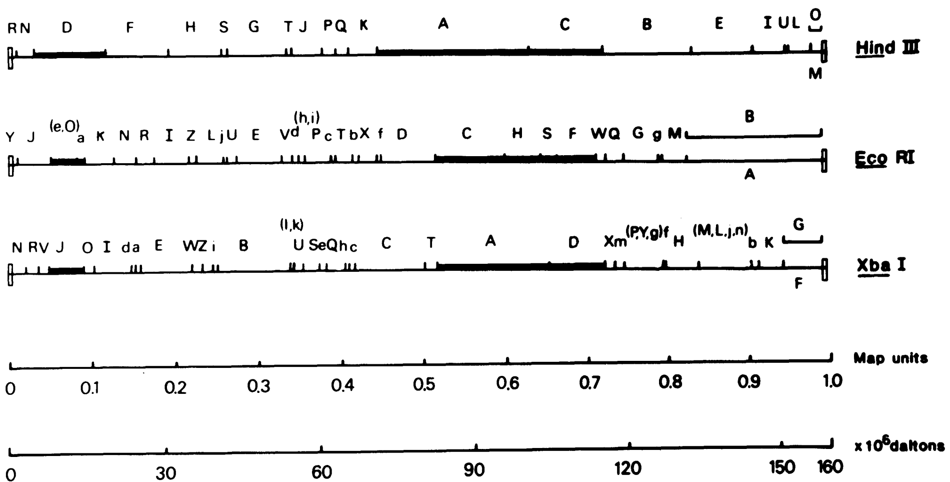

Fig. 1. Location on the GPCMV restriction endonuclease maps of the regions of DNA sequence between GPCMV and HCMV DNA. Dark bars indicate fragments showing some homology with HCMV AD169 DNA. The map without the dark bars is reproduced with permission from the American Society for Microbiology (36).

The region of the GPCMV genome which shares sequence homology with HCMV AD169 *Hin*dIII-E was further analyzed. The HCMV AD169 *Hin*dIII-E fragment contains the DNA sequences associated with transformation by HCMV AD169 DNA and also contains the major immediate early (IE) genes (39,40). HCMV AD169 *Hin*dIII-E hybridized with GPCMV *Hin*dIII-D; *Eco*RI-e, -O, and -a; and *Xba*I-J. Experiments were carried out to test whether the apparent hybridization between GPCMV and HCMV DNAs was blocked by the presence of high G + C content DNA, such as *Micrococcus luteus* DNA (71% G + C). Sonicated, denatured vector DNA (pBR322) was also added to prehybridization and hybridization buffers to decrease background hybridization. For experiments, 0.2, 0.1, and 0.05 µg of cloned GPCMV *Hin*dIII-D were cleaved with *Hin*dIII, subjected to electrophoresis on 0.5% agarose gels and transferred to nitrocellulose filters. When total GPCMV DNA isolated from virions was used as probe, the results obtained in the presence and absence of *M. luteus* DNA were indistinguishable, indicating that addition of the high G + C content DNA did not block authentic hybridization. Similar results were observed when HCMV *Hin*dIII-E was used as a probe (Gao and Isom, unpublished data). This finding indicated that the DNA sequence homology between GPCMV *Hin*dIII-D and HCMV *Hin*dIII-E fragments was not simply due to high G + C content regions binding to each other, but rather to authentic base homology.

To study sequence homology between GPCMV and HCMV DNAs, the T_m or the stringency of hybridization was altered (Fig. 2). GPCMV *Hin*dIII-B, which did not show any hybridization at T_m -25°C with HCMV AD169 total DNA or with HCMV *Hin*dIII-E fragment was used as the control. As expected, the GPCMV *Hin*dIII-D fragment hybridized with itself at the same intensities but not to GPCMV *Hin*dIII-B, under the three different hybridization conditions. When HCMV *Hin*dIII-E was used as a probe, it hybridized with GPCMV *Hin*dIII-D but not with *Hin*dIII-B at T_m -25°C, which confirmed previously obtained results. However, when the stringency of hybridization was increased by elevating the formamide concentration to 57% (T_m -15°C), no hybridization was detected between HCMV AD169 *Hin*dIII-E and GPCMV *Hin*dIII-D. This finding indicated that HCMV AD169 *Hin*dIII-E and GPCMV *Hin*dIII-D possess sequence homology between 83 to 90% of their base pairs since they form thermally stable hybrids at T_m -25°C but not at T_m -15°C. In contrast, under the condition of T_m -40°C, HCMV *Hin*dIII-E not only hybridized with GPCMV *Hin*dIII-D but also with GPCMV *Hin*dIII-B, although at a lower intensity.

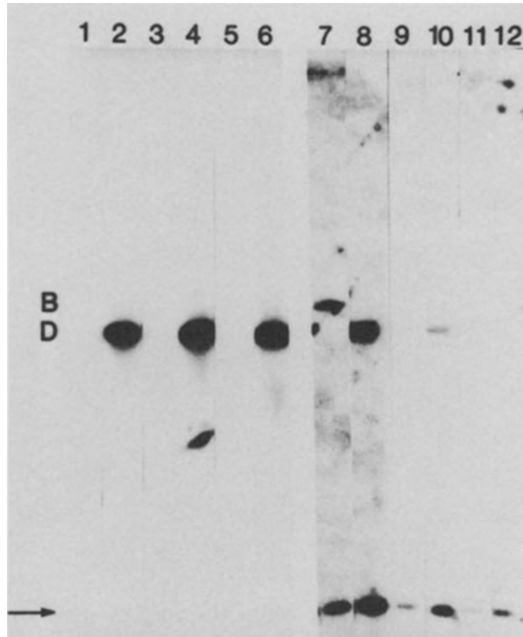

Fig. 2. Effect of reaction conditions on the hybridization between GPCMV HindIII-D and HCMV HindIII-E. For experiments, 0.05 μg of cloned GPCMV HindIII-B (lanes 1, 3, 5, 7, 9, and 11) and 0.05 μg of cloned GPCMV HindIII-D (lanes 2, 4, 6, 8, 10, and 12) were cleaved with HindIII, subjected to electrophoresis on 0.5% agarose gels, and transferred to nitrocellulose filters. ^{32}P-labeled cloned GPCMV HindIII-D (lanes 1-6) and ^{32}P-labeled cloned HCMV AD169 HindIII-E (lanes 7-12) were used as probes for the hybridizations at T_m -40°C (lanes 1 and 2, 7 and 8), T_m -25°C (lanes 3 and 4, 9 and 10) and T_m -15°C (lanes 5 and 6, 11 and 12). The arrow indicates the location of the pBR322 DNA band.

Transcription of IE, early and late RNAs

The library of cloned GPCMV DNA fragments and the physical maps made it possible to examine GPCMV transcription. GPCMV IE RNA was defined as the RNA isolated from cells at 4 hr pi in the presence of cycloheximide. Virus and cellular protein synthesis were inhibited by cycloheximide (200 μg/ml). In the absence of cycloheximide, multiple cell proteins were observed in lysates from mock- and virus-infected cells but no virus-specific proteins could be detected. GPCMV IE RNAs synthesized from infected GPEF cells in the presence of cycloheximide comprised three size classes of abundant transcripts of approximately 3.4, 2.8 and 1.5 Kb and several minor

classes of transcripts. The DNA coding regions for the IE RNAs were determined by the hybridization of cDNA (both oligodeoxythymidylic acid- and randomly-primed) synthesized from the GPCMV poly A^+ IE RNA fraction to cloned fragments (Fig. 3). The abundant IE transcripts originated from HindIII-D, -G, and -B. Hybridization also was detected to the neighboring fragments, HindIII-F and -H, HindIII-E, -I, and -L but the intensity of these bands was less.

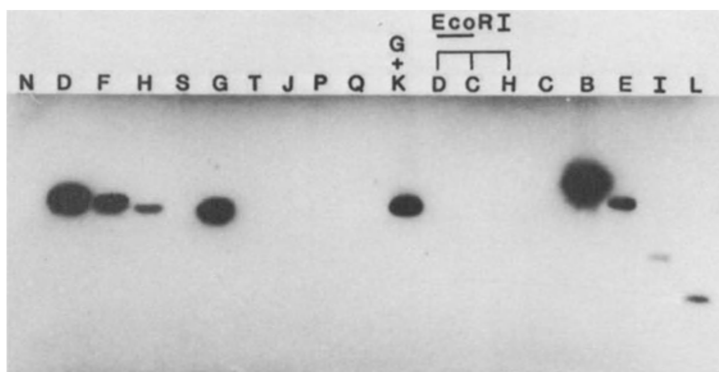

Fig. 3. Southern blot of IE cDNA probes hybridized to cloned fragments of GPCMV DNA. GPCMV HindIII and EcoRI fragments were arranged according to their map positions. Recombinant plasmids were cleaved with respective restriction endonucleases, separated on a 0.5% agarose gel, transferred to a nitrocellulose filter and hybridized to ^{32}P-labeled cDNA synthesized from poly A^+ IE RNA.

To determine the size classes of RNAs encoded by specific regions, northern blot hybridizations of the IE poly A^+ RNAs were carried out. The 3.4 Kb-size class RNA could be detected with HindIII-D and -B probes and at low abundance with HindIII-E and -I probes. The 2.8-Kb size class RNA could be detected with HindIII-D and -B probes, and weakly with HindIII-E and -I probes. The 1.5-Kb size class RNA could be detected with HindIII-D, -G, -T, and -E probes. Hybridization to HindIII-T was detected using northern blot hybridization but not using the cDNA method. Minor size class RNAs were also detected.

When early GPCMV RNA was analyzed by northern blot hybridization, 5 size classes of RNAs (5.6, 5.1, 2.8, 2.1, and 1.5 Kb) predominated. RNAs of 9.5 and smaller than 1.0 Kb also were detected at a lower abundance. When the *Hin*dIII-D fragment was used as a hybridzation probe for the IE and early RNAs, respectively, three size classes of IE RNA were detected at 3.4, 2.8, and 1.5 Kb, but only the 1.5-Kb size class was seen early; that is, two size classes of RNA synthesized from the *Hin*dIII-D region decreased in abundance when transcription switched from IE to early time after infection. cDNA synthesized from GPCMV early poly A^+ RNA, hybridized to all fragments except *Hin*dIII-K and the smallest fragment *Hin*dIII-T. *Hin*dIII-N, which was not expressed at IE times, and *Hin*dIII-L, which was expressed but at a low abundance at IE times, showed the highest degree of hybridization at early times after infection. At late time after infection, heterogeneous bands of RNA ranging in size from 5.0 to smaller than 1.0 Kb were detectable. Four size classes of RNAs of approximately 9.5, 8.6, 7.3, and 6.9 Kb were also seen. At late times after infection, RNAs were transcribed from all the cloned fragments. Transcription also originated from the terminal fragment, *Hin*dIII-M, as detected by the hybridization of acid-primed cDNA to *Hin*dIII-cleaved total GPCMV virion DNA. Failure to see hybridization to the other terminal fragment (*Hin*dIII-R) suggests that at late times either this fragment was expressed at low levels or was not expressed at all. Hybridization to *Hin*dIII-K and -T could be detected only using cDNA probes synthesized from late RNAs.

More than 70% of the IE transcripts were derived from the *Hin*dIII-D, -G, and -B fragments. Early RNAs were transcribed from 16 out of 18 cloned fragments but 35% of the early RNAs were derived from the *Hin*dIII-N and -L fragments. Late RNAs were transcribed from recombinant DNAs representing 99% of the virus genome. Different patterns of percent of hybridization occurred at IE, early, and late times after infection indicating temporal regulation of GPCMV transcription (Fig. 4).

IN VITRO REPLICATION

In vitro cultivation of GPCMV was first accomplished in 1957 in the fibroblast cells of explant cultures of guinea pig embryo muscle (41). The progression of cytopathic effects was slow, with the majority of cells eventually developing large, elongated, often kidney shaped eosinophilic intranuclear inclusion bodies. The virus was passed at first using ground in-

fected cells and later using cell-free fluids. GPCMV also replicated in explant cultures of salivary gland tissue from infected animals. Repeated attempts to replicate GPCMV in human cells failed. The GPCMV propagated in this study was designated strain 22122 and was used in the biological and molecular studies described above.

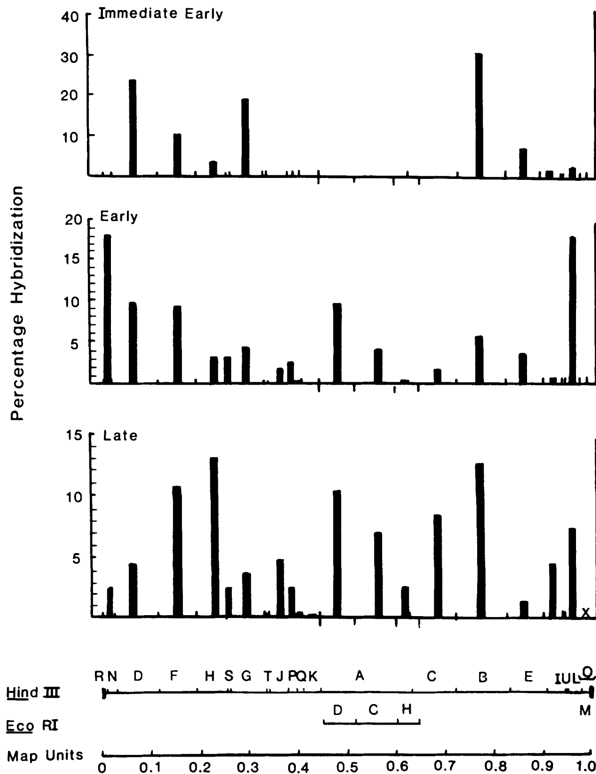

Fig. 4. Relative amount of hybridization of RNAs to GPCMV DNA. The percent of transcription from various regions of GPCMV HindIII and EcoRI physical maps was determined from densitometer scans of autoradiograms of hybridizations of cDNA synthesized from IE, early, and late poly A^+ RNA to cloned virus DNA fragments. (x) Hybridization of cDNA synthesized from late poly A^+ RNA to the terminal HindIII-M fragment from total virus DNA was observed but the percentage of hybridization was not calculated.

A more detailed analysis of GPCMV replication in GPEF cells showed that maximum virus yields were obtained 5 days pi (42). GPCMV replication in GPEF cells was detected as early as 16 hr pi by the presence of nonviral tubular structures in the nucleus (42-44). The tubular structures were

detected before the appearance of intranuclear inclusions and nucleocapsids. Nucleocapsids, nucleocapsids associated with dense matrices, enveloped dense virions, and dense bodies without virus capsids were seen in the cytoplasm. GPCMV antigens were detected on viral capsids and on electron-dense amorphous matrices but not on tubular structures (45).

Although GPCMV did not replicate in guinea pig kidney cells in culture (42), GPCMV replicated in hepatocytes in culture (46); therefore, it is not possible to make the generalization that GPCMV will not replicate *in vitro* in an epithelial cell. When hepatocytes in primary culture were infected with GPCMV, the yields were below those previously reported for GPEF cells and the replication cycle was slower with the eclipse period lasting 3 to 4 days.

To date, GPCMV has been shown to replicate only in cells of guinea pig origin. GPCMV did not replicate in rabbit kidney cells, human embryonic kidney cells, human diploid lung fibroblast cells, mouse embryo fibroblast cells, and primary green monkey kidney cells (42). When mouse NIH3T3 cells were infected with GPCMV, IE GPCMV was not expressed, indicating that GPCMV did not even abortively infect NIH3T3 cells (Gao and Isom, unpublished data). It has recently been demonstrated that GPCMV also replicates in 104C1 cells (34,35,47). When the replication of GPCMV in 104C1 cells was compared to that in GPEF cells using *in situ* hybridization as well as more conventional techniques, GPEF cells were found to be considerably more sensitive to infection than 104C1 cells (48). GPCMV infection of 104C1 cells remained localized to foci of infected cells and the rapid spread of GPCMV usually seen in GPEF cells was not observed in 104C1 cells. Viral antigens were expressed in less than 25% of the 104C1 cells and low levels of GPCMV replication were evident. In 104C1 cells, GPCMV could be detected earlier during the course of infection when *in situ* hybridization was used than when the methods of antigen detection, virus isolation or cytopathology were employed. Biotin-labeled hybridization probes prepared from recombinant plasmids containing GPCMV fragments were used to detect nucleic acids by *in situ* hybridization. Specific hybridization was detected in both cell types whether a single GPCMV recombinant DNA fragment or a mixture of fragments was used as probe.

The availability of cells in culture that support the productive replication of GPCMV has made it possible to determine the time course of GPCMV DNA synthesis. Approximately the same results were obtained using

three different techniques. By electron-microscopic autoradiography viral DNA synthesis began at approximately 18 hr pi and cellular DNA synthesis was inhibited prior to the onset of virus DNA synthesis (48). Results from in situ hybridization showed that GPCMV DNA was first detected at 16 hr pi (47). Similarly, when the kinetics of [^{3}H]thymidine incorporation into virus and cellular DNAs were measured using cesium chloride gradient centrifugation, (a) incorporation of radioactive label into GPCMV DNA was detectable at 16 but not at 12 hr pi, and (b) cellular DNA synthesis declined and became undetectable by 8 hr after the onset of virus DNA replication (Gao and Isom, unpublished data).

CONCLUSION

The species specificity of HCMV prevents the study of HCMV in animals and necessitates finding an appropriate animal model. HCMV has numerous roles in human disease. Infection of the immunocompromised host, transmission by transfusion, and transplacental transmission of HCMV to the fetus result in high morbidity and mortality rates. HCMV can persist or become latent in the human host awaiting reactivation. To date, it is not known which human tissues or cells harbor HCMV in an inapparent state. The similarities in the pathogenicity of GPCMV and HCMV in their respective hosts are impressive and include transient viremia and a mononucleosis syndrome in the healthy human adult; interstitial pneumonia, disseminated infection, and susceptibility to superinfection in the immunocompromised human patient; and transmission by blood transfusion. The similarities between congenital CMV infection in humans and guinea pigs are equally striking. The risk of congenital CMV infection following primary maternal infection during pregnancy has been estimated at 58% in humans and was shown to be 54% in guinea pigs (28). In both species, virus is isolated from the same organs and tissues, viruria is seen and the disease in the fetus or newborn ranges from subclinical to severe generalized infection. Similarities between HCMV and GPCMV at the molecular level are just beginning to be studied. The size and G + C content of the two virus DNAs are very similar, but the structural organization of the GPCMV genome is considerably less complex than that of HCMV. The finding that some sequence homology exists between GPCMV and HCMV DNAs is of considerable interest and requires further investigation. It would be of particular interest to determine whether parallels in pathogenicity can be related to functional sim-

ilarities at the level of gene expression. The molecular cloning of the GPCMV genome has generated reagents that can be used to examine the mechanisms of pathogenicity in the animal at the molecular level. The use of in situ hybridization and other molecular techniques will make it possible to identify the cell types in which the GPCMV genome persists, establish in what state the genome exists when not expressed in these cells, and investigate the mechanism of reactivation of gene expression.

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NUCLEOTIDE SEQUENCE COMPARISON OF SIMIAN ADENOVIRUSES SA7P AND SA7: IMPLICATION FOR THE CLASSIFICATION OF SA7P

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ABSTRACT

A simian adenovirus strain isolated in Moscow was originally characterized as SV20. Subsequently, on the basis of immunological data and restriction-enzyme analysis it was retyped as an antigenic variant of simian adenovirus SA7 (S16), and named SA7P. Recently, the E1A genes of SA7P and of the authentic SA7 have been sequenced. In this paper the two sequences are compared, and found to diverge by 5%. The same degree of divergence applies to the amino acid sequences of the predicted E1A proteins. This relatively large difference combined with the comparative immunological and restriction-analysis data constitutes sufficient cause to classify simian adenovirus SA7P as an independent new type e.g. S25.

INTRODUCTION

Adenoviruses(Ad) are not only studied for their pathogenic effect they exert in their natural hosts, but also because they can elicit a malignant response in animals in which they normally do not multiply. In addition, the adenovirus has provided molecular biologists with a model system, that has yielded some singularly interesting new concepts. For instance, the phenomenon of eukaryotic mRNA splicing was first described for the late RNAs of human Ad type 2 (Ad2). Till now, the role of Ads in cell transformation and oncogenesis has been studied in particular for the human adenovirus type 2, 5 and 12. From these investigations it became clear that only a small region of the 36kb Ad genome is responsible for oncogenic transformation. This region consists of the E1A and E1B transcription units which are located at the extreme left 11% of the linear Ad DNA. The nucleotide sequences of these genes were determined (1) for Ad12 (group A - highly oncogenic), for Ad7 (2) (group B - weakly oncogenic), and for the highly related serotypes Ad2 and Ad5 (3,4)) group

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C - non-oncogenic). When compared, the ElA and ElB sequences from group A, B and C showed an overall homology of about 55% (5,6), whereas the two group C representatives among themselves were more than 98% homologous.

When one of us (R.A.G.) embarked on a study of the transforming genes of simian Ads he chose to investigate a strain that had been isolated in 1965 in the Institute of Polyomyelitis and Viral Encephalitides in Moscow from a culture of African Green Monkey kidney cells and typed at the time as simian adenovirus M7 (SV20). At the beginning of this investigation he did not have at his disposal a standard strain, but when at the Moscow Institute of Virology such a strain was obtained from Dr. Kalter (USA), it became possible to recheck the virus type. It was then established, that the strain which up to that time has been described as SV20P actually was not at all related to the standard SV20, but showed a much greater resemblance to simian AdSA7. On the basis of restriction analysis, serological data and heteroduplex electron microscopy (7), SV207 was renamed SA7P (the P does not stand for prototype but for Polyomyelitis and refers to the Institute where it was first isolated), "an antigenic variant of SA7".

At the department of Medical Biochemistry of Leiden University the primary structures of the leftmost XhoI fragment and of the inverted terminal repeats (ITRs) of SA7P were determined (8). The simian virus DNA sequence showed extensive homology with the corresponding human virus sequences. This homology allowed us to recognize the exons of the ElA region and to situate the XhoI site flanking the segment to the 3' side to the ElB 21kD reading frame. Somewhat earlier, Skripkin et al. had sequenced the ITRs of the authentic SA7 (9), and a short while later, Kimelman et al. (10) reported the nucleotide sequence of the ElA region for this virus. In this paper we will compare the ElA sequences of SA7P and SA7, and review some other properties of the two viruses. On the basis of these comparisons, we will discuss whether SA7P is a correct designation for the strain studied by us.

Comparison of the SA7P and SA7 ElA sequences

Figure 1 shows the sequences of the ElA genes of the simian Ads SA7P and SA7 printed underneath each other. The SA7 sequence is that reported by Kimelman et al. (10), which lacked the three 5' terminal nucleotides. The latter were derived from a paper of Skripkin et al. (9) who determined the inverted terminal repeats (ITR) of SA7. These authors observed

```
   1  CATCATCAAT AATATACCTT ATTTGGGAAC GGTGCCAATA TGCTAATGAG GTGGGCGGAG TTTGGTGACG TATGCGGAAG TGGGCGGAGC AAGGGGCGGG
      ***** **                                                                              *          * *
   1  ---TCTATAT AATATACCTT ATTTGGGAAC GGTGCCAATA TGCTAATGAG GTGGGCGGAG TTTGGTGACG TATGCGGAAA TGGGCGGAGT TAGGGGCGGG

      ---TCTATAT Kimelman et al. (10)
      CATCATCAAT Skripkin et al.  (9)
      ----ATCAAT Tolun et al.    (12)
                                                             enhancer 1                     ↓ end ITR
 101  G-----CGAG AGGCGGGGCT TTTGGTAGGC GTGGCCGGGC GTGGGAACGG AAGTGACGTC GGGGGCGCGC CGGACGTGAC GTGTTTTGAG GCGTTTTAAA
       *****  **      *     ***  **  *    *                           *                *        ** * *    *
  98  GTTTGGCGGT AGGCGTGGCT ---GGG-GGA GTGTCCGGGC GTGGGAACGG AAGTGACGTA GGGGGCGCGC CGGAGGTGAC GTCGTGTGGG GAGTTTTAAA
                                                                                                      ↑ end ITR
                                                                                   enhancer 2
 196  CCGGAAGCAA GGTATTTTAA ACGCTTGCA- GCGCAATTTT GCCGGTTTTG GCGCGAAAAC TGATAAAAAG CGGAAGTTCG GTTAATCATT AATTTTTACG
                                     *             *
 194  CCGGAAGCAA GGTATTTTAA ACGCTTGCAA GCGCAATTTT GTCGGTTTTG GCGCGAAAAC TGATAAAAAG CGGAAGTTCG GTTAATCATT AATTTTTACG

 295  ATAGGGAGGA ATATTTACCG AGGGCCGGTG AACTTTGAGC GATGACGCGG TGGTTTCGTT ACGTGGCACC ACCACGCGAC TGCTCAAAGT CCCCGTTTAT
                                                   *
 294  ATAGGGAGGA ATATTTACCG AGGGCCGGTG AACTTTGAGC GGTGACGCGG TGGTTTCGTT ACGTGGCACC ACCACGCGAC TGCTCAAAGT CCCCGTTTAT

                     TATA                              ↓ cap                                    E1a protein start
 395  TGTCTAGGTG AGGGTATTTA AACCGGCTCA GACCGTCAAG AGGCCACTCT TGAGTGCCCG CGAGAAGAGC TTTCTCCTCT TTCGCTGCGA AAATGAGACA
                                         *
 394  TGTCTAGGTG AGGGTATTTA AACCGGCTCA GAACGTCAAG AGGCCACTCT TGAGTGCCCG CGAGAAGAGC TTTCTCCTCT TTCGCTGCGA AAATGAGACA
                                                       ↑

 495  CTTGGCGTTG GAGATGATGT CTGAACTGCT GGATTTAGGA CTGGATACCA TCGATAGCTG GCTGCACACC GAATTCGCGC CGGTACCGAC GGGGGTGAGT
                   *     *                                    *   *                     ***           *
 494  CTTGGCGTTG GAAATGATTT CTGAACTGCT GGATTTAGGA CTGGATACCA TTGATGGCTG GCTGCACACC GAATTTCGGC CGGTACCGGC GGGGGTGAGT

 595  CATAACATGT CGCTGCACGA AATGTACGAC CTGGACGTTA CCGGCCAGGA GGATGAGAAC GAAGAGGCGG TAGACGGTGT TTTTTCCGAT GCGATGCTCC
                                                                                           *
 594  CATAACATGT CGCTGCACGA AATGTACGAC CTGGACGTTA CCGGCCAGGA GGATGAGAAC GAAGAGGCGG TAGATGGTGT TTTTTCCGAT GCGATGCTCC

 695  TGGCCGCTGA AGAGGGAGTA GAAATGCCTA GTCTTTATTC TCCGGGACCT CTGGTTGGGG GAGGTGAGAT GCCTGAGCTA CAGCCTGAGG AGGTAGATCT
             *   *      *              *                                      **         *  *               *
 694  TGGCCGCGGA GGAGGGAATA GAAATGCCTA ATCTTTATTC TCCGGGACCT CTGGTTGGGG GAGGTGCAAT GCCTGAACTT CAGCCTGAGG AGGAAGATCT

                                                     ↓ splice donor 12S mRNA
 795  TTTCTGCTAC GAAGATGGCT TCCCTCCCAG TGACTCTGAG GAAGGTGAGC ATTCGCAGGT GGAGACGGAA CGTAAAATGG CGGAGGTGGC GGCGGCAGGT
                                                                              *                     *       *
 794  TTTCTGCTAC GAAGATGGCT TCCCTCCCAG TGACTCTGAG GAAGGTGAGC ATTCGCAGGT GGAGACAGAA CGTAAAATGG CGGAGGCGGC GGCAGCAGGT
                                                     ↑

 895  GCGGCGGCGG CCGTCCGCGG GGAGCAAGAT GACTTTCGCT TAGACTGTCC TAGCGTACCT GGCCATGGCT GTAGCTCCTG TGACTACCAT CGCAAAAATA
                    **  **                                                                                      *
 894  GCGGCGGCGG CCGCGCGGCG GGAGCAAGAT GACTTTCGCT TAGACTGTCC TAGCGTACCT GGCCATGGCT GTAGCTCCTG TGACTACCAT CGCAAAACTA

                                                                          ↓ splice donor 13S mRNA
 995  GCGGCTGTCC TGAAATTCTG TGCTCGCTGT GCTATCTGAG GGCTAACAGC ATGTTTATTT ATAGTAAGTG AATTTTT-CT ACTAACTTTC TCGCTGTGTG
                                                                                 *        *                 *
 994  GCGGCTGTCC TGAAATTCTG TGCTCGCTGT GCTATCTGAG GGCTAACAGC ATGTTTATTT ATAGTAAGTA AATTTTTTCT ACTAACTTTC TCGTTGTGTG
                                                                          ↑
                                                                splice acceptor 12S, 13S mRNAs ↓
1094  TTTGCTCGCT CGTTCGCT-- -AACTGCTGG GGTGCTTGGT GTTGGGACTG AGCTTACAGG TATTTTTCTC TGTAATTTTC CACAGGTCCA GTTTCTGACT
                   *     ** *                  *                              *                 *
1094  TTTGCTCGCT CGCTCGCTCG CAACTGCTGG GGTGCTTGCT GTTGGGACTG AGCTTACAGG TATTTT-CTC TGTAATTTTC CATAGGTCCA GTTTCTGACT
                                                                                                   ↑

1191  CTGAGCCAGA CGAGCCTGAC TCCACAACAG CTGATTCAAA TCATGGCAGC CCGCCAACCC TTCGCTGCAC CCCACCCAGG GACTTGCCGC GACCTGTGCC
                             *
1193  CTGAGCCAGA CGAGCCCGAC TCCACAACAG CTGATTCAAA TCATGGCAGC CCGCCAACCC TTCGCTGCAC CCCACCCAGG GACTTGCCGC GACCTGTGCC

1291  AGTGAAGGCC TCTCATGGCA AGCGCCCAGC GGTGAACAGC TTGCATGACC TCATAGAGGA GGTTGAACAA ACAGTACCTT TGGACCTGTC CCTAAAGCGC
                          *
1293  AGTGAAGGCC TCTCCTGGCA AGCGCCCAGC GGTGAACAGC TTGCATGACC TCATAGAGGA GGTTGAACAA ACAGTACCTT TGGACCTGTC CCTAAAGCGC

                  stop E1a proteins                              poly(A) signal
1391  TCTAGGAGCA ATTAGGGTCA TAAAACCCCT CCCCTTCCCC TTAAGTTATA AGGAAATAAA AAGATTAACT GGATTCTTTG TGCCT
                         *                                 *
1393  TCTAGGAGCA ATTAGGGTTA TAAAACCCCT CCCCTTCCCC TTAAGTTATC AGGAAATAAA AAGATTAACT GGATTCTTTG TGCCT
```

Fig.1. The aligned sequences of the ElA genes of SA7P (upper rows) and SA7 (lower rows). Gaps resulting from alignments shifts have been filled with dashes. The asterisks denote the positions where the two sequences differ from each other. A number of strategic sites has been indicated.

a heterogenicity of the terminal eight residues: the sequence given here (CTATCTAT) is present in the majority of SA7 DNA molecules, whereas a minority has the sequence CATCATCA. This string (which is encountered most frequently at the end of adenovirus genomes.; see ref. 11) was the one found for SA7P, and partly also by Tolun et al. (12) in their incomplete sequence of SA7.

In order to obtain an optimum alignment the sequences had to be shifted slightly in some instances. These interventions were so minor that we did not have to resort to a computer to determine the optimum alignments. The gaps resulting from the applied shifts have been filled with dashes. They occur only in non-coding sequences, i.e. the 5' non-coding region and the ElA intron. The asterisks between the two sequences denote the positions where the strains differ.

The ElA regions of the human Ads encode families of coterminal messengers (13S, 12S and 9S) which differ from each other in the amount of material removed by RNA splicing. Kimelman et al. (10) have characterized the 13S and 12S homologues for SA7 ElA. They have coding space for proteins of 28.9 kD and 21.0 kD, respectively. The third mRNA encoded by the ElA gene of human Ads (9S RNA) has not (yet) been found. In SA7P DNA the splice donor and acceptor sites for the 138 species could be predicted on the basis of the homology with its human counterparts (8). The 12S RNA splice donor could not be predicted in this way, but this splice site can now also be designated since the SA7 and SA7P sequences are well conserved in strategic regions.

A superficial inspection of Fig.1 shows that the differences between SA7P and SA7 are evenly distributed along the ElA region; as already mentioned, insertions and deletions occur only outside the coding areas. It is noteworthy that, apart even from the difference in the 8 terminal nucleotides, the ITRs are among the least conserved tracts. If a comparison between SA7P and SA7 had been limited only to the ITRs one might have concluded straight away that the two viruses belong to different types.

We also compared the protein-coding regions of the SA7P ans SA7 13S RNAs codon by codon. To this end, we developed a computer program which in two related genes screens pairs of corresponding codons to establish which, if any, positions in each codon pair are occupied by different bases. In addition, this program classifies the encoded amino acid pairs according to the degree of their relatedness. The result of this scree-

ning is given in Table 1. It shows (in row "Total") that of the 266 codon pairs compared, 25 are different. There are 13 cases of a third-letter change (column 4), 12 of which were silent. There are three instances of codons in which two nucleotides are altered. In all, the SA7P and SA7 29kD proteins diverge at 13 positions; in 7 cases the change is conservative (row: Similar), and in the remaining 6 the amino acids differ strongly (row : Different).

Table I. Codon-by-codon comparison of the ElA genes of SA7P and SA7.

	NNN ***	NNN **	NNN * *	NNN **	NNN *	NNN *	NNN *	NNN	Alt	Tot
Total	241	4	5	13	2	0	1	0	25	266
Identical	241	0	0	12	0	0	0	0	12	253
Similar	0	2	3	1	0	0	0	0	7	7
Different	0	2	2	0	0	1	0	0	6	6

Each pair of codons at corresponding positions was classified according to its degree of conservation. In addition, it was arranged according to whether the encoded amino acids were identical, related or totally different. The asterisks underneath the triplets NNN indicate the conserved positions. E.g., of the 266 compared codon pairs 241 were identical (***), and of course encoded identical amino acids, while there were 4 pairs with a change in the first letter (**). In two instances, this difference resulted in a pair of similar amino acids, and in the other two the amino acids were totally unrelated.

In Fig. 2, the amino acid sequences of the predicted proteins are compared. We see that the 13 differences described in Table I mainly involve the N-terminal half of the proteins. Whereas the portions encoded by the second exon differ only in the residue, the N-terminal 180 amino acids do so at 12 positions, i.e. they diverge by 6.7%. At this stage one should keep in mind that the ElA proteins of the human Ad types 2 and 5 diverge by less than 2%. The differences between these two serotypes are less drastic: of the 5 changed positions, 4 contain similar residues and only one constitutes a radical change (ser-pro).

```
   1  MRHLALEMMS ELLDLGLDTI DSWLHTEFAP VPTGVSHNMS LHEMYDLDVT GQEDENEEAV
              *              *      *    *
   1  MRHLALEMIS ELLDLGLDTI DGWLHTEFRP VPAGVSHNMS LHEMYDLDVT GQEDENEEAV

  61  DGVFSDAMLL AAEEGVEMPS LYSPGPLVGG GEMPELQPEE VDLFCYEDGF PPSDSEEGEH
                      *   *             *         *
  61  DGVFSDAMLL AAEEGIEMPN LYSPGPLVGG GAMPELQPEE EDLFCYEDGF PPSDSEEGEH
                                                                    ↑

 121  SQVETERKMA EVAAAGAAAA VRGEQDDFRL DCPSVPGHGC SSCDYHRKNS GCPEILCSLC
                  *         * *                           *
 121  SQVETERKMA EAAAAGAAAA ARREQDDFRL DCPSVPGHGC SSCDYHRKTS GCPEILCSLC

 181  YLRANSMFIY SPVSDSEPDE PDSTTADSNH GSPPTLRCTP PRDLPRPVPV KASHGKRPAV
                                                                *
 181  YLRANSMFIY SPVSDSEPDE PDSTTADSNH GSPPTLRCTP PRDLPRPVPV KASPGKRPAV
                 ↑

 241  NSLHDLIEEV EQTVPLDLSL KRSRSN
 241  NSLHDLIEEV EQTVPLDLSL KRSRSN
```

Fig.2. Comparison of the predicted translation products of the ElA 13S mRNAs of SA7P (upper) and SA7 (lower). Removal of the stretch between the arrows yields the 12S mRNA products.

Restriction analysis of SA7P and SA7 DN A

The relatedness of SA7P and SA7 came to light when the restriction maps for SA7P were compared to those of the prototype strains of SA7 and SV20 (7). Fig.3 show the maps for EcoRI, SalI, HindIII, BamHI and XbaI (13,14). Although these maps of course show that SA7P resembles SA7, they are by no means identical. A few more differences in restriction pattern deduced from the sequence comparison in Fig. 1 are summarized in Table II. Also, the XhoI site which forms the boundary of the SA7P sequences determined by Dekker et al. (8) is absent in in SA7 DNA (14).

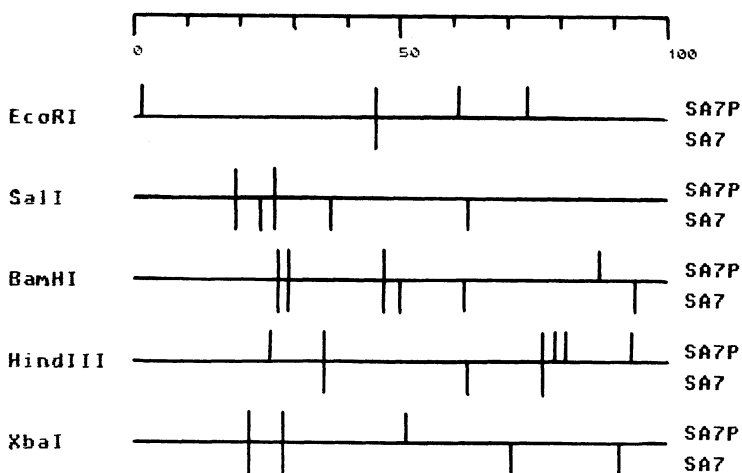

Fig. 3. Comparison of the restriction maps of SA7P and SA7. Where the coordinates of sites were very close, a common site was assumed.

Table II. Restriction site differences between the ElA regions of SA7P and SA7.
Limited to sites occurring one or twice. The numbers give the positions of the sites, the - signs denote the absence of a site.

	SA7P	SA7		SA7P	SA7
AccI	664	-	NotI	-	900
AflIII	174	-	SacII	-	607
BanII	-	1204	SacII	900	-
ClaI	544	-	XmaIII	-	570

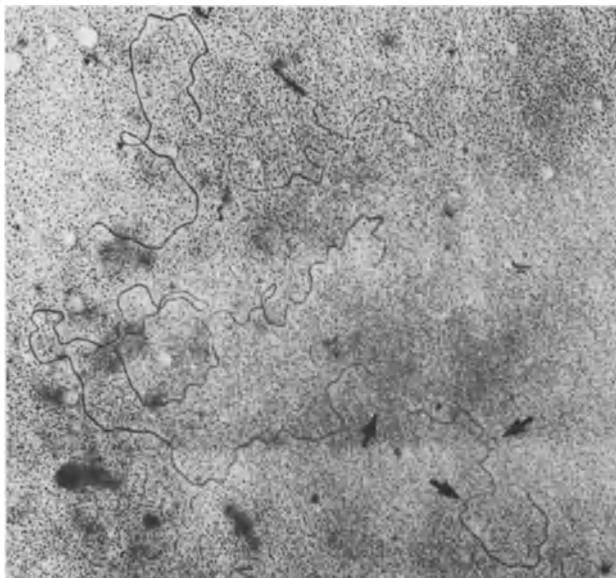

Fig. 4. Electron micrograph of a heteroduplex between SA7P and SA7 DNA. The arrows point at single-strand loops.

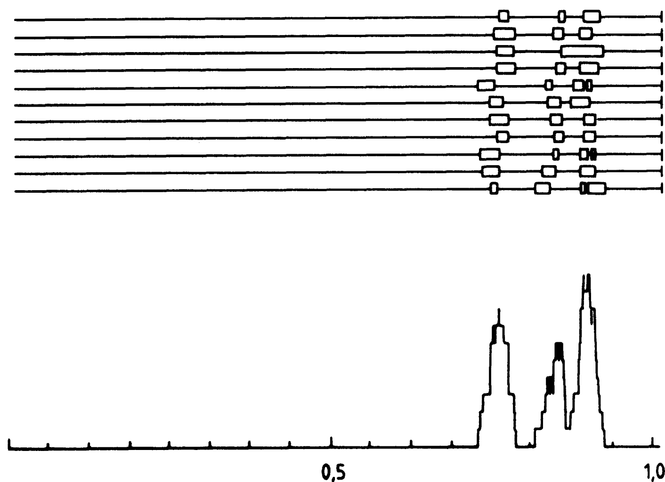

Fig. 5. Distribution histogram of the non-homology regions in the heteroduplexes between SA7P and SA7 DNAs (calculated from 12 heteroduplex molecules).

Heteroduplex analysis and viral proteins

The similarity and differences between the two viral genomes are also evident from the results of the heteroduplex analysis in Figs. 4 and 5, as described by Denisova et al. (7). In this study, the heteroduplex formed between SA7 DNA and the right-terminal SalI-A fragment (26.5-100%; see Fig. 3) revealed 3 zones of non-homology in the right-hand half of the DNAs which had the following coordinates: I: .740-.840; II: .820-.885; and III: .890-.920. Since the left ends of the two viral DNAs which have 95% sequence homology (see above) did hybridize in this experiments, one may deduce that in the regions I-III the homology is less than 95%.

Electrophoresis of the structural proteins of SA7P and SA7 showed that the major hexon protein (pII) and the hexon-associated pIIIa had different sizes whereas polypeptides V, VI and VII had similar molecular weights (ref. 7; not shown here).

Serological characterization of SA7P

Simian Ad SA7P was given its name because of its serological resemblance to authentic SA7. We shall review some of the results of Denisova et al. (7) which led to this typing. First, whilst studying the cross-neutralization of virus SA7 it was found that there was a considerable likeness between SA7 and SA7P; serum against SA7P neutralizes 100 TCD_{50} at a 1:64 dilution, and 1000 TCD_{50} at a 1:32 dilution. Secondly, the neutralization indices of SA7 and SA7P with immune sera (see Table III) shows that 4 neutralizing units of anti-SA7 neutralized 1000 TCD_{50} of SA7P, and, vice versa, 4 units of anti-SA7P neutralized 10,000 TCD_{50} of SA7. This was a clear indication of the serological relatedness of the two viruses; however, Table III also shows that the same amount of

Table III. Determination of the neutralization indices of SA7P and SA7 with immune sera.

Immune sera against:	Virus	Virus dose neutralized: 10^5	10^4	10^3	10^2	10	1	Neutr. Index
SA7P	SA7P	+	+	+	+	+	+	100,000
SA7	SA7P	-	+	+	+	+	+	10,000
SA7	SA7	nd[a]	+	+	+	+	+	10,000
SA7P	SA7	-	-	+	+	+	+	1,000

a) not determined

antiserum neutralized at least 10 times as many TCD_{50} of the virus, against which it has been raised. Thus, the two viruses are clearly akin, but certainly not identical.

CONCLUSIONS

In our studies of virus-induced carcinogenesis we have been working with a strain which we assumed to be simian adenovirus SV20, until we were able to recheck its identity with standard viruses previously unavailable to us. This rechecking prompted us to revise the original nomenclature of our strain and, in view of its marked resemblance to prototype SA7, we proposed the designation SA7P (where P rather unorthodoxly referred to the institute where the virus was isolated). However, when we proposed this name we already noted that although SA7P seemed highly related to SA7, it certainly was not identical (7).

It was found that some SA7P capsid proteins had altered sizes whereas others did not seem to differ in this respect. Heteroduplex analysis revealed three regions where the two DNAs looped out. These non-homologous regions situated in the rightmost quarter of the genomes together comprised about 15% of the genome. Also, serum raised against SA7P was at least 10 times more effective against SA7P itself than against SA7, and vice versa. This factor is of importance since according to the present nomenclature rules it should be at least 16 between viruses in order for them to constitute independent types (unless there are other major biochemical of biophysical differences).

More recently, it became possible to compare the DNA sequences of the inverted terminal repeats and the E1A regions of both viruses. Of course, comparative sequencing could be a great help in establishing questions of identity. In the case of adenoviruses, the inverted terminal repeats might provide a useful criterion for comparing sequences. For two viruses apparently so highly related the ITRs of SA7P and SA7 show a remarkable degree of divergence. Within the family of human Ads several cases are known where different members of one subgroup have identical ITRs (11). The bulk of the E1A region is conserved better, but still differs at 5% of the positions. The 5% divergence is also found in the E1A proteins. The independent human serotypes Ad2 and Ad5 have a homology of better than 98% in this area. The changes in the amino acid compositions of the E1A proteins are also more innocuous for Ad2/Ad5 than for

SA7P/SA7.

The combined weight of these observations has prompted us to reconsider the original proposal that "SA7P is an antigenic variant of SA7". In this paper we therefore ask the adenovirus taxonomists to assign a new name to this virus which would reflect its independence from SA7 (e.g. S25?). Until now, the criteria determining the classification of adenoviruses have been nearly exclusively of a serological nature. This is quite logical, since serological techniques provide a very convenient and reliable method of characterizing virus types. However, the immunogenic properties of a virus are encoded by only a portion of the genome. Information from another part of the DNA should also play a role in taxonomical considerations, and the nucleotide sequence should be the ultimate arbiter.

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DEER PAPILLOMAVIRUSES

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ABSTRACT

Many species of deer are infected with papillomaviruses, as is common for most mammalian species. The papillomavirus family is relatively species specific. The deer papillomaviruses (DPV) induce papillomas, fibropapillomas and fibromas in their natural host and fibromas in Syrian hamsters. Those causing cutaneous fibromas and fibropapillomas also induce similar lesions in the lungs of affected deer or hamsters. These 50-55 nm diameter viruses are nonenveloped and contain a double-stranded, covalently-closed DNA genome. The DPV genome is organized in a colinear fashion with other well characterized animal and human papillomavirus genomes. The white-tailed deer, mule deer, and European elk papillomaviruses (all of which induce fibropapillomas or fibromas) will morphologically transform mouse fibroblasts in tissue culture.

INTRODUCTION

Papillomaviruses induce a variety of benign tumors in their natural hosts. These include papillomas, fibropapillomas, fibromas, keratoacanthomas, and possibly other types (1). In addition, there is mounting evidence that some of the human papillomaviruses induce malignant tumors (squamous cell carcinomas) of the skin and lower genital tract (2,3).

The most extensively studied papillomaviruses are those which naturally infect domestic cattle. At least 6 distinct types have been characterized and these have been classified into two groups based on the morphology of the lesions induced and the physical characteristics of the viruses (4). The group A bovine papillomaviruses (BPV types 1, 2, and 5) have genomes approximately 7.9 kilobases (kb) in length, have extensive regions of cross homology between each other but not members of group B, and induce cutaneous fibromas and fibropapillomas in cattle. The genomes

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of group B bovine papillomaviruses (BPV types 3, 4 and 6) are 7.4 kb in length and induce cutaneous and esophageal papillomas.

Since members of the deer family (Cervidae) are ruminants like cattle (members of the family Bovidae) and member of both families often exist in similar habitats, it is not surprising that the papillomaviruses which infect deer have many of the same physical and pathological properties as the BPV. In fact, the genomes of DPV and BPV-1 share between them many highly conserved regions (5). Although many cervids are naturally affected by papillomaviruses (Table 1), the virus and associated tumors have only been studied extensively in a few. Many of the clinical features and molecular characteristics of these viruses are the same and will be reviewed here.

EPIDEMIOLOGY

The white-tailed deer (*Odocoileus virginianus*) is the most common deer native to North America. In the northeastern part of the United States, up to 10% of the deer are affected with cutaneous fibromas caused by a papillomavirus (6). In other areas of the northeast and midwest, less than 1% of the deer are affected (7, J.P. Sundberg, unpublished results). These studies were done several years apart and changes in the deer population and habitats or differences in field evaluation procedures between researchers may account for the large discrepancy. Lesions are found almost exclusively in young males (1.5-2.5 years of age) and are located primarily on the haired skin around the eyes and mouth, although any part of the body may be affected. The fibromas are usually solitary and less than 1 cm in diameter, however, in rare cases, individual animals may have over 20 tumors up to 7 cm in diameter (6,7) (Figure 1).

In the western part of the United States, mule deer (*Odocoileus hemionus hemionus*) and a subspecies, black-tailed deer (*Odocoileus hemionus columbianus*) are occasionally encountered with cutaneous fibromas. Epidemiological data is limited to scattered case reports but it is probably similar to that of white-tailed deer.

The North American moose (*Alces alces*) also develop cutaneous fibromas of the haired skin. These are most often located on the haired skin over the shoulders of older individuals (4-10 years of age) (8).

In Europe, several of cervids are infected with species-specific papillomaviruses. The disease in European elk (same species as the North American moose, *Alces alces*) has been studied most extensively. Borg (9)

Table 1. Deer species affected by papillomaviruses.

Species	Lesion	Viral DNA Cloned	DNA Sequenced	Oncogenic for Hamsters	Transforms Mouse Cells	Reference
European Elk (_Alces alces_)	Fibropapilloma	Yes	Partial	Yes	Yes	29,30
North American Moose (_Alces alces_)	Fibroma	No	No	Not Tested	Not Tested	8
White-tailed deer (_Odocoileus virginianus_)	Fibropapilloma Fibroma	Yes	Complete	Yes	Yes	5,28
Mule deer (_Odocoileus hemionus hemionus_)	Fibropapilloma Fibroma	Yes	No	Not Tested	Yes	28
Black-tailed deer (_Odocoileus hemionus columbianus_)	Fibropapilloma Fibroma	No	No	Not Tested	Not Tested	26
Red deer (_Cervus elaphus_)	Fibropapilloma	No	No	Not Tested	Not Tested	30
Red deer (_Cervus elaphus_)	Papilloma	Yes	No	Not Tested	Not Tested	M. Favre pers. commun.
Reindeer (_Rangifer tarandus_)	Fibropapilloma	Yes	No	Not Tested	Not Tested	J. Moreno-Lopez pers. commun.

Figure 1. A severe case of cutaneous fibromas in a white-tailed deer.

found 27 cases in 2200 animals examined in Sweden. Sporadic cases with virologic workup have been reported in reindeer (Rangifer tarandus) and red deer (Cervus elaphus), but little is known on the frequency of the disease.

PATHOLOGY

The lesions induced by papillomaviruses in deer have been classified as papillomas, fibropapillomas, and fibromas. True papillomas are rare and reports are limited to a single case in a red deer (M. Favre, personal communication). On gross examination, all three types of lesions present as raised, firm masses, which are usually black to brown. Unpigmented tumors (white) can be found in areas of unpigmented hair. The tumors may be sessile or pedunculated and the large ones often have an ulcerated surface which is infected secondarily. The papillomas have a verrucated surface with a papillary pattern on cut surface. These are usually pigmented. When cut, the fibromas have a thin to moderately thickened

pigmented epidermis which covers a large white firm mass of connective tissue. The fibropapillomas have a broad papillary pattern when cut, consisting of white connective tissue covered by thick, pigmented epidermis. The white connective tissue component is minimal and may not be seen grossly in the true papillomas.

Microscopically, all three tumor types consist of various amounts of proliferating epidermal and dermal components. The papillomas are almost exclusively epidermal and the fibromas are predominately dermal, and the fibropapillomas are in between. The epidermal component is thin on the borders of the lesion and moderately hyperplastic on the uppermost surface of fibromas (Figure 2) while it is uniformly thickened on the other two (Figure 3). The proliferating epidermis is many times thicker than normal, due primarily to thickening of the stratum spinosum (acanthosis) and stratum corneum (hyperkeratosis). Individual or clusters of cells in the stratum granulosum develop degenerative changes typical of the cytopathology of productive infection of papillomaviruses in any mammalian species. The cells swell and the cytoplasm stains poorly, similar to the clear cells (10), pale cells (11), or koilocytes (12) described for other

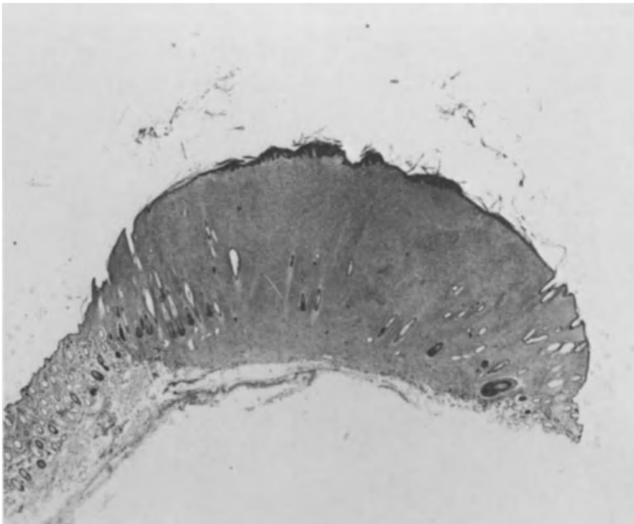

Figure 2. Cutaneous fibroma from a white-tailed deer. The epidermis is thin on the sides and hyperplastic on the top surface. H & E x 15.

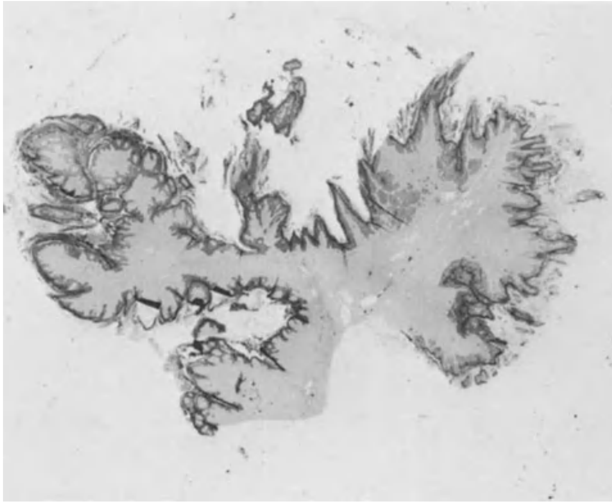

Figure 3. Cutaneous fibropapilloma from a mule deer. The tumor has broad papillary features and consists primarily of fibrous connective tissue covered by a uniformly hyperplastic epidermis. H & E x 15.

species. The cytoplasm contains dark basophilic granules (keratohyalin-like granules) of various sizes, up to the diameter of the nucleus (13). The features of these structures vary between the species and appears to be a specific cytopathic feature of each papillomavirus for all mammals (J.P. Sundberg, unpublished data). The nuclei are often centrally located and may contain an irregular eosinophilic inclusion which is poorly discernable. The stratum corneum may have cells with (parakeratosis) or without (orthokeratosis) retention of nuclei. The retained nuclei in mule deer fibromas are homogeneously dark blue, stain positively for DNA with fuelgen stains, and are filled with crystalline arrays of papillomavirus when examined with the electron microscope (13). The dermal component of papillomas is limited to a thin fibrovascular stalk which supports the hyperplastic epidermis. The dermal component is the most prominent feature of the fibropapilloma and fibroma. Fibroblasts are uniformly separated by thick fasicles of dense collagenous connective tissue (Figure 4). This matrix separates adnexal structures, if they are present at all in the tumors. There is an abrupt change in the collagen matrix at the border of

the tumor (Figure 2). The normal dermal connective tissue is loose and irregular.

White-tailed deer with severe cutaneous fibromatosis have been found to have many discrete, firm, white nodules protruding from the surface and deep in the parenchyma of the lung (14,15). Pulmonary fibromas have also been found in European elk (16) and elk papillomavirus DNA has been detected in tumor extracts by Southern blot hybridization (17). Microscopically, the pulmonary nodules were typical fibromas consisting of loose to dense bundles of collagenous connective tissue. These were present in alveoli attached to the wall by a narrow stalk (17). Virions have not been observed in the pulmonary lesions in either the white-tailed deer or elk even though virions were present in the cutaneous lesions (14,17).

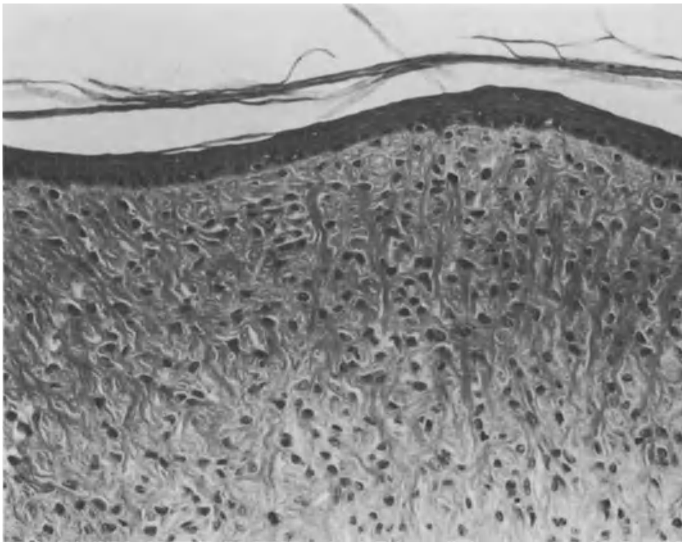

Figure 4. Cutaneous fibroma from a white-tailed deer. A thin epidermis covers a uniform population of fibroblasts separated by bundles of dense collagen. H & E x 200.

Negatively stained preparations of homogenates of tumor epidermis contain virus particles with a circular outline (Figure 5). Full and empty capsids can be distinguished. The surface of the full capsid is composed of

capsomeres about 7 nm in diameter, with an axial hole approximately 2.5 nm in diameter. The virions measure 50-55 nm in diameter. In thin sections, the virions measure 34-38 nm in diameter (Figure 6) (13,18).

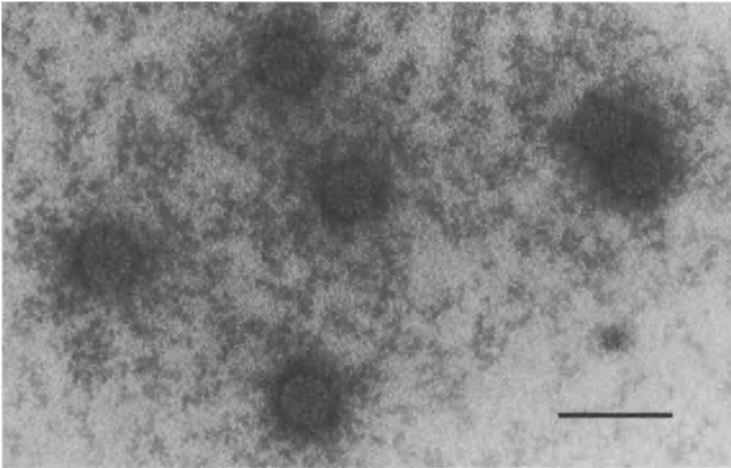

Figure 5. Negatively stained virus particles from a white-tailed deer fibroma. Bar = 100 nm.

TRANSMISSION

The method of natural transmission of DPV is not known, however, it has been speculated that bucks in the rut will rub their antlers on trees to remove the velvet and possibly contaminate the site. The next buck to use that tree may infect himself (19). Tatoo equipment has been associated with transmission in field cases (20).

Experimental transmission of DPV to white-tailed deer has been accomplished several times. Partially purified extracts of epidermis removed from white-tailed deer fibromas were used to inoculate 6-month-old hand-reared fawns of the same species. Intradermal, subcutaneous, and tatoo routes were effective. Lesions, 2-3 cm in diameter, developed in 2 weeks in one study and persisted for 7-10 weeks (21). In another trial, fibromas first appeared 7 weeks after inoculation by scarification and the lesions, 2-3 cm in diameter, persisted for 8 weeks (22,23). Fibromas continued to enlarge on 1 of the 5 deer, forming a semiconfluent mass, 10 months after inoculation. Individual tumors on this animal were 3 cm in diameter and resembled natural cases (23).

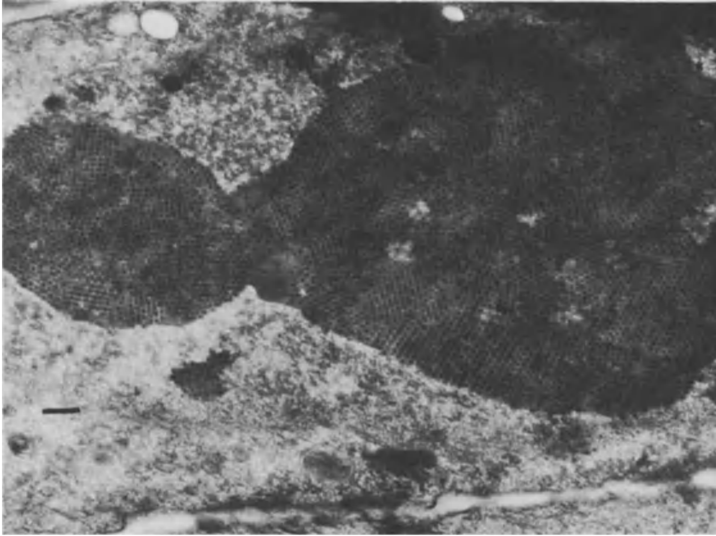

Figure 6. Intranuclear inclusion of virions within a cell in the stratum corneum of a mule deer fibroma. Bar = 300 nm.

Serial evaluation of experimentally induced lesions revealed that following healing of the traumatized site, there was localized fibroblast proliferation. Epidermal proliferation was mild and a late feature of tumor development. Lymphocytic perivascular aggregates and hyalinization of collagen were initial features preceding regression. White-tailed DPV structural antigens were not detectable in serial biopsies tested by indirect immunofluorescence, however, viral DNA was isolated beginning 7 days after inoculation (24). All inoculated deer developed hemagglutination inhibition titers against white-tailed DPV (21).

Papillomaviruses which induce fibromas and fibropapillomas in white-tailed deer and European elk have been used successfully to induce cutaneous and subcutaneous fibromas in Syrian hamsters (25). The fibromas develop 6 months or longer after subcutaneous inoculation and morphologically resemble fibromas in deer. Long-term studies of these white-tailed DPV-induced lesions show subcutaneous implantation of fibromas throughout the skin associated with pulmonary metastatic foci.

Unsuccessful attempts have been made to transmit white-tailed deer fibromas to rabbits, guinea pigs, sheep, calves, monkeys, and horses

(23,25). Attempts to induce fibromas in white-tailed deer by application of BPV to scarified skin or by intradermal injection were also unsuccessful (23).

DETECTION OF VIRAL PARTICLES OR ANTIGENS

Papillomavirus as well as viral structural antigens can be detected relatively easily by several methods. Virus-like particles are only observed within cells of the stratum granulosum and stratum corneum of the epidermis. The affected cells correspond to those with detectable cytopathology as described above. In thin sections, the particles are approximately 35-40 nm in diameter (Figure 6). Fine features of the virion are best visualized in negatively stained preparations where the particles are 50-55 nm in diameter (Figure 5) (13).

Viral antigens can be detected in frozen sections by indirect immunofluorescence or in paraffin sections by either the peroxidase-antiperoxidase or avidin-biotin techniques. Tissues can be screened for the presence of virus using papillomavirus group-specific antiserum, then typed with the appropriate type-specific antiserum. The white-tailed deer and mule deer papillomaviruses cross react serologically. This has been confirmed by immune electron microscopy (26,27).

Purified or partially purified papillomaviruses from white-tailed deer or mule deer fibromas will hemagglutinate mouse red blood cells but not red blood cells from sheep, swine, cattle, human, rabbits, hamsters, or rats (Figure 7) (28). Serum from deer can be used to block the reaction (21).

CHARACTERIZATION OF DEER PAPILLOMAVIRUSES

Papillomaviruses have been isolated from cases of cutaneous fibromatosis in white-tailed and mule deer. SDS-polyacrylamide gel electrophoresis analysis of virions indicated no molecular weight differences in the major structural proteins. Although analysis of restriction endonuclease digestion products indicated minor differences in cleavage patterns, the deer papillomaviruses were indistinguishable by liquid phase hybridization and restriction enzyme cleavage maps indicated most of the sites were similar (28). The virus DNA exists within fibroblasts as intact extrachromosomal genomes (29) as well as within virions forming intranuclear inclusions in epidermal cells.

The genome of the DPV isolated from a white-tailed deer was cloned into pBR322 and the entire nucleotide sequence of 8,374 base pairs was

Figure 7. White-tailed DPV hemagglutinates mouse red blood cells but not those of other species tested.

determined. The overall genetic organization of the DPV genome was similar to that of other papillomaviruses. All significant open reading frames were located on one DNA strand, and the locations of putative promotors and polyadenylation signals were similar to those identified in the closely related BPV-1 genome. The DPV genome was approximately colinear with BPV-1 except for a noncoding region separating the early and late regions. The regions of highest nucleotide sequence homology between DPV and BPV-1 were found in the E1 open reading frame coding for the BPV-1 DNA replication function and in the L1 open reading frame, which encodes the major capsid protein of BPV-1 (5).

The European elk papillomavirus was cloned in the BamHI site of the pBR322 vector and found to be approximately 8.1 kb in size. Cross homology between this viral genome and those of BPV-1 or BPV-2 could be detected by Southern blot hybridization only under conditions of low stringency. Partial sequencing and comparison to BPV-1 revealed similarities in the E1 and L1 open reading frames (30,31).

Preliminary characterization of a papillomavirus associated with a cutaneous fibropapilloma from a red deer indicated that it was

antigenically and molecularly related to BPV-1 and BPV-2 but not elk papillomavirus or BPV-4. The DNA molecule was approximately 7.8 kb in length and had a unique restriction enzyme cleavage pattern (32).

IN VITRO TRANSFORMATION ASSAYS

Virions from both white-tailed and mule deer are capable of morphologically transforming mouse fibroblasts. NIH 3T3 and Balb 3T3 mouse cells are transformable by these viruses. Although BPV readily transforms mouse C127 cells and forms discrete foci (33), these cells are resistant to DPV transformation. Transfected viral DNA shows this same pattern of cell susceptibility (W.D. Lancaster, unpublished results). Analysis of cellular DNA obtained from NIH 3T3 or Balb 3T3 transformants show episomal DPV sequences in high copy number (29, W.D. Lancaster, unpublished results). The inability of DPV to transform C127 cells is the result of a property intrinsic to the DPV genome. C127 or Balb 3T3 cells can be selected for neomycin resistance after co-transfection with DPV DNA and a plasmid carrying the neomycin-resistance gene. These cells contain DPV DNA sequences; however, in C127 cells the viral sequences are integrated while they remain episomal in Balb 3T3 cells. In addition, only Balb 3T3 cells show the transformed phenotype (W.D. Lancaster, unpublished results).

Virus from European elk fibropapillomas also morphologically transforms mouse fibroblasts (31). Cell transformation occurs at the same rate and efficiency as BPV-1. Analysis of cellular DNA by blot-transfer hybridization shows episomal viral DNA at high copy number similar to that seen with BPV-1 (33) and DPV (29).

MALIGNANCIES

Papillomaviruses have been implicated or confirmed as the etiology of squamous-cell carcinomas in many species (1). These malignancies have been reported in sika deer (34), white-tailed deer (35), and other cervids. Papillomavirus antigens have not been detected in these tumors (26), which is a common finding in other species. Since these cases are relatively rare and those cases which are diagnosed are not properly preserved for molecular studies, the role, if any, of the DPV in these malignancies remains unknown.

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18

PARVOVIRUSES

Julia F. Ridpath and William L. Mengeling

The viruses comprising the family Parvovirdae are characterized by having a linear, single-stranded, deoxyribonucleic acid (DNA) genome, an icosahedral, nonenveloped, 20 ± 2 nm diameter capsid (Fig. 1), a relatively high buoyant density (1.37 - 1.45 g/cm^3 in cesium chloride), and a marked resistance to a number of potentially degradative environmental factors such as heat, pH extremes and harsh chemicals. Based on host specificity and requirements for viral replication, family members are subdivided into three genera, Densovirus, Dependovirus and Parvovirus (referred to in the vernacular as densoviruses, dependoviruses, and parvoviruses).

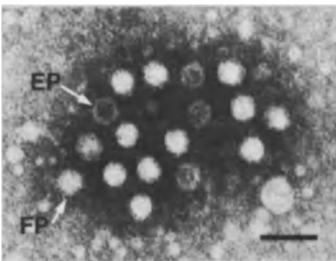

Fig 1. Electron micrograph of porcine parvovirus stained with phosphotungstic acid. (EP-empty particle, FP-full particle). Bar equals 0.5 microns.

Densoviruses infect and cause disease in arthropods (1, 2, 3, 4). They are named in regard to the "dense" intranuclear inclusions that form in infected cells (1). Dependoviruses infect only vertebrates and it is believed that the resulting infections are subclinical (5). They "depend" on coinfection of cells with adenoviruses or herpesviruses for productive replication and for this reason they were at one time commonly referred to as adeno-associated viruses. In contrast, parvoviruses both replicate and cause disease in vertebrates (Table I) and consequently will be the subject of the remainder of this chapter. They are frequently called

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Table I. Animal parvoviruses

Virus	Initial source of virus	Natural host	Major clinical disease	Other possible hosts	Venacular name(s)
KRV	Rat tumor[a]	Rat		Hamster[b]	Kilham Rat Virus
H-1	Human tumor[d]	Rat	Fetal and neonatal death, osteolytic syndrome*	Hamster[d] Rhesus monkey[e]	
MVM	Murine adenovirus stock[f]	Mouse		Hamster[g] Rat[g]	Minute Virus of Mice
HER	Rat tissue[h]	Rat	Hemorrhagic encephalopathy, osteolytic syndrome*	Hamster[i]	Hemorrhagic Encephalopathy of Rats
FPV/ MEV	Feline tissues[j]	Felidae Mink	Panleukopenia, ataxia, gastroenteritis+	Raccoon[k] Coatimundi[l] Ferret[m]	Feline Panleukopenia Virus Mink Enteritis Virus
CPV	Canine feces[n]	Dog	Panleukopenia, myocarditis, enteritis+	Coyote[o] Wolf[p] Fox[q] Cat[r]	Canine Parvovirus Canine Enteritis Virus
MVC	Canine feces[s]	Dog	None reported		Minute Virus of Canines
ADV	Mink tissues[t]	Mink Ferret	Plasmacytosis, glomerulonephritis, arteritis+	Marten[u] Skunk[u] Weasel[u] Fisher[u] Dog[v]	Aleutian Disease Virus
BPV	Bovine feces[w]	Cattle	Enteritis		Bovine Parvovirus HADEN Virus
PPV	Hog cholera virus stocks[x]	Pig	Reproductive failure, embryonic and fetal death		Porcine Parvovirus
GPV	Goose tissues[y]	Goose	Myocarditis, enteritis, hepatitis	Duck (egg)[z]	Goose Parvovirus Derzsky's Disease Virus Goose Plague Virus
LPV	Rabbit feces[aa]	Rabbit	None reported		Lapine Parvovirus Rabbit Parvovirus

* Syndrome expressed in hamster, an experimental host.

+ Virus produces disease in adult animals.

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autonomous parvoviruses because they replicate independently of other viruses. Their replication does, however, depend on functions associated with late S or early G_2 phases of the cell cycle. Thus they have a propensity for mitotically active tissues and often cause disease only in prenatal and neonatal animals of a susceptible species.

ISOLATION

The first virus unequivocally identified as a parvovirus was isolated by Kilham and Olivier (6) during an attempt to isolate a tumorigenic polyoma-type virus from rats. The small DNA virus which they described has since been come to be known as Kilham rat virus (KRV). The isolated virus (KRV) had a cytopathic effect in rat embryo tissue cultures, agglutinated guinea pig red blood cells, was resistant to ether and was heat-stable. It did not produce tumors or any other clinical manifestation when tested in rats, mice and hamsters under their experimental conditions. A year later Toolan et al. (7) reported that a filterable agent isolated from transplanted human tumors was able to cross the placenta and cause a syndrome of deformities in newborn hamsters characterized by "small size, flattened fore-face or microcephalic domed head, protruding eyes and tongue, absent or abnormal teeth and fragile

bone structure." The causative agent was found to be a virus which they named H-1 after the human epithelial (HEp 1) tumor from which it was isolated. Retesting of KRV in a more concentrated form demonstrated that it too was capable of causing a similar developmental anomaly in hamsters and suggested that the two viruses may be related (8). A comparison of these two viruses published in 1963 by Moore (9) revealed that while they shared a number of physiochemical and biologic properties, they were serologically distinct. She further suggested that they represented a new group of DNA viruses.

In the years following these early reports, many other autonomous parvoviruses have been identified. Several were isolated fortuitously as contaminants of stocks of other viruses (10, 11, 12). Others were first isolated from tissues or excretions of seemingly healthy animals (13, 14, 15), or animals that were immunosuppressed with cytotoxic drugs (16). Although, most of the autonomous parvoviruses are now known to be associated with clinical disease, the unequivocal demonstration of their causal role has often been problematical; due, at least in part, to the relatively narrow temporal range of host susceptibility. In some cases, such as feline panluekopenia (17) and Aleutian disease of mink (18, 19), well defined clinical syndromes were recognized long before their association with autonomous parvoviruses was established.

Host range

The parvoviruses as a group have a fairly broad host range having been isolated from humans, rodents, cats, dogs, cattle, swine, geese, chickens, rabbits, horses, mink and raccoon. Individual members of the genus however, are quite host specific and with few exceptions antigenically distinct autonomous parvoviruses are restricted to one or more members of the same animal family (Table I).

As is the case in vivo, parvoviruses are rather exclusive in their range of host cells for cultivation in vitro and consequently are generally propagated in primary, secondary and in some cases permanent cell lines derived from their natural hosts (see 20 for review). While parvoviruses do not replicate readily in cells derived from animals outside their natural host range, parvovirus host range variants may be selected, however, by serial passage of virus in relatively unsusceptible

cells. For example, while it has been demonstrated that the natural host for PPV is the pig (21, 22) and that under normal conditions it is not transmissible to man (23), Hallauer et al. (24) found that the majority of parvoviruses isolated as contaminants of permanent human cell lines were serologically indistinguishable from PPV. Further, Cartwright et al. (25) later succeeded in adapting PPV to human cell lines. In our laboratory, we have been able to adapt PPV to both feline and bovine cell cultures. Host adaptation has been suggested as an explanation for the explosive outbreaks of a new strain of parvovirus in dogs in the late 1970's. While worldwide outbreaks of canine parvovirus (CPV) were reported in 1978 (26, 27, 28), retrospective serology indicated that CPV did not exist in dogs before 1976 (29). While its origin remains a matter of speculation its similarity to FPV has led to the suggestion that either a wild-type or live attenuated vaccine strain of FPV may have mutated to become virulent in dog and then was spread in a biological product designed for veterinary use (30, 31).

Propagation and isolation in the laboratory

Due to the dependence of parvoviral replication on mitotically active cells, cultures are usually infected when they are in subconfluent monolayers. Another aspect of the link between parvoviral replication and host cell cycle is that at low m.o.i.'s several cycles of cell division may be required to generate detectable levels of progeny virus and thus serial passage of infected cultures may be necessary.

Parvoviruses may also persistently infect cell cultures. If only a relatively small number of cells are infected at any one time, the cytopathic effects may go unnoticed (24). Thus it is possible for parvoviruses to be undetected contaminants in cell cultures. The undesired presence of latent parvoviruses may be eliminated by including parvovirus antisera in the culture (32).

Baby hamsters inoculated within 24 to 48 hr after birth have been used as experimental hosts for several parvoviruses including MVM, KRV, H-1 and LuIII (for review see 2). Infection of baby hamsters with these viruses results in impairment of bone and tooth development leading to a group of deformities termed the osteolytic syndrome. In contrast, inoculation of adult hamsters gives rise only to the development of viral

antibodies. Hamsters are not susceptible to FPV (33), mink enteritis virus (MEV) (33) or PPV (34) and no naturally occurring parvovirus has of yet been isolated from hamsters.

Parvovirus virions tend to adhere tightly to cell membranes with dissociation of virus occurring at high pH, at low ionic strength and/or in the presence of EDTA (35, 36). Following pelleting of the cellular debris, virions may be further purified and concentrated by filtration (220 nm) (35) or precipitation with 25 mM $CaCl_2$ (36) followed by density gradient centrifugation. In such preparations, two to four major bands may be detected. When four bands are observed as described by Johnson (4), the particles banding at 1.30-1.32 g/cm^3 represent empty capsids. The particles which band at 1.35-1.37 g/cm^3 are heterogeneous and represent a mixed population of variant particles containing incomplete genomes. The major band at 1.39-1.42 and the minor band at 1.45-1.47 are made up of infectious particles. Clinton and Hayashi (38) demonstrated, in MVM preparations, that infectious particles isolated from "heavy" (1.47 g/cm^3) and "light" (1.42 g/cm^3) bands penetrate and replicate equally well in L-A9 cells, but the heavy particles adsorb more slowly to these cells. The heavy particles also were defective in their adsorption to guinea pig red blood cells as measured by hemagglutination activity (39). The only difference detected between the two types of particles was that in the light particles the 72,000-dalton major structural protein found in the heavy particles had been cleaved to 69,000 daltons. They theorized that the light particles represented virions in the "activated" state. Similar observations have been made with parvovirus LuIII (40) and ADV (41). Often only two main bands are reported (see 20 for review). The upper band (1.30 to 1.32 gm/cm^3) consists mainly of empty capsids and capsids with incomplete genomes, while the lower (1.38 to 1.45 gm/cm^3) is made up mainly of infective viral particles (Fig. 2). Once purified, the virus may be frozen at -70° C and stored indefinitely.

IDENTIFICATION AND CHARACTERIZATION

A property of the parvoviruses which has been valuable in their characterization is their varied ability to agglutinate erythrocytes from different animal species. The majority of parvoviruses described thus far agglutinate erythrocytes of at least one animal species (notable

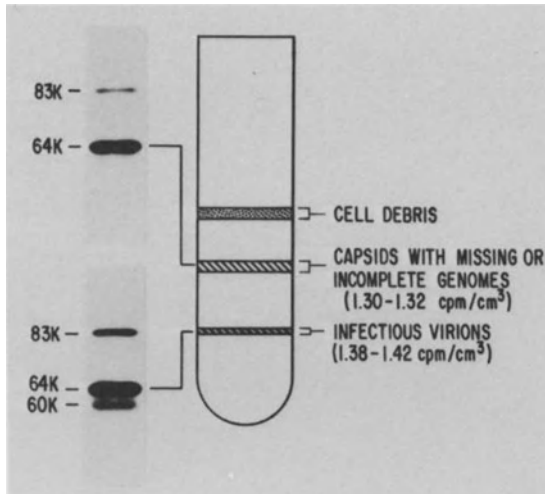

Fig 2. Purification of PPV by gradient centrifugation.

exceptions are ADV and goose parvovirus). Preliminary identification of known parvoviruses can be made by their specific hemagglutination pattern under standardized conditions. While some parvoviruses are capable of hemagglutination at higher temperatures more consistent results are obtained at 4°C (2). The reaction is reversible (42, 43) and virus can be eluted from the red blood cells without destroying the receptors by raising the pH to 9 (44) or in some cases raising the temperature to 37°C (2). Hemagglutination ability is not linked to infectivity and thus empty capsids can not be distinguished from full capsids on this basis.

Hemagglutination inhibition using specific antisera is also often used to characterize parvoviruses and has been used to establish antigenic relationships among the parvoviruses as discussed below. Because nonspecific inhibitors are often present in sera, most procedures call for pretreatment of the sera with kaolin (45). The method most widely used for identification of parvoviruses is hemagglutination combined with hemagglutination inhibition with known parvo-positive sera. Serum neutralization has also been used for identification and to establish antigenic relationships.

Immunofluorescence using parvovirus strain specific antisera has been used for diagnosis, antigenic comparisons and studies of parvovirus replication. Because with the immunofluorescence procedure the antisera is exposed to a broader spectrum of virus coded epitopes, antigenic relationships are sometimes detected with this procedure that are not apparent with serum neutralization or hemagglutination inhibition (Table 2 and Table 3). This is likely due to interaction with nonstructural proteins and epitopes not presented on structural proteins when the virion is fully assembled.

Other tests used for the identification and/or quantification of parvovirus antigens include complement fixation (46, 47, 48, 49, 50), counter-immune electrophoresis (51, 52, 53), immunodiffusion (54, 55), enzyme immunoassay (56, 57), latex agglutination test (58) and radioimmunoassay (59).

Table 2. Antigenic relatedness of selected autonomous parvoviruses tested by SN*

Antiserum	Virus								
	MVM	KR	H-1	HER	PPV	CPV	FPV	BPV	GPV
Mouse anti-MVM	1280	<10	<10	<10	<10	<10	<10	<10	<10
Rat anti-KR	<10	640	10	10	<10	<10	<10	<10	<10
Pig anti-PPV	<10	<10	<10	<10	160	40	40	<10	<10
Dog anti-CPV	<10	<10	<10	<10	<10	10240	1260	<10	<10
Cat anti-FPV	<10	<10	<10	<10	<10	5120	10240	<10	<10
Calf anti-BPV	<10	<10	<10	<10	<10	<10	<10	40	<10
Goose anti-GPV	<10	<10	<10	<10	<10	<10	<10	<10	2560

*Titers expressed as the reciprocal of the maximum serum dilution that reduced infectivity by more than 99% when compared to infected controls; <10 = infectivity not reduced more than 99% with the lowest dilution of serum tested. All of the preexposure sera were <10 for all of the autonomous parvoviruses included in the study. (Table reprinted in part from Mengeling et al., J. Gen. Virol., 67:2839, 1986.)

Table 3. Antigenic relatedness of selected autonomous parvoviruses tested by FA*

Antiserum	Virus								
	MVM	KR	H-1	HER	PPV	CPV	FPV	BPV	GPV
Mouse anti-MVM	2560	20	5	5	<5	<5	<5	<5	<5
Rat anti-KR	320	5120	640	5120	160	160	80	<5	<5
Pig anti-PPV	80	80	40	80	2560	40	40	<5	<5
Dog anti-CPV	160	160	320	320	160	1280	640	<5	<5
Cat anti-FPV	80	160	80	80	40	640	320	<5	<5
Calf anti-BPV	<5	<5	<5	<5	<5	<5	<5	160	<5
Goose anti-GPV	<5	<5	<5	<5	<5	<5	<5	<5	640

*Titers expressed as the reciprocal of the maximum dilution of serum that resulted in unequivocal, specific anti-viral fluorescence; <5 = no reaction at lowest dilution of serum tested. All of the preexposure sera were <5 for all of the autonomous parvoviruses included in the study. (Table reprinted in part from Mengeling et al., J. Gen. Virol., 67:2839, 1986.)

Assay systems in culture

There are a number of assay systems in use for determining the presence of parvoviruses in cultured cells. They are all dependent upon the presence of replicating cells in subconfluent monolayers. One of the earliest described was the visual detection of cytopathic effects (CPE) in unstained monolayers. In general the CPE of parvoviruses can be described as cytocidal. The sequence of observed events have been reported by a number of investigators (9, 12, 15, 16, 34, 60, 61, 62, 63, 64). In general CPE is characterized by rounding of cells, pyknosis, cell lysis and detachment of the cell from the surface of the culture vessel. As described for PPV (64), portions of the lysed cells often remain attached which gives the affected culture a ragged appearance. The complete destruction of the culture occurs only if the culture was infected at a sufficiently high multiplicity of infection. At lower multiplicities of infection the virus infected cells round up, detach and are lost from the cell layer. The monolayer, though thinner than control cultures, may then appear uninfected. Under these conditions the detection of CPE is

transient and may be difficult to reproduce (61, 65, 66). Cellular changes may also be observed in histologically stained monolayers (H & E, May-Gruenwald-Giesma or Feulgen). Detectable at an earlier stage than CPE, the first visible histologic changes are observed 8 to 12 hr after infection in nonsynchronized cultures (67). Assay of stained cultures involves counting inclusion bodies in the nuclei of infected cells. These inclusions represent the accumulation of parvovirus proteins.

In terms of sensitivity and time involved, one of the best methods for detecting parvovirus infected cells in culture is the fluorescent antibody (FA) technique, also known as immunofluorescence microscopy. It has the additional advantage of making it possible to detect the early accumulation of virions in the cytoplasm which can not be shown with other light microscopy methods. Plaque titration, while requiring more time than the fluorescent antibody technique, generally yields higher infectivity titers and is more reproducible (20).

Relationships between autonomous parvoviruses

Antigenic relationships. Most autonomous parvoviruses are host specific with antigenically distinct strains being restricted to one or more members of the same animal family. However, there seems to be a fair degree of cross reactivity expressed between the members of this group with the extent of the cross reactivity observed being dependent upon the serological test used. In general, more extensive cross reactivity is observed with fluorescence antibody techniques than with such techniques as serum neutralization (SN) or hemagglutination inhibition (HI) (Table 2 and 3). The strongest cross reaction observed with all three tests is that between FPV, CPV and MEV. MEV is assumed to be closely related to FPV, based on the cross protection observed between the two viruses and similar biological properties, and is frequently referred to as a strain of the latter (68, 69, 70, 71). Serologic comparisons of CPV, FPV and MEV by hemagglutination (HA), HI, SN and agar gel precipitation indicate that these viruses are very similar (72, 73) and that while antigenic differences do exist, they are subtle and most clearly defined using monoclonal antibodies (73). Other relationships detectable by SN and/or HI include cross reactions between PPV and CPV (74, 75) and H-1, HER and KRV (77).

A much more extensive list of antigenic relationships has been established by FA (Table 3). As discussed above this difference in detection of cross reactivity is likely due to interaction of antisera with epitopes of nonstructural proteins or with epitopes that are lost or not available for binding when the virion is assembled in its native configuration. Cotmore et al. (76) have suggested that the antigenic cross reactivities observed between parvoviruses are due to reaction with nonstructural proteins. They reported that the nonstructural proteins of MVM were precipitated by antisera for several autonomous parvoviruses, whereas, structural proteins are precipitated only by anti-MVM serum. It has also been shown by immunoprecipitation however, that there is a reciprocal relationship between the structural proteins of PPV and those of both CPV and FPV and that anti-FPV sera reacts with structural protein of MVM (77) suggesting that not all shared epitopes are associated with nonstructural proteins. These combined results would seem to suggest that the antigenic relationship among the parvoviruses reflect a multiplicity of epitopes on both the structural and nonstructural proteins which are shared to different degrees and which may not be presented in the final conformation of the proteins in the infective complete virion. In contrast to the cross reactivities between the rodent parvoviruses, FPV and its host range variants and PPV, no antigenic relationships have been reported for BPV, goose parvovirus (GPV), ADV and other known autonomous parvoviruses.

Genomic relationships. The autonomous parvoviruses appear to be highly similar in regard to secondary structure, replicative intermediates, length and translational maps (78, 79, 80, 81, 82). Comparison of the published complete nucleotide sequences for H-1 (79) and MVM (80) and the partial sequences published for CPV (78) and FPV (81) reveal extensive similarity in the primary nucleotide sequences of these viruses. The similarity indexes (83), based on primary nucleotide sequences, range from 95% for FPV vs CPV to 63% for FPV or CPV vs MVM (the index for MVM vs H-1 was 76%). Within the genus, KRV, H-1, H-3, MVM, LuIII, FPV, MEV, CPV, B19 and PPV appear to be closely related as judged from DNA-DNA hybridization studies, restriction site mapping and heteroduplex mapping (84, 85, 86, 87, 88, 89, 90, 91, 92), while the relationship between ADV and BPV and other autonomous parvoviruses seems

to be more distant. Cotmore and Tattersall (85) demonstrated that the observed homology between MVM and B19 was dispersed across the genome. They suggested that this homology reflected the remnants of a series of repeated sequences in a common ancestral virus from which MVM and B19 separately diverged. In contrast, comparison of the homology between PPV and CPV done in our laboratory revealed that the homology was not uniform across the PPV genome but was highest across an area between 1.85 and 2.7 Kb from the 3' end (84). If correlation can be made between the translational maps of rodent parvoviruses (70, 90, 93) and PPV, this protein of the PPV genome would code for both structural and nonstructural proteins. The existence of homology within this area between viruses with different host specificities raises the possibility of conserved functions and antigenic sites for both structural and nonstructural proteins. Observed homologies may represent sequences involved in common mechanisms for viral protein packaging and transport. Lederman et al. (94) have observed that a sequence located just to the left of the initiating methionine for the smallest coded capsid protein is conserved in MVM, H-1, CPV, FPV and to a lesser extent in BPV. They have noted that this sequence bears similarity to the T antigen nuclear transport signal, and on the basis of this observation have proposed that this sequence codes for the nuclear transport of viral proteins.

Adaptation and evolution

The combined results of comparisons of translational maps, antigenic cross reactivity and genome homology, discussed above, suggest that the members of the genus parvovirus may have evolved from a common ancestor. The evolution of parvoviruses may follow a pattern of adaptation suggested by the presumed divergence of CPV from FPV (30, 31). That is, an existing parvovirus strain gains entrance to a previously parvovirus free species and establishes a low level of replication. Adaptation of the virus occurs as it replicates in the new host until a new strain of virus emerges which is readily able to replicate in the new host. The significance of parvovirus adaptation becomes apparent when the potential for the occurrence of adaptation is examined. Parvoviruses seem to be ubiquitous and are not easily destroyed by conventional methods of sterilization. They are a frequent contaminant of primary and permanent

cultures of cells from various animals and man. A study conducted by Hallauer and co-workers (24) revealed that 38 out of 43 strains of permanent human cell lines were contaminated with parvoviruses. The majority of the viral isolates were serologically related to PPV. It was proposed that these PPV related viruses were transmitted to the cultures by the use of trypsin derived from the pancreas of PPV-infected pigs. If the adaptation of parvoviruses does indeed occur, the propagation of cell cultures persistently infected with parvoviruses would seem to be an ideal system in which the process could take place.

It has been proposed that rodent parvovirus contamination of monoclonal antibody preparations for use in human therapy may pose a health hazard (95). Bass and Hetrick (96) have reported that both H-1 and KRV are able to replicate in human B lymphocytes in culture and thus should be considered as possible human pathogens. The intravenous administration of monoclonal antibodies contaminated with these two parvoviruses would allow them direct access to a susceptible human cell type. From the veterinary biologics standpoint, dissemination of parvoviruses via vaccines prepared in contaminated cell cultures offer another avenue of adaptation. For example, because of the occurrence of transplacental infection with PPV, cells prepared from porcine fetal tissues may be infected with PPV (97, 98). At present vaccines for canine infectious hepatitis, infectious bovine rhinotracheitis, parainfluenza and bovine viral diarrhea are federally licensed for production in primary porcine tissue cultures. When testing for PPV in vaccines produced in this manner was initiated in 1979-1980, 25% were found to be contaminated with PPV (D. L. Croghan, National Veterinary Services Laboratory, Ames, IA, personal communication). Further, it should be noted that the two parvovirus strains which have been arisen in modern times, MEV and CPV, were characterized by severe and often fatal illness in the newly susceptible host. These observations suggest the possibility of serious outbreaks of parvovirus associated disease resulting from new strains arising in the lab. In light of this, it becomes important to identify and control the presence of parvoviruses in cell cultures and biological products.

The early studies of the congenital and prenatal deformities induced by KRV led Margolis and Kilham (99) to propose that the virus preferentially infected actively dividing cells. Later studies by Tennant and Hand (100) using X-irradiated rat embryo cells demonstrated that KRV replication was dependent upon one or more cellular functions generated during the S phase of the cell cycle. Subsequently this requirement has been shown to be a common feature of the members of the genus parvovirus. The exact nature of this function(s) has yet to be established. Rhode (101) reported H-1 virus antigen synthesis occurs at a fixed time interval after the onset of S-phase in parasynchronous cultures, independent of the time of infection preceding S-phase. He hypothesized that synthesis of the viral DNA by host cell mechanisms that are restricted to cells in S-phase were a prerequisite to further events in the viral replication process. In a somewhat similar vein, it has been suggested that the S-phase dependency of the parvoviruses may be due to host cell factors necessary for the conversion of single-stranded viral DNA to the double-stranded replicative form (RF) (102, 103).

Interaction of virus and host cells

While active cell cycling is an absolute requirement for host cells it is not the only determinant of susceptibility to parvovirus infection. Several mechanisms have been proposed which would limit parvovirus lytic infection to specific classes of growing cells. In studies of the interaction of MVM with differentiated cells it was demonstrated that this virus required a host cell factor that was not expressed in teratorcarcinoma stem cells (104, 105), but is expressed as a function of differentiation in fibroblasts (105). Tattersall proposed a mechanism involving a system for transporting viral antigen across the nuclear membrane which was only present in differentiated cells (105). Linser et al. (106, 107, 108), on the basis of binding of MVM to susceptible and resistant cells (cloned from cells surviving long-term infection with MVM in monolayer culture), proposed that the virus initiated infection by binding to a specific receptor on the cell surface and further that this receptor might be the determining factor in the cytotropism observed for

parvovirus infection. This type of mechanism has been described for a number of other viruses (109, 110, 111, 112). In contrast, experiments comparing the replication of the prototype strain of MVM with it immunosuppressive variant strain, MVM(i), suggests that the strain-dependent target cell specificity of MVM is mediated by intracellular factors (113, 114). These reports suggested that the selection operated before or at viral RF DNA synthesis. In our laboratory, inoculation of permissive (embryonic swine kidney) and nonpermissive (bovine embryonic spleen) cells with PPV revealed that while PPV enters the nonpermissive cells, the inoculated virus is not cleared from the cytoplasm as it is in the permissive cell type (Fig. 4). This suggests that, in this system, selection may be based on the host cell's ability to uncoat the virus. As these reports indicate, the recognition between host cell and virus is as yet unclear and is worthy of further study.

Course of infection

The lytic cycle of parvovirus infections takes 16 to 24 hr to complete (61, 101, 102, 115). The temporal sequence of events has been studied by a number of authors for a variety of parvoviruses: CPV/FPV (59, 116), H-1 (107, 117), KRV (115, 118, 119), LuIII (120), MVM (62, 102, 121), PPV (21, 122, 123) and BPV (124, 125). A general sequence has emerged from these reports. The initial step in infection is the penetration of the virus into the cell. As discussed above, this may occur through the action of a specific receptor with virus (106, 107, 108) or by pinocytosis (109). FA and electron microscopy (EM) studies indicate that the whole virus enters the host cell. Little information is available on the uncoating process in the parvovirus genus. It has been observed in the dependovirus genus that the virus DNA becomes sensitive to DNase only as it enters the nucleus (126), suggesting that uncoating occurs as the virus penetrates the nucleus. Once in the nucleus, parental single stranded DNA is converted to RF. Following production of RF, by 2 to 6 hours, viral capsid proteins accumulate in the nucleus. Progeny single-stranded DNA is synthesized and simultaneously packaged into capsids. Virus remains mostly cell associated until late after infection (Fig. 3).

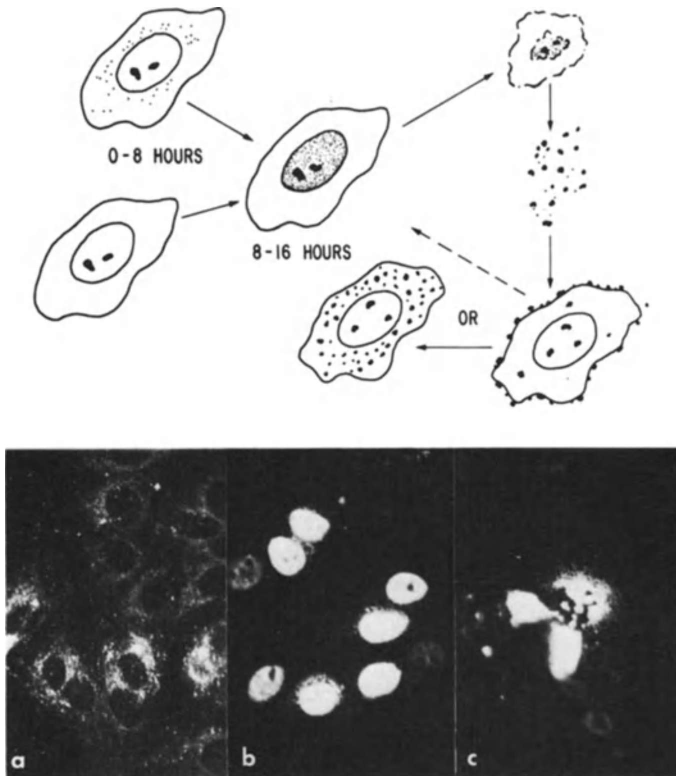

Fig 3. Lytic cycle of parvoviruses.
Upper line drawing represents schematic of lytic cycle as observed by immunofluorescence. Micrograph a represents a fetal swine kidney culture 1 hr after inoculation with a high m.o.i. of PPV, b is a fetal swine kidney culture 15 hr post inoculation and c represents PPV virions being released from an fetal swine kidney cultures 24 hr post inoculation. All cultures stained with anti-PPV porcine serum directly conjugated with FITC.

Several authors have reported an early cytoplasmic fluorescence observed between 4 to 6 hr after infection (115, 116, 120, 122). Since this fluorescence is only observed at high multiplicities of infection, is not blocked by inhibitors of DNA synthesis (120), and is also seen in cells which are not permissive for viral replication (Fig. 4), it is

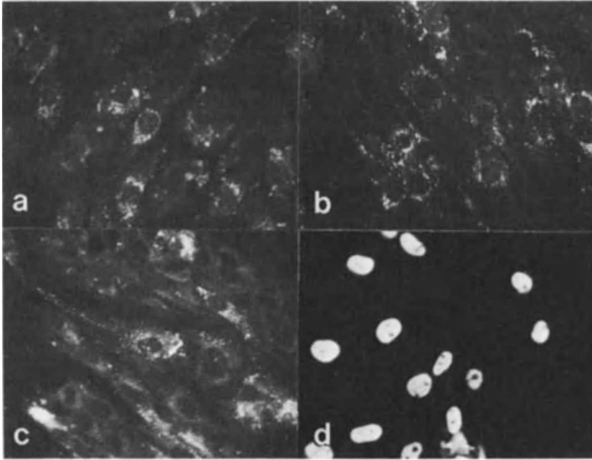

Fig 4. Uptake of PPV in permissive and nonpermissive cells. Micrographs a and b represent bovine fetal spleen cells 1 hr and 15 hr post inoculation with PPV respectively. Micrographs c and d represent fetal swine kidney cells 1 hr and 15 hr post inoculation with PPV respectively. All cultures stained with anti-PPV porcine serum directly conjugated with FITC.

probably of inoculum origin as discussed by Mengeling (21) and Hirasawa et al. (116).

DNA replication

Similar to the dependoviruses, the single-stranded viral DNA of the parvoviruses possess hairpin duplexes at both ends of the genome (127, 128, 129). Unlike the dependoviruses, the 3' and 5' hairpin duplexes of the parvovirus genomes differ in size and sequence (128, 129). The conversion of parental single-stranded DNA to RF is thought to be initiated by a self-priming mechanism via the 3'-terminal palindromic sequence (for review see 82) and involve the participation of a virus coded product (130, 131). It has been proposed that parvoviruses replicate via a rolling hairpin model (132) similar to the model proposed for linear chromosomal DNA replication (133). While monomer and dimer duplex forms of viral DNA have been identified as intermediates in the replication process (82, 120, 134, 135, 136), the exact details of replication have yet to be established. Several proteins have been identified which seem to be involved in the replication process. A

protein has been identified which is covalently bound to the 5' terminus of the RF (138). Several authors have suggested that this protein serves as a site-specific nickase necessary in parvovirus replication (138, 139, 140). Analysis of temperature-sensitive mutants (141), deletion mutants (131) and defective particles (130) have revealed that for H-1 viral capsid proteins and a viral protein termed RF rep are also required for replication.

Organization of the viral genome

Parvoviruses code for at least two structural polypeptides ranging in size from 60,000 to 87,000 and two or more nonstructural polypeptides with molecular weights from 15,000 to 85,000 (Table 2). If the reported molecular weights of the virally encoded proteins are correct, the coding sequences required exceeds the coding capacity of the approximately 5000 base parvoviral genome. This dilemma is overcome by the existence of overlapping and nested genes (79, 80, 139, 142). The general scheme for the translational map of parvoviruses can be seen in Figure 5 (79, 80, 139, 142, 143, 147). An exception to the general rule may be BPV. While it is similar in genome size and secondary structure to other autonomous parvoviruses, preliminary analysis of BPV mRNA indicates that its translational map may differ from those which have been determined for other parvoviruses (144, 145, 146). Analysis of translational maps for H-1 and MVM (79, 142, 143) reveal that these viruses produce four major transcripts which are read from two overlapping transcription units with separate promoters. In the MVM genome these promoters are located at 4 and 39 map units (142, 147). The promoter located at 39 map units (P39) is activated by the protein whose mRNA is transcribed from the promoter at 4 map units (P4). In MVM replication there are four virus-specific mRNA species (76, 79). All four mRNAs are spliced and share a common 3' terminus. Parvovirus infections do not produce early mRNA (148). Two of the mRNAs code for structural proteins and two for nonstructural proteins (149). The most abundant mRNA codes for a viral structural protein which appears late in infection. The mRNAs are coded primarily in two large open reading frames spanning the left and right halves of the genome (80). While there is some overlapping, the structural proteins are coded for by

sequences in the right hand side of the genome and the nonstructural proteins are coded for in the left hand side of the genome (143).

PATHOGENICITY

As stated earlier, parvovirus replication is restricted to mitotically active cells and this fact is reflected in their pathogenicity. The rapidly growing tissues of neonates provide excellent conditions for the replication of the virus, leading to symptoms such as ataxia and osteolytic syndrome. Parvoviruses may cross the placenta and infect fetal tissue resulting in abortion, mummification, stillbirth, and malformation. The ability to induce transplacental infections is widespread among parvoviruses and indeed, the larger share of recognized diseases caused by parvoviruses are related to intrauterine infections. In general, infection of adult animals is subclinical (Table 1). Notable exceptions are ADV and the closely related viruses CPV and FPV/MEV. When disease does appear in adult animals as a result of parvovirus infection, it is due to infection of rapidly proliferating tissues (e.g. lymphoid tissue, the lining of the gut). While the most important determining factor for the pathogenic action of parvoviruses is their predilection for actively dividing cells, different syndromes may expressed depending on the age of the animal, mode of infection, and presence of other infective agents. This is illustrated by the confusion that arose regarding the identity of the parvovirus which caused ataxia in kittens. Early studies, using adult cats, failed to demonstrate neurotropism associated with FPV (150). Later Kilham and Margolis (151) isolated a parvovirus associated with feline ataxia, a disease of kittens originally thought to be a genetic defect. Johnson et al. (152) later demonstrated that the causative virus was identical to FPV and that the different syndromes observed were due to differences in the age of the infected animal and the mode of infection.

TRANSMISSION

Animals infected with parvoviruses have been shown to shed virus in their feces, urine, milk, saliva, and nasal secretions (see 20 for

Table 4. Parvovirus proteins

Virus	Minor structural proteins 1	Minor structural proteins 2	Major structural proteins 1	Major structural proteins 2	Nonstructural proteins 1	Nonstructural proteins 2	Ref.
MVM	83000 (VP-1)	-----	64000 (VP-2)	62000 (VP-3)	83000 (NS-1)	24000 (NS-2)	a, b
H-1	88000 (VP-1)	-----	68000 (VP-2')	65000 (VP-2)	92000 (NCVP-1')	84000 (NCVP-1)	c, d
KRV	81000 (A)	-----	64000 (B)	60000 (C)	-----	-----	e
PPV	83000 (A)	-----	64000 (B)	60000 (C)	86000 (NS-1)	-----	f, g
FPV	77500- 79500 (A)	-----	63000- 63500 (B)	61500- 63000 (C)	-----	-----	h
CPV	82300 (VP-1)	-----	67300 (VP-2')	63500 (VP-2)	-----	-----	i
BPV	80000 (VP-1)	72000 (VP-2)	62000 (VP-3')	60000 (VP-3)	28000 (NP-1)	-----	j, k
LPV	96000 (A)	85000 (B)	75000 (C)	70000 (C')	25000 (F)	22000 (G)	1
ADV	85000 (p85)	-----	75000 (p75)	-----	71000 (p71)	-----	m

Terms in parenthesis under molecular weights refer to authors designations of proteins.

a) Tattersall et al. J. Virol. 20:273, 1976. b) Cotmore et al. Virology 129:333, 1983. c) Paradiso J. Virol. 39:800, 1981. d) Paradiso J. Virol. 52:82, 1984. e) Peterson et al. In: Replication of Mammalian Parvovirus (D.C. Ward and P. Tattersall, eds.) pp. 431-445, 1973. f) Molitor et al. J. Virol. 45:842, 1983. g) Molitor et al. 55:554, 1985. h) Carman and Povey Vet. Micro. 8:423-435, 1983. i) Paradiso et al. J. Gen. Virol. 62:113-125, 1982. j) Lederman et al. J. Virol. 48:10, 1983. k) Lederman et al. J. Virol. 49:315, 1984. l) Matsunaga and Matsuno J. Virol. 45:627, 1983. m) Bloom et al. J. Virol. 43:608, 1982.

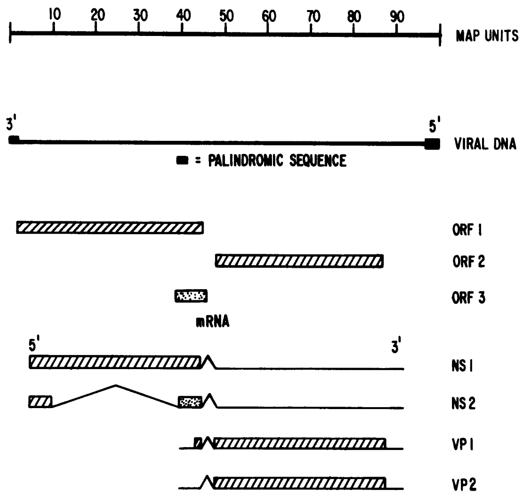

Fig 5. Schematic representation of proposed parvoviral translational map. Three open reading frames (ORF) used to generate two messengers for nonstructural proteins (NS) and two for structural proteins (VP).

review). Both acutely ill and clinically healthy animals may shed virus. An apparently healthy immune animal may serve as a reservoir in that virus may coexist with circulating antibodies (153, 154). Once the virus has been shed into the environment, it may persist in an infective state for a long period of time due to its resistance to breakdown by pH, enzymes, detergents, and temperature. While the main route of infection in a given population is probably horizontal via oral or nasal exposure, vertical transmission via transplacental infection also may occur. The vertical transmission of parvoviruses is important in that a large share of the recognized diseases brought about by these viruses are related to

intrauterine infection. The isolation of PPV from semen and testicles of infected boars (64, 155, 156, 157) has led to the suggestion that PPV may also be sexually transmitted. Transmission of GPV has been reported at the level of the ova (158) but not as of yet for other parvoviruses. While it appears that the zona pellucida may prevent infection of the embryo with MVM (159) and PPV (160), infectious virus bound to the zona pellucida may be a source of infection in tranplantation of embryos (161).

IMMUNITY AND CONTROL

Because it appears that neonates are at high risk in parvovirus infections, passive immunity is important in prevention of clinical disease. Passive immunity resulting from maternally derived antibodies has been demonstrated for KRV (162), PPV (163), FPV (17) and GPV (164) and ranges from 3 weeks for goslings to 24 weeks for suckling pigs. While protective of the young animal, passively acquired antibodies may block the development of active immunity (164, 165, 166). The development of active immunity has been studied for the rodent parvoviruses (62, 167, 168), PPV (163), CPV (72, 169), and FPV/MEV (153, 170). In general, antibodies appear 4 to 7 days after inoculation. Antibody titers will increase until a plateau is reached. If virus is still being produced, titers will remain near this level, if viral production has ceased, titers will drop to a lower level. This lower titer level is effective in preventing the induction of overt disease upon reexposure to virus. Vaccination has proven effective in prevention of parvovirus-associated disease. At present, vaccines are available for FPV/MEV, CPV, PPV, and GPV. In addition to development of active immunity, general hygienic measures may also reduce the incidence of disease. This includes disinfection of all animal areas with compounds such as chlorine bleach which effectively inactivates parvoviruses, and segregation and testing of all new animals for parvoviruses.

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19

WOODCHUCK HEPATITIS VIRUS

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ABSTRACT

Woodchuck hepatitis virus is the animal hepadnavirus which most closely resembles human hepatitis B virus in genomic organisation, in the nucleic acid and amino acid sequences, in the clinical symptoms and in the close association with subsequent appearance of hepatocellular carcinoma. The woodchuck disease is remarkable for the high incidence of hepatocellular carcinoma in chronically infected animals and the very rapid onset of the malignancy.

THE DISCOVERY OF WHV

By the middle of the 1970's, the causative agent of human B-type viral hepatitis (HBV) had been identified (1) and characterised (2,3). Several unique properties of this virus were described, including the fact that the viral genome contained within infectious virions consisted of a DNA template which was only partially double-stranded (4). When the virions were incubated in the presence of deoxynucleoside triphosphates, an endogenous DNA polymerase activity could be observed (5). Summers et al. (6) used this property to screen animals for the presence of DNA polymerase-containing particles that used a small circular endogenous DNA template, in order to establish an animal model of the human disease. The first animals to be found with sera positive by these criteria were American East Coast marmots or woodchucks (Marmota monax). Subsequent studies showed that the viral particles were morphologically very similar to HBV particles and that the topology of the DNA template was identical (7). Woodchuck hepatitis virus (WHV) therefore became the second member of the class of DNA viruses which is now known as hepadnaviruses. Since then, ground squirrel hepatitis virus (GSHV, 8) and duck

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hepatitis virus (DHBV, 9) have been added to the family as distinct but related viruses. More recently, a similar virus has been described for tree squirrels (10), but whether this is a distinct virus remains to be established. However, WHV remains the viral model which most resembles human B-type hepatitis in clinical pathology and in the high incidence of subsequent onset of primary hepatocarcinoma.

THE HOST

Woodchucks are rodents of the family of the Sciuridae, to which also belong both ground and tree squirrels. Six species of Marmota are known, 3 North American (_M. monax, M. caligata_, _M. flaviventis_) and 3 euro-asiatic (_M. marmota, M. bobak_, _M. candata_). _Marmota monax_ ranges from North Carolina to upper New England on the East coast, across the northern and central United States and southern Canada, dipping down into Louisiana, and continuing up to Alaska. However, natural infection with WHV seems largely confined to an area centered around Pennsylvania.

M. monax free of natural infection can be experimentally infected with virus preparations (7). However, an attempt to infect the European alpine woodchuck, _M. marmota_, with WHV was unsuccessful (11), although the age of the animals captured, their state of semi-hibernation when infected, and the relatively small number of animals tested perhaps leaves the question open. Similarly, attempts to infect ground squirrels which are susceptible to GSHV with WHV were also negative (12). Ponzetto et al. (13) have reported that a chimpanzee infected with WHV, while showing no viremia, did mount a weak anti-WHc response, indicating a sub-clinical infection. Only one animal was involved and liver samples were unavailable, so that experimental infection of chimpanzees with WHV remains doubtful. WHV, like the other members of the hepadnaviruses, therefore seems to have a very limited host range.

VIRAL PARTICLES

When particles from infected woodchuck sera are centrifuged on CsCl isopycnic gradients and assayed for protein or for

endogenous DNA polymerase activity, a profile similar to that observed with human HBV-infected sera is obtained. The major protein peak bands at a density of 1,20 g/cm^3 and electron microscopy shows it contains small spherical (about 25 nm) and tubular structures (6, 7). The peak of DNA polymerase activity bands at a higher density and consists of larger (about 50 nm) spherical particles with an electron-dense core, strongly resembling HBV Dane particles. Nucleocapsid cores can be isolated from these particles by Triton X-100 treatment or from infected liver cells (7). Serological cross reactions (7, 14 and see below) show that, as with HBV, the smaller spherical and tubular particles consist of excess surface antigen, WHsAg, while the nucleocapsid cores contain a distinct core antigen, WHcAg. DNA can be extracted from the viral or core particles. After labelling in the endogenous DNA polymerase reaction, extracted DNA was heterogeneous (6), indicating incomplete filling-in of the single stranded regions. Complete filling-in by addition of AMV polymerase results in conversion to two forms, a circular and a linear form, both of which are slightly larger than the equivalent HBV forms (6). DNA extracted from DNA polymerase-containing particles from woodchuck sera and examined by electron microscopy consist of approximately 90% of a relaxed circular form (7). The structure of the WHV genome contained in viral particles is therefore similar to that of HBV, i.e. a partially double-stranded partially single-stranded DNA molecule, maintained circular by a cohesive end formed by the 5' terminii of the two strands and with a heterogeneity in the 3' end of the short strand (see Fig. 1). For HBV (15), DHBV (16) and GSHV (17) it has been reported that the 5' terminus of the long (or minus) strand is blocked by a covalently attached protein which has not yet been characterised. Such a protein has not been reported in the literature for WHV. However, the putative importance of such a protein which appears to serve as the primer for minus-strand synthesis (16) and the close resemblance of WHV to the other hepadnaviruses makes it most likely that the protein exists. Similarly, a protein kinase activity associated with HBV (18) and GSHV (19) core particles has been described, but there is no description of such an activity

associated with WHV core particles.

THE GENOMIC SEQUENCE

The genome of WHV has been molecularly cloned in E.coli (20) and two different isolates have been fully sequenced (21, 22). Full sequences are also available for HBV (23 - 27), GSHV (28) and DHBV (29, 30). The first isolate sequenced, WHV1, is 3308 nucleotides long, while WHV2 is 3320 nucleotides long. Such size differences, always different by a multiple of three, are also observed for different HBV or DHBV isolates. WHV2 differs from WHV1 in 117 base pairs (bp), including one deletion of 3bp and one insertion of 15bp. The two isolates therefore differ by less than 4%, which is well within the range of variation expected for viruses in animals caught in the wild. In fact, the variation is about the same as that found for HBV isolates of the same subtype adr (22) while variations between isolates of different subtypes are much larger. When the sequences of WHV are compared with GSHV, a difference of about 15% is observed. Although initial hybridisation results (6) suggested that there was little homology between the HBV and WHV genome, the sequence shows that, except for two regions, the homology ranges from 62 to 70% (21). GSHV appears to be less closely related to HBV, with an overall nucleotide homology of 55% (28), and DHBV is even more distant, showing only about 40% homology with HBV (29, 30).

The genetic organisation of the genomes of the three mammalian viruses are identical (Fig. 1). The plus strand has no apparent coding capacity. Although some of the published sequences contain sizeable open reading frames (ORF's), there is no consistancy of position and often no suitable initiation codon. The minus strands of all three viruses possess four ORF's capable of coding for proteins larger than 10kDa. The viruses use all three reading frames, allowing overlapping of genes, although at no point the three phases are used simultaneously. 51% of WHV is overlapped, and the total coding capacity of the four ORF's is 1671 aminoacids. With the possible exception of the PreC region (see below), the first ATG of each ORF appears to be used for initiation of proteins so that the coding capacity is efficiently

used. Overlapping of genes to improve coding capacity is obviously desirable for a small virus, but may introduce evolutionary constraints. However, of the 117 differences between WHV1 and WHV2, 67 (57%) occur in overlapped regions as do 47% of the differences between WHV2 and GSHV.

The Pol region. This region covers 80% of the genome and can code for a protein of 879 aminoacids in WHV1. The second ATG codon occurs 511 codons after the first and therefore it is most likely

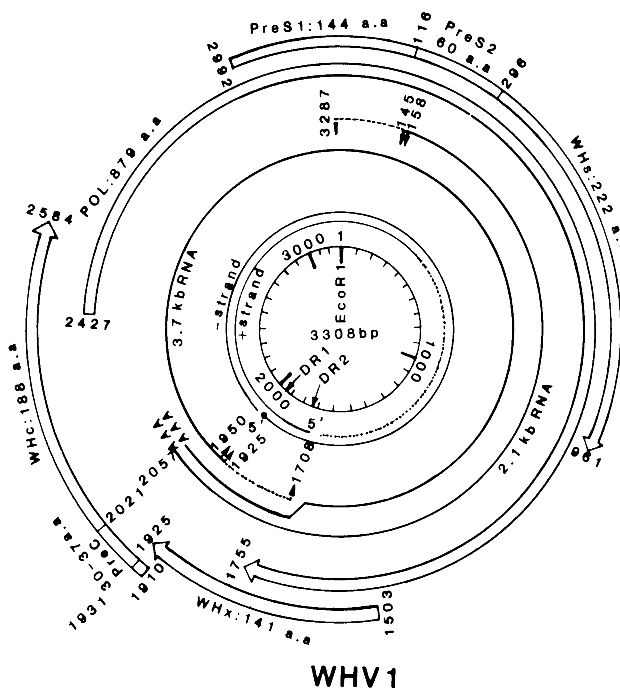

Fig. 1. The genomic organisation and transcriptional map of WHV1. From the inner circle : map units with the unique EcoRI site taken as position 1/3308 and with the positions of DR1 and DR2 indicated : representation of the DNA in virions with the 5' terminal protein of the minus strand (42), and the heterogeneity of the 3' terminus of the plus strand indicated by the dotted line : transcriptional map with the positions of known start sites indicated, minor transcripts indicated by the dotted lines : the four coding ORF's with the first ATG, important internal ATG's and the stop codon being indicated. The DNA and protein data are taken from ref.21 and the transcriptional data from ref.33

Table 1. Comparison of the ORF's of the mammalian hepadnaviruses

VIRUS / REGION	WHV_2		GSHV		HBV ayw	
	base changes total(%)	a.a. changes total(%)	base changes total(%)	a.a. changes total(%)	base changes total(%)	a.a. changes total(%)
Pol	82 (3.1)	40 (4.6)	436(16.5)*	198(22.5)*	N.C.	N.C.
Surface Ag	10 (1.5)	3 (1.4)	58(8.7)*	23(10.4)*	188(28.2)	85(38.3)
PreS1	15 (3.5)	7 (4.9)	103(23.8)	37(25.7)	N.C.	N.C.
PreS2	7 (3.9)	3 (5.0)	38(21.1)	15(25.0)	N.C.	N.C.
core Ag	7 (1.2)	1 (0.5)	65(11.6)*	17(9.1)*	184(32.6)	57(30.3)
PreC	1 (1.1)	1 (3.3)	1(1.1)	1(3.3)	12(13.3)	5(16.7)
x Ag	20 (4.8)	14 (9.9)	60(14.5)	36(26.1)	145(34.3)	74(52.5)

Comparison of the nucleotide and aminoacid differences between the coding regions of WHV1 (21) and those of WHV2 (22), GSHV (28) and HBV ayw (23). Values marked with * are taken from ref.22 and compare WHV2 with GHSV. The other values are calculated by us. The comparison of the PreC region was done starting from the second ATG of the WHV1 ORF. Deletions or insertions, regardless of size, are treated as single events for both base and aminoacid changes. Percentages are calculated using the size of the WHV1 ORF or the size of its predicted aminoacid sequence. N.C. - not calculated.

that translation starts with the first ATG, presuming that the region is translated as a free standing protein (see below). A similar large ORF is found in all hepadnaviruses, and is assumed to code for the viral polymerase, initially on the basis of the size of the region (23), and more recently on the basis of amino-acid homologies with other RNA dependent DNA polymerases (see below). The Pol region overlaps completely or partially the other three ORF's. The PreS and WHs region is completely overlapped. Apparently it is the PreS-WHs aminoacid sequence which is privileged. Of 31 base changes noted between WHV1 and WHV2 in the overlap, 26 involve positions 1 and 2 of Pol codons while only 13 involve positions 1 and 2 of PreS-WHS codons. In the overlap of the WHx region, Pol is slightly favoured, 8 out of 16 base changes involve positions 1 and 2 of Pol codons against 10 for WHx. There are no base changes in the overlapped portion of WHc. Overall, the

Pol regions of WHV1 and WHV2 are relatively well conserved, and when WHV2 is compared with GSHV, although the differences are much larger, the WHV Pol region shows good homology with the GSHV Pol region (Table 1).

The PreS and WHs antigen (WHsAg). This region can code for a total of 426 aminoacids. By analogy with HBV, the region coding for the major structural protein found in full and empty viral particles, WHsAg, starts with the third ATG of the ORF, and would code for a protein of 222 aminoacids. Proteins starting from the first and second ATG's of the ORF are known (see below) and so WHV, like HBV, possesses a PreS1 and a PreS2 region, coding for 144 and 60 aminoacids respectively. The WHsAg region is very well conserved in all three mammalian viruses (Table 1). The PreS regions are more variable, and frequent insertions and deletions make comparison of the PreS regions of WHV and HBV difficult (21). Comparison of the PreS1 and PreS2 regions of WHV1 with those of WHV2 and GSHV shows that in both cases the PreS regions are more variable than the respective S Ag regions and that globally, the PreS1 and PreS2 regions vary to the same extent (Table 1). However, in the last third of the PreS1 region, from the EcoRI site up to the PreS2 ATG at position 116, the two WHV isolates are completely homologous. When the PreS1 region is compared with GSHV, the base changes are evenly distributed along the entire region, but in the last third, these lead to relatively few amino-acid changes. The variability of the PreS1 region therefore lies principally in the N-terminal part, which includes the 15 base pair insertion found in WHV2 and not in WHV1 or GSHV and a three base pair insertion found only in GSHV. The PreS regions of different subtypes of HBV are also less well conserved than their corresponding HBsAg region (22, 25). Features that are conserved in all hepadnaviruses are the presence of a large hydrophobic stretch in the surface antigens and at least one potential glyco-sylation site (Asn-x-Thr) in the surface antigen region and one in the PreS2 region.

The PreC and WHc region. This region can code for a total of 225 aminoacids. On the basis of the observed size of HBcAg, the second ATG of the HBV region was proposed as the site of initiation of HBcAg (23). Comparison of the HBV and WHV sequences

shows that it is the third ATG of the WHV region which would serve as initiator for WHcAg. Sequence homology indicates that the second ATG of the GSHV C region is the probable initiation site for the core antigen of that virus. The genomic organisation of DHBV is different from the mammalian viruses in that its C region, identified by aminoacid homology at the carboxy-terminus is much larger than those of the other viruses, and may represent a fusion of X and C regions (29). The three mammalian viruses therefore apparently initiate their c antigens, which are found in core and viral particles, at an internal site of the ORF, leaving open the possibility that a larger PreC protein can also be made. There are indications that such a protein may exist and may be important in the viral life cycle and in pathological effects (see below). Several sequenced isolates of HBV do not have a PreC region because of stop codons situated between the ATG of HBcAg and the upstream ATG (25, 31). However, in infectivity tests, an HBV isolate containing a PreC region was infectious while another isolate without a PreC region was not (32). While the lack of infectivity cannot be attributed unambiguously to the lack of the PreC region, the working hypothesis is that a PreC region and a PreC protein are part of the normal viral life cycle, at least in mammals. If this is so, then which of the two ATG's located upstream of the WHcAg gene in both WHV1 and WHV2 is used ? Starting with the second ATG, there is good sequence homology when compared with the region downstream from the single PreC ATG of both HBV and GSHV. It is therefore likely that this is the initiator codon for a woodchuck PreC protein. However, the use of the first ATG cannot be excluded, since it is present in both WHV isolates and also because the HBV PreC ATG occurs just before the end of the HBxAg gene, as does the first ATG of the WHV PreC ORF. This ATG may therefore be considered structurally homologous to the HBV PreC ATG.

The core antigen region is well conserved in all three mammalian viruses, better even than the surface antigen genes (Table 1), at least at the aminoacid level. The PreC region, starting with the second ATG of the WHV ORF, is even better conserved, and is the most homologous region at both the

nucleotide and aminoacid level of all the mammalian hepadnaviruses. In the short distance separating the first two ATG's of WHV, there is one base change which does not lead to an amino acid change, but in the corresponding region of GSHV, there are 6 base changes which involve not only the introduction of a stop codon but also the transformation of the first ATG to ATA. This indicates that even if WHV uses the first ATG, the N terminus of the PreC region is not essential for viral replication. We have preliminary results suggesting that this is so.

The WHxAg region. The fourth ORF of WHV can code for a protein of 141 aminoacids. It is the least conserved region of the three mammalian viruses (Table 1) and, as we have mentioned above, DHBV does not possess a free standing X ORF. The HBV X gene is longer by 9 codons than the WHV X gene, at the carboxy terminus, but the GSHV X gene product is shorter than that of WHV by loss of three codons at the carboxy terminus. It would seem that HBV has extended the X gene to ensure overlap with the PreC region while GSHV has doubly ensured that such an overlap does not happen. It is also interesting to note the effect of the base changes on the amino acid sequence. For example, although the percentage of base changes between WHV1 and either GSHV or HBV are similar for both the C gene and the X gene, the percentage of amino acid changes nearly doubles for the X gene as compared with the C gene (Table 1).

Putative control elements. Several sequences are thought to play an important controlling rôle in the hepadnavirus life cycle. First, all four sequenced viruses contain two short direct repeats, called DR1 and DR2 (see Fig. 1), which are found at or near the 5' terminii of the minus and plus strands respectively of DNA extracted from virions. Their role in viral replication will be discussed later. In DHBV, the direct repeats are 12 bases long, while in HBV, GSHV and WHV2 they are 11 bases long. The WHV2 and GSHV direct repeats are identical, while those of HBV differ at one position. The direct repeats of WHV1 are only 10 bases long, the difference occuring because at what would be the first position of DR2, a G has been substituted for a T.

Another DNA structural feature which may play a role is the

presence of two closely spaced inverted repeats, which can form a stem and loop structure, in all the hepadnaviruses. In the mammalian viruses, the inverted repeats are imperfect, while in DHBV they are perfect. The structure is identical for WHV1, WHV2 and GSHV while that of HBV differs by one base. In DHBV, the structure is located between the direct repeats, but in the mammalian viruses it is situated outside the direct repeat region. The constant feature is that in all the hepadnaviruses, the stem and loop structure is situated within the PreC region. It is possible that this structure modulates expression of the PreC and C proteins.

TRANSCRIPTION

Viral transcription has been studied in chronically infected woodchucks (33). Poly $(A)^+$ RNA hybridising with WHV DNA probes fall largely into two families, one having a size of about 2.1 kilobases (kb) and the other a size of about 3.7 kb. These families represent 45% and 40% respectively of hybridisable material. About 10% of the hybridisable material is found in forms larger than 3.7 kb (5.6 and 6.9 kb). Poly $(A)^-$ RNA gives a hybridisation pattern showing a smear lower than 3.7 kb with some discrete bands in the 1-2 kb region. Presumably this represents degraded material having lost the poly (A) tail.

S1 mapping showed that the poly (A) tail is added to all transcripts at position 2057 (± 5), 20 bases downstream from the variant polyadenylation signal (TATAAA) found at position 2036-2041 on the WHV1 genome, just after the start of the WHc gene. The 3' end of viral transcripts has also been mapped to this location in all the other hepadnaviruses (34-36).

S1 mapping and mapping by primer extension show that the 5' ends of the 2.1 kb RNA are situated mainly at positions 145 (± 10) and 158 (± 10). Primer extension indicates that there is a minor transcript starting at position 3287 (± 20). Both major 2.1 kb transcripts can code for WHsAg (see Fig. 1). The minor transcript can code for a PreS2 protein. There was no indication of an independent transcript starting before position 2992 which would code for the PreS1 protein.

The size of the 3.7 kb transcript, even if one assumes a poly (A) tail of 100-200 bases, indicates that it is larger than the viral genome. This is confirmed by S1 and primer extension mapping. These show that the major start sites for the 3.7 kb RNA occur at positions 1925 (± 10) and 1950 (± 10), with a minor start site at 1708 (± 20). The 3.7 kb RNA's are therefore not matured at the first polyadenylation signal, while the 2.1 kb transcripts are. This is a feature of all the hepadnaviruses. The ability of the 3.7 kb RNA to be efficiently matured only at the second polyadenylation signal is essential for the expression of various viral proteins and for production of the template for viral replication (see below). This may imply that polyadenylation requires further signals upstream of the 5' start sites of 3.7 kb RNA. These signals would be present only before the second polyadenylation signal of 3.7 kb RNA, but would also be present in the 2.1 kb RNA. Both major 3.7 kb species can code for the WHc protein, the Pol protein, and, eventually, the WHx protein. The WHx protein could also be coded for by the 2.1 kb species, but there is no evidence for this. A separate minor transcript for the HBx protein has been reported (37). At present, there is no evidence for a specific WHx mRNA, and the question remains open. Similarly, a PreS1 protein could be coded for by 3.7 kb RNA, but the existance of a minor specific mRNA cannot be excluded. The major RNA species starting at position 1925 (± 10) could code for a PreC protein starting at ATG 1931, but a PreC protein starting at ATG 1910 would probably have to be coded by the minor RNA starting at 1708 (± 20).

What are the promotors for the different viral transcripts? Again, the answers are ambiguous, since the hepadnaviruses apparently use "unconventional" regulatory sequences. Cattaneo et al. (38) have identified two regions upstream of the major HBs mRNA start site which are homologous with the sequence of SV40 late promotors rather than with the "traditional" TATA box. WHV contains a similarly placed sequence, although the homology with SV40 is weaker. It has been proposed that a sequence found in HBV preceeding the PreS1 region, TATATAA, could be part of the promotor elements for a PreS1 mRNA (39). However, this sequence, per se, does not exist at or near this position in WHV1, WHV2 or

GSHV and a specific PreS1 mRNA has not been identified for these viruses. The promotor for the 3.7 kb RNA family (or core promotor) has been tentatively placed for HBV as the sequence TACATAA (39). However, in WHV1, WHV2 and GSHV, not only has this sequence been almost entirely deleted, but also it would have been situated about 150 bases from the start sites of the major 3.7 kb transcripts.

A transcriptional enhancer element has been described for HBV, situated about 300 bases from the beginning of HBx (40). There are no published reports of such an element for WHV, but in the region concerned, WHV1, WHV2 and GSHV conserve the sequence found in HBV, GTGTTG, which is similar to the consensus enhancer sequence (40), surrounded by two very GC rich regions. Tur-Kaspa et al. (41) have shown stimulation of HBV enhancer activity by glucocorticoids and have identified a sequence NCAANNTGTYCT, outside of the enhancer region, which is similar to the consensus glucocorticoid receptor binding site. However, at the equivalent position in WHV1, WHV2 and GSHV, this sequence is extensively modified away from the consensus sequence.

There is no hard evidence for the splicing of hepadnavirus mRNA's.

REPLICATION

There are no detailed published descriptions of WHV replication. However, WHV contains all the features thought to be important for replication, and a model can be constructed based on results obtained with DHBV (42-44), and GSHV, including partial results with WHV (45).

The template for the first round of DNA synthesis would be one of the 3.7 kb transcripts. This template is terminally redundant, and the DR1 sequence is found at both ends of the molecule. Minus-strand synthesis is primed by the protein found attached to the 5' terminus of minus strands. The 5' end maps within the DR1 sequence. Which DR1 is used is not known. The simplest model assumes initiation within the 3' DR1 and continuous synthesis up to the 5' end. However, it is also possible that synthesis could initiate at the 5' DR1 followed by strand

switching to the 3' end of the template just upstream of the second DR1. The advantage of the latter model is that a combination of the 5' DR1 and the cap normally found at the 5' terminus of eukaryotic mRNA's could serve as the signal for positioning of the protein primer. The 3' end of minus strand DNA maps to a point several bases upstream of DR1 which means that minus strand DNA is itself terminally redundant by 7-8 bases. DNA synthesis is accompanied by degradation of the RNA template, presumably by RNase H activity which is probably of cellular origin. At the end of minus-strand synthesis, a small RNA oligo is released which consists of the DR1 sequence and the short sequence up to the 5' end of the RNA molecule. This oligo then hybridises to the minus-strand DNA at DR2 and serves as primer for plus strand synthesis. These steps all occur within the intracellular core particles. At some point after initiation of plus strand DNA, the core particles are transformed into mature viral particles and exported. Minus-strand DNA synthesis is interrupted, leading to the partially single-stranded nature of virion DNA and the heterogeneity of the 3' terminii of plus-strand DNA. The final step would occur after the infection of hepatocytes, involving removal of the plus-strand 5' RNA oligo and of the terminal redundancy and covalently attached protein of the minus strand, completion of the plus strand and covalent closure to produce a supercoiled DNA molecule which would serve as the transcriptional template.

THE PROTEINS

The proteins of WHV have been extensively studied, especially their relationship to the corresponding HBV proteins. Dot matrix analysis of the three major viral proteins, the polymerase, the core antigen and the surface antigen, clearly reveals continuous amino acid homology over the entire lengths of the HBV and WHV proteins (29). WHx and HBx share little aminoacid sequence homology except at the N-terminus where 24 of the first 30 aminoacids are shared and a stretch near the end of the proteins where 16 out of 23 amino acids are shared. Schaeffer and Sninsky (46) have compared the hydrophilicity profiles and the predicted secondary structures of HBV and WHV viral proteins, and find that

even for WHx and HBx, which share little amino acid homology, the predicted probable secondary structures are very similar.

The polymerase. The polymerase proteins of the hepadnaviruses not only show amino acid homology amongst themselves, but more interestingly they show homology with retroviral reverse transcriptases. The most salient feature is a nonapeptide common to WHV1, WHV2 and GSHV which shares seven amino acids with a similar nonapeptide found in RSV and MoMLV reverse transcriptases. Using this nonapeptide as the basis of alignment of the different aminoacid sequences, other homologies between the hepadnavirus polymerases and retrovirus reverse transcriptases can be found both upstream and downstream from the peptide (29, 47). In a very interesting article on a common origin of retroviruses and hepadnaviruses, Miller and Robinson (48) have also described homologies between retroviral gag proteins and hepadnaviral core proteins, raising the possibility that the polymerase found in viral particles will turn out to be a fusion protein coded by parts of both the core and Pol ORF's. This would seem to be the case in the hepadna-related CaMV plant virus system (49), and Will et al. (50) have reported finding polypeptides in several human hepatocarcinomas that react with both anti-HBc and anti-Pol sera.

Anti-viral agents which specifically affect the polymerase would be useful for treatment of viral hepatitis, and in a series of studies, Hantz et al. (51, 52) and Nordenfelt et al. (53, 54) have compared the properties of WHV and HBV polymerases and the "in vitro" and "in vivo" effects of different anti-viral agents. The requirements of the WHV and HBV polymerases are similar with respect to the optimum pH and ionic strength of the incubation medium and the need for magnesium. The polymerases are inhibited "in vitro" by trisodium phosphono-formate (PFA) but not by phosphono-acetic acid (PAA). However, in chronically infected woodchucks, PFA appears to have no effect on viral particle formation as measured by serum DNA polymerase assay (54). Treatment of chronically infected woodchucks with vidarabine monophosphate for 10 days results in a large decrease of serum DNA polymerase levels but which increase again after the end of treatment (52).

WHs and PreS proteins. The serological relationship between WHsAg and the surface antigens of the other hepadnaviruses has been studied extensively. It was evident soon after the discovery of WHV that WHsAg was antigenically cross-reactive with HBsAg (14). Commercial tests designed to detect HBsAg, such as AusRIA II, can be used to detect WHsAg. WHsAg is also antigenically cross-reactive with GSHsAg (55), but DHBsAg appears to be antigenically distinct from the mammalian surface antigens (56). Cote et al. (57) have used monoclonal antibodies to define the antigenic relationships between the mammalian hepadnavirus surface antigens. Of 11 murine monoclonal antibodies directed against WHsAg, 3 were specific to the woodchuck protein, and 8 also cross-reacted with GSHsAg of which one also cross-reacted with HBsAg (57). The 11 antibodies could be divided into five groups which recognise nonoverlapping antigenic sites of WHsAg (56). One group consists of the single antibody which recognises all three surface antigens, two other groups recognise GSHsAg while two groups of antibodies are specific for WHsAg.

WHV particles contain two major proteins which migrate on SDS-polyacrylamide gels with apparent molecular weights of 22 and 25 kDa. Tryptic peptide mapping shows that they are closely related and that p25 is probably a glycosylated form of WHsAg (58). Other larger peptides are also observed, which contain tryptic peptides common to WHsAg as well as several other unique peptides, suggesting that those polypeptides are coded from the PreS1 and PreS2 ATG's. This has been confirmed by Schaeffer et al. (59). They raised polyclonal antibody sera against a polypeptide containing the PreS1 and PreS2 junction (mostly PreS1) cloned and expressed in E. coli. These sera recognised four proteins of apparent molecular weights of 33, 36, 45 and 47 kDa in infected woodchuck sera, corresponding to a PreS1 and PreS2 protein and their glycosylated forms. The sera could immunoprecipitate viral particles containing endogenous DNA polymerase activity, indicating that the PreS determinants are found on the surface of mature virions. The sera can also detect similar proteins in infected ground squirrel sera, showing that the two viruses share common PreS determinants. PreS proteins are thought to be involved

in attachment of viral particles to hepatocytes, but the exact mechanism is controversial. HBV viral particles can bind to artificially polymerised human serum albumin (HSA) but not to monomer HSA or polymerised BSA, and this property is ascribed to the PreS2 region (60). HBV particles would bind to a similar factor in human blood for which hepatocytes carry a surface receptor. The HBV particles would therefore attach to hepatocytes by a "sandwich" mechanism and the entire complex would be internalised into the hepatocyte. Neurath et al. (61), however, have concluded that HBV particles can attach directly to hepatocytes, and that this attachment involves mainly PreS1 determinants. There are as yet no published results concerning this problem in any of the other hepadnavirus systems. However, either mechanism could explain the host range of WHV.

WHcAg and PreC protein. As we have mentioned above, the core antigen is the best conserved major protein of the hepadnaviruses. Dot matrix analysis shows a characteristic "arrow tail" pattern when WHcAg is compared with HBcAg (29). This is due to numerous small basic repeats which confer a protamine - like structure to the carboxy - terminus and which is presumably involved in core antigen-DNA interactions. The major difference between HBcAg and WHcAg is the intracellular localisation - in infected human hepatocytes, immunofluorescence staining for HBcAg is largely nuclear while in woodchucks, WHcAg staining is largely cytoplasmic as is also the case for the ground squirrel and ducks (13). The reason is not known, but in infected human livers, HBcAg staining in those hepatocytes which are actively replicating HBV appears to be cytoplasmic (62). WHcAg can also be detected in full viral particles, but only after detergent treatment. From the beginning, it was evident that the WHcAg/anti-WHc and HBcAg/anti-HBc systems were strongly cross reactive, which could be demonstrated by immuno-diffusion, haemagglutination and immuno-electronmicroscopy (14), although the lack of reliability of the commercial Corab kit in detecting anti-WHc (63, 64), suggests that anti-WHc has a relatively low affinity for HBcAg.

The core antigen is also involved in another antigenic system, the e system. HBeAg is a soluble antigen found in the

serum of hepatitis patients during the replicative phase of viral infection. Seroconversion to anti-HBe positivity generally indicates the end of active viral replication. HBeAg has been purified from the blood and has been shown to consist mainly of HBcAg which lacks the protamine-like tail (65). HBcAg can also be converted to HBeAg by various denaturing techniques. The HBe epitopes are therefore masked on normal HBcAg. Woodchucks also possess a WHeAg/anti-WHe system which cross reacts with the human system (66).

The putative PreC protein may also be involved in the e Ag system. Ou et al. (67) have shown that when DNA capable of expressing either human PreC protein or HBcAg are transfected into Cos cells, HBcAg staining is cytoplasmic when the core construct is used, but is membrane associated when the PreC construct is used. In addition, the material showing HBeAg reactivity is excreted into the medium when the PreC construct is used, while HBcAg remains intracellular. The presence of core determinants in the cell membrane is important, because neither WHV nor HBV appear to be cytopathic, and destruction of infected hepatocytes appears to be mediated by cytotoxic T cells probably directed against membrane-associated core Ag determinants (68).

WHxAg. We and others have shown that some human hepatitis patients harbour antibodies directed against HBxAg protein (69, 70). We have also shown that a woodchuck serum can immuno-precipitate HBx protein cloned and expressed in E. coli (69). Symmetrically, we have also observed that a human serum which strongly recognises HBxAg in western blots also weakly recognises WHxAg cloned and expressed in E. coli (A.K., unpublished results). Woodchucks therefore possess a WHxAg/anti-WHx system which cross-reacts with the human system.

The role of the WHxAg is unknown. It is tempting to think that it is the as yet unidentified protein covalently attached to the 5' terminus of the minus strand, but there is no evidence for this. Miller and Robinson (48) have speculated that the X gene may be of recent cellular origin and that the x protein may be analogous to the transactivating protein found in HIV.

PREVALENCE, PATHOLOGY AND CELLULAR TROPISM.

Exposure to WHV occurs early in life and 50% of feral woodchucks captured in the endemic area of the United States are either WHsAg carriers or have undergone seroconversion to anti-WHs (71, 72). The proportion of chronic WHsAg carriers is high. Seronegative animals can be experimentally infected with WHV (7, 73, 74), inducing acute hepatitis in some cases, but the infections are usually self limiting with rapid seroconversion to anti-WHs and anti-WHc. Chronic WHsAg status is rare in these animals, probably related to their age at the time of infection.

Hepatic lesions seen in infected woodchucks are similar to those seen in HBV infected humans (73, 75). They range from a normal liver or one containing only minor portal and/or parenchymal lesions which can also be seen in some seronegative animals, through moderate and then severe portal and parenchymal lesions and finally hepatocellular carcinoma (HCC). Hepatic inflammation can range from mild to severe.

The incidence of HCC in naturally infected woodchucks is high, especially in the chronic WHsAg carrier population (73, 75), although Wong et al. (76) have also reported HCC in some anti-WHs-positive animals. Unlike HCC in humans, HCC in woodchucks is not associated with cirrhosis and the animals are also usually serum DNA polymerase positive (75). Both phenomena are probably related to the extremely rapid onset of HCC in woodchucks.

Although major pathological effects are restricted to the liver, low but significant amounts of WHV DNA have been found in the kidney and spleen of infected animals (6) as well as WHsAg but not WHcAg (73). Korba et al. (77) have also detected WHV in peripheral blood lymphocytes and bone marrow of chronically infected woodchucks. There was no sign of integration of WHV DNA into the genome of the lymphocytes and the DNA existed mostly in multimeric forms, with little sign of the replicative intermediates normally found in liver cells.

INTEGRATION AND HEPATOCELLULAR CARCINOMA

In almost all human and woodchuck HCC's studied, viral DNA integrated into the host chromosome has been identified. Analysis

of integrated viral DNA isolated from woodchuck tumours shows that the viral genome is extensively rearranged (78), apparently more so than equivalent HBV integrations. Rogler and Summers (79) have cloned a viral integration from the liver of a chronically infected animal which did not develop HCC, and in this case, the viral genome was not rearranged but about 500 base pairs were deleted. Kaneko et al. (80) investigated primary HCC tissue which contained integrations and also extrachromosomal replicative-intermediate WHV DNA, but when the tumours were transplanted or established in cell culture only integrated DNA was found. As a general rule then, integrated WHV DNA cannot serve as a pregenome for the production of extrachromosomal replicating viral DNA or the production of infectious viral particles, although some viral antigens may be expressed.

Although viral integration appears to be a prerequisite for HCC, the actual mechanism of carcinogenesis is unknown. The hepadnaviruses do not appear to contain an oncogene and, with the exception of one human HCC (81), viral integration does not seem to occur near a known cellular oncogene. The virus does not seem to integrate at a specific site or sites, and in the case of the integration in the chronically infected liver, there was no rearrangement of the cellular DNA at the site of integration. Recently, Möröy et al. (82) have shown that in 3 out of 9 woodchuck HCC's, the c-myc oncogene locus was rearranged. However, again there was no apparent physical association between integrated viral sequences and the oncogene.

CONCLUSIONS

WHV is the animal virus which most closely resembles HBV in genomic organisation, nucleic and amino acid homologies and in the pathological consequences of viral infection. However, many fundamental aspects of the hepadnaviruses have not been studied in the woodchuck system, no doubt because of the scarcity of experimental breeding colonies and perhaps also because of difficulties arising from the fact that the animal enters into profound hibernation for many months. It is somewhat surprising that there are no published studies of the effect of this

hibernation, during which body temperatures drop from 36° to only 8°C, on the course of viral infection.

WHV also very closely resembles GSHV, but the pathological outcomes are strikingly dissimilar. Infected ground squirrels show few clinical signs of acute hepatitis and although GSHV-associated HCC has recently been reported (83), the incidence is much lower than in woodchucks, and squirrels develop HCC much later in life than do woodchucks. The overall genetic organisation of WHV and GSHV is very similar, with the exception that there can be no overlap of the GSHV X and PreC genes. This may modulate X or PreC gene expression, or both. Another possibility is that the profound hibernation of woodchucks (ground squirrels also hibernate, but less deeply) provokes liver changes which increase the likelihood of viral integration or favour the expansion of tumour cells.

The past few years have seen a rapid increase in our knowledge of fundamental aspects of hepadnavirus life cycles, but several important features are, at best, only imperfectly understood. The recent advent of cell culture systems capable of replicating HBV and producing apparently complete viral particles (84) will help in resolving some of these problems. However, study of some of the pathological aspects of viral hepatitis will still require use of the captive animal.

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GROUND SQUIRREL HEPATITIS VIRUS

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ABSTRACT

Ground squirrel hepatitis virus (GSHV), found in Beechey ground squirrels in California, is 1 of only 4 well-characterized members of the hepadnavirus family. Studies of GSHV since its discovery in 1979 have confirmed the genetic, antigenic, morphologic and biologic characteristics of this virus family, originally defined by its prototype: hepatitis B virus (HBV). GSHV shares with HBV a liver tropism and an ability to cause persistent infection in some individuals exposed to the virus. There is a high frequency of development of primary hepatocellular carcinoma in long-term carriers of both GSHV and HBV. Use of fresh tissues from GSHV-infected squirrels has been invaluable in defining the various steps of hepadnaviral replication. The GSHV-ground squirrel model was recently used for the first in vivo tests of the genetic organization of hepadnaviruses. This animal model of HBV should continue to be useful in the future for studies of still unknown steps of viral infection requiring fresh tissues or tissue culture, and in testing the effects of new antivirals and immune modulators on mammalian hepadnavirus infection.

INTRODUCTION

Ground squirrel hepatitis virus (GSHV) was the second hepatitis B-related virus found in non-primate animal species. The discovery of GSHV in Beechey ground squirrels in 1979 (1) followed that of woodchuck hepatitis virus (WHV) (2) in eastern woodchucks by a year and was the result of a search for a hepatitis B-like virus in California relatives of the woodchuck.

The relationship of the ground squirrel virus to hepatitis B was based on the following observations: 1) Sera from some ground squirrels contained particles with the unique physical and morphological properties of hepatitis B virus (HBV). 2) These particles, like HBV, harbored a DNA polymerase which repaired a single-stranded region in a viral DNA similar in size to the 3200 base pair (bp)

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HBV DNA. 3) Most of the repaired GSHV DNA in virions was held in circular configuration by cohesive ends resembling those of HBV DNA. 4) Finally, the surface antigen of the ground squirrel virus particles was found to cross-react with that of hepatitis B virus.

Despite the continued considerable search for virus in the sera of many animal species, GSHV remains one of only 5 members of the hepadna (for "hepatic" and "DNA") virus family. HBV, WHV, and GSHV were joined in 1980 by duck hepatitis B virus (DHBV) infecting domestic ducks (3) and in 1986 by a less well-defined virus found in common gray tree squirrels, named tree squirrel hepatitis virus (THBV) (4).

HBV, the prototype hepadnavirus infecting man, has been studied since the initial observation of its surface antigen by Blumberg and coworkers in 1965 (5). However, the unique molecular and biological features distinguishing HBV from other viruses were difficult to study in a virus that infects only man and two rare and expensive primates (6). The discovery of HBV-related viruses infecting non-primates coincident with the amazingly rapid development of genetic cloning and engineering has allowed exploration of the HBV family at an unexpectedly fast pace. All the hepadnaviruses but THBV have been cloned, sequenced, and their genomic structure defined and compared both to each other and existing viral and cellular sequences. Hepadnavirus replication and tissue tropism have been elucidated using the abundant fresh tissues available in the animal models. The course of virus infection in the animal models has been studied and compared to HBV. Studies of intervention with antivirals are increasing as the models become more clearly defined. Understanding the pathology associated with the various animal models of HBV has taken several years of observation of carrier animals. The unique association of HBV with both hepatitis and primary hepatocellular carcinoma now appears to extend to WHV and GSHV and their hosts, with hepatitis detectable in DHBV and THBV carriers. Both the latter viruses may eventually be associated with liver cancer when more years of observation are completed.

The two goals of this chapter are to describe ground squirrel hepatitis virus and to show how the study of this virus has contributed to knowledge of hepatitis B and the hepadnaviruses.

NATURAL OCCURRENCE AND HOST RANGE

GSHV infection in the wild has only been detected in the original host, Beechey ground squirrels (Spermophilus beecheyi) (1,7-9), which are found on the Pacific Coast of North America ranging from Baja California in Mexico north into the state of Washington in the United States. The known geographical range of the virus is extremely limited, being confined to squirrels living on the grounds of Stanford University, Stanford, California and adjacent areas. Ground squirrels three miles distant from the virus-infected population have neither virus nor antibody to viral antigens in their sera. We have been unable to detect virus in hundreds of sera of this squirrel species from many other parts of the state of California, the major area of Beechey ground squirrels. Virus has not been found in other species of ground squirrel in California or in 7 animal species trapped in the area endemic for GSHV. The reasons for the strict geographical isolation of GSHV are not known. While the travels of both man and the domestic duck explain the worldwide spread of HBV and DHBV, the natural occurrence of the other hepadnavirus of wild animals, WHV, is restricted to a geographical range of a few states on the East Coast of the United States (10).

The host range as defined by injection of virus into various animal species is as limited with GSHV as with HBV. Experimental transmission of GSHV by injection of whole virus has resulted in a GSHV viremia only in the original host and in one other member of the sciuromorph family, the chipmunk (a Eutamias species) (9). No viremia resulted from injection of GSHV into several more closely related Spermophilus species, nor into several non-members of the squirrel family: Sprague-Dawley rats, Syrian hamsters, guinea pigs, New Zealand rabbits, and 2 strains of mice (7,8).

The development of new techniques may change our understanding of host range in this virus family. Though no viremia has been documented when GSHV is injected into woodchucks, another member of the squirrel family, viremia did occur when cloned GSHV DNA was injected directly into the liver of woodchucks, utilizing a technique first established with HBV DNA in a chimpanzee (11). It is not yet clear whether infection using viral DNA and not whole virions is successful in this heterologous system because of an evasion of the immune system or because the attachment and entry steps of viral infection are circumvented. This genetic approach to exploring the various levels of host range may give us a better understanding of host susceptibility. Infection of unrelated laboratory animals with GSHV or even HBV DNA is an exciting possibility.

VIRUS STRUCTURE

Electron microscopy shows the GSHV virion to be about 47 nm in diameter, slightly larger than the 42 nm HBV virion (1). Like HBV, the GSHV virion consists of a nucleocapsid containing a DNA genome and at least 2 enzymatic activities. The nucleocapsid is surrounded by an envelope containing lipid, protein, and probably carbohydrate.

Viral genome.

The nucleic acid of the ground squirrel hepatitis virion was observed in the initial publication to be DNA of about 3200 bp in length, partially single-stranded, and held circular by cohesive ends (Fig. 1). Like HBV DNA, the long, complete strand has a protein bound to the 5' end (12). The virion DNA was subsequently cloned into plasmids and the restriction pattern of several isolates determined (13,14). Regions homologous to the coding regions for HBV envelope surface antigen and nucleocapsid core antigen were localized by annealing radiolabeled HBV DNA probes specific to the previously determined individual antigen coding regions.

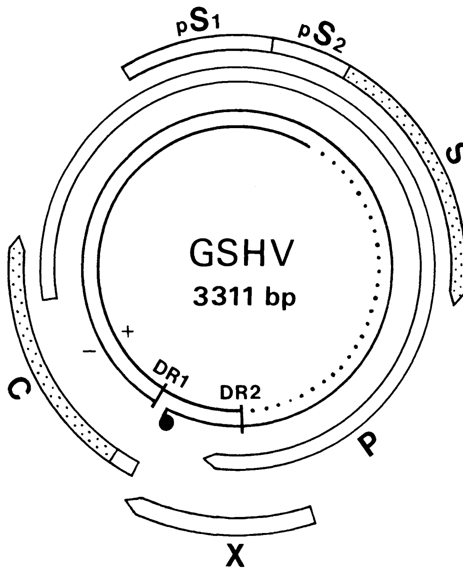

Fig. 1. Genetic organization of GSHV. The long (-) and short (+) strands of virion DNA are shown in the inner circles, with the dashed line representing the heterogeneous 3' ends of the short strand. DR 1 and DR 2 are the locations of the direct repeats. The wide arrows show the positions of the open reading frames.

The nucleotide sequence of GSHV DNA was published in 1984 by Seeger and coworkers (15). The DNA of the sequenced clone was 3,311 base pairs long and contained four major open reading frames (ORFs) in an arrangement previously described for HBV and WHV (see Fig. 1). The S and C ORFs code for the previously mentioned surface and core antigens respectively. A long ORF (P) is presumed to code for the viral polymerase. The remaining short ORF is termed the X ORF, since its product has not yet been identified. As with the three other hepadnaviruses, an additional ORF precedes and is contiguous with the S ORF of GSHV DNA and is known as the pre-S coding region. As predicted by earlier DNA hybridization and serology experiments (16,17), GSHV is closely related to WHV, the hepadnavirus infecting another member of the sciuromorph family, with a nucleotide homology of 82% and an amino acid homology of 78%. GSHV is related to HBV less closely, but still significantly, with nucleotide and amino acid homologies of 55 and 46%.

Analysis of the sequence of GSHV DNA revealed the presence of two 11-nucleotide direct repeats separated by 211 nucleotides. These sequence features, similarly present in the other hepadnaviruses, play an important role in viral replication as will be discussed later. Seeger and coworkers, using primer extension and S1 nuclease protection studies (18), located the three fixed termini of the long and short GSHV DNA strands, adding information about a mammalian hepadnavirus to several findings observed with DHBV (19-21). The 5' end of the long strand (after removal of the bound protein and linearization) was mapped within the first direct repeat sequences (DR 1) mentioned above, with the 3' end located only 8 nucleotides upstream. The long strand therefore has a terminal redundancy of 9 nucleotides and is longer than the cloned GSHV genome by the same amount. The 5' end of the short strand was determined to reside 232 nucleotides upstream from the 5' end of the long strand and 7 nucleotides upstream of the second direct repeat (DR 2). The 5' end of the short strand is not DNA, however, but consists of 17 or 18 ribonucleotides covalently linked to DNA. This short 5' sequence of RNA will be further discussed under Viral Replication. The 3' ends of the short strand are heterogeneous, with the short strand only spanning 40 to 60% of the long strand.

In summary, the GSHV genome is similar in size, physical structure, and sequence features to the genomes of other hepadnaviruses. Other than the actual DNA sequences, the only known variation in hepadnavirus genome structure is in DHBV, which appears to lack the X ORF.

Viral envelope.

In the HBV genome, the S gene encodes a 25 kilodalton (kDa) polypeptide which is found in the virion envelope (reviewed in 22). This surface antigen (HBsAg) elicits protective antibody appearing at the end of acute HBV infections. The most abundant forms of virus particles in sera during HBV infections are not complete virions, but are defective spherical and tubular forms 22 nm in diameter which contain only viral envelope and no nucleocapsid. These defective forms known as sAg particles also exist in GSHV infections, with the tubular forms particularly abundant and elongated into a filamentous form (1). These numerous unusually long filaments are not seen in the other hepadnavirus infections.

Analysis of purified GSHsAg particles showed them to have a buoyant density similar to that of HBsAg particles, and to contain a pattern of polypeptides similar to those of HBsAg particles on SDS-PAGE (23). In HBV this pattern consists of two major polypeptides, the 25 kDa species encoded by the viral genome and a 29 kDa species, plus several slower migrating minor polypeptide species ranging to 65 kDa. All the polypeptides can be precipitated individually by antibody to HBsAg. The 29 kDa polypeptide is a glycosylated form of the 25 kDa polypeptide. In similar analysis of GSHsAg particles, the major polypeptides migrate as 23 kDa and 27 kDa species. The sAgs of GSHV, HBV, and WHV are all cross-reactive, with the strongest reactivity between those of GSHV and WHV (16).

It has now been shown that the 31 to 45 kDa polypeptides in HBV particles showing HBsAg reactivity are either glycosylated or unglycosylated products of the regions encoded both by various portions of the pre-S ORF as well as the entire S gene (24-26). Polypeptides encoded by the complete pre-S have both the pre-S_1 and the pre-S_2 regions in addition to the S region, while other polypeptides have only the pre-S_2 region adjacent to the S gene. In HBV, the pre-S_2 region encodes a polyalbumin binding site thought to be involved in viral infection (27-29). Polypeptide species containing pre-S_1 appear to be more numerous on complete virions than on defective particles (24-25). There is evidence that the pre-S polypeptides are more immunogenic than the S polypeptides alone (25,30,31) and that anti-pre-S is a neutralizing antibody (32). The presence of 45 and 47 kDa pre-S encoded polypeptides in GSHV particles has been demonstrated by Schaeffer and collaborators (33) using Western blot analysis with an antibody to a bacterially-synthesized fusion molecule containing

89 WHV pre-S-encoded amino acids. A similar analysis by Persing and coworkers (34) using bacterially-synthesized GSHV pre-S_1 identified a 43 kDa polypeptide which carried pre-S_1 determinants. The Persing group observed 3 species of polypeptides ranging from 40-49 kDa in purified GSHsAg particles using silver staining and SDS-PAGE. Some of these polypeptides may have been reactive to the anti-pre-S sera used by the Schaeffer group.

All studies of the GSHV envelope to date indicate that it is similar in physical, chemical, and antigenic properties to that of HBV. It should be possible to use GSHV as a model of HBV in studies of two relatively unexplored areas, neutralization of hepadnaviruses and viral attachment and entry into the host cell.

Nucleocapsid.

The nucleocapsid of HBV is seen by electron microscopy to be a sphere of about 27 nm in diameter (35). These nucleocapsids or cores can be isolated from virions by removal of the virus envelope with a nonionic detergent (36). An antibody to the surface of these cores (anti-HBc) is produced early in infection. When analyzed by SDS-PAGE, these HBV cores contain a major polypeptide of 19 kDa (37,38) which is encoded by the C ORF (39,40). Similarly sized particles with the same antigenic reactivity can be isolated from the liver of infected individuals.

GSHV virions contain a nucleocapsid of a size similar to that of HBV (1). Antibody to HBV cores cross-reacts with GSHV cores isolated from virus-infected ground squirrel liver with the procedures used for HBV cores. These GSHV cores also contain one major polypeptide component, which migrates on SDS gels slightly faster than the HBV core polypeptide (42). As with HBV, the GSHV core particles contain the viral DNA and a DNA polymerase activity. In addition purified core particles of both GSHV and HBV have an associated protein kinase activity which phosphorylates the core polypeptide predominantly at serine, but also at threonine residues (43). Therefore, while the precise size and sequence of GSHV and HBV cores have been shown to differ, no morphological or functional differences in the nucleocapsids have been detected.

VIRAL REPLICATION

Following the seminal work by Summers, Mason, and coworkers on hepadnavirus replication in ducklings infected with DHBV (44,45), work with GSHV-infected ground squirrels has confirmed that in the mammalian

hepadnaviruses as well, replication occurs through reverse transcription of an RNA intermediate. In addition, the GSHV model has been used by Seeger, Enders, Ganem, Varmus, and coworkers to elucidate many additional steps of hepadnavirus viral replication. Those interested in a detailed discussion of hepadnavirus replication and replication of GSHV in particular are encouraged to read the excellent review published recently by this group (18).

GSHV-infected ground squirrel liver, like all hepadnavirus-infected liver, contains viral DNA species not seen in virions (12,46). A protein-free supercoiled viral DNA in the nucleus of infected cells is thought to be formed from incoming virion relaxed circular DNA and to serve as the template for synthesis of viral RNA. Relaxed circular viral DNA with variable amounts of repair of the short strand is found in the cytoplasm along with single-stranded species ranging from the lowest limit of detection on a Southern blot of an agarose gel to the full-length 3300 nucleotide species. All of these cytoplasmic DNAs appear to have covalently-bound protein and all are found in core particles. Use of phage-derived single-stranded probes of hepadnaviral DNA has shown that the short strand of virion DNA is plus-stranded, like viral mRNA. The long strand is therefore minus strand DNA as is the single-stranded viral DNA found in infected liver.

Plus-stranded viral RNAs found in the liver are three 3.5 kb species with terminal redundancies of 130-160 nucleotides, and 2.3 kb RNAs (47). All these RNAs are polyadenylated at the same location and appear to be unspliced. The 2.3 kb RNAs cover the S gene and possibly the pre-S_2 region, providing the mRNA for expression of some of the sAgs. The other ORFs are complete only in the 3.5 kb RNAs; expression of the remaining genes is assumed to be from some form of these RNAs. Enders and coworkers have recently determined that the shortest of three 3.5 kb RNAs is found predominantly in the core particles (48). This species is assumed to be the template for reverse transcription of the minus or long strand of viral DNA. The primer for this transcription appears to be the protein bound to the 5' end and the transcriptase is assumed to be the polymerase originally found in virions. Degradation of the RNA template, by a yet undescribed mechanism, leaves an approximately 18-nucleotide RNA oligomer which serves as a primer for plus-strand synthesis and which remains in the virion after export from the cell. The direct repeats (DRs) play a role in viral DNA synthesis. The RNA oligomer remaining at DR 1 moves to DR 2 before extension of the strand by the polymerase, thus forming the cohesive ends of the

virion DNA. By mutating both GSHV DNA DRs by 1 base and infecting animals with viral DNA with one or the other mutation, Seeger and collaborators have shown that the plus strand begins with an RNA oligomer having the sequence of DR 1 but continues with DNA synthesized with the sequence of DR 2 (18). This experiment further indicated that successful viral replication of GSHV is not dependent upon the two DRs being of strictly identical sequence.

The biosynthesis of GSHV core or surface proteins has not yet been described, and the products of the presumed polymerase and X genes have not been isolated from infected cells or virions of any of the hepadnaviruses. The function of the X gene product is totally unknown as is the role or source of the nucleocapsid protein kinase. It is also unknown whether the protein bound to the minus strand is coded by a viral or cellular gene.

TISSUE TROPISM

GSHV is predominantly a hepatotropic virus. Although little liver pathology was observed in early studies of GSHV infection, viral cores containing DNA polymerase activity were found to be as abundant in GSHV-infected ground squirrel liver as in HBV-infected liver (42). A study by Ganem and coworkers (7) of various tissues of GSHV-infected squirrels demonstrated that hybridizable viral DNA was found in highest amounts in the liver (600-6000 genome-equivalents per cell). GSHV DNA equivalent to less than 1% of that in the liver was found in other tissues. Trace amounts of viral DNA found in the kidney and spleen may have been from contamination with the virions in the blood or possibly from low-level replication as has been demonstrated in DHBV-injected ducklings (49). With an assay sensitivity of 2 viral genome-equivalents per cell, no viral DNA was detected in the pancreas, contrary to results found in DHBV infection (50). Replication in lymphocytes, reported to occur in the other mammalian hepadnaviruses (51,52), has not yet been examined.

COURSE OF THE VIRUS INFECTION

GSHV infection in the ground squirrel has been monitored by measuring viral DNA polymerase, GSHsAg, anti-GSHs, and anti-GSHc levels in sera or by detection of hybridizable viral DNA in the serum or liver (1,7,8). In our study of experimental transmission of GSHV (8), virions in sera were measured by DNA polymerase assay, anti-GSHc by enzyme immunoassay, and both GSHsAg and anti-GSHs by radioimmunoassay. All antibody assays were done using reagents

purified from GSHV particles or ground squirrel sera. The sAg assay was a commercial assay for HBsAg. GSHV-infected sera was injected into Beechey ground squirrels having no detectable markers of GSHV, and serum samples were taken at two week intervals. As with experimental transmission of HBV, experimental infection of ground squirrels with GSHV led to three types of response: 1) self-limited or transient GSHsAg-positive infection, 2) GSHsAg-positive infection which became persistent, and 3) primary anti-GSHs and anti-GSHc responses without detectable GSHsAg or virion DNA polymerase. Anti-GSHc was a marker that appeared in all ground squirrels with primary GSHV infection and never appeared when other species were injected with GSHV. The higher the dilution of inoculum the longer was the delay before appearance of anti-GSHc. Onset of infection ranged from 4 to 16 weeks after inoculation. There were proportionally fewer self-limited and more primary antibody responses without GSHsAg detection (45%, 45%) than in one study of experimental transmission of HBV (70%, 23%) (53). These differences may be due only to the lower numbers of individuals studied or the less frequent blood sampling of the squirrels, rather than an actual difference in response of humans and squirrels to hepadnavirus injection.

In naturally-infected chronic carriers of GSHV, the levels of virion-associated DNA polymerase activity are higher by 10-20 fold than those of non-experimental HBV carriers. These high levels appear to be due to large amounts of virions in the blood rather than increased DNA polymerase activity per virion, as the amount of hybridizable viral DNA in natural GSHV infections is also high. Why GSHV infections are of such a high titer is not known. Clearance of viremia in naturally-occurring persistent infection is a rare event: only 1 of 39 carrier ground squirrels studied over a 2 year period resolved their viremia and became positive for anti-GSHs. This animal had been trapped only a few months prior to seroconversion, however, and the infection may have been acute and not chronic. Replicating forms of GSHV DNA were found in the liver of all of 7 squirrels trapped as carriers and observed in captivity for 4 to 6 years until death. Thus viremia probably continued in all these animals which survived until late middle or old age. We have never observed a carrier squirrel to have GSHsAg but no viral DNA or DNA polymerase activity in the blood in late infection, as is seen in HBV infection of man. The longer life span of man compared to that of a squirrel may explain these differences, though a difference in other host or virus genetics cannot be excluded. Prenatal

transmission of GSHV infection from mother to offspring was examined in one study of 6 newborn squirrels born to an animal with a high titer of GSHV (54). Due to the small size of the newborns, livers and not sera were tested for markers of GSHV. No viral DNA was detected in any of the newborns, suggesting that transmission of virus from mother to offspring does not occur *in utero*.

In a recent study by Persing, Varmus, and Ganem (34), peptides encoded by both the pre-S and the X regions of the GSHV genome were produced in bacteria. Sera from GSHV-infected and postresolution squirrels were used to immunoprecipitate radiolabeled, bacterially-synthesized pre-S and S encoded peptides. Only the postresolution sera precipitated the pre-S peptide, with 6 of 12 anti-S positive and 2 of 7 anti-S negative animals showing an anti-pre-S response. It is not known whether antibody to these pre-S determinants or to the synthesized S determinants contribute to either resolution of infection or immunity to reinfection. Four of 12 postresolution ground squirrel sera immunoprecipitated bacterially-synthesized peptide from the X region, indicating that this coding area is expressed during GSHV infection.

PATHOLOGY OF GSHV INFECTION

Early studies of GSHV infection indicated that this hepadnavirus was less pathogenic than either HBV or WHV (7,8). Our accumulated data from 65 captive squirrels observed over a period of six years supports these early observations. However, significant to moderately severe hepatitis is associated with GSHV infection. The coded study showed that 52% of GSHV carriers had hepatitis rated significant or greater at necropsy, while only 6% of uninfected ground squirrels had significant hepatitis. Unlike observations of WHV and HBV carriers (55), severe hepatitis extending beyond the portal area and into the parenchyma of the liver is rarely seen in GSHV infection. The cirrhosis associated with HBV infection of man but not WHV infections has not been seen in GSHV infections.

The primary hepatocellular carcinoma (PHC) associated with HBV infection in man usually occurs after 10-30 years of persistent infection and in only some of those infected with HBV (reviewed in 56). WHV-infected woodchucks are more likely to have PHC, since about 1/3 of captive carriers develop liver tumors per year (55,57). To examine the association of GSHV with PHC, we have observed virus-infected and uninfected ground squirrels held in separate quarters

since 1980. Some animals have been added into the colony since its inception and some squirrels were experimentally infected and studied. Necropsies were done on all animals at death and histology performed under code. For 4 years no PHC was observed and GSHV received the reputation of being a non-pathogenic virus. There has been a dramatic change in the number of tumors observed in our colony in the past 2 years, however, with PHC emerging as the predominant type of tumor (58).

Tumors have appeared only in squirrels estimated to be 4.4 years of age or older. In this age group tumors developed in 9 of 15 carrier squirrels, 4 of 5 postresolution squirrels, and 4 of 13 GSHV marker-free squirrels. There have been a total of 10 cases of PHC (59% of all neoplasms): 8 of these were in carrier animals, 2 in convalescent, and none in GSHV-free animals. These observations indicate that PHC is associated with GSHV infection. Tumors other than PHC have been varied, with no tumor type predominating. The average age of the squirrels when PHC is detected is about 6 years, older than the average age of WHV carriers when PHC develops, but considerably younger than that of HBV carriers. Of the carrier animals developing PHC, one was an experimentally-infected animal, while the others became GSHV carriers before capture. High molecular weight viral DNA was detected in the tumor DNA of 3 carrier animals analyzed by Southern blots. This high molecular weight viral DNA is assumed to be integrated into host DNA, as has been observed in tumors of other hepadnaviruses (reviewed in 59).

PHCs appearing in the 3 younger squirrels (ages 4.5 to 5.4 years) were all single small tumors, and those in the 7 older squirrels (ages 6.0 to 8.0 years) were all multinodular. The diameter of the major tumor was generally larger in older squirrels. The histological type of all but one PHC was trabecular, with the arrangement of the tumor cells maintaining some aspects of liver architecture. The remaining PHC was a more anaplastic medullary hepatocellular carcinoma, and this tumor arose in one of the squirrels with anti-GSHs and not GSHsAg. Trabecular PHC is the type of liver tumor most often associated with both HBV and WHV (55,57). Thus development of PHC, especially of the trabecular type, appears to be a common element of long-term mammalian hepadnavirus infection.

EVOLUTIONARY ASPECTS OF GSHV

As previously discussed, the closest known relative of GSHV by nucleotide sequence homology and antigenic cross-reactivity is WHV. As might be expected, the hosts of these viruses are also closely related, being 2 different genera of the same Sciuridae family. The viruses might have diverged as their hosts diverged, or were selected or adapted after entry into a host somewhat related to the original host. Whether the newly-discovered tree squirrel hepadnavirus is the same as either GSHV or WHV or more closely related to GSHV than WHV will be determined by more complete characterization of this hepadnavirus-like element. All the hepadnaviruses have a similar genetic and morphologic structure despite their varying degree of relatedness.

The relationship of GSHV and the hepadnaviruses to the retroviruses and retrovirus-like endogenous DNA elements has been emerging over the past few years, as the viral genomes and DNA elements have been sequenced and computer programs developed to compare sequences. Following the original observations that there was a reverse transcription step in hepadnavirus replication (44) as well as amino acid sequence homology between the putative polymerase protein of HBV and a conserved region of the reverse transcriptase of retroviruses (60), Miller and Robinson examined the DNA sequences of 13 hepadnavirus isolates in greater detail (61). They found that the most highly conserved sequence of the hepadnavirus genomes, positioned near the initiation site for minus DNA strand synthesis, is homologous over 67 nucleotides to the U5 region in retroviral long terminal repeats. Furthermore, 98 amino acids of the core protein of HBV share a significant 41% amino acid homology with the p30 nucleocapsid protein of type C retroviruses. From these and additional analyses the authors suggest that the hepadnaviruses and retroviruses have a common evolutionary origin, with the former arising from deletions of a retrovirus or retrovirus-like ancestor.

USE OF GSHV AS A MODEL OF HBV INFECTION IN MAN

The many ways that study of GSHV has advanced knowledge of HBV and the hepadnavirus family have been described in the preceding sections. The concordance of the morphology and structure of GSHV with the other hepadnaviruses has helped to define the virus family. Development of PHC in long-term carriers of GSHV has affirmed the association of hepadnaviruses with liver cancer and provided hepadnavirus-associated tumor materials for study and

comparison to HBV PHC. Use of fresh tissues from GSHV-infected ground squirrels has been invaluable in defining the various steps of hepadnaviral infection and also in determining whether phenomena discovered in the avian hepadnavirus, the most divergent of the 4 well-defined hepadnaviruses, are also present in the mammalian viruses. It is with the GSHV-ground squirrel model that the genetic organization of hepadnaviruses was first tested in vivo by mutating the genome then observing virus progeny resulting from injection of the altered DNA directly into the host liver. We have also demonstrated the usefulness of GSHV carrier ground squirrels for evaluating the effects of antivirals (62).

Some limitations of the GSHV model of HBV have been defined by the early studies. Beechey ground squirrels are only available in certain locations of the West Coast of the United States. Naturally-infected animals are no longer easy to obtain because the original locale of high incidence has been taken over by man. Production of chronic viremia occurs in only 10% of the experimental animals. Acute viremia does not always result from injection of GSHV into susceptible squirrels, as many animals respond with only a primary antibody response to both surface and core antigens. The lapse of time between injection of high titer virus into ground squirrels and development of viremia is several weeks, with a great deal of variation seen among individual squirrels (7,8). Injection of similar amounts of DHBV into young ducklings usually results in a persistent viremia in only a few days, with little variation in response among the ducklings (64). It is difficult to obtain newborn ground squirrels that might be more readily susceptible to persistent virus infection, as there is no breeding facility for these animals, and newborn squirrels in the wild remain underground until they are 2 months old.

Nevertheless, there are many ways in which the GSHV model of HBV can be useful in the future. The DHBV model uses animals that are more readily available and more easily infected than the other animal models, but there is considerable variation between birds and mammals and between the avian and mammalian hepadnaviruses. Experimental observations of DHBV must be confirmed in one of the mammalian systems before application to hepadnaviruses in general and HBV in particular. GSHV is as convenient a mammalian model as any available. There is a continuing use for GSHV in studies of viral replication and this animal model provides a needed source of fresh material for studying the relatively uncharacterized DNA polymerase, protein kinase, and DNA-bound

protein associated with hepadnaviruses. Tissue culture of DHBV in primary hepatocyte cultures has recently been accomplished (64). Similar cultures of GSHV should also be possible, allowing study of the initial adsorption and uncoating phases of mammalian hepadnavirus infection by virions. These studies are not possible when infection is done by introduction of cloned DNA into cultured cells (65). Finally, the high titer persistent GSHV infection of ground squirrels should continue to be useful in studying the effects of new antivirals and immune modulators on mammalian hepadnavirus infection.

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DUCK HEPATITIS B VIRUS

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ABSTRACT

Duck hepatitis B virus (DHBV) is a member of the hepadna virus family. In this review we have attempted to summarize the current knowledge of the molecular biology of DHBV and its use in hepadna virus research. DHBV has attracted considerable attention because ducks are the only domesticated animals in which hepadna viruses can be studied. Major advances in the understanding of the life cycle of hepadna viruses have been obtained by using DHBV-infected animals. The basic principles and many details of hepadna virus replication have been discovered using DHBV-infected ducks. Infection of hepatocytes in vitro has been achieved so far only with duck liver cells. Important knowledge came from studies on tissue and cell tropism, genome structure, gene organization and gene expression mechanisms. In addition, there is increasing evidence for the further potential of this animal system for studying pathogenicity, host range, evolution, viral receptors, and viral therapy.

INTRODUCTION

The hepatitis B virus family (*hepadnaviridae*) includes four viruses which have been studied in detail on the molecular level (1). The prototype of this family is the human hepatitis B virus (HBV). Closely related viruses have been identified in woodchucks [*Marmota monax*, WHV (2)] and ground squirrels [*Spermophilus beecheyi*, GSHV (3)]. Related viruses seem to exist also in tree squirrels, stink snakes, and kangaroos (4). The Pekin duck hepatitis B virus (DHBV) is

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the only avian hepadna virus described so far. Although DHBV is less related to HBV than the other mammalian hepadna viruses (5), DHBV-infected animals represent a highly attractive system to study hepadna viruses. In contrast to woodchucks and ground squirrels, Pekin ducks are domesticated animals readily available from commercial sources, they are easy to house, and breed, and closely related domesticated animal species are available for host range and viral disease-related studies. Thus it is not surprising that a growing number of laboratories is using this animal system.

Often up to 10% or more of Pekin ducks from commercial breeds are naturally infected with DHBV. The primary route for maintenance of DHBV is by vertical transmission through the egg (6). Horizontal transmission is rare if it occurs at all (7). Experimental infection can be very efficiently achieved by intraveneous injection of DHBV containing serum into 15-to 17-day-old duck embryos or by intrahepatic injection into one-day-old ducklings (7). Cloned viral DNA can induce viral infection after injection into the livers of one day old ducks (8). Thus, it is possible to study biological effects of in vitro manipulated genomes in vivo. It is also possible to establish and to infect primary liver cell cultures of ducks with DHBV (9).

Major advances in the understanding of the life cycle of hepadna viruses have been obtained by using DHBV-infected animals (10). Most important, the basic principles and many details of hepadna virus replication have been elucidated by using DHBV core particles and virions. In addition, many studies with this animal system have provided important knowledge concerning tropism, genome structure and gene organization, and gene expression of hepadna viruses. In this review we attempt to summarize the present knowledge of the molecular biology of DHBV and its use in hepadna virus research.

INCIDENCE AND HOST RANGE

Duck Hepatitis B Virus seems to be widespread in commercial duck flocks all over the world. Initially, duck hepatitis B viruses were discovered in serum samples from domestic ducks collected in the People's Republic of China (11, 12) and thereafter in commercial flocks of Pekin Ducks from the United States (13). In 1984, we observed DHBV in German Pekin ducks (8), and in 1985 Cova et al. reported a DHBV infection rate of 1-6% in Pekin ducklings in France (14). In addition, DHBV was identified in Pekin ducks in Australia (Freiman, J., pers. communication) and in ducks from Taiwan (12).

Serum samples from other domestic ducks such as Khaki Campbell and Indian Runner were also found to contain DHBV-like viruses (12). Furthermore, wild migrating mallards (Anas platyrhynchos), which are the common ancestors of domestic ducks (15), were found to be infected with DHBV (16). This suggests that DHBV may have a high incidence in many domesticated ducks derived from mallards.

During the second half of the 19th century Chinese Pekin ducks were imported to the U.S and Europe and were used to create the current domestic duck breeds (17). The genetic drift of DHBV in these different breeds of ducks has been studied by cloning and sequencing of the viral genomes. The analysis revealed a relatively close relatedness of the American and German isolates (5.6% base exchanges) whereas Chinese DHBV isolates differed from both by approximately 10% (Sprengel, et al. unpublished). This indicates a high flexibility of DHBV genome sequences without interfering with viability and host range as shown by experimental transmission of German, American, Chinese and Mallard DHBV isolates to European Pekin ducks (Sprengel et al. unpublished and 16).

Recently, we have identified DHBV-like viruses in geese (*Anser domesticus*). The genomes of some of these isolates have been cloned and partially sequenced. The nucleotide sequences revealed low sequence variability compared to the

German and American DHBV strains (Sprengel et al., unpublished) suggesting that geese are infected by a DHBV derivative. By experimental infection we could demonstrate the infectivity of these "DHBV" strains in both ducks and geese. In addition, several strains of geese have been infected successfully by DHBV (18). Geese [subfamily *Anserinae* (19)] and ducks (subfamily *Anatinae*) belong to the same family (*Anatidae*), and may have become infected horizontally by DHBV. Unexpectedly, Muscovy ducks [not descendents of mallards but belonging to the same subfamily (*Anatinae*) (20)] could not be infected with DHBV (18).

NATURAL AND EXPERIMENTAL TRANSMISSION

The major route for DHBV transmission is vertical, through the eggs laid by viremic ducks. Supporting evidence was obtained by analysis of the progeny derived from infected dams. Almost 100% of the embryos and ducklings were infected (21). During vertical infection maternal DHBV virions are passively transferred to the egg together with the liver-derived yolk-sac proteins. The yolk-sac membrane may then transport the virus from the yolk to the developing embryo which then becomes infected (6, 21). In the embryo, DHBV DNA synthesis was observed at about day 6 of incubation, coinciding with the formation of the liver (21). From day 8 of incubation and continuing throughout embryonic development, subviral particles reflecting viral replication have been observed. Recently, the presence of DHBV-specific transcripts similar to those identified in the infected duck liver was identified in yolk-sac tissue (Tagawa, M. pers. communication). This suggests that DHBV is transcriptionally active and probably replicates in yolk-sac tissue.

Horizontal transmission is rare (7), if it occurs at all. We and others (16) have not observed a single clear-cut case of horizontal transmission of DHBV from viremic to virus-free ducks when housed together for up to three months after hatching. Experimentally, DHBV can be efficiently trans-

mitted by inoculating DHBV-infected serum directly into the livers of one-day-old ducklings. Approximately three weeks after injection close to 100% of the injected animals develop high titered viremia (Sprengel, et al. unpublished). A lower efficiency of infection is obtained by intraveneous injection of the virus. Infection of animals older than 3 weeks is only rarely achieved (7, 22) presumably because of immunological defense mechanisms. Immunological tolerance to DHBV particles is probably responsible for the high chronic carrier rate observed after vertical transmission and experimental infection of embryos. Ducklings developing from embryos injected with DHBV intraveneously at days 12 to 15 almost always become chronic carriers (7, 23). Infection of the embryonic liver occurs very rapidly. Six hours after infection the supercoiled, closed circular (CCC) form of the viral genome (template for transcription, see below) has been detected. Replicative intermediates and viral transcripts are present 24 hrs after experimental infection (24).

MORPHOLOGICAL STUDIES

The morphology of viral particles present in sera and infected liver tissue has been studied by electron microscopy. Viral particles of serum purified by sedimentation and banding to equilibrium on sucrose or CsCl density gradients can be separated into two fractions (12, 13). The high density fraction (1.16 g/cm^3) contains a relatively uniform population of spherical particles approximately 40 nm in diameter which represent complete virions. They consist of an outer membrane and an inner core structure harbouring the viral genome. The lower density fraction (1.15 g/cm^3) consists of pleomorphic, roughly spherical particles ranging from 35 to 60 nm which represent empty viral particles. In contrast to HBV, no filamentous forms of surface antigen particles seem to exist. A similar study was performed with liver specimens from infected ducks (25). Complete and empty viral particles

of similar sizes were located within the hypertrophied cisternae of the endoplamic reticulum. In addition, naked cores 35 - 37.5 nm in size were localized in the nuclei, free in the cytoplasm, and also near or on the cisternal membrane of the endoplasmic reticulum. From these studies it was concluded that complete viral particles are probably formed by protrusion of the core particles through the endoplasmic reticulum with simultaneous encapsulation by a coat derived from the endoplasmic reticulum. By immunofluorescence staining most of the DHBV core reactivity has been localized in the cytoplasm (26) whereas that of HBV is mainly in the nucleus (27). The reason for this difference is not clear.

GENOME STRUCTURE

DHBV has an unusual genome structure that is characteristic of hepadna viruses. It is a small (3021 bp), circular, partially single stranded DNA molecule of fixed polarity with neither strand covalently closed (Fig. 1). This unusual genome structure is a consequence of the mode of DHBV replication that depends on reverse transcription of the RNA pregenome (28). The long strand (minus strand) begins at position 2537 (29) and is terminally redundant by 6 to 8 bp (10). The short strand (plus strand) has also a fixed 5`-end [pos. 2488 (30)] but varies in length at its 3'-end. In the majority of DHBV virions the plus strand is close to full length but some particles contain a plus strand of appr. 50% genome length (8, 12). The relaxed circular conformation of the genome is maintained by a short cohesive overlap (50 bp) between the 5'-ends of the two DNA strands (Fig. 1). A protein is covalently linked to the 5'-end of the DNA minus strand (31), and a short capped oligoribonucleotide (18 to 19 nucleotides in length) is linked to the 5'-end of the DNA plus strand (30). The protein and the oligoribonucleotide appear to serve as primers for DNA synthesis (see below).

After entry of the virus into the cell, and the partially single-stranded DNA is converted into the CCC form which represents the intracellular form of the genome (32).

GENOME ORGANIZATION AND VIRAL PROTEINS

The genetic organization of DHBV was deduced from the analysis of the nucleotide sequences of two cloned DHBV genomes (33, 34). Both clones were shown to be infectious after injection into the livers of one-day-old ducklings (8, 34). As is known for all other hepadna viruses, only the DNA minus strand seems to code for viral proteins. It encodes conserved open reading frames (ORFs) of considerable length whereas on the DNA plus strand no long open reading frames are found. On the DNA minus strand three overlapping ORFs defined as C, Pre-S/S and P are present. In analogy to mammalian hepadna viruses they are thought to encode the viral nucleocapsid protein, two envelope proteins, and the DHBV polymerase, respectively.

The C-reading frame spans 915 nucleotides (pos. 2518-412) and codes for the core antigen. It is unclear whether the first, second or both AUGs are used as translation initiation codons. In DHBV the first AUG cannot be used extensively for expression of the major core protein since the putative C m-RNA starts 6 to 8 nucleotides downstream of the first AUG of the C-ORF (35). Because of sequence homologies to the HBV pre-C region and its similar location, the region between the two AUGs has been designated as DHBV precore region (34). Strikingly, the precore region is conserved in the DHBV-3 and -16 genomes (34), in two Chinese and one goose DHBV isolates (Sprengel et al., unpublished). It is therefore tempting to speculate that a corresponding protein may be expressed from a minor transcript initiating upstream of the precore AUG codon.

The S-reading frame (pos. 693-1785) encodes the viral envelope proteins. It is divided into two sections, the pre-S region (pos. 693-1284), and the S-region (pos. 1284-1785).

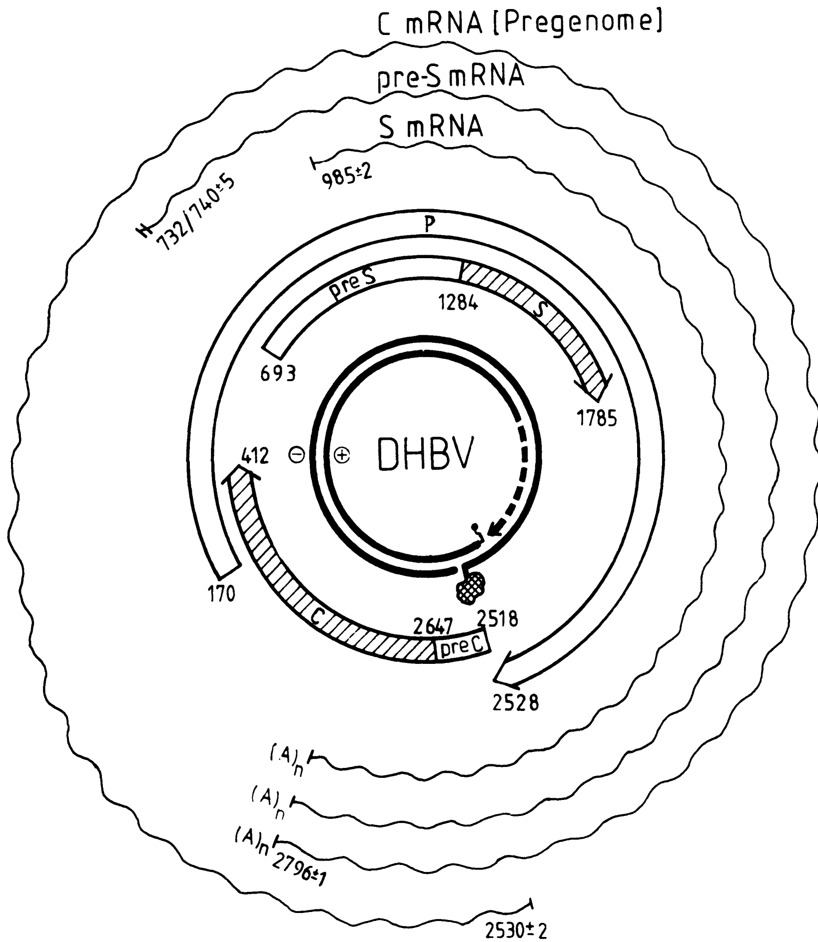


Fig. 1: Structure and genetic organization of the DHBV genome. The inner circles represent the two DNA strands of the virion encapsidated genome. The protein linked to the 5'-end of the minus strand and the small RNA primer at the 5'-end of the plus strand are indicated. The open arrows represent the three large open reading frames encoded by the minus strand of the DHBV genome. The size of the open reading frames was deduced from cloned DHBV-3 DNA (34). The three major DHBV specific transcripts are drawn as wavy lines. Positions of 5'- and 3'-ends of the transcripts and the first and last nucleotide of the open reading frames are indicated by numbers.

The S-region encodes a protein of 167 amino acids (MW. 18170 d), consistent with the size of the major viral surface antigen of about 17.5 kd as detected in infected ducks (36). The first AUG (pos. 693) of the pre-S region seems not to be used for synthesis of a pre-S/S fusion protein since it is not present on the pre-S m-RNA (35). Instead, the second AUG (pos. 801) is likely to serve as initiation codon to produce a pre-S/S protein of appr. 36 kd. A protein of this size has been identified in vivo (37, 38). In contrast to the mammalian hepadna viruses which all seem to express two pre-S proteins (39), comparative DNA sequence and viral protein analysis revealed only one pre-S protein.
The P-reading frame probably encodes the viral polymerase. In genome DHBV-16 this frame begins at position 20 (33). In isolate DHBV-3 a point mutation in position 108 creates a translation termination codon (34). Therefore, the initiation codon at position 170 may be the start of the P-ORF. The 5'- and 3'-ends of the P-ORF overlap by 80 and 4 codons, respectively, with the carboxy- and aminoterminal end of the C-ORF. This leads to a completely overlapping gene organization and leaves no space for an intergenic region. Although the gene organization of DHBV and the mammalian viruses is very similar, there is one striking difference: DHBV contains no fourth reading frame (X-ORF) between the carboxy-terminus of the P-ORF and the beginning of the C-ORF. Formally, fusion of the X- and C-reading frames of mammalian hepadna viruses could explain this difference and the larger size of the DHBV core protein. However, there are no significant DNA or protein sequence homologies between the X-ORFs and the DHBV C-ORF. In addition, the fusion hypothesis is not consistent with the location of the regulatory sequences involved in replication, the position of the precore region and the C m-RNA promoter of DHBV.

CONSERVED DNA AND PROTEIN REGIONS ON THE DHBV GENOME

Functionally important DNA sequences in the DHBV genome and protein domains in DHBV encoded polypeptides have been searched for by comparative sequence analysis of two virus genomes (34, Figure 2).

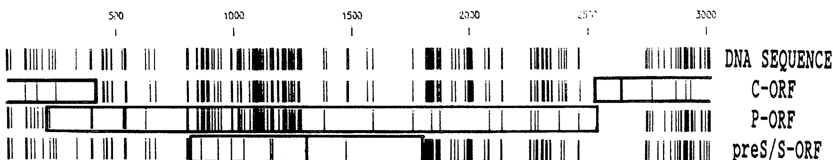


Fig. 2: Sequence comparison of two cloned DHBV genomes [DHBV-16 (33) and DHBV-3 (34)]. Two nucleotide sequences have been aligned and the base exchanges are indicated by vertical bars (row 1). In addition, amino acid seqences predicted from all possible reading frames (A, B, and C) of both genomes have been aligned, and the amino acid exchanges are displayed (rows 2 to 4). Long open reading frames known or predicted to code for proteins are boxed (C-ORF within frame A, P-ORF within B, and pre-S/S-ORF within frame C). The second AUG of the pre-S ORF which is likely to be used in translation of the pre-S protein delimits the beginning of this ORF. The scale is given in bp on top of the figure. The distribution of the vertical bars reveals conserved (few or no bars) and variable regions (many bars).

On the DNA level the two DHBV sequences published [DHBV-16 (33) and DHBV-3 (34)] show an overall sequence variability of 5.6% (34). Base exchanges are not uniformly spread along the genome (Figure 2. lane DNA). Conserved DNA sequence regions were found to be located at the carboxy-terminus of the C-frame and within the S-region. This is not unexpected because they both overlap with another ORF, the P-ORF.

In regions encoding a single polypeptide three additional highly conserved regions are found. They are located at the start site for the pre-S ORF (region 1), at position 2200-2250 in the P-ORF (region 2), and at the origin for DHBV replication (region 3). All three regions are thought to encode important regulatory signals for viral replication or transcription. Region 1 and 2 are predicted or have been shown to harbour regulatory signals for pre-S m-RNA transcription, RNA pregenome synthesis, and viral replication. In region 3 an enhancer consensus sequence (AGTGTTTGCT) can be identified which may play a role in the life cycle of DHBV. For HBV a functionally active enhancer sequence has been detected experimentally in an analogous position (40). With one exception, high DNA sequence variability is restricted to regions which do not overlap with other ORFs. The region which does not follow this rule encodes the pre-S ORF overlapping with part of the P-ORF. Unexpectedly, this region shows similar or even higher sequence variability than regions encoding a single protein. Most of the base substitutions lead to amino acid changes preferentially in the protein predicted from the P-ORF, whereas the pre-S is as conserved as other protein coding regions of DHBV (Figure 2. lane P-ORF and lane S-ORF, respectively). This suggests that in this part of the DHBV genome only the pre-S frame has protein coding function, whereas the overlapping part of the P-ORF is unlikely to encode an enzymatically active polypeptide. It may function as a spacer between different domains. The product of the S-ORF and the middle part of the P-ORF are highly conserved. The C- and the pre-S protein show similar protein sequence variability.

VIRAL TRANSCRIPTS AND PROTEINS

DHBV transcription in infected liver tissue has been studied by Northern blot analysis, S1-, and Exo VII- nuclease protection assays (35, 12). All transcripts detected by Northern blot analysis are transcribed from the minus-strand DNA consistent with the conclusion drawn from the DNA sequence analysis. Three major polyadenylated transcripts (3.5 kb, 2.3 kb and 2.1 kb in size) have been characterized. According to the position of the first AUG codons downstream of their 5' ends, the 3.5 kb RNA is refered to as C m-RNA, the 2.3kb as pre-S m-RNA, and the 2.1 kb RNA as S m-RNA. By nuclease mapping, the viral sequences in these transcripts are about 3.3 kb, 2.06 and 1.8 kb long. All three transcripts are synthesized in approximately equimolar amounts, and they are all unspliced. They initiate at three different sites but they are processed and polyadenylated at a common site (coterminal transcripts) located at the amino terminal end of the C-ORF (pos. 2800 +/- 15). Very recently we haved mapped the 3'-end of these transcripts more precisely by cloning and sequencing of DHBV specific c-DNAs to position 2796 +/- 1 (Schneider R., pers. communication), which is 13 nucleotides downstream of the processing/polyadenylation signal sequence AAUAAA.

The smallest RNA initiates 100 nucleotides upstream of the initiation codon of the S-ORF and is used for the production of the major DHBV surface protein (36). The 5'-end of the middle sized RNA is slightly heterogeneous (pos. 732 and 740 +/- 5) and is located approximately 40 to 50 nucleotides downstream of the first AUG of the pre-S region. This RNA may serve as template for expression of the 36 kd pre-S/S fusion protein found in the serum of infected ducks (37). The C m-RNA starts 13 to 15 nucleotides downstream of the precore AUG and is likely to be used for expression of the major core protein (translation-initiation codon at pos. 2647). For synthesis of this transcript the unique processing/polyadenylation signal described above must be

ignored at least once. This transcription readthrough seems to be very efficient during C m-RNA synthesis because no transcript of appropriate length corresponding to processing at the first passage of the signal has been detected. In contrast, readthrough of the same signal is very rare at the second passage through the signal sequence as during synthesis of the S and pre-S m-RNAs. Thus, the recognition of the processing/polyadenylation signal seems to depend on the initiation site and conformation of the transcripts. The C m-RNA is the only transcript of more than genome length which can be reverse transcribed into a genome length DNA genome. Indeed, there is currently more convincing evidence for its function as a RNA pregenome than as template in translation (see below).

No m-RNA initiation sites were found in front of the P-frame. Minor transcripts from which the polymerase protein may be expressed may exist but have not been detected. Due to similarities in gene organization of hepadna- and retroviruses another possibility on the mechanism of hepatitis B polymerase expression has been suggested. As for retroviruses (41, 42), the DHBV polymerase may be synthesized in the form of a precursor protein containing both core and polymerase sequences from which the active enzyme is release by proteolytic cleavage. Indirect evidence for this possibility has been presented for HBV (43). The only possible messenger RNA for synthesis of a core-polymerase fusion protein that is known so far would be the C m-RNA.

As for the P-ORF no transcript initiating upstream of the precore region has been identified. In contrast, such transcripts have been detected as relatively major species in GSHV and WHV infected liver tissue (44, 45).

Three minor polyadenylated transcripts (6.6 kb, 5.5 kb, 5.0 kb in size) corresponding to 1-5% of the major RNA species were also detected in DHBV infected livers. These species probably arise from single and double readthroughs of the

unique processing/polyadenylation site during synthesis of the major transcripts. Low amounts of two nonpolyadenylated RNA species, 3.0 kb and 1.7 kb in size, were also found. The biological functions and significance of all minor transcripts are not known.

Signals involved in regulation of DHBV transcription have not been defined functionally. Promoter like sequences (TATA boxes) precede the C m-RNA by 32 nucleotides and the pre-S m-RNA by 23 and 31 nucleotides but not that of S m-RNA. A consensus enhancer sequence is found about 300 bp upstream of the core promoter (pos. 2214-2234).

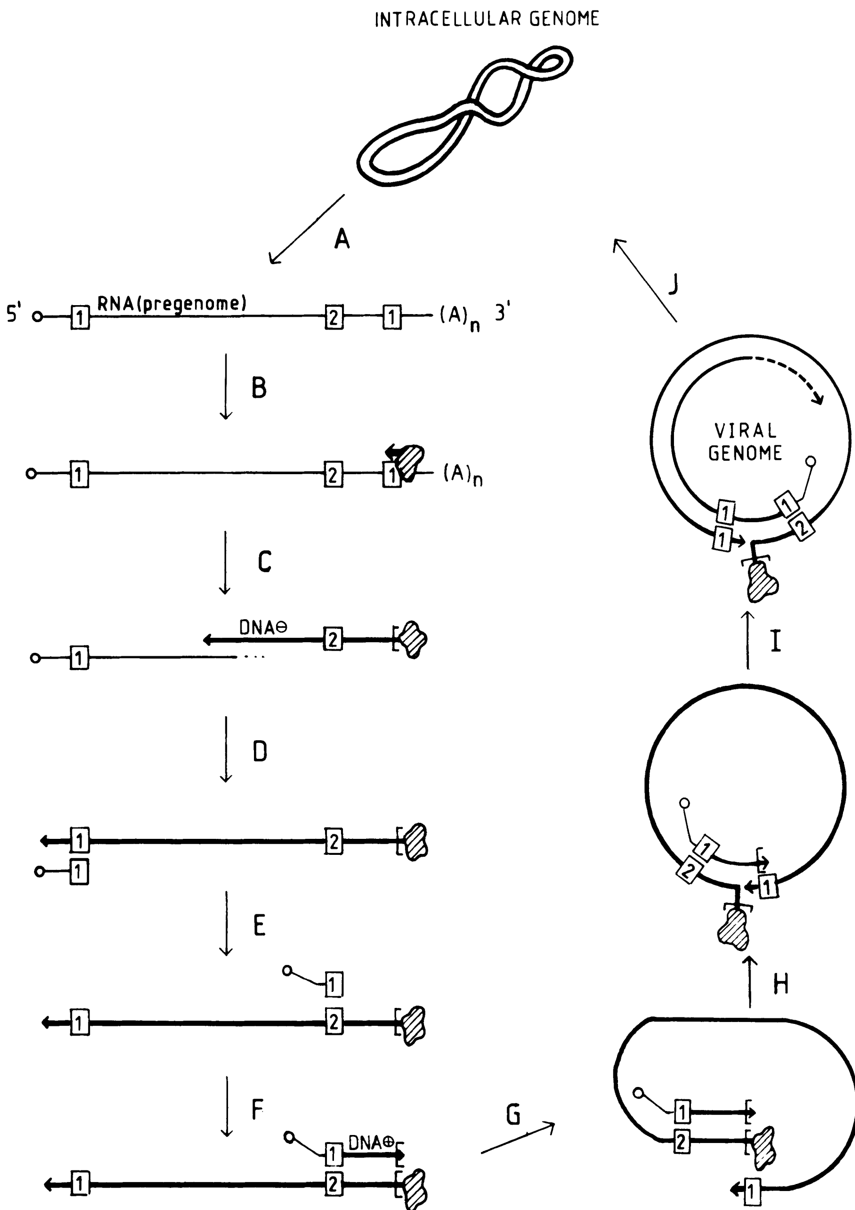
VIRAL REPLICATION

One of the most interesting aspects of hepadna viruses is their unusual mode of replication involving reverse transcription. The basic principles and many details of the replication strategy of hepadna viruses have been discovered using the DHBV/duck system (9, 28, 29, 30, 31, 35, 46), and the current model of the replicative cycle of hepadna viruses is summarized in Fig. 3. The key features of the replication mode are (i) repair synthesis of the viral genome, (ii) synthesis of an RNA pregenome, (iii) reverse transcription of the pregenome to produce DNA minus strands, and (iv) DNA plus-strand synthesis by copying the DNA minus

Fig. 3: Schematic representation of the replication pathway of DHBV (model). Step A: the RNA pregenome is transcribed from the CCC form of the viral genome. The direct repeat sequences DR1 and DR2 are indicated by boxes numbered by 1 and 2. Step B: the protein primer binds to repeat sequence DR1 (only one of the two possibilities is shown) and DNA minus strand synthesis is initiated. Step C: elongation of DNA minus strands and concomitant degradation of the RNA pregenome. Step D: completion of DNA minus strand synthesis. All but the first 18 nucleotides of the RNA pregenome are degraded. Step E: transfer of the RNA primer to the DR2 sequence. Step F: elongation of the DNA plus strand up to the 3'-end of the DNA minus strand. Step G: circularisation of the replicative intermediate. Step H: intramolecular template switch. Step I: elongation of the DNA plus strand. Step J: conversion of the partially single stranded genome into the CCC form.

Fig. 3:

REPLICATION PATHWAY OF HEPADNAVIRUSES



strand. Semiconservative replication of viral DNA does not take place at any step of the replication cycle (32).

After infection of the hepatocyte the partially double-stranded circular viral genome as present in the virions is converted to a double-stranded, superhelical, circular, intracellular form (CCC-form) by repair synthesis. To achieve this, the gap of the partially single-stranded viral DNA has to be repaired by a polymerase, the genome-linked protein has to be released by a protease or an endonuclease, the terminal redundancy of the DNA minus strand has to be removed by an exo- or endonuclease, and the oligoribonucleotide linked to the DNA plus strand must be removed by RNase H or by DNases after strand displacement. Finally, the DNA strands have to be covalently closed by a ligase. These steps have to occur during or after the release of the virion encapsidated genome within the cell. The intracellular CCC form of the viral genome is used as template for transcription of the viral RNAs as it appears prior to synthesis of viral RNAs and replicative intermediates, and as about 50 copies are present only in the nuclei of infected liver cells (7, 24). One of the viral transcripts of more than genome length serves as RNA pregenome to produce full-length viral DNA genomes. Shortly after or even before initiation of reverse transcription the RNA pregenome is encapsidated into core particles [usually referred to as immature core particles (28)]. Thus, reverse transcription and DNA synthesis take place during virus maturation within the virus particle on its way out of the cell. The signals and proteins involved in encapsidation, and the conformation of the RNA after encapsidation are not known.

The 3.5kb C m-RNA is the only virus-specific transcript of more than genome length which could serve as RNA pregenome. This transcript is terminally redundant by 270 nucleotides

and carries two potential initiation sites for DNA minus-strand synthesis. After binding of the primer protein, DNA minus-strand synthesis could either start close to the 5'-end or at the 3'-end of the RNA pregenome within a repeat sequence designated DR 1 (Fig. 3) according to location of the 5'-end of the DNA minus strand (29, and Will, H. unpublished). Initiation at the 5'-end would lead to synthesis of only a few nucleotides of DNA minus strand and would then require a template switch for elongation. Initiation at the 3'-end of the RNA pregenome would not require a template switch for synthesis of a complete DNA minus strand. Whether both or only one initiation site is used remains to be determined. After initiation, reverse transcription proceeds up to the last 5'-terminal nucleotide of the RNA pregenome as indicated by mapping the 3'-end of the DNA minus strand (30). This leads to a terminal redundancy of a few nucleotides in the DNA minus strand as described for GSHV and HBV (47, 48). During DHBV DNA minus-strand elongation the pregenome is degraded to a large extent except for the first 18 5'-terminal ribonucleotides. This short oligoribonucleotide is capped and serves as primer for DNA plus-strand synthesis (30). The capping of the primer supports the assumption that the pregenome may also function as m-RNA in protein synthesis (see above). In order to be able to serve as primer in DNA plus-strand synthesis the short oligoribonucleotide must to be transferred from DR1 to the second direct repeat sequence DR2 (Fig. 3). Only 12 out of the 18 ribonucleotides of the primer are homologous to DR2 (Fig. 4), which is sufficient for its stable association with virtually all viral genomes. The mechanism of the transfer of the primer and its unusual stability is unclear. The DNA plus-strand initiation synthesis comes to a halt after approximately 65 nucleotides when reaching the 3'-end of the DNA minus strand. At this stage, three DNA strands are meeting at the initiation site of DNA minus-strand synthesis and may form a triple helix structure (Fig. 3

and 4). This may play a role in circularisation of the replicative intermediate. For elongation of the DNA plus strand a template switch is required. This is made possible by the short terminal redundancy (about 7 nucleotides) in the DNA minus strand The AT rich sequence in the triple strand region (Fig. 4) probably facilitates the intramolecular template switch. Elongation of the DNA plus-strand can now occur and will gradually increase the stability of the circular conformation. The circularised replicative intermediates with growing plus strand synthesis of different length represent virion encapsidated genomes. The corresponding virions can infect hepatocytes and start a new round of DHBV replication.

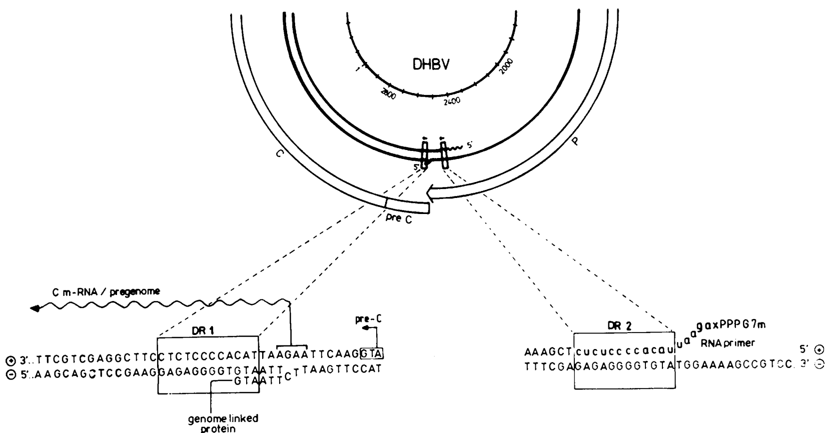


Fig. 4: The cohesive end region of the DHBV genome as encapsidated in the virion. Open boxes indicate known genes and open reading frames. The two small boxes with arrows on top represent the direct repeat sequences on the DNA strands important for replication. The DNA sequences in the region of both direct repeats are shown below. The sequence of the RNA primer is indicated in small letters.

EXTRAHEPATIC INFECTIONS

The tropism of HBV for liver cells may not be absolute, as is suggested by the location of the surface antigen (HBsAg) of HBV in extrahepatic tissues from human patients. The range of cell types and organs prone to HBV infection has not been studied in sufficient detail. For example, replication and gene expression of HBV proteins in extrahepatic cells has never been convincingly demonstrated. DHBV infected duck tissues have been successfully used to address these questions.

In order to screen for extrahepatic sites of DHBV replication various organs of viremic ducks were analysed quantitatively for viral DNA. Very high amounts of viral DNA have been detected in the liver and bile duct epithelia, intermediate amounts in the pancreas, low amounts in the heart, the kidney, and the spleen (12). The significance of the low amounts of viral DNA could not be evaluated for all organs and might be due to the virus present in the blood supplies of these organs. As shown by DNA and RNA hybridization m-RNA and replicative intermediates are present in small amounts in the pancreas and the kidney of infected ducks (12), indicating gene expression and replication in at least some of the pancreas and kidney cells. The types of extrahepatic cells infected have been identified using immunofluorescence staining for viral antigens and cell specific antigens and hormones (26, 49, 50, 51, 52). In the pancreas, DHBV antigens have been localized to endocrine and exocrine cells, and in the kidney they were associated with glomeruli and a subpopulation of tubular cells. The glomeruli-associated DHBsAg appeared to be extracellular and bound in immune complexes. Moreover, viral antigens were also detected in cortical and medullary cells of the adrenal gland. Whether the tropism of DHBV and possibly other hepadna viruses for extrahepatic cells has pathological consequences remains to be determined.

PATHOGENICITY

HBV and WHV have been shown to cause a variety of liver diseases, and chronic infection is strongly associated with the development of primary liver carcinoma (27). Although far from being proven, there is accumulating evidence that DHBV infection may have similar consequences for its host.

Most ducks kept indoors show only mild or no signs of hepatitis (53). In contrast, severe to moderate hepatitis was observed in some but not all ducks kept indoors that were inoculated in ovo or at 1-day post-hatching. One possible explanation for this result is to assume that vertically infected ducks may be immunologically tolerant to viral proteins whereas experimentally infected ducks are not. In support of this assumption, antibodies to DHBV have never been detected in vertically infected ducks (Will, unpublished) but seem to be induced in experimentally infected ducks (Halpern, M.S. pers. communication). Therefore, as supposed for HBV (27), also DHBV-related hepatitis may be mediated by immune response mechanisms.

Studies with infected ducks kept indoors seem to contrast with studies on ducks kept in outdoor flocks (54). Severe forms of hepatitis including cirrhosis and a multicentric hepatocellular carcinoma were observed in a highly infected flock of Chinese ducks. There are several possible explanations for this discrepancy. It may be due to a higher pathogenicity of the chinese DHBV isolates, different genetic and immunological factors may play a role, and finally, aflatoxins or other hepatotoxins may contribute to or cause these liver diseases. None of these possibilities can be excluded so far.

The mechanism of carcinogenesis used by mammalian hepadna viruses is not clear. However, viral DNA integrated into host chromosomes is believed to play a role because it is found almost always in primary liver carcinoma (PLC) but only infrequently in chronically infected liver tissue, and not at all in acutely infected tissue (27). Integrated DHBV

DNA has never been found in acutely or chronically infected ducks of Western countries (53, and Will, unpublished). However, it has been found in a hepatocellular carcinoma from a Chinese duck (55) suggesting that DHBV behaves like the mammalian hepadna viruses in relation to hepatocarcinogenesis. Recently, integrated DHBV DNA from duck hepatocellular carcinoma has been cloned and partially sequenced (Imazeki, F. pers. communication). As usually observed in HBV and WHV PLC tissues, also the integrated DHBV DNA was highly rearranged with inverted repetitions both of viral and host sequences. Further studies are definitely needed to investigate the possible association of DHBV infection with liver disease and hepatocarcinogenesis.

Infection of different species of ducks or related animals is another strategy currently used to study DHBV pathogenicity. Goslings chronically infected with DHBV exhibited both liver inflammation and an altered hepatocellular morphology (18). Similar studies in other closely related animal species may become a powerful tool to study hepadna virus-related diseases.

VIRAL THERAPY

Various treatments have been used in an attempt to alter the natural course of the carrier state in human patients but none has been proven to be efficacious. DHBV-infected ducks seem to become a useful animal system for such studies as chronically infected ducks are readily available for experimental investigations and antiviral drug effects can be more easily monitored. In particular, the titer of DHBV in serum is usually 100 to 1000 times higher [up to 10^{11} virions/ml as detected by DNA dot blot hybridization (8)] than in human chronic carriers, which greatly facilitates the quantitative evaluation of drug effects on virus production.

Potential antiviral effect of two compounds has been tested in the DHBV/duck system. Sodium suramin, a potent inhibitor of reverse transcriptases of a number of retroviruses, has

been shown to inhibit effectively and irreversibly the DHBV polymerase activity associated with core particles from infected livers and circulating virus (56). In a second report, foscarnet, a new antiviral compound known to inhibit a number of reverse transcriptases of retroviruses as well as DNA polymerases of herpesviruses, HBV and WHV, has been tested (57). With this drug a dose-related decrease in serum and intrahepatic DHBV DNA during treatment, with a rapid return to baseline values after the cessation of treatment has been observed. The degree to which these results can be extended to the mammalian systems remains to be determined.

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RETROVIRUSES

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ABSTRACT

The pathogenic properties of retroviruses are reviewed. Emphasis is placed on some recent developments that correlate D-type retroviruses with immunosuppression in monkeys. Special attention is given to infections and disease caused by lentiviruses including newly isolated simian retroviruses that induce simian acquired immunodeficiency syndromes. In general, an attempt has been made to review retroviral disease with respect to genomic structure and the corresponding functions of viral gene products. Retroviruses are subdivided into those coding for an oncogene, the sarcomaviruses, and those that do not encode any oncogenes, like mouse mammary tumor virus whereas bovine leukemia virus is dealt with from the point of view of the trans-acting activator gene. In addition, the disease-specific production of unintegrated feline leukemia virus variant DNA in feline acquired immunodeficiency syndrome (FAIDS) is covered.

INTRODUCTION

Retroviruses are the only RNA viruses that can cause neoplastic tumors in many different vertebrates. It is remarkable that retroviruses also induce a wide variety of nonproliferative diseases besides tumors. The spectrum of pathogenic syndromes manifested by retroviruses ranges from slow, degenerative diseases that affect neural and hematopoietic tissues to proliferative fatal diseases. Among those syndromes are anemia, pneumonia, osteopetrosis, arthritis, encephalitis, wasting disease, lymphadenopathies, renal fibromatosis, and different kinds of tumors, to name a few. Most frequently, tumors are leukemias and lymphomas; but carcinomas and sarcomas are also rather often found to be induced by retroviruses.

One of the primary interests of retrovirologists has been the elucidation of the mechanisms that underly retroviral oncogenesis. In this respect, progress has been made towards identifying the genomic regions responsible for the pathogenic

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effects of retroviruses. This development was mainly due to the fact that the primary structures of several retrovirus genomes have been deciphered which in turn allowed to deduce the primary structures of the viral gene products. This constitutes a first, but fundamental step towards clarifying their functions. This development coupled with the ingenious use of identifying the essential viral regulatory signals for transcription has led to the detection of functional oncogenes. The characterization of viral oncogenes (v-onc) in turn opened the path for the discovery of their cellular homologs, the proto-oncogenes (c-onc). This in turn paved the way for correlating the c-oncogenes with hormone receptors, growth factors and hormones. Today, it seems obvious that oncogenes are components of cellular signal chains that include mitotic signal sequences, cytoplasmic effectors and transducers as well as nuclear regulatory proteins. In broad terms, this hypothesis may lead to a better understanding of the multistep process that underly the cellular mechanisms involved in oncogenesis on the molecular level.

Because of the broad spectrum of pathogenic effects induced by retroviruses described above, the subdivision of the following review is partly based upon the conventional taxonomy of retroviruses. The retrovirus family has been provisionally classified into three subfamilies, the oncovirinae, the lentivirinae, and the spumavirinae (1).

The oncovirus subfamily is by far the largest group and has been studied in great detail. It encompasses the C-type RNA viruses most of which can cause tumors in suitable hosts. However, based on recent data on the primary structures of many virus genomes and their corresponding gene products that were used to construct meaningful phylogenetic trees, it has become obvious that B- and D-type RNA viruses as well as Rous sarcoma virus (RSV) should be classified as another subfamily different from many oncoviruses such as mouse leukemia virus (MLV), feline leukemia virus (FeLV) and reticuloentholiosis virus (REV) (2). The provisional classification of the retrovirus family into five subfamilies or genera that is based on a phylogenetic tree that has been derived from the primary structures of the corresponding pol gene products will be referred to in this review as shown in table 1(3).

In this chapter, human diseases that are caused by retroviruses, e.g. the human immunodeficiency virus (HIV, LAV, HTLV-III, AIDS virus) and the human T-cell lymphotropic viruses (HTLV-1 and -2) will not be covered. Nor will the simian T-cell lymphotropic viruses (STLV) be dealt with, since they are the subject of another chapter (chapter 29) of this book. The molecular biology of the retroviruses will be referred to when it is relevant for the induction of the disease.

Table 1. Provisional classification of the retrovirus family

Subfamily (Genus)[a]	Prototype	Other members
Oncoviruses	Mouse leukemia virus mouse sarcoma virus	feline leukemia virus reticuloendotheliosis virus
Type D-retroviruses[b]	Mason-Pfizer monkey virus Rous sarcoma virus[d]	squirrel monkey retrovirus (mouse mammary tumor virus[c])
HTLV-/BLV	human T-cell lymphotropic virus-1	bovine leukemia virus
Lentiviruses	visna virus	human immunodeficiency virus
Spumaviruses	human spumaretrovirus	simian spumaretrovirus

[a]The issue of whether these five groups should be subfamilies or genera has not been resolved.

[b]The type D-retroviruses are very heterogeneous, so that a further subdivision is likely.

[c]Mouse mammary tumor virus has conventionally been classified as a type B-retrovirus; however, its genomic sequence has a higher degree of homology with other D-type retroviral genomes, particularly in the pol region.

[d]Rous sarcoma virus has unfortunately been classified as the prototype oncovirus; but due to its high degree of sequence homology with Mason-Pfizer monkey virus, its phylogenetic placement must be revised (2,3).

On the other hand, some more recent developments, e.g. the pathogenic effects of lentiviruses and type-D virus will be reviewed in respect to their peculiar way of interacting with the host immune system and their genomic structures. Both groups of viruses are of special interest, since they cause a wide spectrum of slow, degenerative diseases in sheep, goats, horses, and monkeys that can be regarded as model systems for understanding the underlying mechanisms of persistent viral infections in man.

DISEASES INDUCED BY RETROVIRUSES THAT DO NOT ENCODE AN ONCOGENE

D-type retrovirus and simian immunodeficiency syndromes

The prototype of the type-D RNA viruses subfamily is Mazon-Pfizer monkey virus (MPMV). It was originally isolated from a breast carcinoma of a female Rhesus monkey in 1970 (4). When new-born rhesus monkeys were inoculated with MPMV, tumors were not found after careful post-mortem examination even after several

years. However, the infected monkeys developed a wasting disease that was accompanied by opportunistic infections. Histopathological examination revealed thymic atrophy and lymphadenopathy (5). Based upon the combined occurrence of a thymic target cell and opportunistic infections, it was assumed that MPMV had a T-cell-specific immunosuppressive effect. Attempts to induce disease in thirteen macaques with D-type retroviruses related to MPMV that were initially isolated from monkeys with the simian immunodeficiency syndrome (SAIDS) failed although different D-type retroviruses, generally termed simian retroviruses (SRV-1, SRV-2) were repeatedly isolated from macaques at different American primate centers with symptoms characteristic for SAIDS (6-11). This dilemma could be explained by assuming that there seems to exist different forms of the SAIDS syndrome that are associated with the occurrence of three exogenous retroviruses. SRV-2 occurs in association with retroperitoneal fibromatosis in the absence of lymphomas and encephalopathies. The closely related SRV-1 occurs in the presence of SAIDS but without retroperitoneal fibromatosis. The third form of SAIDS is associated with STLV-III (now called SIV-III) that can induce lymphomas and encephalopathies in the absence of retroperitoneal fibromatosis. SIV-III has not yet been characterized in detail, but seems to be the true simian equivalent of HIV (as discussed later), the etiological agent of human AIDS, since it shows significant sequence homology to HIV in contrast to SRV.

Two of the type-D retroviruses, MPMV and SRV-1, have been characterized in detail, therefore a meaningful comparison is possible (2,12). The comparison revealed that SRV-1 and MPMV have 91.9% sequence homology at the nucleotide level. All open reading frames are similarly organized and even the env proteins share 88.5% of their amino acid residues. It has been safely concluded that MPMV and SRV-1 are strains of the same virus (2).

Another remarkable feature of the D-type retroviruses is the fact that in both envelope (env) proteins the immunosuppressive peptide region that comprises 35 amino acid residues is perfectly conserved. It has been shown by Ciancolo et al. (13) that a synthetic seventeen residue-long peptide that represents a subregion of this "immunosuppressive peptide" that is located in the transmembrane part of murine and feline env proteins inhibits the lymphocyte proliferation of an interleukin-2-dependent murine cytotoxic T-cell line. Other peptides representing different regions of viral proteins were inactive. Since this peptide is also well conserved in a number of different retroviruses, such as RSV, HTLV-1 and II, BLV, Mo-MLV, FeLV and last not least the REV-A strain of reticuloendotheliosis virus that all have immunosuppressive effects on their hosts, it can be concluded that the 'immuno-

suppressive peptide' is responsible for an initiating role in the development of different diseases, e.g. the lymphoproliferative disease induced in turkeys or the SAIDS-like syndromes in monkeys that is usually accompanied by various opportunistic infections. It is assumed that this peptide sequence acts by blocking the induction of lymphocytes in response to antigens or mitogens resulting in an immunodeficiency of the host (13). The immunodeficiency even if transitory can facilitate the expression of viral oncogenes if present. It is interesting that lentiviruses and spumaviruses (14) do not have this 'particular' immunosuppressive peptide sequence. Instead their corresponding env glycoproteins must possess peculiar, but sequences different from the immunosuppressive region described above, that ensures the corresponding cell tropism. In the case of visna virus, the target cell seems to be the monocyte/macrophage cell (15) (see under lentiviruses).

In summary, one can conclude that since all attempts to induce SAIDS by experimental infection of MPMV, SRV-1 and other D-type retrovirus strains failed, these viruses can be regarded as viral co-factors and as viral immunosuppressive agents. On the other hand, the true SAIDS syndrome can be induced into suitable monkeys by infecting them with the recently discovered new retrovirus isolates SIV-III (STLV-III) which seem to be closely related to HIV according to immunological analysis. This will be described in a later section.

DISEASES ASSOCIATED WITH FELINE LEUKEMIA VIRUS (FAIDS)

FeLV can cause neoplastic and degenerative disease in cats. More pet cats die from the degenerative FeLV-induced diseases than from various kinds of tumors that can also be induced by FeLV. Anemias (erythroblastosis and erythroblastopenia) are degenerative diseases of erythroid cells, thymic atrophy of kittens. Primary lymphoid depletion is a non-neoplastic disease of lymphocytes and granulocytes. Myeloblastopenia is a degenerative disease of granulocytic leukocytes. There are other forms of pathogenic syndromes of cats that include thrombocytopenia of megakaryocytes, a glomerulonephritis of the kidney, and various kinds of secondary immunosuppressive diseases. In most cases like i.e. anemia, the mechanisms by which FeLV induces anemias is unknown. In the cases of secondary immunosuppressive diseases that are associated with FeLV, soluble FeLV gp70, p15 (E) and some gag antigens have been detected as immunocomplexes with IgG in infected animals (16). In analogy to D-type retroviruses, it is remarkable that the immunosuppressive domain of the FeLV env protein is located in the p15 (E) part and could at least be partially responsible for the immunosuppressions observed. It is of interest

that about 45% of cats with chronic diseases, such as infectious perinitis, stomatitis, gingivitis, and with abscesses are infected with FeLV (16).

It has been pointed out by several authors that the clinico-pathological features of feline AIDS strikingly resembles those observed in human AIDS patients. The similarities include the cytopathic effect of FeLV and HIV on selected T-cell populations, a reduction in granulocyte leukocytes accompanied by hemorrhagic lymphadenopathies and anemia. Recently, a FeLV-FAIDS variant D was found at 10-50 copies per cell as unintegrated viral DNA in bone marrow cells of cats with FAIDS (17). Since this disease-specific and tissue-specific production of unintegrated FeLV DNA has also been observed in cells from several AIDS patients, it has been suggested to use FAIDS cats as model system for the evaluation of antiviral compounds.

Mouse mammary tumor virus

Mouse mammary tumor virus (MMTV) is a slowly transforming virus which induces predominantly mammary carcinomas after a relatively long latent period of 6 to 12 months. Thus, MMTV is different from the acutely transforming oncoviruses (formerly classified as C-type RNA viruses) that can induce neoplastic disease within shorter time periods, sometimes as early as one week after experimental infection.

For several reasons, MMTV has been the subject of intense research over the last decades. 1) It is a replication-competent retrovirus that can induce carcinomas in the apparent absence of a virus encoded oncogene. 2) MMTV is milk-transmitted from mother to offspring in mice strains with a high incidence of mammary tumors and it has been shown subsequently that the MMTV proviral genes are inducible by glucocorticoid hormones. 3) MMTV expression is tissue-specific and developmentally regulated. 4) Its genome has an unusually long LTR of 1328 nucleotides that seems to encode a protein, but the function of this protein is still unknown. These attractive aspects have been in the center of molecular biological investigations in many laboratories (see review 18).

MMTV is produced in the mammary epithelial cells of lactating animals. By using virus-specific probes, it has been shown that the MMTV-induced carcinomas acquired new proviral elements that were integrated at many different sites within the host genomes. Since tumor induction by MMTV requires a long latency period, the transformation of susceptible cells seems to be relatively rare and to occur by an indirect mechanism. A type of insertional mutagenesis has been postulated as the basic mechanism for MMTV tumorigenesis. This assumption is supported

to some extent by the fact that many MMTV-induced tumors contain one proviral copy in a limited region of the mouse chromosomal DNA. At least two independent, but common regions of integration have been identified that flank the newly acquired MMTV proviral elements. They were termed int-1 and int-2 (19).

A detailed analysis has been shown that they both int loci encode cellular oncogenes that are not directly related to any of the known v-oncogenes. However, the RNAs of the int genes are expressed in tumor cells, but not in normal mammary tissues. Thus, it seems likely that the proviral insertion at one of these preferred sites is responsible for activating the int gene expression. Furthermore, it has been shown that the int-1 mRNA encodes a 41-Kilodalton protein (20). The primary structure of the murine int-1 protein has several features it shares with growth factors or hormone receptors (21), e.g. a hydrophobic region of 46 amino acid residues, four glycosylation sites and a cysteine-rich domain. Analysis of the human counterpart of the int-1 mammary oncogene revealed that the human int-1 gene product is almost completely conserved in its protein sequence when compared to the mouse int-1. It is unknown whether abnormal expression of this interesting cellular gene contributes to mammary oncogenesis in man.

The aspect of hormonal regulation and tissue-cell specifity of MMTV have been actively pursued recently. The glucocorticoid responsive elements of the MMTV genome have been identified and are extremely valuable for studying the hormonal control of transcription in general. Moreover, the hormone-independent enhancer elements responsible for the enhancement of cellular genes that are located proximal to the proviral copy are being studied intensively. One interesting result sheds light on the tissue-specificity of a MMTV provirus that was found in a renal adenocarcinoma cell line. Refined analysis of this proviral DNA showed that it contained a striking difference in the U3 region of the two LTRs when compared to the U3 region of a MMTV provirus that had induced a mammary carcinoma (22). The observation that an altered LTR (in the U3 region) of a given retrovirus is accompanied by a change in target cell specificity has been documented for other retroviruses as well (23). In addition, it has been shown that foster-nursing of GR mice on C57/BL mice which eliminates the milk-born source of the exogenous GR virus allows to reveal that certain endogenous MMTV loci do also activate neighbouring int loci (24).

Bovine leukemia virus (BLV)

BLV is the causative agent of enzootic bovine leukosis and produces clonal tumors of the B-cell lineage after a long latency period of one to eight years (25).

Under natural conditions, BLV is transmitted from infected to healthy cattle mainly horizontally as well as congenitally. Since BLV does not encode any oncogene and since, furthermore the integrated proviral DNA is apparently not expressed in tumors, it is completely unknown how the leukemia develops. Southern blot analysis of bovine tumor DNA shows that BLV proviral DNA sequences are detectable in all tumors. In many cases, the BLV provirus has suffered deletions, more frequently at the 5' half but not necessarily the 5'-LTR of the genome. However, the tumor cells that contain BLV as a provirus do not express it. The mechanism of this repression is unknown (25). It is of interest that the 3' part of the BLV genome seems to be intact in tumor DNA of cows (26). It has been suggested that integration of the BLV provirus could result in a down-regulation of the transcription of adjacent cellular genes that are assumed to be important in the control of growth. However, analyses of 28 BLV-induced tumors with respect to a preferred site of integration into the host chromosomal DNA revealed that the cellular sequences belong to different chromosomes.

It should be mentioned that sheep can be infected experimentally with BLV. Infected sheep develop a T-cell lymphoma. Since BLV encodes a tat-like protein that has been shown to be necessary for viral replication (27), and since the corresponding gene is located at the 3' end part of the BLV genome, it might be worthwhile to develop antiviral strategies directed against the activity of this virus-specific protein. It seems probable that BLV can act as viral immunsuppressor and thereby contributes to bovine leukosis, since its env glycoprotein possesses the typical immunosuppressive domain mentioned above. It remains to be seen whether or not the tat-like activity of BLV is required for virally induced bovine leukosis.

DISEASES INDUCED BY LENTIVIRUSES

Over the past yeas, the biology and molecular biology of lentivirus subfamily of retroviruses has gained momentum, since the nucleic acid sequence analyses of the human immunodeficiency virus (HIV, HTLV-III, LAV) and of the visna virus (VIV), the prototype lentivirus, has surprisingly revealed that they apparently belong to the same subfamily (28,29). Both viruses share many common features even at first glance that support this notion. They both cause characteristic slow infections, have similar cytopathic effects in cell cultures, and the electron-microscopic morphology of the virion structure is similar. In addition, both VIV and HIV have large env glycoproteins (983 and 859 amino acid residues, respectively), and their genomes have extensive sequence homologies in the gag and pol regions. Both

viruses use lysine-tRNA as the primer for viral DNA synthesis. Judging from the published nucleotide sequences three lentivirus genomes, it is notable that there are remarkably similar in their genomic organization (Table 2). Each lentivirus contains overlapping gag and pol genes in different reading frames and a non-overlapping env gene (30). All three lentiviruses contain short open reading frames located in the pol-env intergenic region and at the 3'-end of the env gene. Moreover, both VIV and HIV have a novel type of viral-encoded proteins in common that are transacting activators of transcription, the so-called tat-genes or proteins. There is at least one important biological difference between VIV and HIV; the target cells of VIV are monocytes of sheep, whereas human helper T-cells are the target cells of HIV. CAEV DNA has been shown to have sequence homology with visna virus genome in the gag, pol and part of the env regions. This result justifies the conventional classification of CAEV as a lentivirus (31,32).

Quite recently there have been reports from different laboratories that succeeded in isolating novel monkey retroviruses that antigenically and in other biological aspects are close relatives of the AIDS virus and will probably be grouped into the lentivirus subfamily (36,37). Table 2 summarizes the properties of known lentiviruses. It is noteworthy that the genomes of lentiviruses are larger than the genomes of either the onco-viruses, the type-D viruses, or the HTLV/BLV subfamily. The genomes of spumaviruses, another subfamily of retroviruses, are even larger than those of the lentiviruses (R.M. Flügel, unpublished). Although spumaviruses that are also called foamy viruses have been isolated from patients suffering from leukemias, nasopharynx carcinomas, and the de Quervain syndromes, in laboratory animals inapparent infections were found (1).

The term 'slow infection' was coined by the Iceland physician Sigurdsson to characterize the unusually long periods of incubation of up to ten years or more that were observed in natural visna virus infections of sheep. The second feature of either an experimental or natural infection by lentiviruses is that the animals develop an immune response to the extracellular virus, but not to the persisting viruses that seem to evolve into variants of the originally infecting strain. These virus variants and/or mutants apparently persist in many organs and circulate in blood and tissue fluids. The third characteristic of a VIV infection is that after a prolonged period of virus persistence, the animals become short of breath, pratically paralyzed and usually die of wasting (visna) (33). The virus replicates in the choroid plexus and alveolar macrophages (visna virus is expressed in monocytes and in glial cells); however, virus replication is limited, unproductive, and restricted

Table 2. Properties of lentiviruses

Virus	Genome size[a]	Natural hosts	Disease	Reference
VIV	9.202	sheep, goats	pneumonia, meningo-encephalitis	(28,35)
PPV	?	Sheep, goats	pneumonia	(33)
CAEV	9.900	Goats, sheep	arthritis, pneumonia, encephalitis	(34)
ZZV	?	sheep	pneumonia, meningo-encephalitis	(35)
HIV	9.198 to 9.212	man	AIDS	(29,35)
SIV	?	monkeys	Simian AIDS	(36,37)
EIAV	9.252	horses	anemia, fever, glomerulo-encephalitis	(30)

[a] in nucleotides

VIV = visna (maedi) virus
PPV = progressive pneumonia virus
CAEV = caprine arthritis-encephalitis virus
ZZV = zwoergerziekte virus
HIV = human immunodeficiency virus
SIV = simina immunodeficiency virus
EIAV = equine infectious anemia virus

to a few cells (35). This situation is quite different from virus replication in cell cultures.

To explain this difference, A. Haase has suggested a peculiar mechanism for visna virus replication in vivo (35). According to the Trojan horse mechanism, a mobile sheep monocyte harbors VIV and expresses it only at a negligible level if at all, but conveys the virus to other sites without detection and without being neutralized by circulating antibodies. In situ hybridization of VIV RNA in the cerebrospinal fluid revealed a restricted level of viral RNA expressed in monocytes. The notion that altered env glycoproteins gradually emerge in infected animals was shown by Narayan et al. who found point mutations in the env glycoproteins that had been isolated from persistently VIV-infected sheep (38). Thus, it has been suggested that antigenic variation is the means of hiding the virus and shielding it from the appropriate immune response of the host animal.

In the case of EIAV, the mechanism for in vivo virus dissemination by antigenic variation is well documented. Montelaro et al. showed that several new virus isolates can be obtained from a pony infected with a distinct single EIAV strain. Each newly

isolated EIAV substrain cannot be neutralized by antibody that neutralized the inoculated, previous virus isolate. This result was borne out by sequence studies of the EIAV env gene and of the env glycoproteins (30,39). Since there is also evidence for extensive antigenic variation in the env glycoproteins of HIV, it seems that in the case of VIV this sort of mechanism plays a role at least in part.

The pathological changes that are observed after a VIV infection consist mainly of the destruction of specific cells and tissues. The brain lesions are characterized by demyelinations. By combining in situ hybridization and antibodies against distinct brain cells, it was recently shown that the oligodendrocyte cell is one of the neural target cells of VIV. It has been suggested that the destruction of brain cells is caused by the inflammatory host immune response (35).

As an exogenous virus, VIV is transmitted by the respiratory route between animals and from mother to lambs via the gastrointestinal route in the colostrum. Since the mothers carry neutralizing antibodies and VIV conceals from host immune defences in the central nervous system, vaccinated sheep are not protected. However, it seems possible that novel vaccines can be developed on the basis of the tat gene products that are required for lentiviral replication. In addition, the use of anti-sense viral RNA has been suggested for an alternative way to control lentivirus infections and disease.

Much less is known about some other lentiviruses, e.g. PPV, ZZV (Table 3) that cause slowly progressive and inflammatory diseases of the central nervous system. The syndromes include chronic arthritis of the joints, progressive pneumonia of the lungs, and leukoencephalitis. Whereas the older isolates of CAEV completely lyse ovine or caprine fibroblasts, certain new CAEV field isolates establish persistent infections in fibroblasts of sheep or goats (40). Although not characterized in detail, this biological difference seems to be a consequence of genomic differences. Those lentiviruses that cause latent infections can be activated experimentally into a replicative-competent virus (41). The different pathogenic behaviour of lentiviruses that have been passaged in cell cultures when compared to those lentiviruses that were propagated in vivo is distinct and typical. It sets the lentiviruses apart from the type D-viruses, since the immunosuppressive effect of i.e. MPMV has remained stable when compared to the original MPMV isolate despite many in vitro passages (56).

Quite recently several groups reported on the isolation and serological characterization of simian retroviruses that are closely related to the human AIDS virus. One isolate designated as STLV-III was obtained from sick macaques at the New England Regional Primate Center (42). Its close relatedness to HIV was shown

by using human sera positive for HIV-antibodies. This sera immunoprecipitated all major gag and env proteins of STLV-III, that were almost identical in size to those of HIV (43). Consequently, this virus, now proposed to be termed as SIV, should be provisionally classified as a lentivirus. The virus shows T-cell tropism, a morphology similar to that of HIV and was isolated from macaques with SAIDS or lymphomas (43). Experimental infection of six rhesus monkeys with SIV (STLV-III) resulted in the death of four animals within 160 days (36). The four monkeys showed similar immunological abnormalities including a decrease in $T4^+$ peripheral blood lymphocytes. The four animals developed a wasting disease, a primary retroviral encephalitis, and opportunistic infections. Although the incubation period of only a few weeks was observed and no lymphadenopathy found, the large dose of virus administered intravenously might explain the rapid progression of disease. It is noteworthy that SIV-infected chimpanzees do not develop simian AIDS.

Another simian retrovirus that was recently isolated from wild-caught African Green monkeys showed cross-reactivity with antibodies directed against HIV. Biological properties of this novel virus, designated as STLV-(AGM) are very similar to HIV, including the ultrastructural morphology, the growth characteristics in cell culture, and the sizes of the major gag and env proteins when analyzed by gel electrophoresis under denaturing conditions (44). Of major significance could be that natural STLV-(AGM) infection of African nonhuman primates does not lead to disease. This can be explained by assuming that Cercopithecus aethiops is the natural host of this virus. However, when different species of monkeys are infected, SAIDS can be induced with this retrovirus.

This assumption is supported to a certain extent by reports from two different American Primate Research Centers (37,45). Another retrovirus, STLV-III/Delta was isolated from rhesus monkeys and from asymptomatic sooty mangabeys (Cercocebus atys). When the tissue homogenates from seropositive mangabeys were inoculated into rhesus monkeys, the animals developed SAIDS. Again, analyses of the ultrastructural morphology and of the major gag and env proteins revealed the close relatedness of STLV-III Delta to HIV. A comparison of the gag proteins of STLV-III MAC, STLV-III-Delta, and HIV shows similarities and only minor differences (37). Again, it is noteworthy that STLV-III-Delta seems to be prevalent in mangabeys, but induces SAIDS in another non-human primate. Similar findings were reported in the Yerkes Regional Primate Research Center (45). The new isolate STLV-III-MANG was obtained from peripheral blood cells of 14 of 15 healthy mangabeys. It was shown to be lytic for human $OKT4^+$ cells. All other properties described above for STLV-III-Delta hold for STLV-III-MANG (37). It is interesting

that these mangabeys had been housed together with African Green monkeys. Further work is necessary to demonstrate the role of these viruses in simian and human AIDS. This also holds for some new isolates of retroviruses from non-human primates and humans that both antigenically seem to be different from SIV-III and from HIV (46,47).

DISEASES INDUCED BY ONCOVIRUSES THAT ENCODE AN ONCOGENE

The number of reports and even reviews on the detection and identification of retroviral oncogenes has been in full blossom. There have been a number of excellent reviews on viral and cellular oncogenes (1,19,48). Table 3 summarizes some data on viral oncogenes, including some v-oncs that were recently described, i.e. kit, sea etc. Table 3 does not contain numerous non-viral oncogenes that were discovered by using DNA transfection techniques and DNAs from various tumors that arose spontaneously or were induced by chemical carcinogens. The cellular oncogenes include among others met, neu, trk, lca, and syn. It is not within the framework of this review to discuss the relatedness of the c-oncs to the v-oncs. Nor does Table 3 contain those oncogenes that are activated by MMTV and were dealt with under that section.

Several attempts have been made in the past to correlate the retroviral oncogenes with each other or to classify them according to disease syndromes or types of tumors or their subcellular location. However, these efforts have been hampered, since the functions of many retroviral oncogenes remain unknown. It seems that in a not too remote future, v-oncs will be divided into related families together with their proto-oncogenes from which they are evolutionarily derived. At this point, it is still obscure to what extent viral and cellular oncogenes can be considered as having an identical function. It seems as if either the structure of a proto-oncogene and/or its expression must be altered before it becomes a true oncogene in the sense that it can exert its transforming and tumorigenic potential. The changes in the proto-oncogenes that are required seem to be subtle and manifold, e.g. different point mutations in the case of v-ras or truncations at various positions in the case of the v-erb-B gene that result in an abnormal expression of the corresponding gene products when compared to the cellular homolog, the EGF-receptor.

Since most, if not all, hormone and cell-surface receptors of growth factors possess an intrinsic protein kinase activity, it seems obvious that those retroviral oncogenes that do show a high degree of amino acid homology to cellular protein kinases, belong to the same, large family of protein kinases. Table 3 contains fifteen different viral oncogenes that can be grouped into the superfamily of protein kinases.

Table 3. Retroviral Oncogenes

v-onc	v-onc origin	virus disease	v-onc protein[1]	subcellular location[2]	v-onc function
abl	mouse	preB-cell leukemia	p90-160 gag-abl	plasma membrane	tyr-protein kinase
	cat	sarcoma	p98 gag-abl	"	
erb-B	chicken	erythroblastosis	gp65 erbB	"	tyr-protein kinase EGF receptor
fps	chicken	sarcoma	p205-170 (5)	"	tyr-protein kinase
fes	cat	"	p85-110 (3)	"	"
fgr	cat	"	p70 gag-actin-fgr	"	unknown
sea	chicken	fibrosracoma erythroleukemia	gp70-gp85 (2)	"	tyr-protein kinase
ros	"	"	p68 gag-ros	"	tyr-protein kinase
src	"	"	pg60 src	inner side of plasma membrane	"
yes	"	"	p80-90 gag-yes (2)	plasma membrane	"
fms	cat	"	gp120-180gag-fms (3)	"	CSF-1[4]
raf	mouse	"	p75-gp90gag-raf (2)	unkown	protein kinase
mht/mil	chicken	", carcinoma	p100gag-mht	"	"
rel	"	reticuloendothe-liosis, erythro-leukemia	p64rel	cytoplasmic	"
mos	mouse	sarcoma	p34-85env-mos (4)	"	"
kit	cat	sarcoma	p80gag-kit	plasma membrane	CSF-1 receptor truncated PDGF-receptor[5]
erb-A	chicken	unknown	p75gag-erbA	cytoplasmic	estrogen receptor
fos	mouse	osteosarcoma	pp55-p75(gag)-fos	nuclear	unknown
sis	monkey, cat	sarcoma	p28env-sis,p76gag-sis	unknown	PDGF-like
myc	chicken	sarcoma, car-cinoma, myelocytoma	p58-200gag-myc (5)	nuclear	nucleic acid binding

myb	chicken	myeloblastosis	p45myb,p135 gag-myb-ets	nuclear	unknown
ets	"	sarcoma, carcinoma	p135gag-ets-myb	unknown	unknown
ski	"	squamous carcinoma	p110-p125gag-ski	nuclear	unknown
ras	rat	sarcoma, erythroleukemia	pp21(Ha) (4)	plasma membrane	GTP binding GTPase

[1] Number in parenthesis signifies number of different protein species of a given v-onc that was originally derived from different isolates of retrovirus strains.

[2] Predominant subcellular locations at which the onc proteins are detectable in transformed or tumor cells

[3] EGF = epidermal growth factor

[4] CSF-1 = macrophage colony stimulating factor

[5] PDGF = platelet-derived growth factor

Actually there exist even more cellular members in this family, but viral homologs have not been found for everyone. The v-onc protein kinases can be subdivided into those enzymes that specifically phosphorylate tyrosine rsidues and protein kinases that transfer the y-phosphate group of ATP to either serine or threonine residues. The latter group of enzymes include the following v-oncs: raf, mil/mht, mos, and rel; the group of tyrosine-specific kinases include: abl, erb-B, fps, fes, ros, src, yes, fms, and probably kit. Thus, these enzymatic activities are not only fascinating from an evolutionary aspect, but may also allow to perform specific assays in order to identify them and differentiate between them.

A fascinating candidate for membership in this family is the v-kit gene product. Its extracellular domain resembles the lymphocyte colony stimulating factor (CSF-1) receptor (49,50). Another part of v-kit has close structural similarity to the PDGF-receptor (platelet-derived growth factor). The sis oncogene, on the other hand, is almost completely homologous to one of the polypeptide chains of PDGF. This provides an excellent example of how different oncogenes can be part and parcel of the same signal chain that trigggers events that finally lead to a cancer cell. It has been shown independently that PDGF is an extracellular polypeptide growth factor that promotes early signals in the membrane with a subsequent mitogenic response (51).

Another group of oncogenes that has attracted the interest of many research groups is the ras oncogene. It has been detected and found in many different animal and human tumors. The ras protein has the ability to bind guanine nucleotides, and its cellular homolog c-ras has the capacity to hydrolyze GTP. A point mutation at the GTP binding site of c-ras was found to decrease this GTPase activity. This feature is reminiscent of how the G-proteins of mammalian cells mediate regulation of adenlyate cyclase. In general, cellular guanine nucleotide binding proteins mediate signal transduction through stimulation of a receptor protein, a G-protein releases GDP and binds GTP. Once GTP is bound, the G-protein regulates the function of an effector molecular, usually an enzyme or an ion channel. Since the ras oncogene has a weak protein homology to certain G-proteins, it has been suggested that it might function in a similar way as a signal transducer. However, ras has also amino acid sequence homology to the elongation factor Tu, so it is still an enigma how the ras gene can induce cellular transformation and induce tumors. This is particularly remarkable in view of several reports that found amino acid substitutions at certain positions (i.e. at residue 116 in Ha-ras) that drastically reduced both the guanine binding capacity and the GTPase acitvity. These substitutions, however, did not diminish the transforming ability of the activated ras genes (52,53).

A third group of oncogenes about which less is unfortunatley known comprises the following genes: myc, myb, ski, and fos. It has been assumed that they are nuclear regulatory molecules, since there is evidence that they can be found predominantly in the nuclei of transformed cells. It is, however, completely obscure how they might function in viral oncogenesis. The myc oncogene is unusually interesting, since it has been detected in various tumors in different forms. There is evidence that the myc protein plays a direct role in DNA synthesis, e.g. antibodies raised against myc reversibly inhibit DNA synthesis (54). It is noteworthy that at least one of the nuclear oncogenes, v-fos is unusual in that its proto-oncogene c-fos can induce cellular transformation when brought under the transcriptional control of a LTR. Thus, c-fos can be oncogenic without tampering with its coding sequence provided the 3'-noncoding region is disconnected (51). Most, if not all nuclear oncogenes are modified post-transcriptionally, and in the case of fos associated with a particular cellular protein. The mechanism of the cell-specific transforming potential of c-fos and v-fos is complex and has recently been reviewed in depth by R. Müller (55).

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DISTRIBUTION OF HUMAN T-CELL LEUKEMIA VIRUS TYPE- I (HTLV-I) FAMILY AMONG NON-HUMAN PRIMATES

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ABSTRACT

The seroepidemiological surveys of human T-cell leukemia virus type-I (HTLV-I) family were conducted among free-ranging Japanese monkeys, Asian and African non-human primates kept in cages and sampled in the field study. Seropositive animals to HTLV-I family in nature belonged to macaques originated from various localities in Asia. Grivet monkeys and Anubis baboons including those hybrid offspring between Anubis and Hamadryas were seropositive for HTLV-I family in Africa. As a result, HTLV-I family was proved to be widespread on Asian and African Continent. Thus the wide geographic and phylogenetic distribution of HTLV-I family in nature among various species of catarrhines suggests that the introduction of this virus family into primates occurred in ancient times and the HTLV-I family was considered as a common retrovirus among catarrhines.

INTRODUCTION

A new human T-cell leukemia virus known as the first human oncogenic retrovirus was initially found from T-cell leukemia/ lymphoma patients (1-3). This virus was first named human T-cell leukemia virus (HTLV) in U.S.A., while in Japan, it was named adult T-cell leukemia virus (ATLV) because of its close association with a new disease entity adult T-cell leukemia (ATLV) (4). In this paper this virus is designated as human T-cell leukemia virus Type-I (HTLV-I).

One of the striking characteristics of ATL is geographical clustering restricted to south-western Japan (5) and specific antibodies to ATL-associated antigens (ATLA) synthesized by HTLV-I producer cells were detected in most but not all ATL

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patients (2). It was suggested that ATLA are HTLV-I associated antigens (3,6) and a high incidence of ATLA antibodies has been documented in adults in ATL-endemic areas by seroepidemiological studies (7). Thus, the serum antibody to ATLA was considered a marker for the virus infection. Further seroepidemiological studies revealed the foci of its endemic areas in humans as being in Japan, the Caribbean Basin and West Africa (7-14). These characteristics of this virus mentioned above prompted the authors to examine whether primates (Table 1) other than man are infected with HTLV-I or its relative virus(es). The following seroepidemiological studies have been carried out using non-human primate serum or plasma specimens taken from mainly those animals living under natural conditions to avoid the possibility of artificial infection.

The Japanese monkey, a unique non-human primate in Japan, is distributed all over this country (except Hokkaido Island) regardless of ATL-endemic or non-endemic areas. The natural occurrence of the antibody to HTLV-I was shown among free-ranging Japanese monkeys living in ATL non-endemic areas. The age dependency and familial-clustering in seropositive incidences were also revealed in the presence of the ethological data on this Japanese monkey troop (15-17).

The previous result required further studies on other primate species. To outline the distribution of this virus among primates, subjects from various primate species: prosimian, New World monkeys, Old World monkeys and apes, kept in cages were examined for the presence of antibody to HTLV-I. All the seropositive subjects belonged to Old World monkeys and apes, while none of prosimians and New World monkeys were seropositive for HTLV-I. According to this evidence, further surveys were carried out on Old World monkeys and apes living in Asia and Africa.

Materials from various primates, three genera (Macaca, Presbytis, and Hylobates) with 20 species were obtained by the field studies in Southeast Asia and South Asia. Seropositive subjects for HTLV-I in nature belonged only to macaques originated from various localities (18,19). No animals except macaques

were antibody seropositive. As for the African primates, materials from three genera (Cercopithecus, Papio and Gelada), four species (grivet monkey (C. aethiops aethiops), Anubis baboon (P. anubis), Hamadryas baboon (P. hamadryas) and gelada (Theropithecus gelada)) were obtained by the field studies in Ethiopia. Seropositive subjects for HTLV-I were found among grivet monkeys and Anubis baboon including those hybrid offspring between Anubis and Hamadryas baboons but not among pure-Hamadryas baboon nor gelada (20).

The wide distribution of this virus among various macaques in nature suggests that the introduction of this virus into primates occurred in ancient times. The HTLV-I family was proved to be the common primate retrovirus among catarrhines.

Table 1.Brief classification of primates with emphasis on the Old World monkeys

Primates	Genus
Prosimia	Lemur, Galago, Nycticebus
Simia	
Ceboidea	
(New World monkeys)	
Callithricidae	Callithrix, Saguinus
Cebidae	Saimiri, Ateles, Cebus, Aotes
Cercopithecoidea	
(Old World monkeys)	
Cercopithecidae	
Cercopithecinae	
Cercopithecini	Cercopithecus, Erythrocebus
Papionini	Papio, Macaca, Theropithecus
Colobinae	Colobus, Presbytis
Hominoidea	
(Apes and Man)	
Hylobatidae	Hylobates
Pongidae	Pongo, Gorilla, Pan
Hominidae	Homo

MATERIALS AND METHODS

Sera

Eighty-eight serum or plasma specimens were obtained from a free-ranging Japanese monkey troop consisting of 126 individuals in Nagano Prefecture (Honshu) where ATL in humans is rare (5). Since this Japanese monkey troop has been followed

ethologically for the last thirty years, their age, sex, and mother-infant relations have been studied and recorded. The age of the subjects (31 males and 57 females) ranged between 1 and 25 years old (mean age: 6.5).

A total of 898 serum of plasma specimens were collected from 38 species of non-human primates kept in cages. Those specimens consist of 50 prosimians (Lemur, Galago, Nycticebus and Tarsius), 132 New World monkeys (Callithrix, Saguinus, Saimiri, Aotes, Ateles and Cebus), 579 Old World monkeys (Papio, Theropithecus, Cercopithecus, Macaca and Erythrocebus) and 147 apes (Hylobates, Pongo, Gorilla and Pan).

A total of 1118 sera were obtained from feral Asian primates in South and Southeast Asia. In South Asia, sera were collected from 14 bonnet monkeys (Macaca radiata), 19 liontailed macaques (M. silenus) and 4 Nilgiri langurs (Presbytis johnii) in India, and 253 toque monkeys (M. sinica), 24 Hanuman langurs (P. entellus) and 8 purple-faced langurs (P. senex) in Sri Lanka. In Southeast Asia, sera from 316 crab-eating monkeys (Macaca fascicularis) (117 from Thailand and 199 from Malay Peninsula), 137 stump-tailed macaques (M. arctoides) (Thailand), 131 pig-tailed macaques (M. nemestrina) (35 from Thailand and 96 from Malay Peninsula), 30 rhesus monkeys (M. mulatta) (24 from Bangladesh and 6 from Thailand) and 16 gibbons (Hylobates spp.) (Thailand) were collected during the field study of the Japan - U.S. Cooperative Science Program. In Indonesia, sera were obtained from 166 so-called Celebes macaques including seven species (21), M. maura, M. tonkeana, M. hecki, M. ochreata, M. nigrescens, M. nigra and M. brunnescens in Sulawesi (Celebes). The age of the animals was estimated from their dentitions (see footnote to Table 5). Names and numbers of the subjects were listed in Table 4 and their localities are mapped in Fig. 3. The blood was collected from the animals and sera were separated and stored in a freezer before use.

A total of 983 serum or plasma specimens obtained from non-human primates under natural conditions in Ethiopia were examined for the antibody to HTLV-I. In Table 6, the numbers and species are listed and their localities are mapped in

Fig. 6. Sera from 192 grivet monkeys were collected from three different troops. Ninety-seven pure-Anubis baboons (*Papio anubis*) from 5 troops, 40 pure-Hamadryas baboons (*P. hamadryas*) from 1 troop and 502 hybrid baboons between Anubis and Hamadryas baboons (22) from 8 troops in Awash, totaling 639 baboon samples were obtained. Sera from 152 gelades (*Theropithecus gelada*) were collected from 4 herds (troops) and their detailed descriptions were alredy recorded (23).

Antibody detection

Sera (not inactivated) were examined for antibody to HTLV-I at a dilution of 1:10 in phosphate-buffered saline (PBS) to reduce the non-specific reaction in the indirect immunofluorescence test. Specific antibody to HTLV-I antigens can be detected at this dilution even among non-human primates. MT-1 cells (24), of which a minor population expresses HTLV-I specific antigens, were smeared on a slide, dried, fixed in acetone for 5 min at room temperature and used as target antigens. The MT-1 cell smears were first treated with diluted test samples at 37°C for 30 min, and then washed with PBS. They were incubated with FITC-labeled anti-monkey IgG goat serum (Cappel Laboratories, U.S.A.) at 37°C for 30 min. After rewashing, they were mounted with buffered glycerol and examined for specific antibody to HTLV-I under fluorescence microscope. When the positive reaction was detected at this dilution (1:10 in PBS), the animal was regarded as seropositive and then titer of the antibodies was examined.

RESULTS

HTLV-I family among free-ranging Japanese monkeys

The positive-staining cells in the indirect immunofluorescence test showed a fluorescence pattern in seropositive HTLV-I human serum similar to that obtained in seropositive Japanese monkey plasmas. A characteristic feature common to these sera was that fluorescence distributed mainly throughout the cytoplasm and polynuclear giant cells showing brilliant fluorescence.

Table 2. Result of anti-HTLV-I antibody test among Japanese monkey (No. positive/No. tested (%))

Age group	Result			
(years old)	Male	Female	Total	GMT[1]
1- 4	2/16	2/23	4/39 (10.3)	52.8
5- 9	3/14	5/19	8/33 (24.2)	29.3
10-14	1/ 1	2/ 5	3/ 6 (50.0)	31.7
15-	-	7/10	7/10 (70.0)	56.6
Total	6/31 (19.4)	16/57 (28.0)	22/88 (25.0)	41.1

1. GMT: geometric mean titer of seropositive subjects.

Table 2 and Fig. 1 show age and sex distribution of antibodies to HTLV-I seropositive Japanese monkeys and their titers of antibodies, respectively. Twenty-two subjects (25.0%) including six males and 16 females gave positive results in this test and their age ranged between 2 and 25 years (mean age: 10.4). The proportion of seropositive Japanese monkeys increased with age (Table 2). The age dependent incidence of seropositive individuals in the Japanese monkey resembles that of healthy human subjects in ATL-endemic areas (2). The youngest age group (1-4 years old) showed low incidence of HTLV-I seropositive (10.3%). In the adult age group (over 5 years old), the seropositive frequency for HTLV-I reached 36.7%. Moreover, seven of ten subjects (70%) over 15 years old were seropositive for HTLV-I. The titers of antibodies to HTLV-I reached x320 in one female subject and an overall geometric mean titer (GMT: $10^{(\Sigma \log Ti)/n}$, Ti=titer) was x41.1.

Five subjects were successfully captured again after one year and were reexamined for the antibodies (Fig. 1). One seronegative female subject was found to change to positive between 7 and 8 years of age, while one female remained seronegative. The titers of two seropositive subjects increased, however, that of one old female subject (21 years old) was unchanged.

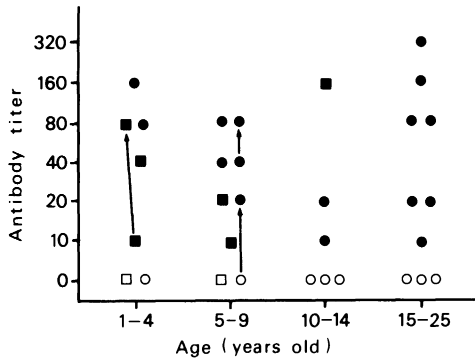

Fig. 1. Titer of antibodies to HTLV-I among Japanese monkey. Arrows indicate the changes of the titers in one year (■: male, ●: female).

Antibodies to HTLV-I were present in 19.4% of males and 28.0% of females among the Japanese monkey subjects. A sexual difference in HTLV-I seropositive incidence has been observed in humans in ATL-endemic areas (25), however, the difference in seropositive frequency between male and female Japanese monkeys was not statistically significant (χ^2=0.813, d.f.=1, P> 0.45).

Since Japanese monkeys have promiscuous sexual relations, the father of the infant could not be identified. The pedigree of maternal families in the troop, however, was recorded in the

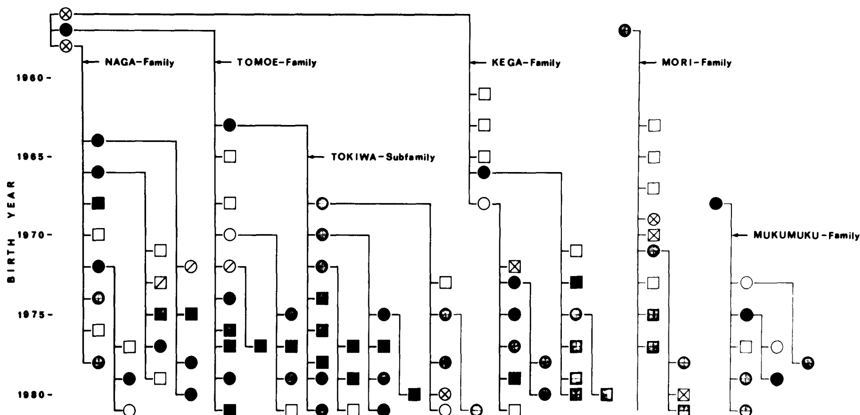

Fig. 2. Pedigree of Japanese monkeys and the results of HTLV-I antibody test. ■ ●, positive male and female; ▩ ◍, negative male and female; □ ○, not tested; ⊠ ⊘, dead and not tested; ⊠ ⊗, left the troop and not tested.

ethological studies because the mother-infant relation is tight and clear. Three major maternal families, the Naga-, Tomoe- (including the Tokiwa-subfamily) and Kega-families, are shown in Fig. 2 with the results of the immunofluorescence test. The seropositive frequency of these three families were 42%, 24% and 23%, respectively. Fig. 2 also represents two other small families, Mori- and Mukumuku-family which were shown to be HTLV-I sero-negative and -positive, respectively. These results based upon the pedigree suggested familial-clustering of HTLV-I seropositive incidence in Japanese monkey families.

HTLV-I family among caged non-human primates

Seropositive animals were found in 96 individuals belonging to four genera (*Cercopithecus*, *Papio*, *Macaca* and *Pan*), eight species as shown in Table 3. All the seropositive animals belonged to catarrhines (Old World monkeys and apes), while all of platyrrhines (New World monkeys) and prosimians tested were seronegative.

Table 3. Prevalence of antibody to HTLV-I among caged non-human primates

Primates			Result[1]
Prosimia			
		Lemuridae	0/ 5
		Lorisidae	0/ 22
		Tarsidae	0/ 13
Simia			
	Platyrrhini		
		Callithricidae	0/ 65
		Cebidae	0/ 67
	Catarrhini		
		Cercopithecidae	82/579
		Hylobatidae	0/ 12
		Pongidae	14/135

1. number positive/number tested.

HTLV-I family among Asian primates in the wild

The localities of Asian primates used in this study are mapped in Fig. 3. Among the three genera tested (*Macaca*, *Presbytis* and *Hylobates*) antibody to HTLV-I was found only in macaques caught in South and Southeast Asia. Three macaque species

in South Asia, M. silenus, M. radiata and M. sinica, were antibody positive at a frequence of 5.3%, 28.6%, and 17.3%, respectively.

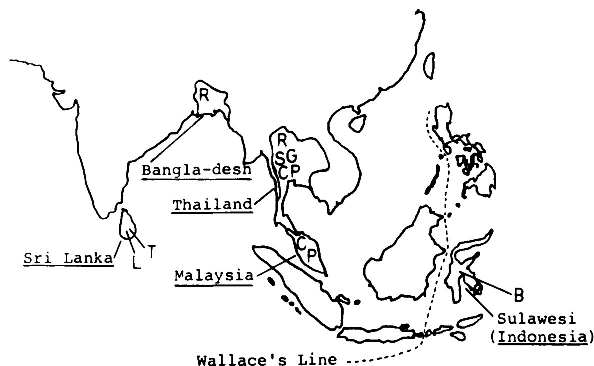

Fig. 3. Localities of origin of the animals examined in this study. Capital letters indicate the sites where the animals were captured. B, Celebes macaques; C, crab-eating monkeys; G, gibbons; L, langurs; P, pig-tailed macaque; R, rhesus monkey; S, stump-tailed macaque; T, toque monkey; M, bonnet monkey; N, lion-tailed macaque.

The results with 248 toque monkeys of which age and sex were determined by morphological observation are shown in Table 4. The seropositive frequency clearly indicated age dependency and reached 28.2% in adult animals. The sexual difference in the incidence was statistically not significant.

Table 4. Antibody to HTLV-I among toque monkeys[1]

Sex	Age group[2]				
	A	YA	J	INF	Total
Male	12/ 33	6/15	4/ 68	0/1	22/117 (18.8)
Female	17/ 70	1/17	4/ 44	-	22/137 (16.8)
Total	29/103 (28.2)	7/32 (21.9)	8/112 (7.1)	0/1 (0.0)	44/248 (17.7)

1. number positive/number tested (% positive).
2. A (adult), having full dentition; YA (young adult), having the permanent M2; J (juvenile), having permanent I_1,I_2 and M_1; INF (infant), completion of milk teeth.

(X^2=0.171, d.f.=1, $P > 0.7$). The result with each toque monkey troop examined is shown in Fig. 4 to indicate the geographical distribution of seropositive incidence in Sri Lanka. No apparent geographical clime was found in this incidence. None of the 36 langurs examined were seropositive. In Southeast Asia, Celebes macaques in Indonesia and the following monkeys in

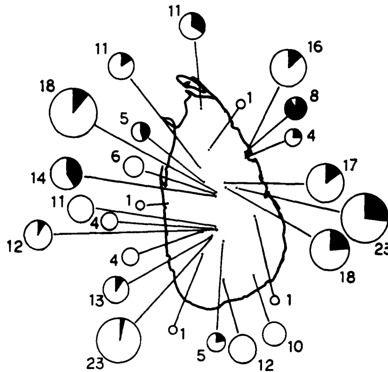

Fig. 4. Distribution of HTLV-I antibody among toque monkey in Sri Lanka. The number attached to the circle represents the sample size and the black segment indicates the seropositive frequency. (Reprinted with permission from ref. 19).

Thailand, crab-eating monkeys, stump-tailed macaques and rhesus monkeys, were antibody positive and their seropositive frequencies were 16.9%, 1.3%, 1.5% and 3.3%, respectively. No seropositive individuals were detected among pig-tailed macaques and gibbons in this area (Table 5).

Among the 166 Celebes macaques tested, 28 (16.9%) were antibody positive. Table 6 shows the results summarized by age and sex which were determined by morphological observation. Adult Celebes macaques showed a seropositive rate of 36.4%; this incidence was comparable to that of four Japanese monkeys. The age dependency of the incidence is also presented in Table 6. A significant sexual difference in the incidence was observed 24.6% for females and 12.4% for males (X^2= 4.101, d.f. = 1, $0.01 < P < 0.05$). No geographical, that is, no interspecific clime, in seropositive incidence was oberved among Celebes macaques (Fig. 5).

Table 5. Materials examined for the antibody to HTLV-I in South and Southeast Asia

Genus and species	Name	Locality[1]	Result[2]
M. fascicularis	Crab-eating monkey	T and M	4/316
M. arctoides	Stump-tailed macaque	T	2/137
M. nemestrina	Pig-tailed macaque	T and M	0/131
M. mulatta	Rhesus monkey	B and T	1/ 30
M. radiata	Bonnet monkey	India	4/ 14
M. silenus	Lion-tailed macaque	India	1/ 19
M. sinica	Toque monkey	Sri Lanka	44/253
Celebes macaques[3]		Sulawesi	28/166
Presbytis entellus	Hanuman langur	Sri Lanka	0/ 24
P. senex	Purple-faced langur	Sri Lanka	0/ 8
P. johnii	Nilgiri langur	India	0/ 4
Hylobates spp.[4]	Gibbons	T	0/ 16

1. T: See Fig. 3. Thailand, B: Bangladesh, M: Malaysia.
2. number positive/number tested.
3. Macaca nigrescens, M. nigra, M. hecki, M. tonkeana, M. maura, M. ochreata, M. brunnescens.
4. Hylobates lar, H. concolor, H. pileatus.

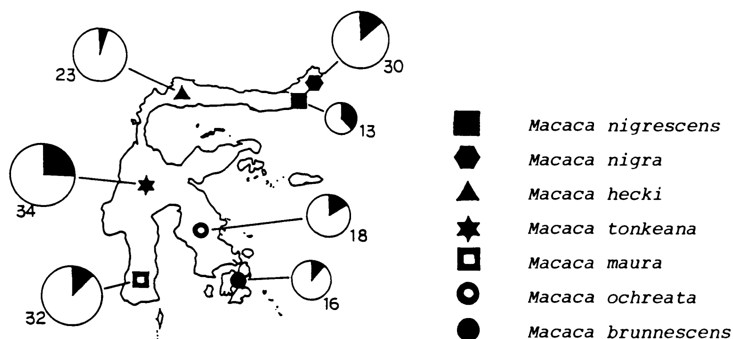

Fig. 5. Distribution of HTLV-I-antibody among Celebes macaques in Sulawesi. The number attached to the circle represents the sample size and the black segment indicates the seropositive frequency.

Table 6. Antibody to HTLV-I among Celebes macaques[1]

Sex	Age group[2]				
	A	YA	J	INF	Total
Males	2/ 5	2/20	8/66	1/14	13/105 (12.4)
Females	2/ 6	4/16	7/30	2/ 9	15/ 61 (24.6)
Total	4/11 (36.4)	6/36 (16.7)	15/96 (15.6)	3/23 (13.0)	28/166 (16.9)

1. number positive/number tested (% positive)
2. See footnote to Table 3.

HTLV-I family among African primates in the wild

The result with each species is represented in Table 7. Only grivet monkeys, pure-Anubis baboons and Anubis-Hamadryas hybrids were seropositive, while non of pure-Hamadryas baboons and geladas were seropositive (Table 7).

Out of 192 grivet monkeys, 54 subjects from three different troops were seropositive (28,1%) (Table 8). The seropositive frequency among adults was 35.6% and sexual difference in the incidence was not significant (males: 28.8%, females: 26.9%) (X^2=0.0807, d.f.=1, $p > 0.8$). Fig.6 shows the sampling site and the results. The seropositive frequency of each troop ranged between 20.0% and 42.1%.

Table 7. Materials examined for the antibody to HTLV-I in Africa

Name	Genus and species	No. troops	Result[1]
Grivet monkey	*Cercopithecus aethiops aethiops*	3	54/192
Baboon[2]	*Papio* spp.	14	72/639
Gelada	*Theropithecus gelada*	4	0/152

1. number positive/number tested.
2. *Papio anubis*, *P. hamadryas* and their hybrid.

Table 8. Result of anti-HTLV-I antibody test among grivet monkeys[1]

Sex	Age group		
	Adult	Young	Total
Male	29/ 67	7/58	36/125 (28.8)
Female	7/ 34	11/33	18/ 67 (26.9)
Total	36/101	18/91 (19.8)	54/192 (28.1)

1. number positive/number tested (%).

Among 639 baboons tested, 72 subjects were seropositive (11.3%). The seropositive frequency among adults was 19.2%. The frequency of each troop between 0% and 46.5% and between 0% and 65.4% in adults. These baboons were classified into three categories and Table 9 shows the seropositive frequency of each category. The seropositive frequencies of pure-Hamadryas and pure-Anubis baboons were 0% and 7.2%, respectively, while that of hybrid groups was 12.9% on the average. One of the hybrid troops showed 46.5% which was the highest among baboon troops. All the geladas from these four herds were seronegative in this study.

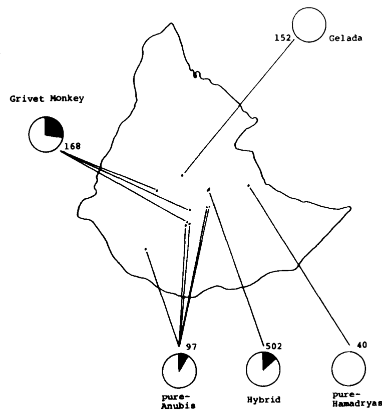

Fig. 6. Results of immunofluorescence test for detecting antibody to HTLV-I in Ethiopia. The numbers by the circles represent the numbers of sample and the black segment in the circle indicates seropositive frequency. (Reprinted with permission from ref. 20).

Table 9. Result of anti-HTLV-I antibody test among baboons[1]

Age group	Category			
	pure-Anubis	hybrid	pure-Hamadryas	Total
Adult	2/18 (11.1)	49/228 (21.5)	0/19 (0.0)	51/265 (19.2)
Young	5/79 (6.3)	16/274 (5.8)	0/21 (0.0)	21/374 (5.6)
Total	7/97 (7.2)	65/502 (12.9)	0/40 (0.0)	72/639 (11.3)

1. number positive/number tested (%).

DISCUSSION

The present seroepidemiological study shows the natural occurrence of HTLV-I antibodies among Japanese monkeys, which belong to Catarrhines. Age dependency and familial-clustering of seropositives which are similar to those in humans were also revealed among seropositive free-ranging Japanese monkeys. Moreover, the present data based on the caged primates that confirms and expands the former reports (17,26) indicates that the HTLV-I-like agent is prevalent only among catarrhines.

Family Cercopithecidae (Old World monkeys) is divided into two subfamilies, Colobinae and Cercopithecinae (Table 1). Seropositive animals have been found only in the latter subfamily. All the subjects belonging to subfamily Colobinae living both in Africa and in Asia were seronegative for HTLV-I. In Sri Lanka, even though toque monkeys and Hanuman langurs exist systematically in some areas, the former is HTLV-I antibody positive and the latter is negative. The same phenomenon was found between lion-tailed macaques and Nilgiri langurs which share the same habitat in South India. These results suggest the absence of interspecific infection between them and of the participation of vectors which transmit this virus indiscriminately.

As for hominoids, seropositive animals were found among chimpanzees, gorilla (29), and recently gibbon (30). As the number of orangutans tested is small, a conclusion cannot be drawn from the results with apes at this moment. However, distribution of seropositive animals among apes suggests that an ancestral hominoid was infected with the HTLV-I family.

The present data show the wide geographical distribution of the HTLV-I family in Asia and Africa. The geographical range of seropositive macaque species covers most part of South and Southeast Asia and that of guenons (*Cercopithecus aethiops*) including three subspecies and baboon species (*Papio anubis* and *P. ursinus*) covers most part of the subsaharan African continent (Fig. 7).

Present results among Papionini are of much interest. There are both seropositive (pure-Anubis baboon and hybrid

baboon) and seronegative (pure-Hamadryas baboon and gelada) groups in it.

Fig. 7. Geographical distribution of anti-HTLV-I antibody in humans and non-human primates. Circles are areas where HTLV-I is endemic in humans. Dotted parts indicate the areas where seropositive non-human primates were detected. The parts surrounded by dotted lines indicate the areas where non-human primates inhabit.

Moreover, hybrid baboons between Anubis baboon (seropositive species) and Hamadryas baboon (seronegative species) are seropositive for the HTLV-I family. The virus in hybrids must have been derived from Anubis baboons but not from Hamadryas baboons, because the latter species was considered to be negative for the HTLV-I family. This suggests that the HTLV-I family can be transmitted from the virus-positive groups to the new animal groups by reproductive processes and that the Hamadryas baboon is susceptible to this virus family.

As for the modes of HTLV-I transmission in humans, participation of virus-carrying cells was suggested in the transmission between spouses via semen and from mother to infant via breast milk (34,35). In this connection, two mating patterns of baboons; (i) seropositive male Anubis x female Hamdryas and (ii) seripositive female Anubis x male Hamadryas are available to confirm whether the HTLV-I family is transmitted via semen and/or via breast milk. More knowledge of the route of virus

transmission will be attained by the longitudinal ethological observation and virological examination of these hybrids and their parental animals.

The evidence that Celebes macaques are antibody positive is important, since it means that the HTLV-I family is prevalent in areas beyond Wallace's Line which biogeographically separates the Papuan and Malayan provinces from each other (Fig. 3). This suggests that introduction of the HTLV-I family into macaques occurred in ancient times before the beginning of macaque speciation of which the date is estimated at about 6-8 million years ago by molecular biological studies (28). If one assumes its introduction after the divergence of macaques, repeated virus introduction which might have occurred in various places and niches are required to explain the present distribution of the HTLV-I family among macaques.

Collectively, there are virus positive and negative groups among catarrhines. To explain HTLV-I family distribution which reflects primate phylogeny, two possibilities can be proposed for the prevalence of the HTLV-I family among catarrhines. First, this virus family infected the ancestral catarrhines and persisted during the evolution of host animals, whereas some lineages lost it. Second, several lineages acquired the virus independently in their own habitats during their evolution. The first possibility seems more likely, because the wide phylogenetic distribution of the HTLV-I family among catarrhines suggests its introduction into ancestral catarrhines, moreover, the HTLV-I family is not always transmitted from generation to generation as shown by the seroepidemiological and pathological studies (33). If this is the case, the virus introduction occurred between the separation of ancestral simians into platyrrhines and catarrhines of which the date is estimated as 35 million years ago and the separation of catarrhines into Cercopithecoidea and Hominoidea of which the date is estimated as 20 ± 3 million years ago (36). Those lineages, such as Colobinae, gelada and Hamadryas baboon, that are HTLV-I negative must have lost this virus family during their evolutionary processes. Nevertheless, any markers which dis-

tinguish the virus-positive species from negative ones are not found at this moment.

In conclusion, the HTLV-I family would be generally regarded as a common primate exogenous retrovirus family among catarrhines, because all the virus-infected species were found only among catarrhines, while none of platyrrhines and prosimians were virus positive. Isolation of viruses belonging to the HTLV-I family from various primates is indispensable in the search for the origin of the HTLV-I family.

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24

INFLUENZA A VIRUSES IN MAN AND ANIMALS: THE MOLECULAR BASIS OF EVOLUTION, EPIDEMIOLOGY, AND PATHOGENICITY

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INTRODUCTION

Influenza viruses are classified into three types, A, B and C according to their different biological and biochemical characteristics. Recent studies have indicated that influenza C viruses differ from influenza A and B viruses in their genomic structure, in the type and distribution of the biological activities of their viral glycoproteins, and in the substrate specificity of their receptor destroying enzymes (1, 2). These differences provide the basis to classify influenza C viruses separately from the A and B type viruses. Of the influenza A and B viruses, which differ in the antigenicity of their inner components, the A type viruses appear to be the most important disease agents. They have been found in natural infections in various mammalian species, including man, pig, horse, mink, seal, whale and in a wide variety of different avian species. In mammals, influenza viruses usually cause an acute respiratory disease, whereas in birds, depending on the virus strain and the host, the infection may lead to an inapparent or a systemic fatal disease, known by the term fowl plague.

The wide-spread distribution of influenza A viruses in mammals and birds, their high mutation rates, and their ability to interact genetically with each other, favour the appearance of "new" influenza viruses. The purpose of this report is to review recent developments concerning the variability of influenza A viruses and their genetic interactions which represent the basis for understanding the evolution, as well as the epidemiology and pathogenicity of these viruses.

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STRUCTURE OF INFLUENZA A VIRUSES AND FUNCTION OF THE PROTEINS

Influenza A viruses are usually spherical particles, 80 to 120 nm in diameter. Stalk-like projections or "spikes" are observed on the surface of the virus particles by negative staining in electron micrographs (Fig. 1).

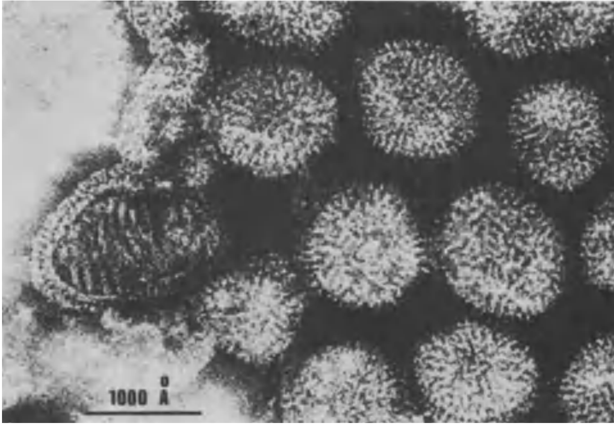

Fig. 1. Electron micrograph of an influenza A virus. On the left a partially disrupted particle can be seen which shows the internal organisation of the virus.

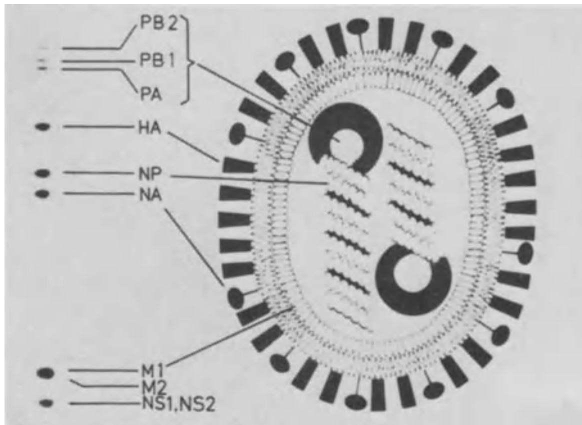

Fig. 2. Structure and structural components of influenza A virus (right) and assignment of the protein gene products to viral genes which are separated by polyacrylamide gel electrophoresis (left). The virus particle is enclosed by a lipid envelope in which the viral glycoprotein hemagglutinin (HA) and neuraminidase (NA) are embedded. Within the lipid envelope lies the membrane or matrix (M) protein. The polymerase complex

consisting of the P proteins (PB2, PB1 and PA), the nucleoprotein (NP) and associated with the viral RNA, is located on the inside of the virus particle. In addition, at least three virus-encoded non-structural proteins M2, NS1 and NS2) are found in infected cells.

Influenza A viruses possess a genome consisting of 8 separate single stranded RNA segments of negative polarity, which can be resolved by polyacrylamide gel electrophoresis (Fig. 2). These RNA segments encode 7 proteins found in the virion (PB2, PB1, PA, HA, NP, NA, and M1) and also 3 non-structural proteins (M2, NS1 and NS2) that are present only in the infected cells. PB1, PB2, PA, and NP are associated with the viral RNA forming the helical nucleocapsid which shows polymerase activity. The nucleocapsid is surrounded by a lipid bilayer which is lined on the inner side by the membrane protein M1. The M1 protein which is encoded by the RNA segment 7 appears to have a decisive function in the maturation of the virions at the plasma membrane. RNA segment 4 and segment 6 encode the glycoproteins neuraminidase and hemagglutinin, respectively, which comprise the spikes protruding from the envelope (3).

Neuraminidase

The neuraminidase (NA) represents the receptor-destroying enzyme of the virus particles which hydrolytically cleaves the glycosidic bonds joining the keto group of N-acetylneuraminic acid to D-galactose or D-galactosamin (4). It acts on neuraminic acid containing cell-surface glycoproteins and glycolipids and prevents virus particles from being adsorbed to inhibitory mucopolysaccharides, for instance in the respiratory tract. It also desialates viral glycoproteins thereby preventing progeny virions from aggregation (5).

Antigenically, the NA is strain-specific and displays antigenic variation among each subtype and major antigenic differences between the subtypes. Although NA does not induce the production of neutralizing antibodies, NA specific antibodies inhibit the release of virus particles from infected cells (6, 7) and protect the host against infection possibly by preventing spread of the virus (8 - 10).

Electron microscopic examination of NA reveals mushroom-shaped structure with head and stalk (11, 12). The head consists of 4 co-planar subunits (13), with the enzymatically active site and the variable antigenic domains (14). The complete amino acid sequences of NA of several

virus strains are known and the three-dimensional structure of pronase-isolated NA heads of two virus strains has been determined (15).

Hemagglutinin

The hemagglutinin (HA) is the major surface glycoprotein of the virus (16). It accounts for about 25% of the viral protein. The hemagglutinin mediates the initial steps in virus infection and induces the production of neutralizing antibodies in the infected host (17).

The HA spike is a trimer of non-covalently linked identical monomers (18). Each monomer consists as two chains, HA_1 and HA_2, linked by a disulfide bond. The amino acid sequence of many subtypes has been determined (16) and the three-dimensional structure of the H3 subtype has been identified by X-ray crystallography (19). The HA molecule contains two major regions: a triple-strand coil of alpha helices and a distal globular region of antiparallel beta sheets. The cell receptor binding site and the variable antigenic determinants are located on the globular domain (20). The carbohydrate side chains are linked by N-glycosidic bonds (21).

The HA glycoprotein is synthesized as a precursor HA which is cleaved by posttranslational limited proteolysis into the larger amino terminal fragment HA_1 and the smaller carboxyterminal fragment HA_2 (17, 22). Proteolytic cleavage of HA involves the sequential action of a trypsin-like endoprotease and a carboxypeptidase N, both of which are of cellular origin (23 - 25). Cleavage is essential for the virus to be infectious. The infectivity of virions which contain an uncleavable HA can be increased by trypsin treatment *in vitro*. Differences in the amino acid sequences at the cleavage site are of particular importance for the biological properties of the virus (see below).

NOMENCLATURE

Based on their genetic and antigenic differences, 13 HA (HA) and 9 NA (N) subtypes of influenza A viruses can be discriminated. All subtypes are represented among avian influenza viruses. Only the subtypes H1, H2, H3, H4, H7, H10 and H13, and 8 N-subtypes have been found so far in man and the other mammals (Table 1). Each of these subtypes continue to circulate primarily in birds in many possible HA-NA combinations (Table 2).

Table 1 **Occurrence of H and N subtypes of influenza A viruses in man and animals**

Subtype	Hosts	Subtype	Hosts
H1	man, pig, whale, birds	**H6**	birds
H2	man, birds	**H7**	horse, seal, birds
H3	man, horse, pig, birds	**H8**	birds
H4	seal, birds	**H9**	birds
H5	birds	**H10**	mink, birds
		H11	birds
		H12	birds
		H13	whale, birds

Subtype	Hosts	Subtype	Hosts
N1	man, pig, birds	**N5**	seal, birds
N2	man, pig, whale, birds	**N6**	birds
N3	whale, birds	**N7**	horse, seal, birds
N4	mink, birds	**N8**	horse, birds
		N9	whale, birds

The system of nomenclature includes the type of the virus, the host origin (except for human influenza viruses), geographic origin, strain number, and the year of isolation. The H- and N-subtypes are given in parantheses, e.g. A/mink/Sweden/1/85 (H10N4) or A/USSR/92/77 (H1N1).

Table 2 **HN-Subtype combinations of influenza A viruses in man and lower mammals**

subtype	host	Literature
H1N1	man	103
H1N1	pig	103
H1N2	pig	31
H1N3	whale	40
H2N2	man	103
H3N2	man	103
H3N2	pig	103
H3N8	horse	103
H4N5	seal	39
H7N7	horse	103
H7N7	seal	38
H10N4	mink	34
H13N2	whale	41
H13N9	whale	41

In birds nearly all possible HN-combinations have been found.

NATURAL INFECTIONS OF INFLUENZA A VIRUSES

A detailed coverage of the clinical manifestations and the pathological alterations will not be given. The reader is requested to refer to the specialized literature.

Primates

Influenza A viruses of the subtype H1, H2 and H3 can infect man and many species of old and new world primates. However, only gibbons,

baboons and chimpanzees have been found to be naturally infected with epidemic human strains (26 - 28). Age and immune status of the host are important factors in the outcome of an infection. The route of infection, the clinical picture, and the pathological alterations are similar among the species of primates. In most cases infections are airborn. After an incubation period of 1 to 2 days an infection, primarily of the upper respiratory tract and the major central airways occurs. Pneumonia is responsible for about 50 per cent of the increased mortality rate. This is mainly because of bacterial co-infections, but in some instances the virus infection itself may lead to pneumonia. The pathology is usually characterized among non-complicated viral infections by desquamation of the epithelium of the nasal mucosa, larynx and tracheobronchial tree. Viral antigen is present predominantly in the epithelial and mononuclear cells.

Lower mammals

In lower mammals the disease is also mainly confined to the respiratory tract with clinical and pathological signs similarly to those seen in human influenza.

Swine influenza was first observed in the United States during the human pandemic of 1918/19 and has since remained in the swine population in different parts of the world. The causative virus (A/swine/Iowa/15/30, H1N1) has been shown to be antigenically similar to the virus responsible for the human pandemic (29). This classical swine influenza virus is responsible for the most prevalent respiratory disease in pigs, with about 25% of animals having evidence of infection in North America. It has been shown that the virus could persist throughout the year in the pigs.

Outbreaks of swine influenza in Europe since 1980 have been associated with virus isolates serologically and genetically more closely related to the H1N1 virus isolated from birds, rather than to the classical swine influenza virus (30).

In 1980 an influenza virus was isolated from pigs in Japan where swine influenza is enzootic. The virus was identified as H1N2 strain (31). It is tempting to speculate that this virus represents a reassortant between the classical swine influenza virus (H1N1) and the H3N2 subtype of human influenza viruses.

In addition, there are several reports that H3N2 viruses, which are antigenically identical to human strains, also infect swine, but do not cause any clinical signs of the disease. There is evidence that H3N2 strains can persist in pigs after they have disappeared from the human population.

Equine influenza, first described in 1843 (32), is caused by 2 different subtypes of influenza viruses, A/equine/Prague/1/56 (H7N7) and A/equine/Miami/1/63 (H3N8). Both viruses continuously circulate among horses throughout the world. Both viruses can be present within one stable of horses (33). Nevertheless, neither virus has shown marked antigenic variations, although minor changes could be found among the two subtypes.

An influenza H10N4 virus was isolated from **mink** during a devastating outbreak of influenza in several farms on the south coast of Sweden in 1984. The morbidity was 100% and thousands of mink died from the outbreak. After an incubation period of 2 - 5 days, the most pronounced clinical signs were found to be anorexia, sneezing and coughing, and nasal and ocular discharges. Post-mortem examinations revealed acute interstitial pneumonia with alveolar involvement (34).

Obviously mink are susceptible to several influenza A viruses, including human H3N2 and H1N1 subtypes and several avian subtypes (35, 36). However, clinical signs of disease were not evident among the infected mink.

Influenza virus in **seals** was first isolated when approximately 20% of the harbor seal (Phoca vitulina) population of the east coast of the United States died in 1979/80 because of a severe respiratory infection (37). Pathological studies indicated consolidation of the lungs typical of primary viral pneumonia. The isolates (A/seal/Mass/1/80) were antigenically characterized as H7N7 (38). In humans, the virus causes uncomplicated conjunctivitis.

A second type of influenza virus, with lower mortality, was isolated from the common seal in 1982. This virus,presumably persisting in the seal population, was found to be of the H4N5 subtype (39).

In addition, there have been reports of influenza viruses in **whales**. Lvov et al (40) isolated an influenza virus which was identified as H1N3 virus from lung samples of a striped whale belonging to the family Balaenopteridae. More recently, influenza viruses of the subtypes

H13N2 and H13N9 were found by Hinshaw et al. (41) in suspensions of disrupted lung and hilar node of a pilot whale (Globicephala melaena) stranded in October 1984 on the New England coast. It is of particular interest that both viruses were isolated from the same whale. The antigenic, genetic, and biological comparisons of both viruses, with other influenza A viruses, suggests that the two H13 isolates probably originated from gulls.

Birds

Since Schäfer (42) (1955) first showed that fowl plague virus is an influenza virus, an increasing number of other influenza viruses have been isolated from different avian species, including domestic and wild birds in many areas of the world (43).

The majority of the avian influenza viruses, which have representatives in all of the 13 H- and 9 N-subtypes, induce an asymptomatic infection which is restricted to local sites in the mucosal membranes of the respiratory tract and the gut. Others cause a relatively mild chronic respiratory infection, particularly in turkeys. Some strains among the H5- and H7-subtypes, however, are highly pathogenic. Infection with these viruses leads to a rapid, fatal disease with involvement of the central nervous system and with death occurring within 2 to 7 days. The variation in pathogenicity among avian influenza viruses depends on both the virus and the host.

Most avian influenza viruses with almost every possible combination of the H- and N-subtypes, were isolated from apparently healthy feral ducks. The vast array of viruses seems to circulate continuously in a single population of ducks. The viruses replicate in the epithelial cells of the intestine and high concentrations of infectious virus are excreted in the feces. Since the viral infectivity is highly stable in water, water fowls have a very efficient mode of transmitting avian influenza viruses. (44).

THE HEMAGGLUTININ AS A DETERMINANT FOR PATHOGENICITY

As already mentioned, activation of the HA by posttranslational proteolytic cleavage into the fragments HA_1 and HA_2 is essential for infectivity of influenza viruses. It is generally accepted that cleavage is necessary for virus penetration by triggering fusion of the viral envelope with cellular membranes (45). The NH2 terminus of HA_2, resulting

from the specific cleavage reaction, is hydrophobic and conserved among the HA subtypes. The fusion capacity is expressed only at low pH and it has been shown that under these conditions the HA molecule undergoes a conformational change (46) which might permit the fusion peptide to insert into the cell membrane and thus facilitates membrane fusion.

Since the HA-activating trypsin-like protease is a cellular enzyme, the infected cell type determines whether the HA is cleaved or not (17). The HAs of all the mammalian influenza viruses and the non-pathogenic avian influenza viruses, which cause a local infection, are susceptible to proteolytic cleavage only in restricted cell types. On the other hand, the HAs of pathogenic avian viruses among the H5 and H7 subtypes, causing a systemic infection, are cleaved by proteases present in a broad range of different host cells (47). Thus, there are differences in host range, resulting from differences in HA cleavability which can be correlated with the pathogenic properties of the virus. The differences in cleavability are due to differences in the structure of the haemagglutinin cleavage site (48, 49). Sequence analyses have revealed that the HA_1 and HA_2 fragments of the HA molecule of the pathogenic avian influenza viruses are linked by several extra basic amino acids (Fig. 3).

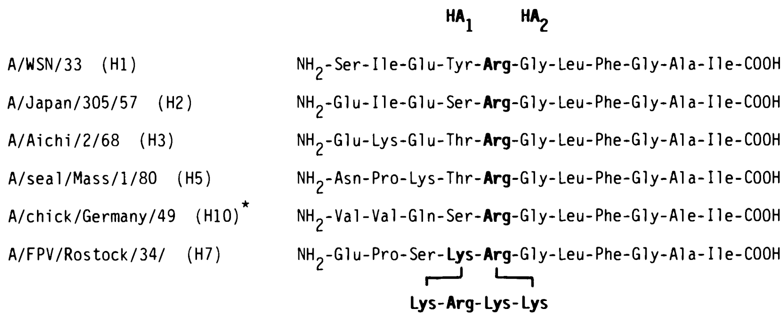

Fig. 3. The cleavage site of hemagglutinins of several influenza A viruses. The amino acids eliminated in the cleavage reaction are indicated by heavy letters (22;*H. Feldmann, personal communication).

This is in contrast to the HA of all the other influenza viruses in which the HA is cleaved only in a few cell types. They have a single arginine residue at the cleavage site that is eliminated by the cleavage reaction (17, 22). Although the HA of all influenza viruses is cleaved by the same general mechanism it has to be assumed, however, that differences exist in the specificity of the proteases involved in the proteolytic activation of the hemagglutinin (25, 50).

These data indicate the important role of the influenza virus HA in pathogenicity. If the HA is cleaved in a restricted number of cell types, the infection will be confined to localized areas of the host. In mammals this type of infection affects the respiratory tract, whereas in birds it is likely to be clinically inapparent. On the other hand, cleavability of the HA in a wider range of different host cells, as is the situation in the pathogenic H5 and H7 viruses, permits a rapid production of infectious virus particles in all organs and thereby spread in the organism, resulting in a systemic fatal disease (22, 51, 52).

SYNERGISM BETWEEN BACTERIA AND INFLUENZA VIRUSES

Combined viral-bacterial pneumonia in man is apparently three times more common than primary viral pneumonia (53). Besides a number of different bacteria, _Staphylococcus aureus_ is most commonly involved. It is generally thought that virus infection in a given tissue favors growth conditions for bacteria. On the other hand, it was recently found that _Staphylococci_ exert a decisive influence on influenza virus replication in the respiratory tract and promotes the development of influenza pneumonia. Some _Staphylococcus aureus_ strains have been shown to secrete a protease capable of activating the HA by proteolytic cleavage _in vitro_. The presence of the bacterial enzymes in cell-culture media enabled the virus to undergo multiple growth cycles. Thus, co-infection of mice with _Staphylococcus_ enhanced the virus titer in the lung enormously, resulting in a fatal disease with extended lesions in lung tissue (54). These findings may explain the high fatality rate seen after co-infection with _Staphylococcus aureus_ in man (55, 56). It is reasonable to assume that severe cases of influenza in man, such as was found during the 1918/19 pandemic or progressing disease in animal species as observed in several occasions, might occur by a similar synergistic effect initially between low pathogenic viruses and relatively harmless and ubiquitous microorganisms.

GENETIC VARIATION OF INFLUENZA VIRUSES

One of the most remarkable properties of influenza viruses is their high genetic variability. It became clear that, by genetic reassortment between viruses of different subtypes, progeny with new biological characteristics could arise (57). It is also known that influenza viruses tolerate high rates of mutation, and thus it is to be expected that new sub-populations arise continuously. From such genetically heterogenous virus populations, variants with altered phenotypes could be selected by environmental factors of the host.

Gene Reassortment

The segmented genome of influenza virus can theoretically yield 256 different gene combinations when two different viruses co-infect a single cell. This means that through gene reassortment, progeny viruses could arise, with genomes containing genes from both parental viruses. These reassortants can, therefore, have completely new characteristics, such as altered antigenicity, host range specificity or pathogenicity. If the HA gene is exchanged and the immunogenicity of the virus is drastically altered, this reassortment is called **antigenic shift**. It is relatively easy to produce such reassortants, with high frequency, experimentally *in vitro* and *in vivo*, even between human and animal influenza viruses of different origins (57, 58).

Studies on field strains of avian influenza viruses provided circumstantial evidence that gene reassortment can occur continuously in nature. As already mentioned, antigenic analysis of different isolates showed that viruses representing nearly every possible combination of the surface glycoproteins have been isolated. It also has been shown that field strains of avian viruses, possessing the same HA-NA-combinations may have differences in the composition of the other genes (59).

Gene reassortment has also been indicated as the most likely mechanism for the origin of new mammalian influenza viruses, such as the H7N1, H10N4 and the recently isolated European swine influenza virus H1N1, which caused influenza in seals, mink and pigs, respectively. There is good evidence that all these viruses evolved by reassortment involving genes of avian influenza viruses.

By genome analysis it became apparent that human subtypes also arise by reassortment. It is well known that the subtype H1 (1918/19 - 1957) was replaced by subtype H2 in 1957 which in turn was superseded in

1968 by subtype H3. In 1977 subtype H1 appeared again, the genes of which were identical to those of the earlier subtype H1 (60). Genetic analysis of the gene composition of the H2 subtype showed (Fig. 4) that the NS, M, NP and the PB_2 gene were transferred from an H1N1 strain to another virus. The latter virus, of unknown origin, donated the HA, NA and the other two P genes. In the H3-subtype, only the HA gene was exchanged. This gene has a base sequence which is, to a large extent, identical to an HA gene known to exist before in a horse (H3N8) and an avian (H3N8) influenza virus (61). Therefore, it was concluded that this gene was acquired by reassortment from an animal virus.

Influenza Virus

RNA – Segments	H1	H2	H3
1 (PB2)	-----	-----	-----
2 (PB1)	-----	———	———
3 (PA)	-----	———	———
4 (HA)	-----	———	━━━
5 (NP)	-----	-----	-----
6 (NA)	-----	———	———
7 (M)	-----	-----	------
8 (NS)	-----	-----	------

Fig. 4. Gene derivation of human H1, H2 and H3 subtype influenza A viruses. The H2 viruses have taken four genes from the H1 viruses, and the H3 viruses have taken the HA gene from an animal influenza virus.

Studies on antibody prevaling in man have shown that viruses containing H3 but N8 circulated among humans around the turn of the century (62). It is probable that over a period of 60 or more years, the H3 virus, by a series of reassortment events, had cycled between H3N8 virus in man, H3 viruses in animals and appeared in man again as the H3N2 subtype.

The most recent example of reassortment involving human influenza viruses occurred about 1977/78 when the H1N1 appeared during an ongoing H3N2 epidemic (63). The subsequent epidemics were caused almost exclusively by H1N1 viruses, however, the analysis of their genome revealed

that they contained a mixed gene composition: Four to five genes of the H1 subtype virus were replaced by the corresponding genes from the H3-virus (64, 65).

Taken together, these observations suggest that one important mechanism by which "new" influenza viruses could arise is by segmented genomic reassortment between viruses of different origin. These reassortants must be capable of overcoming the host limitations, to become transmissible in the novel host population, and to exhibit pathogenic properties. Therefore, it should not be surprising that nature requires many "throws of the dice" before new influenza viruses appear.

Gene Reassortment and Pathogenicity

It has been repeatedly shown that by gene reassortment tissue specificity of influenza viruses can be altered (66, 67) and that these changes can be correlated with alterations in pathogenicity (68, 69).

Studies with reassortants obtained *in vitro* revealed that in viruses constructed artificially in this way, pathogenicity is of polygenic nature (70 - 73). This conclusion was reinforced by genetic analysis of a large number of reassortants obtained from fowl plague virus (highly pathogenic for chicken) and other non-pathogenic influenza viruses from mammals and birds. Taken together (57), the data indicate that the HA gene is a necessary, but not the sole, factor involved in pathogenicity. For each reassortant, a specific gene constellation, responsible for the pathogenic properties, might be required, which is dependent upon the parent virus strains used and the particular genes that were exchanged. Therefore, there is no general rule for which gene or genes have to be replaced in order to increase or attenuate pathogenicity. Nonetheless, besides the HA gene, the genes coding for the polymerase complex seems to be particularly important for pathogenicity.

Reassortants obtained from parental viruses both non-pathogenic for a particular host can assume pathogenic properties for that host (66, 74) or in contrast, reassortment between highly pathogenic parent strains may lead to non-pathogenic viruses (75).

Restoration of pathogenicity is possible through extragenic suppression of defects in viral genes by reassortment (76, 77). In such suppressor reassortants it is to be expected that the defective gene or gene product co-operates with the gene product of the replaced gene. Furthermore, initially nonpathogenic reassortants can mutate into pathogenic

ones under special conditions (78). It is not yet known by which mechanism of mutagenesis this could occur or how high the reactivation rate is.

Evidence that, in nature, reassortants with an optimally functioning genome composition are selected by environmental factors in the host organism could be obtained with avian influenza viruses. It was found that the body temperature of the bird (42°C) represents the selective barrier for the formation of reassortants pathogenic for chickens (79). In contrast to pathogenic reassortants, the non-pathogenic ones were found to be unable to grow at the elevated temperature. As a consequence of double infection _in vitro_ at 41°C, reassortants are selected that are exclusively pathogenic for chickens if the appropriate HA is present in the virus particles.

Genetic Alteration by Mutation

The mutation frequency of influenza viral genes, for any single nucleotide, appears to be in the range of 10^{-5} per replication. Thus, besides reassortment mutation provides an ample source for the selection of naturally occurring variants. Mutations in each gene may influence the viral phenotype as shown with temperature-sensitive mutants (80). In addition, complementation of a mutation in one viral gene may occur between mutants having lesions within the same gene or may arise from suppression of the phenotype by a different gene, pressumably coding for a protein which complements the functional defect in the gene carrying the mutation. Such intragenic complementations (81 - 84) and extragenic suppressor mutations (85) have been found to occur in several of the viral genes.

Since most data are available on mutational changes of the HA gene and its biological consequences, the following brief description will be restricted to this particular gene and its protein product. Mutations of the HA gene could be associated with changes in the antigenicity, the receptor specificity of the hemagglutinin and cleavability of the HA, a prerequisite for infectivity and pathogenicity.

Antigenic Changes by Mutation

The hemagglutinin is subtype specific and induces the production of neutralizing antibodies in the infected host. The virus can escape neutralization by spontaneous mutations resulting in amino acid substitutions of the HA. Successive minor mutations in the HA gene following the

emergence of a new subtype (most pronounced among human influenza virus strains) are called **antigenic drift**. They lead to an accumulation of amino acid changes that alter the antigenic sites in such a way that they are no longer recognized by the preformed immunity. The HA has been sequenced from different field strains resulting from antigenic drift, but it is not possible to deduce from the data which of the sequence changes are responsible for alteration in antigenicity (86).

However, antigenic variants of several virus strains have been selected by growing virus in vitro in the presence of monoclonal antibodies to the HA. The variants which did not bind at all to the antibodies used for their selection were found to possess in most instances changes in the amino-acid sequence in the globular part of the three-dimensional structure of the hemagglutinin molecule. The changes were clustered into 4 distinct regions, as the antigenic sites (A-D) of the HA (86 - 88). In contrast to the HA variants selected with monoclonal antibodies, every new naturally occurring "drift" strain generally had amino acid substitutions in more than one of the 4 antigenic sites of the molecule (89).

Amino acid substitution, at the carbohydrate attachment sites of HA at or close to, the antigenic domains could also lead to changes in antigenicity. This was found to have occurred with strains of the H3 subtypes during antigenic drift, whereby an increased saccharide content at the antigenic sites has been demonstrated (90). It is suggested that attachment of immunulogically indifferent carbohydrates could prevent binding of antibodies to the specific site.

Minor antigenic differences have also been detected in the influenza A subtypes occurring in animals. The evidence, however, points to co-circulation of different variants rather than to gradual antigenic drift. The limited antigenic variation, found among animal influenza viruses, is a feature that distinguishes these viruses from human strains, and antigenic drift may not be an accurate term for antigenic variations among animal influenza viruses. It is not known on which mechanisms these differences are based.

In general, it is assumed that antigenic drift occurs as a result of selection under immunological pressure. There is evidence, however, for an additional selection mechanism for antigenic variants of influenza A viruses that depends upon differences in host cell tropism of viral

sub-populations. After adaptation of a human influenza A virus to MDCK cells, variants were selected which were antigenically distinct from the virus from the same source but grown in chicken eggs. A single amino acid exchange at the antigenic site A was responsible for the drastic change in antigenicity (91). It is tempting to speculate that this host-cell dependent selective pressure mechanism, for generation of antigenic variants, could have implications for the evolution of influenza virus in nature.

Changing in the Receptor Binding Site

The receptor binding site of the HA determines whether 2-3 linked or 2-6 linked neuraminic acid substrates are recognized by the virus as receptor (92, 93). Influenza viruses, at least among the human subtype H3, contain sub-populations that are specific for one or the other receptor linkage. Growth of those viruses in the presence of horse serum inhibitor, for instance, will select for variants recognizing only the 2-3 linkage which might possess altered pathogenic properties (94). Comparison of the amino acid sequence of the wild type and variant HA indicate that they differ only in one amino acid at a site of the HA molecule, proposed to be the receptor binding site (93). Similar results were obtained with a reassortant between a human H3 strain and an avian H2 virus (95). Unlike the parental human virus, the reassortant possessing the H2 hemagglutinin was able to replicate in the duck intestine. Sequence analysis of the HA of the reassortant revealed that the most likely cause for the changed cell tropism of the virus was a substitution of two amino acids at the receptor binding site. This suggests that mutation at the HA receptor binding site can alter tissue tropism and probably species specificity.

Mutations Affecting HA Cleavage

Recent results with a human influenza virus have revealed that susceptibility of the HA to cleavage is not a fixed entity of the molecule but can be altered by adaptation of the virus to a novel host cell, previously non-permissive to the wild type virus. Cleavability of the HA of the adapted virus in the new cell type was attributed to a single amino acid substitution close to the cleavage site. The mutation did not result in a general increase in the susceptibility of the HA to activating proteases. By the amino acid substitution, the HA was activated only by the enzymes present in the particular host cells to which the

virus was adapted, in addition to the original permissive cell types (91). Preliminary studies with a non-pathogenic avian influenza virus (A/turkey/Oregon/71, H7N3) have shown that, following adaptation to chicken fibroblasts variants could be obtained, the HA of which became activated in a broad range of different cell types, similar to the pathogenic avian viruses. With one of the variants, increase in cleavability was correlated with aggravation in pathogenicity for chickens (Rott and Orlich, unpublished results).

It should be noted that the devastating series of outbreaks of avian influenza, in Pennsylvania in 1983, were caused by H5N2 viruses and were based on a similar event (96, 97). Comparison of the isolates from the outbreaks revealed that 2 biologically distinct viruses existed, with different pathogenic properties. Enhancement of pathogenicity was attributed to the selection of a variant. In this variant a cleavage site of the sequence Lys-Lys-Lys-Arg- , i.e. a cleavage site susceptible to ubiquitous proteases, that was present already in the apathogenic precursor strain, was unmasked by a mutation resulting in the loss of an adjacent oligosaccharide.

One can conclude from these findings that the growth of influenza viruses in a novel host presumably allows the selective survival of appropriate mutants with altered biological properties. If the HA cleavability is affected by the mutation, the pathogenic properties of the virus may be altered. Selection for those variants might occur by transmission of a virus from one species to another.

RESERVOIR OF VIRUS

The observations reviewed here illustrate that influenza viruses in man and animals vary considerably and, in spite of the interaction of the viruses and their hosts result in a disease of remarkable constancy in a given host. By mutation and gene reassortment, even between subpopulations of a distinct virus population, new viruses may involve, thereby overcoming the host limitations and cause disease in different species. This mechanism of evolution of influenza viruses would require a reservoir for viruses to persist. The reservoir could, therefore, be involved as a host for different influenza viruses in which genetic interaction could occur and interspecies transfer would be possible. Current information suggests that transmission of influenza viruses occurs frequently between avian species, infrequently between avian and mammalian species

and rarely between mammalian species (44).

Mink, seals and horses can be widely excluded as such a reservoir because these animals are affected only by very few influenza virus strains.

Although there is no direct evidence that man may be affected by viruses from birds, or vice versa, it is clear that birds, with their unusual heterogenous collection of influenza viruses, are a potential reservoir in which new influenza viruses could be created. It should be mentioned again that all the 13 H and 9 N-subtypes were found in birds. Infection in birds represents an ideal host-virus-relationship, because most of the influenza viruses are non-pathogenic for birds and, therefore, persist in avian species. The genetic relatedness of avian virus strains to those appearing in all other species support the possibility that the influenza viruses in birds are the primordial source of all influenza A viruses (44). More recently, the H3 subtypes in man and the avian-like viruses in pigs, mink, seals and whales may have emerged from birds.

Migrating birds, particularly feral ducks, have the potential for disseminating influenza virus. Some avian species such as ducks or quails are remarkable resistant to disease from infection even with strains highly pathogenic for chicken and turkey (98). It has been shown, for instance, that a potentially highly pathogenic virus for chicken could circulate among quails by direct transmission without causing disease. The shed virus, however, caused a fatal infection in chickens when they were housed in contact with the infected quails (99). The results demonstrate how potentially pathogenic avian influenza virus strains can be generated and maintained in a particular species without harm. This pathogenic potential could became fully manifested when a different species of high susceptible birds is exposed to these viruses. Birds such as quails or ducks could, therefore, represent a reservoir for the origin of new epidemics in a different avian species. Furthermore, outbreaks in domestic birds co-incided with the migration of waterfowl. Antigenically similar viruses have been isolated from both the domestic and migrating birds, and also from pigs in the same area (39, 41, 101).

The pig is apparently susceptible to a wide range of different influenza viruses, including human strains. The classical swine influenza virus, presumed to have been passed from man to pigs during the pandemic

of 1918/19, has persisted in the pig population. In some instances it could be transferred to man, although it was epidemically abortive (100). The occurrence of H1N2 virus in pigs in Japan probably was the result of a virus reassortment in pigs. There is also evidence that swine influenza virus infect birds or, conversely, can be transmitted from birds to pigs (39). Owing to the great susceptibility of pigs to influenza viruses of different sources it is likely that pigs may represent an intermediate link between a genuine animal reservoir and man (101, 102).

CONCLUSION

Among the 13 known H-subtypes of influenza A viruses, new genotypes evolve constantly by mutation or genetic reassortment, some of which have distinctly different phenotypes. Alterations in the viral genome affect the antigenicity of influenza viruses, tissue specificity, host range, and their pathological properties. Such genetic changes are because of the plasticity of the segmented structure of the viral RNA resulting in a heterogenous population of viruses. Several of these new genotypes can be maintained within a virus population when transmitted from host to host. The relative dominance of each sub-population will fluctuate depending on the environmental properties of each new host encountered. The mechanisms by which host range limitations can be overcome are poorly understood. However, a limited number of amino acid substitutions will affect the function of the major viral surface glycoprotein and other viral functions. Thus, adaptation to a new host may generate variants by pressures of selection with alterations among several genes.

The creation of new influenza viruses requires a host reservoir in which genetic interplays can take place and where the different virus variants will propagate. There is good evidence that birds, particularly feral waterfowls, provide an environment for influenza viruses which favor the appearance not only for new avian viruses, but also mammalian viruses including human virus strains. The newly formed viruses, which are able to overcome the host barrier, must be able to replicate to high titers with the production of sufficient stable infective virus in the new host, to possess pathogenic properties and to be transmissible. This means that the new viruses have to contain an optimally constructed genome for each particular host. It is, therefore, not surprising that, in nature, rarely do such new influenza viruses appear, particularly in mammals.

In view of these considerations it becomes clear that during evolution influenza viruses were not strictly confined to a particular host species. Influenza has, therefore, aspects of an unique zoonosis which will always be difficult to control.

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25

PARAMYXOVIRUS ISOLATED FROM MIGRATING DUCKS

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ABSTRACT

A total of 47 paramyxoviruses isolated from migrating feral ducks were characterized by immunological, biochemical and genomic analyses. For comparison, 4 additional paramyxoviruses derived from domestic ducks and caged birds were also used. Eight antisera monospecific for the hemagglutinin-neuraminidase protein of reference strains of avian paramyxovirus could clearly differentiate the antigenic relationships amongst the above viruses, indicating that duck-paramyxoviruses are grouped into three serotypes.

Of the 30 identified as paramyxovirus type 1 by antisera monospecific to the nucleoprotein and hemagglutinin-neuraminidase protein of Newcastle disease virus, 14 were further analyzed antigenically by a panel of monoclonal antibodies to hemagglutinin-neuraminidase proteins and they appeared to be grouped into 3 variants. The remaining 17 isolates were divided into 2 serotypes with the following prototypes: avian paramyxovirus type 4/duck/Hong Kong/D3/75; avian paramyxovirus type 6/duck/Hong Kong/199/77. RNA analysis of these viruses by oligonucleotide mapping revealed that duck-paramyxoviruses spread from duck to duck efficiently through a water-borne transmission and they were found to be circulating world-wide along with migration routes of the birds. In addition, it was evident that ducks play an important role for the maintenance of avian paramyxoviruses, suggesting a possible transmission of these viruses to domestic poultry.

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INTRODUCTION

The viruses belonging to the genus Paramyxovirus (1), family Paramyxovirus are distributed in a wide range of animal species from human beings to lower animals and birds (2-10), posing some interesting questions in the natural ecology of these viruses. Over a period of thirty years, the orthomyxo- and paramyxoviruses have been known to share a number of biological properties, such as special affinity for sialic acid-containing receptors on cells, hemagglutinating and neuraminidase activities, requirement of the proteolytic cleavage of a viral glycoprotein for fusion activity, single-stranded RNA genome of negative polarity and the presence of transcriptase in the virion (11,12), suggesting an evolutionary relationship between both virus groups. Coupled with this evidence, extensive surveillance programs of influenza in bird populations, which has been done since the early 1970s, yielded the following results: 1) isolation of a large number of viruses and their subsequent analyses by modern techniques strongly suggested that all influenza A viruses distributed in mammals are related to avian influenza viruses (13-21); 2) the virological investigation considerably influenced the research on avian paramyxoviruses that had been traditionally focused on Newcastle disease virus (NDV).

As a result of influenza surveillance programs, numerous paramyxoviruses other than NDV, in addition to the avian influenza virus, have been isolated from a wide range of wild, caged and domestic birds (22-30). The noteworthy finding was that the majority of the isolates were derived from apparently healthy wild and domestic ducks (10,24,25,27,30). Many reports described biological properties and a wide variation in antigenic characteristics of the viruses, and, in recent years, their classification has been the subject of much discussion (10). Although a recent report described the usefulness of the matrix (M) protein for the differentiation of avian paramyxoviruses (31), classification based on antigenic characteristics of hemagglutinin-neuraminidase (HN) protein is not always consistent with the results obtained by reaction with M protein (32), indicating the necessity for further study.

The extensive review by Alexander (10) and subsequent reports were pertinent to the above subject (30,33), indicating that avian paramyxovirus has been divided into the following nine serologically distinct groups on the basis of hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests (10,30): avian paramyxovirus type (PMV-) 1/NDV; PMV-2/chicken/California/Yucaipa/56; PMV-3/turkey/Wisconsin/68; PMV-4/duck/Hong Kong/D3/77; PMV-5/budgeriger/Japan/Kunitachi/74; PMV-6/duck/Hong Kong/199/77; PMV-7/dove/Tennessee/4/75; PMV-8/goose/Delaware/1053/76, and PMV-9/domestic duck/New York/22/78. In 1985 the authors (33) also characterized antigenic structure of reference strains of avian paramyxovirus by immuno-double-diffusion (IDD) tests with antisera to the HN proteins of reference strains and supported the classification proposed by Alexander (10).

In the present communication we describe and discuss the knowledge obtained mainly from our studies on paramyxoviruses isolated from wild and domestic ducks through the comparison of avian influenza virus.

MATERIALS AND METHODS

The virus isolation was done by inoculation of tracheal and cloacal swabs into the allantoic cavity of 10- to 11-day-old fertile hen's eggs, followed by incubation at 35 C for 3 days. For identification of the isolates, HI and NI, and IDD tests were employed according to the methods described previously (26, 33). Antisera monospecific to the HN protein of avian paramyxovirus were prepared in our previous study (33). Anti-NDV-NP serum was made by intramuscular injection of NP polypeptides separated by polyacrylamide gel electrophoresis. An established line of monkey kidney (LLCMK2) cells were used for characterization of paramyxovirus by growth behavior as described previously (34). Of 56 duck-isolates, 49 were used in the present study. All viruses were grown and purified as described previously (35). The structural polypeptides and genome characteristics of the viruses were analyzed as described previously (35).

RESULTS

Antigenic drift of NDV-like isolates from wild ducks

In the course of a surveillance program for influenza in birds promoted by WHO, we have isolated more than one hundred paramyxoviruses from wild, caged and domestic birds, and they were found to represent 8 distinct serological groups. Fig. 1 shows the electron micrograph of a negatively stained avian paramyxovirus. The helical nucleocapsid with a width of 20 nm is frequently seen in partially disrupted virus particles. As shown in Table 1, a total of 49 viruses isolated from wild and domestic ducks appeared to consist of three antigenic types, showing that NDV stands out very vividly from other avian paramyxoviruses belonging to PMV-4 and PMV-6 serotypes. For serotyping, we used antisera monospecific to the nucleoprotein (NP) and HN proteins.

The antiserum to NP protein developed a single line of precipitation with reference and 4 isolates, (duck/Aomori/24,duck/Niigata/361,duck/Aomori/15,duck/Aomori/75) (Fig. 2), but not duck/Hong Kong/D3/75 (HK-D3) virus used as an antigenically un-

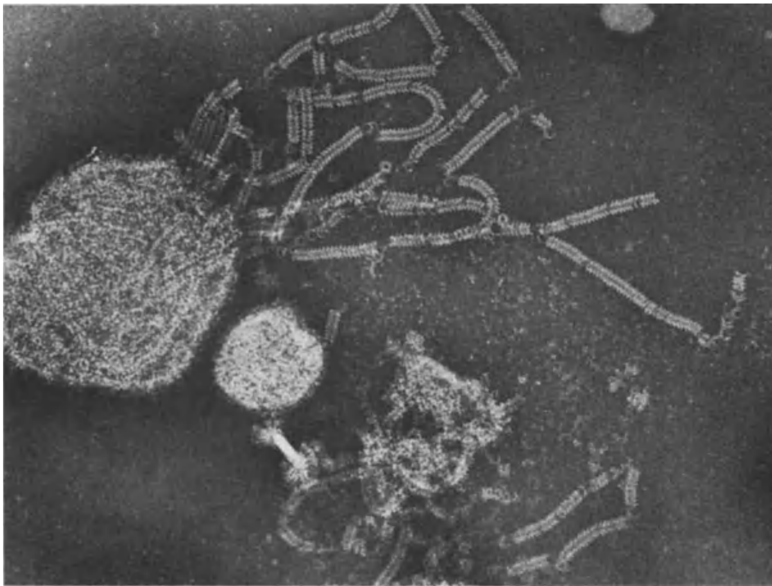

Fig. 1. Electron micrograph of an avian paramyxovirus (PMV-2) stained with phosphotungustic acid.

related virus antigen, indicating that this antiserum is useful for characterization of avian paramyxoviruses. The antiserum to the HN protein also gave a single and distinct line of precipitation which was continuous among the homologous strain and the isolates.

Influenza virus is divided into three distinct types on the basis of the immunological properties of inner proteins such as NP and M, and type A virus is further subtyped based on antigenic difference of surface glycoproteins (HA, NA) (1), but this method has not been adopted in the paramyxovirus family. For this reason, the results obtained in the above IDD test are of interest, suggesting that NP of paramyxovirus may also serve for antigenic differentiation. All viruses were identified as PMV-1 group by HI tests with antiserum monospecific to HN, and these isolates were further analyzed by five monoclonal antibodies. Three monoclonal antibodies (1/29, 5/205, 5/220) to the HN of Miyadera strain were prepared by us (36) and two monoclonals (Taka/2, Taka/3) to HN of Taka virus were kindly provided by Dr. T. Mikami (37). The results with 14 duck-derived isolates, 3 isolates from caged birds, and reference strains are presented

Table 1. Number and serotypes of duck-paramyxoviruses analyzed in the present study

Serotypes#	Number	Place of isolation
PMV-1	22	Japan
PMV-1	2	U.S.A.
PMV-4	14	Japan
PMV-4	4	U.S.A.
PMV-4	1	Hong Kong
PMV-6	6	Japan
Total	49	

Viruses were divided serologically according to the method of nomenclature recommended by Alexander.(10).

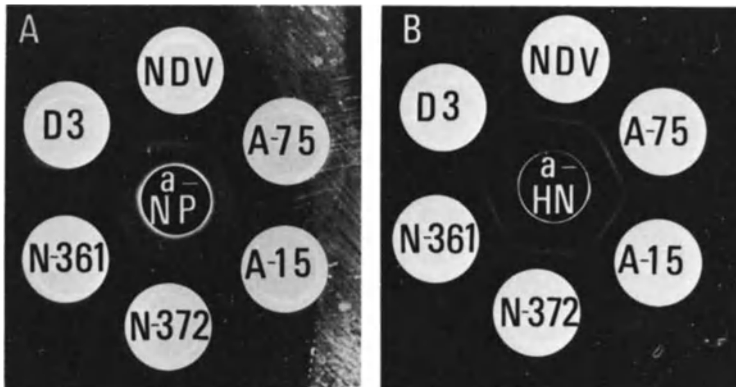

Fig. 2. Antigenic characterization of duck-paramyxoviruses by immuno-double-diffusion tests with antisera monospecific to the isolated NP and HN proteins of NDV. The center wells contain antisera to NP (A) and HN (B), and the outer wells contain the following Triton X-100 treated virus antigens: NDV, Miyadera strain which was used in preparation of the above antisera; D3, PMV-4/duck/Hong Kong/D3/75. The remaining antigens are presented in Table 2.

in Table 2. Three viruses derived from birds other than ducks (B1, parakeet/Tokyo/246/79, parakeet/Tokyo/483/77) seemed to react to low titers with antiserum monospecific to the HN proteins, whereas this antiserum could not detect the marked antigenic differences between the NDV and NDV-like isolates examined. However, the occurrence of a clear antigenic difference in HN molecule of numerous isolates and reference strains of NDV was observed with the monoclonal antibodies, showing a wide variety of HI reaction patterns.

All viruses including three reference strains were divided into five variant groups on the basis of reaction with the five monoclonals. The first variant comprising Miyadera and B1 strains reacted with all monoclonal antibodies used in the HI tests, but the Sato strain belonging to the 2nd variant failed to react with one monoclonal (5/220), suggesting both variant groups are different from each other at least in one antigenic determinant. Although hemagglutinating activity of the third and fourth variants represented by duck/Aomori/24/80 and duck/Aomori/15/80, respectively, was inhibited by 3 out of 5 monoclonals

Table 2. Analysis of the antigenic structure of duck-paramyxoviruses by monospecific antiserum and monoclonal antibodies to HN subunits of Newcastle disease virus (NDV)

Virus strains		HI titers with the following antibodies:					
		Antiserum monospecific	Monoclonal antibodies				
		for HN	1/29	5/205	5/220	2/Taka	3/Taka
Reference strains							
1st variant:	Miyadera	1,024	16,384	8,192	16,384	1,024	256
	B 1	2,048	32,768	8,192	4,096	2,048	512
2nd variant:	Sato	512	8,192	32	–	1,024	256
3rd variant:	duck/Aomori/24/80	4,096	16,384	–*	256	4,096	512
	duck/Aomori/88/80	2,048	16,384	–	64	2,048	256
	duck/Aomori/110/80	2,048	8,192	–	64	1,024	256
	duck/Aomori/171/80	2,048	16,384	–	32	2,048	128
	duck/Niigata/361/80	1,024	16,384	–	64	1,024	256
	duck/Niigata/372/80	1,024	2,048	–	512	1,024	512
	duck/Niigata/412/80	1,024	8,192	–	32	1,024	64
	duck/Niigata/423/80	1,024	8,192	–	128	512	256
4th variant:	duck/Aomori/15/80	4,096	16,384	–	–	2,048	256
	duck/Aomori/24K/80	2,048	32,768	–	–	4,096	128
	duck/Aomori/75/80	2,048	16,384	–	–	1,024	256
	duck/Aomori/99/80	2,048	8,192	–	–	512	128
	duck/Aomori/143/80	2,048	32,768	–	–	4,096	512
	parakeet/Tokyo/246/79	512	8,192	–	–	2,048	128
	parakeet/Tokyo/483/77	512	8,192	–	–	1,024	128
5th variant:	duck/Aomori/96/80	1,024	8,192	–	–	–	–
	parrot/Tokyo/17/78	1,024	16,384	–	–	–	–

*–, less than 32. All antisera and monoclonal antibodies were treated with RDE.

(1/29, 2/Taka, 3/Taka). The remaining one monoclonal (5/220) definitely distinguished the 3rd variant from the 4th. The former (duck/Aomori/24/80-like isolates) reacted with 5/22o but the latter (duck/Aomori/15/80-like virus) did not. Parakeet/Tokyo/246/79 and parakeet/Tokyo/483/77 strains were found to be antigenically similar to duck-viruses belonging to the 4th variant group. The 5th variant, represented by duck/Aomori/96/80 strain appeared to react with only 1/29 which recognizes the epitope conserved in all NDVs examined. These results revealed that the various duck-NDV variants have been constantly co-circulating in many areas of the world, in agreement with the recent report with a large number of NDVs from different species of birds (36,37,38). From a pathological point of view, it is noteworthy that a majority of the NDVs have been isolated from apparently healthy ducks (10,24,25,27,30,39,40-44), suggesting the stable host-parasite relationships.

NDV contains two kinds of glycoprotein on its surface, which are involved in essential biological activities. Of these, one possesses hemagglutinin and neuraminidase activities, and other glycoprotein (F) is responsible for cell fusion, hemolysis and virus penetration. The precursor F protein is synthesized in the infected cells as an inactive form (Fo) that is cleaved by host cell protease to yield F1 and F2 subunits (9,12,45,46). This cleavage step of Fo is an essential event for the expression of virus infectivity by acquisition of cell fusion activity (46). In connection with the above evidence, it was of interest that the presence of the active form of F protein (F1) on the virus particles is a potential indicator for virulence (46). It was recently reported that duck-NDV-like viruses contain F1 glycoprotein (47), in agreement with virus pathogenicity shown in 11-day-old fertile eggs and one-day-old and young (42,43,48) SPF chickens. The natural ecology of the paramyxovirus in the duck seems to be similar to that of the avian influenza virus. Since the beginning of a large-scale influenza surveillance in birds, huge numbers of influenza A viruses were isolated from wild and domestic ducks, but no ducks from which viruses were isolated, died or showed any signs of the disease.

On the origin of world-wide distributed duck paramyxoviruses

In 1976 Webster et al. isolated thirteen hemagglutinating agents from the cloacas of migrating feral ducks shot on the Mississippi flyway and 8 of them were identified as paramyxoviruses (24). Of these, 6 were studied along with viruses isolated from domestic ducks in Japan. Table 3 shows the results of the antigenic analysis of the United States and Japan isolates. Hemagglutinating and neuraminidase activities of all isolates from both countries (presented in the 2nd column of the Table) were strongly inhibited by antiserum monospecific to the HN protein of HK-D3 virus but not by the remaining antisera. The similar levels of HI and NI titers of the homologous strain and 5 isolates suggest that the HN antigen of the above viruses is closely related.

The antigenic characteristics of the above viruses led us to compare the electrophoretic migration pattern of structural polypeptides of references and the isolates. Fig. 3A showed a wide variety of migration patterns. Avian paramyxoviruses contain 6-7 major structural polypeptides with mol. wt. ranging from 80,000 to 34,000, and two of them were identified as glycoproteins by carbohydrate staining (Fig. 3B). Among 11 virus strains, four (duck/Miss/116,320,334, duck/Tokyo/41/78) were similar to each other antigenically as well as electrophoretically, showing that these viruses possessed six major polypeptides with mol. wt. 80,000, 61,000, 57,000, 48,000, 43,000-44,000, and 40,000 (Table 4). Duck/Miss/406/76 was also antigenically closely related to the above 4 strains but slightly different electrophoretically. As reported previously, the electrophoretic pattern of structural polypeptides was more similar among serologically closely related to the above 4 strains but slightly different electrophoretically. As reported previously, the electrophoretic pattern of structural polypeptides was more similar among serologically closely related viruses than between viruses of different serological groups (35,49,50).

In 1978 Nakajima et al. (51) revealed that Russian (H1N1) influenza virus originated from the epidemic strain which had been prevalent in man around 1950 by oligonucleotide mapping.

Table 3. Antigenic analysis of the HN of avian paramyxoviruses isolated from ducks by HI and NI tests with antisera monospecific for the HN proteins of NDV, HK-D3, and HK-199 viruses

Virus	Titers with indicated antisera: HI[a]			NI[b]		
	NDV	HK-D3	HK-199	NDV	HK-D3	HK-199
Reference strains						
PMV-1/NDV (Miyadera)	2,048	–	–	1,024	–	–
PMV-4/HK-D3	–	512	–	–	1,024	–
PMV-6/HK-199	–	–	2,048	–	–	2,048
duck/Miss/116/75	–	512	–	ND#	ND	ND
duck/Miss/319/75	–	1,024	–	ND	ND	ND
duck/Miss/320/75	–	1,024	–	ND	ND	ND
duck/Miss/334/75	–	2,048	–	ND	ND	ND
duck/Miss/406/75	–	4,096	–	ND	ND	ND
duck/Tokyo/41/78	–	512	–	ND	ND	ND
duck/Miss/604/75	2,048	–	64	ND	ND	ND
duck/Miss/330/75	256	–	64	ND	ND	ND
duck/Niigata/346/80	–	512	–	–	1,024	–
duck/Niigata/391/80	–	2,048	–	–	256	–
duck/Niigata/420/80	–	1,024	–	–	1,024	–
duck/Niigata/421/80	–	512	–	–	256	–
duck/Niigata/476/80	–	512	–	–	256	–
duck/Niigata/496/80	–	2,048	–	–	1,024	–
duck/Niigata/498/80	–	512	–	–	256	–
duck/Niigata/505/80	–	512	–	–	256	–
duck/Niigata/383/80	–	–	256	–	–	256
duck/Niigata/397/80	–	–	128	–	–	512
duck/Niigata/400/80	–	–	512	–	–	512

*a –, less than 32. b –, less than 4. #ND, Not done. Values given represent the terminal serum dilution inhibiting the hemagglutinating and neuraminidase activities of viruses examined. All antisera were treated with RDE.

Since then this technique has been widely used to compare epidemic strains in the other viruses. We have, therefore, analyzed

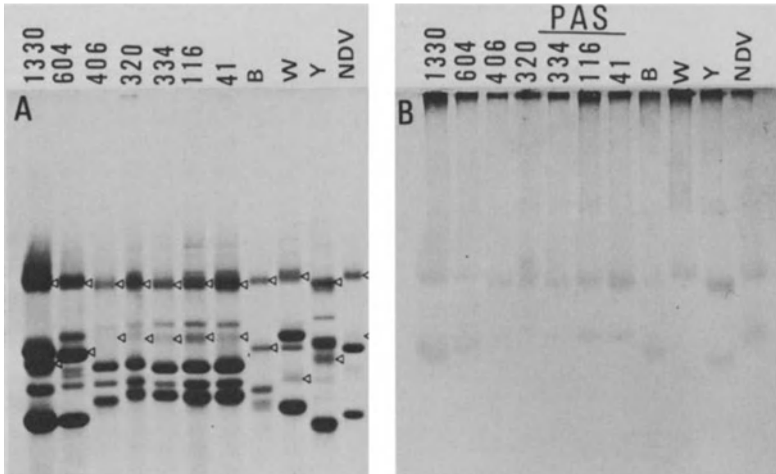

Fig. 3. Comparative analysis of the structural polypeptides of duck paramyxoviruses isolated in Japan, Hong Kong and the United States. The structural polypeptides of isolates and four reference strains were separated by electrophoresis on a 13% polyacrylamide gel. Arrows indicate the position of glycoprotein identified by PAS staining. NDV, Miyadera strain: Y, PMV-2/chicken/California/Yucaipa/5 (Yucaipa); W, PMV-3/turkey/Wisconsin/68 (Wis.), B, PMV-2/finch/N. Ireland/Bangor/73 /Bangor). Other lanes were duck/Miss/75 and duck/Tokyo/41/78 isolates numbered as in Table 3.

the RNAs of six viruses isolated in Hong Kong, Japan and the United States by oligonucleotide fingerprinting to study the genetic relatedness of these avian paramyxoviruses. Oligonucleotide maps produced by six viruses exhibited a marked variability (data not shown). In order to estimate the extent of variation about 40 large ribonuclease T1-resistant oligonucleotide were selected and closely examined. The oligonucleotide analysis revealed that the avian paramyxoviruses isolated from migrating feral ducks in the United States in 1975 can be divided genetically into three groups despite their close similarity in antigenic and polypeptide analyses. The oligonucleotide maps of duck/Miss/334 and duck/Miss/406 were very similar to each other, suggesting a common origin. However, the oligonucleotide maps

Table 4. Molecular weights of the polypeptides of avian paramyxoviruses isolated from ducks and other bird species

Polypeptide No.	Molecular weights ($\times 10^{-3}$)[a]										
	NDV (PMV-1)	Yupaica (PMV-2)	Bangor (PMV-2)	Wis. (PMV-3)	Tokyo/41	116#	334#	320#	406#	604#	1330#
1	80*	76*	79*	80*	80*	80*	80*	80*	80*	80*	80*
2	57*	63	52*	61	61	61	61	61	59	57*	56
3	52	54	43	57	57*	57*	57*	58*	58*	51	52*
4	47	49*	39	54	48	47	47	49*	48	47	47
5	(44)?	43	ND	44*	43	43	43	44	43	43	42
6	36	34	37	37	40	40	40	40	37	35	35

a, Molecular weights of each polypeptides were estimated on the basis of migration of L and H chains of immunoglobulin, chymotrypsin, bovine serum albumin and polypeptides of swine influenza virus (A/swine/Iowa/15/30).
#, duck/Mississippi strains isolated in 1975. *, glycoprotein. Question mark (?) indicates a protein whose status as a viral protein is unclear. ND, Not determined.

of the RNAs of the above two isolates were different from that of Miss/116 and HK-D3 belonging to the 2nd group by only seven to nine spots. On the other hand, the oligonucleotide patterns of the remaining duck/Miss/320 was distinguished from that of either duck/Miss/334 (1st group) or duck/Miss/116 (2nd group), thus placing it in the 3rd group. In addition, the comparison of the oligonucleotide map of duck/Tokyo/41/78 isolated from a domestic duck in Tokyo with those of the above five viruses showed unexpectedly that duck/Tokyo/41/78 virus was closely related genetically to HK-D3 and duck/Miss/116 viruses. In the autumn, different species of more than 10,000 ducks migrate from the North to Japan. From an epizootiological point of view, it appears that PMV-4/HK-like viruses are widely circulated and duck/Tokyo/41/78 was derived from the virus introduced by migrating ducks. These results point to the possibility that wild birds bring epizootics through direct or indirect contact with domestic poultry.

Demonstration of an efficient water-borne transmission of duck paramyxoviruses

Surveillance of apparently healthy migrating ducks in the Northern part (Niigata prefecture) of Japan yielded a large number of hemagglutinating agents, 22 of which were paramyxoviruses. Of these, 11 presented in Table 3 were analyzed immunologically and genetically. As shown in Table 3, antigenic analysis indicated that they were divided into two serotypes, PMV-4 and PMV-6. Antiserum monospecific to the HN of HK-D3 virus specifically inhibited hemagglutinating and neuraminidase activities of eight isolates (Niigata 346, 391, 420, 421,476,496,498, and 505), to similar titers to that of the reference strain, indicating that these viruses possessed HN antigens related to that of HK-D3 virus. Antigenic analysis by HI and NI tests with monospecific antiserum also showed that the HN of the remaining three viruses (Niigata 383, 397, and 400) were antigenically similar to that of PMV-6/duck/Hong Kong/199/77 (HK-199) strain (Table 3). In order to further analyze the HN antigen, IDD tests were done. The eight viruses and HK-D3 gave a single continuous line of precipitation with antiserum monospecific to the HN of

HK-D3 (Fig. 4A and B). The IDD tests revealed close antigenic relationships amongst eight isolates (Niigata 346, 391, 420, 421, 476, 496, 498, and 505) and HK-D3 strain. Similarly, definite precipitation lines were also observed between the remaining three isolates and reference strain of PMV-6 serotype (HK-199) (Fig. 4C). It is the first evidence that antisera monospecific to the HN protein of reference avian paramyxovirus can be routinely used for the detailed characterization of paramyxovirus isolates (33, 35).

In order to follow the origin of these viruses, we compared the RNA genomes of the twelve isolates including one reference strain by oligonucleotide mapping. Fig. 5 shows the oligonucleotide maps of eight isolates belonging to PMV-4 (HK-D3) serotype.

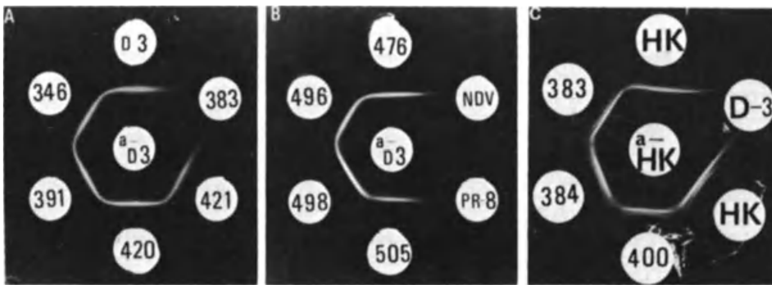

Fig. 4. Antigenic characterization of HN proteins of HK-D3- and HK-199-like virus isolated by immuno-double-diffusion tests with specific antisera. The center wells (a-D3) in A and B contain antiserum to the isolated HN protein of HK-D3 virus. The center well (a-HK) in C contains antiserum to the isolated HN protein of HK-199 virus. The outer wells contain the following Triton X-100-treated virus antigens: D-3, HK-D3; NDV, Miyadera strain of Newcastle disease virus; HK, HK-199; and PR-8, influenza A/PR/8/34 (H1N1) virus, which was used as a strain unrelated to avian paramyxovirus. All viruses except PR-8 were isolated from wild ducks in Japan in 1980 (see Table 3). Each number in the outer wells represents a virus isolate. From Nerome et al.(39).

Genetic relatedness among the RNAs of these isolates was very close, suggesting a possible common origin. To determinate the degree of homology among these viruses, approx. 55 large oligonucleotides distributed below the bromphenol blue dye marker were selected and compared. 51 out of the 55 spots (92%) were

common to all viruses, as indicated by arrows pointing to the left in Fig. 5. As can be seen in the same figure, we could detect two more oligonucleotide spots (indicated by arrows pointing to the top or bottom) common to two groups of the virus, each comprising 3 isolates. Using this quantitative analysis, we determined that only 4% of examined spots were different between Niigata 391, 498, and 346 and Niigata 420, 421, and 505 isolates. In the previous paper (35) we described six avian paramyxoviruses isolated in different areas between 1975 and 1978, and they appeared to be divided genetically into three groups despite close antigenic and biochemical relatedness. However, eight isolates in 1980 described here were shown to have oligonucleotide spots different from those of the formerly characterized viruses.

Finally, we also characterized the RNAs of reference strain (HK-199) and three HK-199-like isolates by the oligonucleotide fingerprinting method. The results in Fig. 6 show that the reference strain, HK-199 produced an oligonucleotide map easily distinguishable from those of the three isolates (Niigata 383, 384, and 400). On the other hand, the latter viruses isolated at the same place and in the same year had similar maps, suggesting their common origin. For more detailed comprison, we examined approx. 50 large oligonucleotide spots. The oligonucleotide spots indicated by arrows pointing to the left showed their presence in other viruses examined. The oligonucleotide map produced by the reference strain appeared to possess only 13 spots common to the three isolates, showing that the former was genetically different from the isolates. A considerable number of common spots (90%) showed that the three isolates (Niigata 383, 384, and 400) belonging to PMV-6 serotype are of the same origin.

Genetic as well as antigenic relatedness of all 11 isolates of PMV-4 and PMV-6 serotypes suggests a rapid and efficient waterborne transmission, once paramyxoviruses are introduced during the migrating season for birds. Recent intensive studies in duck farms in Hong Kong provided circumstantial evidence that influenza viruses are constantly maintained throughout the year, through water contaminated by feces. Markwell & Shortridge (53) also reported the presence of asymptomatic infection in ducks. Recent virological surveillances of influenza in birds and

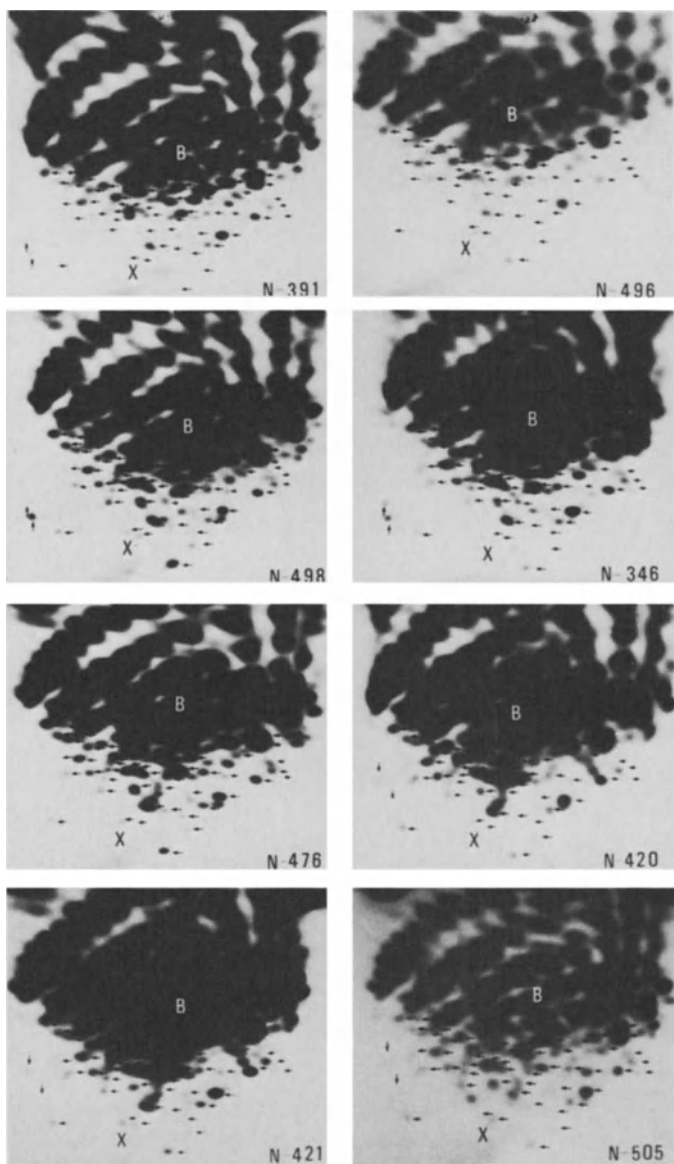

Fig. 5

Fig. 5. Genomic analysis of HK-D3-like strains of avian paramyxovirus by oligonucleotide mapping. The first dimension was performed at pH 3.5 in 10% polyacrylamide gel, and the second dimension was performed at pH 8.0 in a 21.8% polyacrylamide gel. Polynucleotide kinase (Boehringer Mannheim Biochemicals) and [γ-32 P] ATP (amersham Corp.) were used for labeling the 5'-ends of the oligonucleotides prepared by T digestion. The positions of the dye markers, xylene cyanol FF and bromphenol blue, are indicated by X and B, respectively. Arrows pointing to the left represent the large oligonucleotide spots common to all viral RNAs examined. The spots indicated by arrows pointing upward show oligonucleotides present in three isolates (N-391, N-498, and N-346), and arrows pointing downward represent the oligonucleotide spots common to N-420, N-421 and N-505 viral RNAs. All numbers given represent viral isolates (see Table 3). From Nerome et al. (39).

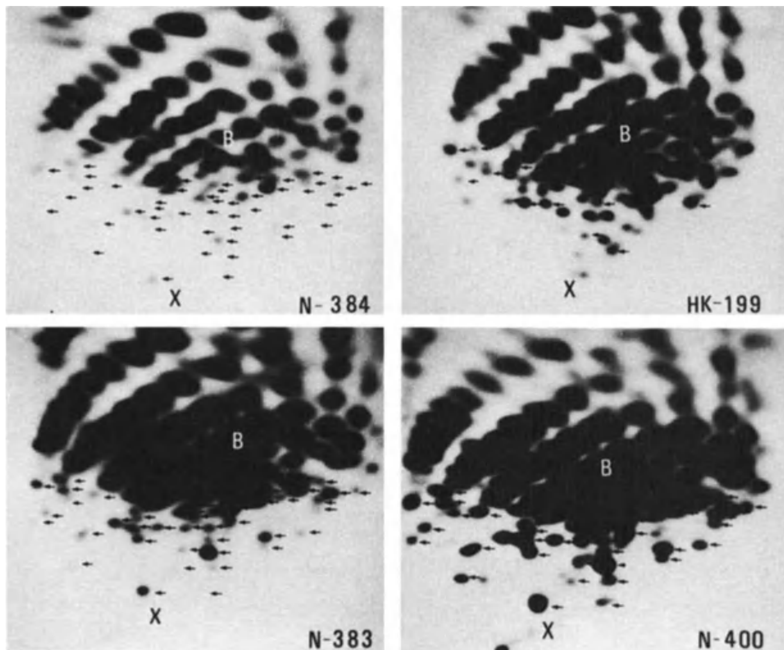

Fig. 6

Fig. 6. Oligonucleotide maps of the reference strain and three HK-199-like isolates recovered from wild ducks in Japan in 1980, Experimental conditions were the same as described in the legend to Fig. 5. The spots indicated by arrows pointing to the left represent large oligonucleotides common to HK-199 virus and HK-199-like virus isolates. HK-199 shows RNA of HK-199 virus, and, N-384, N-383, and N-400 show RNAs of three isolates (see Table 3). From Nerome et al. (39).

subsequent investigation of the viruses strongly suggested that all influenza A viruses distributed in a wide range of natural hosts originated from birds. Furthermore, the finding that the virus isolation rate was as high as 60% on Canadian lakes where ducks were assembled before migration led us to postulate the following: 1) Orthomyxo- and paramyxoviruses are constantly circulating in the duck population by the same mechanism; 2). duck paramyxoviruses may be a key to understanding the evolution of the viruses belonging to the genus paramyxovirus.

In 1983 an unprecendented large-scale influenza outbreak occurred among chickens in the United States and caused damage of some 50 million dollars (53,54). The subsequent epizootiological investigation and virological study based on molecular biology revealed that domestic ducks received H5N2 influenza virus from wild ducks in the small ponds. The outbreak of chicken influenza was attributed to introduction of the virus to chickens in Pennsylvania. The present study also provides evidence that wild duck is a potential reservoir of paramyxovirus (35), constituting a grave menace to the poultry industry.

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RABIES

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Rabies, and its association with the bites of animals, has been known from time immemorial. A passage in the text of the Mesopotamian Laws of Eshnunna, circa 2000 B.C., is frequently cited as being the earliest known reference to the disease: "If a dog is vicious .... and (its owner) does not keep it in, it bites a man and causes (his) death ...." (1). Ancient texts such as these are open to different interpretations, however, and it is apparent that death following savaging by dogs can be due to other causes. A less ambiguous description of the disease may be found in the writings of Aristotle (4th century B.C.): "Rabies drives the animal mad, and any animal whatever, excepting man, will take the disease if bitten by a mad dog so afflicted: the disease is fatal to the dog itself and to any animal it may bite, man excepted" (2). In view of Aristotle's credibility, the specific exclusion of man from the list of susceptible species is puzzling. The suggestion has been made that the disease had only recently established itself and was as yet incompletely known (2). In the 1st century A.D., Celsus, the Roman philosopher and author on medicine, wrote of the "virus" present in the bite of rabid animals. Since the Latin word "virus" can mean "slime" or "slimy liquid", Celsus may have been referring to the frothy, or slimy, saliva of the rabid individual (2,3). It was not until the start of the 19th century, however, that Zinke, a physician in Jena, provided rational experimental evidence that rabies could be transmitted to other animals through the saliva of a rabid dog (2,3). This discovery provided the means for continuous culture of the infectious agent and, together with the pioneering work of Galtier, Roux and others, paved the way for development of Pasteur's vaccines in the 1880's (4).

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Prior to Pasteur's work, prophylactic and therapeutic treatments for rabies ranged from the practical to the bizarre, and the early medical literature contains numerous anecdotal "cures" (4-6). Many physicians recommended immediate cauterization of a bite wound, a procedure that can be traced back to classical times. Pliny, a contemporary of Celsus, recommended the prophylactic removal of the "worm" in a dog's tongue, and cauterization of pustules under the tongues of rabies victims was a procedure reported even as late as 1880. Immersion in sea water was a commonly advocated treatment, and a myriad of drugs and medicines were purported to cure the disease. Even today, however, there is still no effective treatment for rabies once the disease symptoms appear. Nevertheless, in the century since Pasteur developed his vaccines, and particularly in the last two decades, considerable progress has been made in the characterization of the infectious agent and in the prevention of the disease.

THE INFECTIOUS AGENT

Classification and structure

Rabies virus belongs in the family Rhabdoviridae, and is the prototype of a group of serologically related viruses that constitute the genus *Lyssavirus* (7,8). Long considered to exist as a single serotype, classical strains of rabies can be subdivided on the basis of their reactivity with panels of monoclonal antibodies (9,10). Monoclonals can also be used to distinguish between rabies and the rabies-related lyssaviruses (10,11).

As is typical of rhabdoviruses, rabies virions are bullet-shaped particles, approximately 170 nm long and 80 nm wide, with 10 nm spike-like projections (8,12). Virus populations also contain truncated forms, commonly known as DI (defective, interfering) particles, the proportions of which vary with the virus strain and culture conditions used (12-14).

The rabies viral genome consists of a non-segmented, single-stranded RNA molecule, of negative polarity and with a M.W. of 4.6 megadaltons. It exists within the virion in a helical configuration, in close association with an RNA transcriptase (the L protein), and two phosphoproteins designated N and NS. Together, these constitute

the active transcriptase complex that synthesizes mRNAs complementary to the genome. This ribonucleoprotein core, or nucleocapsid, is surrounded by a lipoprotein envelope derived from the lipid bilayer of the host cell membrane and containing viral M (matrix) protein. Projecting from the envelope are the surface spikes, consisting of polymeric forms of viral G (glyco) protein (12).

It was initially thought that rabies virus particles contained two matrix proteins: M1 and M2. However, following identification of the M1 protein as being associated with the RNA it was renamed NS to conform with the nomenclature of the rhabdovirus prototype, vesicular stomatitis virus (VSV) (8,15). Transcriptional mapping studies have indicated that the physical arrangement of the 5 rabies structural genes is analogous to that of VSV: 3'-N-NS(M1)-M(M2)-G-L-5' (12).

Replication

Rabies virus infects many different types of cells *in vitro*. Initial interaction between virus and host cell takes place between the viral glycoprotein spikes and as yet unidentified cellular receptors. There is evidence that rabies uses a rhabdovirus-common receptor (16,17). Treatment with neuraminidase, but not proteases, renders cells of both neuronal and fibroblastic origin temporarily refractory to infection, and sialic-acid rich gangliosides have been reported to be involved in the cellular receptor structure (18). The acetylcholine receptor (AChR) of muscle cells, or a closely related structure, has been implicated by a number of different techniques (19-22). It is apparent, however, that presence of this is not a primary requirement, at least not *in vitro*, since cells with no detectable AChR are susceptible to infection with rabies (17,23).

Viral replication takes place entirely in the cytoplasm of the host cell. Fusion of the viral envelope and cell membrane is probably involved in the entry process, following which the viral transcriptional complex is liberated into the cytoplasm either at the plasma membrane or in acidic prelysosomal endosomes (24,25). The viral RNA is transcribed into 5 polyadenylated monocistronic mRNA species, which together account for almost all the coding capacity of the viral genome (26). Each mRNA codes for one of the 5 structural proteins (27).

Rabies virus-infected cells show little, if any, specific cytopathic effects. Synthesis of DNA, RNA, and protein in infected cell cultures frequently remains unaffected (28,29,33). Viral antigen detectable by fluorescent antibody (FA) staining appears early in the course of replication, and eventually becomes distributed throughout the cytoplasm. The pattern of FA staining varies with the virus strain, ranging from a diffuse fluorescence to large, discrete masses. The larger of these inclusions are morphologically and cytochemically indistinguishable from the classic eosinophilic Negri bodies seen in infected brain cells *in vivo* (30), and consist primarily of an amorphous matrix of viral nucleocapsid material. In the later stages of the infectious cycle, this material becomes filamentous, and appears to condense to form virus particles (31, 76). Virions are also formed, or released, by the process of budding into cytoplasmic vacuoles or from the plasma membrane (31,32).

Various types of persistent infection have been described (32-36). In one study, infected cultures of hamster fibroblasts produced virus in cycles: production of interferon was thought to be the regulating mechanism (35). In another, cultures of rabies-infected cells were passaged over 6 years. There was a progressive decrease in virus excretion until no infectivity remained, although viral antigen was detected throughout. Reactivation of infectious virus, however, was achieved by co-infection with an unrelated virus, distemper (36).

RABIES-RELATED VIRUSES

The concept that rabies virus was a unique antigenic species was first challenged by the isolation in 1958 of a rabies-like virus from the brains of Nigerian fruit bats (37). This virus, designated Lagos bat virus, has subsequently been isolated from fruit and insectivorous bats in the Central African Republic and South Africa (38).

A second rabies-like virus was isolated in 1968 from the viscera of shrews trapped in the district of Mokola, near Ibadan, Nigeria (39) and later from cats, a dog, and rodents in several African countries (38). Mokola virus was also isolated from the central nervous system of two Nigerian girls, causing a fatal infection in one (40).

In 1970, an adult male South African by the name of Duvenhage died of a rabies-like infection five weeks after being bitten on the lip by an insectivorous bat (41). Brain sections revealed typical histopathological lesions of rabies, including Negri bodies, but immunofluorescence tests were negative. A virus isolated by mouse passage was later characterized as being another member of the rabies serogroup (42). Identification of further isolates resembling the Duvenhage virus have since been made: from a Transvaal insectivorous bat in 1981 (11); from bats in northern West Germany captured between 1968 and 1982 (11); from insectivorous bats in Denmark, 1985 (11,43, 44); and from a human case in Finland, 1985 (44,45). First suspected as being due to classical rabies, the human case was that of a 30-year-old zoologist who had been bitten by bats years previously in Malaysia and Switzerland, and in Finland 51 days before onset of neurological symptoms. Prior to this incident, the last cases of human and animal rabies in Finland were in 1934 and 1959 respectively (45).

Lagos bat, Mokola, and Duvenhage viruses are now classified in the genus Lyssavirus as the prototypes of serotypes 2, 3, and 4 respectively, with classical rabies, strain CVS, being serotype 1 (46). Group-specific antigens responsible for cross-reactions of these viruses are nucleocapsid-associated (47).

Two other rabies-related viruses have been identified in Africa: Obodhiang and Kotonkan (48). Both were isolated from arthropods, multiply in insect cells, and appear to have the potential for being transmitted by arthropods. Antibodies to Kotonkan virus have been found in a high percentage of Nigerian domestic animals and rodents, and the virus has been associated with a disease in cattle similar to bovine ephemeral fever (49). Other rabies-related viruses may exist: for example, Bolivar virus from cattle in Venezuela (48), and Nigerian horse virus (50,51), isolated from the brain of a horse with "staggers", a rabies-like disease. Strains such as derriengue from vampire bats (48,52), arctic rabies (11), and oulou fatou of West African dogs (51) are probably variants of the rabies serotype.

The veterinary and public health significance of the rabies-related viruses is presently unclear. Most isolations have been made in widely separated African countries, and it appears likely that the

viruses exist extensively in natural wildlife reservoirs, especially bats and small terrestrial animals. Being less pathogenic than most classical street rabies strains, they may cause largely subclinical and abortive infections in their natural hosts. This factor, and limited contact, may explain why infection in aberrant hosts (including humans and domestic animals) is presently uncommon (38). With ecological changes such as massive increases in human and domestic animal populations, however, this situation may alter. In view of the genetic instability of rhabdoviruses, it is possible that the virulence of these viruses may increase with increasing exposure to aberrant hosts (38). The recent reports of Duvenhage isolates in Northern European bats, and the human fatality in Finland (11,43-45), give cause for concern, since it is apparent that the rabies-related viruses are not confined to the African continent.

PATHOGENESIS

Rabies infections are usually established following introduction of virus-infected saliva into a bite or scratch, although animals can also be infected by the oral (53-57) and nasal (olfactory) routes (53,58-62). It would appear that orally-administered virus gains access through the buccal or pharyngeal mucosa since the infectivity is rapidly destroyed by gastric juice (63). Bat-infested caves may result in infectious aerosols: non-bite transmission of the disease from this source has been reported in a number of animal species, including humans (59,64).

In experimental studies, following deposition in tissue, the virus replicates in striated muscle cells (65,66). The long and variable incubation period of rabies may be determined, at least in part, by persistent infection of local muscle fibers prior to dissemination (53,67-69). However, extensive studies have shown that infection of the peripheral nervous system, exposed in neuromuscular and neurotendinal spindles and motor end plates, is necessary for progression of the disease (19,69,72,76). In a similar fashion, non-bite infections may develop following infection of the sensory endings of epithelial and subepithelial tissues of the skin and mucous membranes, or of the olfactory neurons in the nasal cavity. Viremia

has occasionally been observed in experimental studies, but generally only after inoculation of high concentrations of virus (54,70,71).

Although viral replication in non-neural tissues may occur in natural infections, this has not been demonstrated conclusively to be a prerequisite for infection of the nervous system. Exactly how the virus enters the nerve endings is unclear. Nevertheless, once this has been effected, the virus is carried passively towards the CNS, at a rate of about 3mm/ hour, by retrograde axoplasmic flow (73,74,77). Replication occurs in the cell bodies of the neurons, and budding from perikaryal and dendritic plasma membranes provides a means for cell-to-cell transfer such as across synaptic junctions (75). Additional amplification of the virus probably occurs in the neurons of the dorsal root ganglia, or at the site of entry into the CNS, following which there is rapid progression up the spinal cord to the brain. Localization of the virus in the limbic system results in neuronal dysfunction, manifesting as loss of natural timidity, abnormal sexual behavior, and aggressiveness (67,76,77). In the later stages of the disease the virus spreads throughout the brain, resulting in dysfunction of the higher centers, possibly through impairment of acetylcholine receptors (78). Infection of the hypothalamus and hypophysis (pituitary gland) may result in hormonal imbalance, with consequent growth impairment, dehydration, and immunodepletion (79).

Even in the terminal stages, the brains of rabies-infected animals exhibit very few degenerating neurons compared to the large number of infected ones. Apart from the neuronal intracytoplasmic inclusions characteristic of rabies infections (Negri bodies) (30), perivascular infiltration is the most frequently noted histological change (80). The level of encephalomeningitis varies with the strain of infecting virus, and may be inversely related to the pathogenicity of the latter (79).

Recently, spongiform lesions have been identified in the brains of a number of animal species infected both experimentally and naturally with rabies (81). The close similarity of these lesions to those of the traditional spongiform encephalopathies may indicate similar pathogenetic mechanisms.

The above summarizes the progression of the disease, but does not provide a mechanism for transmission to other animals. Infection may occur following ingestion of infected tissues (56-58), although the importance of this in the natural spread of the virus is uncertain. Even before signs of CNS involvement appear, however, the virus disseminates extraneurally, via the same axonal routes as before. Many tissues of the body are thereby infected: for example, skin biopsies and corneal impressions will frequently reveal rabies antigen, even before death (82-84). From the standpoint of virus perpetuation, however, the most important target organ is the salivary gland. Nerves entering the glands transmit the virus to the epithelial cells. Virions mature abundantly on the plasma membranes of mucus-producing cells (76,77,85), and are released directly into glandular ducts and saliva by normal secretory flow. Titres in saliva in some species may reach 10^6 infectious units/ml or more (86-89). However, neural networks appear to be essential to achieve widespread infection of glandular tissues: after inoculation of street virus into the submandibular salivary gland of skunks, antigen was not detected in epithelial cells without its concurrent presence in the brain (90). There is evidence from skunk studies that viral titers in the salivary glands may be markedly influenced by the immune response (91).

Virulence

Strains of rabies virus vary greatly in their pathogenicity. Wild or "street" strains isolated from naturally occurring cases of rabies can be modified by passage in other animals. For example, the first anti-rabies vaccine developed by Pasteur was a monkey-adapted strain of canine origin, no longer pathogenic for dogs (92). His human vaccine originated in virus from a rabid cow. Passage of this strain in the brains of rabbits resulted in attenuation and a shortening of the long incubation period to a fixed period of about 6-7 days (hence the term "fixed" virus).

Fixed, or modified, rabies virus strains differ considerably in their lethality for adult mice, and the factors involved in determining virulence are probably manifold. One measure of virulence is the dramatic loss in body weight following intracerebral inoculation of pathogenic, but not apathogenic, strains (93).

Attenuation of virulence in adult mice has also been found associated with a single amino acid substitution at position 333 in the viral G glycoprotein, with arginine in the virulent strain being replaced by isoleucine, glutamine, or glycine in the attenuated ones (94-97).

Host responses to infection

Following a bite and deposition of infected saliva, replication of virus at the site of entry appears to be limited. Consequently, the resulting antigenic mass may not be adequate to stimulate the immune response. During its passage along axons, the virus is insulated from immunocompetent cells, and there is probably little further opportunity for host defenses to become activated until the virus becomes entrenched in the central nervous system and large amounts of virus are produced, late in infection. At this stage, any response may be too late, and death generally ensues even in the presence of high levels of circulating antibodies and brain interferon (98,99). Nevertheless, there is abundant evidence that some animals survive natural rabies infection, and the immune response may be at least partly responsible for these cases. Genetic and other factors, however, are undoubtedly involved (100-102).

That the immune system plays a role in protection against rabies is demonstrated by studies showing increased mortalities in immunosuppressed animals (91,103) and the protective effect of rabies vaccines. In animals, a direct relationship can be established between circulating antibody levels and post-exposure protection (104,105). There is also a considerable body of evidence to indicate that cell-mediated immune responses are involved (106-108). The role of interferon is controversial, and it is not clear whether it acts in vivo by its antiviral action or as an immunomodulator. Protection in animals has been achieved by administration of exogenous interferon (109,110) and by interferon inducers (111-113). Although high titers of interferon may be produced in the brain during experimental infection (99,114), this may occur too late for protection. Circulating interferon produced early after virus inoculation, however, may have some effect in controlling the infection (115).

Components of the immune system may also be involved in immunopathological reactions. In some cases, animals that responded

poorly to vaccines have died earlier, following challenge, than non-vaccinated animals given the same challenge (116,117). This "early death" phenomenon is not well understood but appears to be mediated by antibody (118) and may involve immune cytolysis. Lytic antibody has been demonstrated in sera from naturally infected animals and humans (104,106). It has also been shown in vitro that antibodies, through opsonization of immune complexes, can enhance the infection of macrophages by rabies, resulting in the increased production of virus (107,108). These observations indicate that interaction of virus and antibody does not always result in neutralization, and instead may result in consequences detrimental to the host.

Clinical symptoms.

The disease generally takes one of two forms: "furious", with sporadic episodes of rage; or "dumb", in which there is an early progressive paralysis. Both forms almost invariably result in death. In the prodromal stage, there is a change in temperament in the affected animal. A normally lively and sociable dog, for example, may become anorexic, withdrawn, irritable, or restless. This behavior may suddenly change, with the animal becoming highly affectionate. At this stage, the dog may try repeatedly to lick the hands and face of its owner or handler. Since its saliva may contain rabies virus (122,123), this is a particularly dangerous phase. As the disease progresses, the animal may appear to have difficulty swallowing, as if a bone were caught in its throat. Any attempt to alleviate the problem manually exposes the handler to considerable risk, either through a bite or the deposition of virus-infected saliva on mucous membranes or minor scratches (124). The dog's bark becomes high pitched and hoarse, indicating the onset of paralysis. The animal drools saliva. Convulsive seizures and muscular incoordination become apparent, followed by progressive paralysis, usually terminating in death within 7 days of the onset of symptoms (123,124).

In about 25-50% of cases, apparently as a result of limbic lobe dysfunction (76,77), dogs with rabies develop the furious form of the disease (122-124). Affected animals may eat abnormal objects, and during paroxysms of rage, will attack almost anything. It is this form of the disease that permits perpetuation of infection.

The clinical symptoms of rabies in cats are similar to those in dogs, but the disease more frequently takes the furious form - 74% of cases in one study (125). Rabid cats can be more dangerous than dogs, preferring dark places to hide, and often attacking their victims on the head and neck with both teeth and claws (70,124).

Cattle are frequently infected as a result of dog, fox or skunk bites, and may develop the furious or the dumb form (126). Infected animals produce a copious flow of saliva, but rarely attempt to bite. The initial stages of paralysis may produce the symptoms of choking, and, as with dogs, any unsuspecting attempt to alleviate this condition manually may be extremely hazardous. Virus may be present in milk, although no case of rabies in man has been recorded from milking an infected animal or from drinking milk (70). In Central and South America, a paralytic form of the disease follows the bite of infected vampire bats (52). Other domestic animals such as horses, pigs, sheep and goats present clinical symptoms that are broadly similar to those of cattle. Horses tend to develop furious symptoms more frequently, however, and may kick and bite indiscriminately until paralysis sets in (70,127).

EPIZOOTOLOGY AND ECOLOGY.

Rabies exists worldwide and in all climates. The major areas presently rabies-free include Australia and Antarctica, the British Isles, New Zealand, Japan, Taiwan, Hawaii, and other islands in the Pacific and Atlantic (128). The virus is capable of infecting all warm-blooded animals, although the primary reservoirs are feral carnivores and bats. The danger to domestic animals (and humans) stems mainly from contact with these wild animals.

In many parts of the world, dogs provide the major host, and dog and cat bites account for more than 90% of the cases of human rabies (67). Programs to eliminate stray dogs and to immunize pets have resulted in a marked reduction in urban rabies in developed countries. Many tropical countries have benefitted only minimally from these innovations, however, and continue to experience urban canine rabies as their major problem (129,130).

In individual geographic regions, different animal species tend

to predominate as vectors of the disease (51,128,130-133). Within the arctic circle, rabies is enzootic in the arctic fox (Alopex lagopus) which may transmit the disease to sled dogs. Further south, the red fox (Vulpes vulpes) is a major vector in eastern Canada, U.S.A., and central Europe. Another important vector, and the commonest reservoir of wildlife rabies in the United States, is the striped skunk (Mephitis mephitis). Bat rabies is widely distributed worldwide, but is of particular significance in Central and South America: infected vampire bats (primarily Desmodus rotundus) transmit the disease to a wide range of species but primarily cattle, causing tens of thousands of mortalities yearly. In Eastern Europe, the raccoon dog (Nyctereutes procyanoides) is becoming an increasingly important vector, and the wolf (Canis lupus) and other canids carry the disease in Asia Minor. Mongooses are indigenous to Africa, and the Middle and Far East, where they are important reservoirs of rabies. The small Indian mongoose (Herpestes auropunctatus), introduced into the Caribbean in the late 1800's to combat rats in the sugar cane fields, is now a reservoir and major vector in several of the islands (132).

In recent years, the practice of keeping wild animals, such as skunks, foxes, and raccoons, as pets has been increasing in some countries. This has resulted in many persons being exposed and requiring anti-rabies treatment (46). In North America, the urban raccoon (Procyon lotor) presents an increasing public health hazard, particularly in view of its semi-domestication and close habitation with humans. Until the late 1970's, raccoon rabies was recognized as a problem only in the south-eastern United States, but this focus has since expanded dramatically to encompass the mid-Atlantic region (131,133), possibly as a result of importation of infected southern animals (134). In 1983, more than 80% of the 1,903 confirmed cases of animal rabies in West Virginia, Virginia, Pennsylvania, Maryland and Washington, D.C. involved raccoons. Other affected animals included 104 skunks, 101 bats, 32 cats, 21 foxes, 14 woodchucks, 12 cows, 6 dogs, 3 oppossums, 2 beavers, 1 deer, 1 squirrel, 1 horse, and a red panda (133). An increased occurrence of rabies in these other animals is thought to have stemmed from "spillover" from the raccoon outbreak. Although rabies in lagomorphs and rodents is uncommon,

woodchucks (Marmota monax) may have become involved in the mid-Atlantic epizootic as a consequence of competition with raccoons for den sites in areas of intense rabies activity (135).

Resistance to natural infection

Animal species vary greatly in their susceptibility to rabies. Foxes, wolves and certain rodents are considered to be among the most susceptible, with dogs, cattle and humans occupying an intermediate position, and prey birds being among the most resistant. Multiple factors may affect this relationship, however, such as the degree of species adaptation of the infecting strain of virus. The concept of "biotype" has been proposed, which may help explain why rabies in a primary vector species does not become established in other species co-existing in the same ecosystem (136). For example, dogs infected with fox rabies virus (fox biotype) excrete much less virus in their saliva than do foxes, and exhibit furious symptoms much less frequently. The fox biotype, therefore, may infect individual dogs, but the characteristics of infection in the latter species may prevent it from becoming established in the dog population (136,137).

DIAGNOSIS OF RABIES INFECTION

Examination of clinical material

Rabid animals may secrete infectious virus for several days before and after the onset of clinical symptoms, but never before the appearance of virus in the CNS. During the process of viral synthesis, an excess of nucleoprotein is produced, and this material forms the basis of the cytoplasmic inclusions known as Negri bodies (30). Histopathologic methods such as staining fresh brain tissue smears or fixed sections for the presence of these specific inclusions are commonly used in diagnostic laboratories. The most suitable part of the brain is the Ammon's horn of the hippocampus. However, faulty interpretation may result from the presence of non-specific inclusion bodies, or those produced during viral infections other than rabies. Additionally, Negri bodies may not be detectable in all infected animals, particularly in feral species such as skunks and bats (92,138,139). A more reliable microscopic technique is the rabies fluorescent antibody (RFA) test, which involves reaction of tissue

films or sections with anti-rabies globulins tagged with a fluorescent dye (139,140). With appropriate monoclonal antibody panels, this test can also be used to differentiate between rabies strains and the rabies-related viruses (10,11,141,142).

Confirmation of rabies infection requires isolation of virus. Suckling or weanling white laboratory mice are widely used for this purpose, being inexpensive and highly susceptible. Inoculation of test material is made intracerebrally, and the animals are observed for at least 21 days. Presence of rabies in the inoculum causes tremulous muscular activity, incoordination, excitation, and paralysis, with death usually occurring between days 5 and 15. To confirm the diagnosis, brains can be examined for the presence of rabies antigen by the RFA test.

Serological diagnosis

Antibody tests are used primarily to assess the immune status of an animal following vaccination. They are of limited use in the detection of rabies-infected animals, since the immune responses of these vary considerably, and antibodies may be produced only in the late stages of infection, if at all.

The reference method for detection of rabies-specific antibodies is the serum-virus mouse neutralization test (MNT) (139,166). This involves incubation of a constant amount of a standard virus strain with the test serum, which is then inoculated intracerebrally into weanling mice. If the serum contains protective antibodies, the virus challenge is neutralized, and the animals will survive. Testing of serial serum dilutions against the standard virus permits a measure of the antibody titer, the endpoint being related to the highest serum dilution that provides protection against the challenge.

Although the specificity of this test remains unchallenged, it has a number of drawbacks, a major one being that it requires 3-4 weeks to complete. Of the many proposed alternatives, _in vitro_ immunofluorescence-inhibition techniques (138,139,143,146-148) have achieved widest acceptance, since they are much faster and less costly than the MNT while providing generally comparable results (144,145). As with the MNT, the fluorescence-inhibition techniques measure neutralizing antibody, but use cell cultures instead of mice. In one

version, the rapid fluorescent focus inhibition test (RFFIT) (143), hamster kidney cells (BHK-21 line) are added to serum-virus mixtures in glass tissue-culture chambers. Following incubation to permit viral growth, the resulting cell monolayers are fixed in acetone and stained with fluorescein-conjugated anti-rabies globulin. Presence of rabies-specific fluorescence in the cells indicates viral replication and therefore absence of neutralizing antibodies in the test serum.

An increasingly utilized alternative is the enzyme-linked immunosorbent assay (ELISA or EIA) (149-155). This can take various forms, but a common one measures the binding of specific antibody to rabies antigen adsorbed to a solid phase. The antigen-antibody complex is incubated with an anti-species globulin (anti-antibody) conjugated with an enzyme such as horseradish peroxidase or alkaline phosphatase. The extent of binding of the conjugate is proportional to the amount of rabies antibody present, and can be measured colorimetrically by addition of an enzyme substrate which, upon reaction, produces a coloured product.

The ELISA is a versatile technique, and can be used to measure specific antibody isotypes (155), viral antigens (151,153,154) and to differentiate between viral strains (153). A method has been reported that incorporates features of both immunofluorescence-inhibition and ELISA (156). Related techniques that have been applied in rabies serology include the radio-immunoassay, which utilises antibody labelled with a radioisotope (e.g., ^{125}I) in place of the enzyme (47,157), and an assay that uses fluorometry to measure the extent of binding of anti-antibody conjugated with a fluorescent dye (158). All these techniques measure antibody binding rather than virus neutralization, however, and the nature of the antigen used may affect the results obtained. For example, neural tissue vaccines stimulate production of high levels of non-neutralizing anti-nucleocapsid antibodies. If whole virus is used as the antigen, a titer may be obtained that is not indicative of the level of protection attained (159). Since the G protein is the antigen associated with stimulation of neutralizing antibodies, assay procedures using purified G protein instead of whole virus as antigen will provide results that correlate more closely with those obtained by the MNT.

PREVENTION AND CONTROL OF RABIES

Once a virulent strain of rabies virus has established itself in the central nervous system of an infected animal, the outcome is almost always death. There are a few documented cases of human recoveries following intensive care (124,160), and some animals may recover following post-infection encephalitis. At a practical level, however, the only ways of preventing rabies are by pre-exposure immunization or by initiating treatment as soon as possible after exposure.

Prevention of rabies by chemotherapeutic agents has not been achieved with any major degree of success (161-163). More encouraging effects, however, have been obtained with agents that stimulate host defenses. In animals, inducers of interferon such as polyI.polyC have been demonstrated to have a therapeutic effect (111-113). Orally-administered saponins afford significant protection to mice against intracerebral challenge with virulent rabies, apparently by stimulating non-specific T cell responses (164). Saponins have been employed as adjuvants in non-parenteral rabies vaccines, but their use has been limited by their high toxicity. Parenterally, however, saponins are well-tolerated, and strongly potentiate rabies vaccines given by this route (165).

Vaccines

In the original Pasteur treatment, multiple injections were given of suspensions of infected rabbit spinal cord. The virulence of the cords had been reduced by desiccation for varying lengths of time, with the least virulent preparations being administered first. Safer, and more easily prepared vaccines were introduced in 1911, which contained phenol-inactivated virus grown in sheep brain (92,166,167). These Semple-type vaccines are still in use today, particularly in Asia and Africa. However, problems of neuroparalytic reactions from the myelinated nervous tissue stimulated development of vaccines grown in neonatal animals (which have less myelin), and suckling mouse brain vaccine is now widely used in Latin America.

Since the early 1960's, many tissue culture-grown vaccines have been introduced which have completely replaced the nervous tissue vaccines in most developed countries, and are increasingly being used

elsewhere (46,92,167-170). It is now possible to immunize practically all major animal vectors, although economic and other considerations make this a distant prospect. The new generation of "high-tech" vaccines (171,172) will undoubtedly facilitate this progress.

Laboratory and captive animals (domestic and wild) can be immunized with existing vaccines, although modified-live vaccines that are safe in some species may not be entirely safe in another (173,174). Captive wild animals, however, may be incubating rabies when captured, although the disease may take weeks or months to develop. Unless completely isolated, domestic and zoological animals can be infected by contact with rabid wildlife. Even isolation does not ensure safety: non-bite spread of rabies has been reported in an enclosed animal holding facility (60). The threat of rabies to humans and associated animals will remain until the disease is eliminated in its wildlife reservoirs. Encouraging progress is being made in this direction, however: programs directed towards the oral immunization of foxes (by means of vaccine-containing baits) have been under way in Europe and North America for more than a decade (46,92,175-178). Vampire bat rabies has been considerably reduced by a combination of more effective cattle vaccines and the use of anticoagulants (to which the bats are highly susceptible) (46,52). An experimental vaccinia-vectored rabies vaccine has been found to be more effective in immunizing a wider range of wildlife species by the oral route than conventional rabies vaccines (179,180). Continuing progress along these lines gives rise to cautious optimism that the eventual eradication of rabies may become a practical reality.

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REOVIRUSES

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INTRODUCTION

The family Reoviridae derived its name from Sabin who proposed the acronym reoviruses for a group of related respiratory and enteric viruses that were isolated from healthy individuals as well as patients with mild febrile disease and diarrhea (1). Because these viruses typically were not associated with any human disease, they were called respiratory enteric orphan viruses, or simply reoviruses. Members of the presently recognized reoviridae family of viruses are grouped into six genera that display an extremely wide host range (Table 1). However, the members of the reoviridae have similar biochemical and biophysical properties and replication strategies. Among the most fundamental characteristics of the reoviridae is that all members possess a double-stranded RNA genome composed of ten to twelve segments that are enclosed in a virion of about 70 to 80 nm diameter. In addition, all reoviridae replicate in the cytoplasm of their hosts. However, the various members of the reoviridae differ widely in the virulence that they may exhibit toward their respective hosts. Because the orthoreoviruses have provided many insights into the molecular biology of the reoviridae, and because the rotaviruses and the orbiviruses are important agents of disease, this chapter will focus on these three genera of dsRNA viruses. Emphasis will be placed on the fundamental concepts and the new insights which have been gained in understanding their molecular biology and pathogenesis since the excellent monograph edited by Joklik was published in 1983 (2).

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ORTHOREOVIRUSES

All mammalian reovirus isolates so far identified can be classified immunologically as belonging to one of three serotypes: serotype 1, prototype Lang strain; serotype 2, prototype Jones strain; and serotype 3, prototype Dearing strain (2,3). In addition to orthoreoviruses isolated from mammalian hosts, orthoreoviruses have also been isolated from birds. There appear to be five serotypes of avian reoviruses which, like the mammalian reoviruses, have been isolated from both asymptomatic animals and animals with respiratory or enteric illness (4). However, because the mammalian reoviruses were the first to be discovered (1) and because they can readily be grown in cell culture (2), most of our detailed knowledge of the orthoreoviruses is based on studies carried out with the mammalian viruses.

Organization of the Mammalian Reovirus Genome

The structural organization and protein coding-assignments of the mammalian reovirus genome is summarized in figure 1. The reovirus genome consists of ten segments of double-stranded RNA (5) that fall into three size classes: 3 large (L) segments, each about 4500 bp; 3 medium (M) segments, each about 2300 bp; and 4 small (S) segements of dsRNA, each about 1200 bp (6,7). Each of the dsRNA segments is transcribed into a ssRNA of the polarity of mRNA by a virion-associated polymerase (8-10). These ten mRNAs, designated l, m and s for the three size classes, possess the 5'-terminal $m^7G(5')ppp(5')G_pC$ cap structure characteristic of most eukaryotic cellular and viral mRNAs (11,12). However, unlike most eukaryotic mRNAs, reovirus mRNAs are not polyadenylated at their 3'-termini (13). The ten reovirus mRNAs are translated into a series of polypeptides that, with one exception, also fall into three size classes designated λ (large), μ (medium) and σ (small) (14-16). Nine of the reovirus mRNAs appear to be monocistronic (17-19,42); the s_1 mRNA is bicistronic (19-21). The protein-coding assignments of the reovirus RNA segments as summarized in figure 1 were

Figure 1. Organization and protein coding assignments of the reovirus genome.

Table I: The Reoviridae

Genus	Segment Number	Host Range
Orthoreovirus	10	Vertebrates
Rotavirus	11	Mammals
Orbivirus	10	Vertebrates, insects
Cypovirus	10	Insects
Phytoreovirus	12	Plants, insects
Fijivirus	10	Plants, insects

deduced by translation *in vitro* of individual genomic dsRNA species after denaturation (17), by translation *in vitro* of individual mRNA species (19,42), and by analysis of genetic reassortant viruses formed between different reovirus serotypes during mixed infection (18).

Multiplication Cycle of Mammalian Reoviruses

The first step in the multiplication cycle of reoviruses, virion attachment to the host cell, involves a specific interaction between the virion minor outer capsid polypeptide σ_1 (σ_{1a}) and receptors present on the surface of the host cell (22-24). The σ_1 (σ_{1a}) polypeptide, positioned at the twelve vertices of the virion icosahedron (25), is strategically located for interaction with the cell surface receptor. The cellular receptor for σ_1 (σ_{1a}) recognition has been identified with a monoclonal anti-receptor antibody raised against the σ_1 (σ_{1a}) antigen-binding site of a neutralizing monoclonal antibody (26). Further characterization revealed that the cellular receptor for reovirions is a 67-kDa polypeptide structurally similar to the beta-adrenergic receptor (27). The monoclonal anti-receptor antibody blocks virion attachment to and penetration into the host cell (28).

The penetration of reovirions into their hosts appears to occur either by a phagocytic process (29) or by direct penetration of the cell membrane by a form of subviral particle (30). Electron microscopy has revealed that viral particles are detectable in phagocytic vacuoles shortly after infection and subsequently appear within lysosomes where they are uncoated by hydrolytic enzymes (29). Other EM studies indicated that intermediate subviral particles enter the host cell directly without involvement of phagocytic vacuoles (30). The mechanism of host cell penetration is an area of reovirus molecular cell biology in which further information is needed; the process may possibly vary with the cell type and the conditions of infection. Whatever the exact mechanism of penetration, the functional end-result is that partially uncoated virions are found within the cytoplasm of infected cells. The concept of partial uncoating is a characteristic of the reoviridae (2) and, in the case of the mammalian orthoreoviruses, involves the removal of the outer capsid polypeptides σ_3, μ_{1C} and σ_1 to yield the subviral core particle (31,39). The parental dsRNA genome is not found free within the infected host, but rather is conserved within the subviral particle which is composed of the inner capsid core polypeptides λ_1, λ_2, λ_3, μ_1, μ_2, and σ_2 (31-33,39).

The partial uncoating of the parental reovirions to yield subviral particles results in the activation of the virion-associated transcriptase such that complete mRNA transcripts may be synthesized (8-10,31,32,34). The synthesis of viral mRNA catalyzed by the reovirus transcriptase occurs by a conservative mechanism: the parental genome dsRNA remains intact (10,35). Furthermore, only one strand of each genome segment is transcribed (10). The plus-strand mRNA transcript of a reovirus genome segment is identical with the plus-strand of the genome dsRNA segment as established by the identical complete cDNA nucleotide sequences obtained for the serotype 1 Lang s1 mRNA transcribed _in vitro_ (36) and the plus-strand of Lang S1 dsRNA (37). Additional

evidence for the identity of plus strands includes the ability to translate denatured genome dsRNA *in vitro* to yield polypeptide products similar to those obtained *in vivo*, both for the monocistronic genes (17) and the bicistronic S1 gene (21). This result not only indicates that the plus strand of genome dsRNA and the plus strand of mRNA ssRNA are likely identical, but also that intron-exon splicing of transcripts does not occur as part of the biogenesis of reovirus mRNAs. Reovirus particles possess all of the enzymic activities known to be involved in the synthesis and modification of transcripts to yield mature viral mRNA: the dsRNA-dependent ssRNA polymerase (8-10) and the four enzymes involved in cap formation, a nucleotide phosphohydrolase, a guanylyltransferase and two methyltransferases (11,12). The reovirus transcriptase and cap formation activities appear topographically related within the core (38). Although the L1-encoded λ_3 polypeptide appears to govern the pH optimum of the transcriptase (40), definitive assignment of the core enzymic activities to specific core polypeptides has not yet been possible. Transcription of the reovirus genome is detectable by 2 h after infection and reaches a maximum at about 12 h after infection. The relative frequency of transcription of each genome segment varies considerably (14), but the mechanism of regulation of reovirus transcription *in vivo* remains largely unknown.

The translation of reovirus mRNAs is regulated in that some mRNAs are far more efficiently translated than others, both *in vivo* (14,41,43) and *in vitro* (15,16,42,44). Parameters that possibly contribute to the different translational efficiencies of reovirus mRNAs include nucleotide sequences at the -3 and +4 positions flanking the initiation AUG codon (45), and mRNA competition for a message discriminatory protein synthesis factor present in limiting concentrations in infected cells (41). The 5'-cap modification clearly facilitates translation *in vitro* (44,46), although uncapped reovirus mRNA directs the synthesis of the same spectrum of polypeptides as methylated mRNA, albeit with

lower efficiency (44). Only about 10% of the reovirus mRNA present in infected cells is synthesized by transcriptase associated with partially uncoated parental particles; most viral mRNA is synthesized by progeny particles following progeny dsRNA synthesis (48,49). The known products of the translation of reovirus mRNAs include nine polypeptides that are structural components of virions (λ1, λ2, λ3, μ1, μ1C, μ2, σ_{1a} (σ_1), σ_2 and σ_3) and three nonstructural polypeptides found within infected cells but not as part of virion particles (μ_{NS}, σ_{NS} and σ_{1bNS}) (14,17,20,21,39,47). About 90% of polypeptide μ1 is cleaved postranslationally to yield polypeptide μ1C (14).

Synthesis of reovirus progeny double-stranded RNA is dependent upon viral polypeptide synthesis (48). Replication of the reovirus genome proceeds via a conservative mechanism as the parental genome remains intact (35,50). The plus-strand products of the virion-associated transcriptase, in addition to serving as mRNA, also function as template for minus-strand synthesis (50,51). The synthesis of minus strands occurs within virion-like particles; the minus strand products of the replicase remain associated with their plus-strand templates, thereby yielding a progeny dsRNA product (52,53). The virion-like particles which possess the ssRNA-dependent dsRNA polymerase activity resemble viral cores (54); they are likely precursors of mature progeny virions. Little additional information is available concerning reovirus morphogenesis and maturation, an area clearly in need of further study.

Sequences of Mammalian Reovirus RNAs and Genetic Relatedness of the Serotypes

The terminal sequences are identical for all ten of the reovirus serotype 3 genome segments as well as for those genome segments (S1, S2, M3 and L3) of serotypes 1 and 2 that have been analyzed (55-57). The tetranucleotide GCUA- is found at the 5'-terminus and the pentanucleotide -UCAUC is

found at the 3'-terminus of the plus strand of all orthoreovirus dsRNA segments (55-57). The function of the conserved terminal nucleotides is unclear; they may contain recognition signals for initiation of plus-strand RNA synthesis catalyzed by the virion-associated transcriptase, for initiation of minus-strand synthesis catalyzed by the replicase, and/or for assembly of reovirus RNA segments during morphogenesis.

The complete nucleotide sequence of several reovirus genes has been determined from cDNA clones. The complete sequences include those of the S1 gene of serotype 1 (36,37), serotype 2 (37) and serotype 3 (25,37,58); the S2 gene of serotype 3 (59); the S3 gene of serotype 1 (60) and serotype 3 (61); and the S4 gene of serotype 1 (62) and serotype 3 (63). The S1 genome segments of the three reovirus serotypes have diverged far more than any of the other reovirus S-class genome segments. For example, the serotype 1 Lang and serotype 3 Dearing S1 genes are only about 30% related at the nucleotide level, and the S1-encoded σ_{1a} (σ_1) and σ_{1bNS} polypeptides are about 20% related at the amino acid level (25,36,37,58). By contrast, the homology between serotypes 1 Lang and 3 Dearing for the S2, S3 and S4 genes is greater than 85% at the nucleotide level; the S2-encoded σ_2, S3-encoded σ_{NS} and S4-encoded σ_3 polypeptides display greater than 95% identity between serotypes at the amino acid level (59-64). Most of the nucleotide differences between serotypes 1 Lang and 3 Dearing in the coding regions of the S2, S3 and S4 genes are silent differences, most often in the third base position of codons, which do not cause a change in amino acid (59-64). The levels of sequence homology for the S2, S3 and S4 genes of reovirus serotypes 1 Lang and 3 Dearing deduced from nucleotide sequence data (59-64) are considerably higher than might be expected from previous hybridization studies (65) which assessed the RNase sensitivity of +RNA:-RNA hybrids containing the plus strand of one serotype and the minus strand of the other serotype.

The genetic relatedness among the three serotypes of reovirus has also been assessed by quantitation of the serologic relatedness of the cognate reovirus polypeptides (66). These studies revealed that the antigenic determinants on most of the polypeptides of the three reovirus serotypes have been highly conserved during evolution. The most type-specific of all reovirus polypeptides is σ_{1a} (σ_1) as measured by immunologic analysis with polyclonal antibodies prepared in pathogen-free rabbits (66). A number hybridomas secreting monoclonal antibodies directed against reovirus serotype 3 polypeptides have been prepared (67,68). With the exception of the monoclonal antibodies directed against polypeptide σ_{1a} (σ_1) which display type-specific immunoprecipitation activity, the monoclonal antibodies directed against most of the other reovirus polypeptides were able to react with the cognate polypeptides specified by serotypes 1 and 2 as well as by serotype 3 (67). The monoclonal antibodies directed against polypeptide σ_1 (σ_{1a}) also possess type-specific neutralizing activity and type-specific hemagglutination-inhibition activity (68,69); however, the ratios of these activities may differ widely for the various σ_{1a} (σ_1) monoclonals which suggests that distinct functional domains exist on the σ_{1a} (σ_1) polypeptide (68-70).

Functions of the Reovirus-encoded Polypeptides

The S1 genome segment specifies a bicistronic mRNA which is translated in two different reading frames to yield two polypeptides, the minor capsid polypeptide σ_{1a} (σ_1) and the nonstructural polypeptide σ_{1bNS} (19-21) (Fig. 2). The 49-kDa minor outer capsid polypeptide σ_{1a} (σ_1), which represents <1% of the virion protein (39), plays an important role in viral pathogenesis (71). σ_{1a} (σ_1) is the cell attachment protein (22-24), is responsible for induction of type-specific neutralizing antibody (68,69,72), is responsible for generation of type-specific cellular immune responses (73-75), is the viral hemagglutinin (68,69,76), and

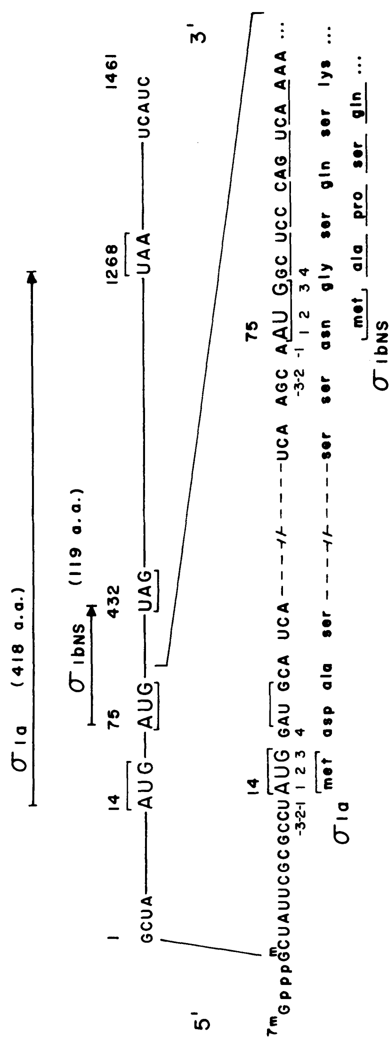

Figure 2. Structure and expression of the bicistronic reovirus s_1 mRNA.

is responsible for association of reovirions with microtubules (77). Genome segment S1 products have been implicated in the inhibition of cellular DNA synthesis (78), a function possibly mediated by the 14-kDa nonstructural σ_{1bNS} polypeptide (21,79).

The S2 genome segment specifies the 38-kDa inner capsid core polypeptide σ_2 (39,59). The specific function of σ_2 is not known; however, it may relate to polymerase activity as group C *ts* mutants which map to genome segment 2 (80) fail to synthesize dsRNA at the restricted temperature (49).

The S3 genome segment specifies the 41-kDa nonstructural polypeptide σ_{NS} (47,60,61). σ_{NS} has been shown to bind single-stranded reovirus mRNA with high affinity (81) and may function during viral morphogenesis to bring together the plus-stranded mRNAs subsequent to synthesis of the complementary minus strands by the ribonucleoprotein particles to yield dsRNA (52,82).

The S4 genome segment specifies a monocistronic mRNA which is translated to yield the 41-kDa major outer capsid polypeptide σ_3. Somewhat more than one-fourth of the virion capsid protein is the σ_3 polypeptide (39,62,63). In addition to the structural role of σ_3 as a major virion component, biochemical studies have established that σ_3 binds to dsRNA (81). Genetic studies have revealed that the S4 gene product is responsible for the inhibition of cellular protein synthesis in lytic infections (83,93) and, in addition, plays an important role in the establishment of persistent infections (84).

The M1 genome segment specifies the 70-kDa inner capsid core poypeptide $\mu 2$ (17,18,39). It is present in minor amounts within reovirions (39); its function is not known. Temperature sensitive mutants have been mapped to M1 (group H) on the basis of intertypic recombinants derived from two ts parents (85), however the biochemical properties of group H mutants have not yet been elucidated.

The M2 genome segment specifies the 80-kDa minor inner capsid core polypeptide μ_1 (17,18,39) and its 72-kDa post-translational cleavage product, μ_{1C} (14). More than 90% of μ_1 is cleaved to yield μ_{1C}. μ_{1C} is a major component of the outer capsid, accounting for about one-third of the virion protein (14,39). μ_{1C} is also modified by ADP-ribosylation and polyadenylation (86). Genetic analyses have revealed that the ability of reoviruses to grow in the intestinal tissue and their responses to pancreatic proteases (87), the attenuation in neurovirulence of reoviruses (88), and the stimulation of suppressor T cells (89) are controlled in part by the M2 gene products. Thus, polypeptides μ_1/μ_{1C} play a major role in determining the virulence of reoviruses. This may be related to the role that the proteolytic cleavage of μ_{1C} plays in the activation of the reovirion-associated transcriptase such that complete mRNA products may be produced (34,40).

The M3 genome segment specifies the 75-kDa nonstructural polypeptide μ_{NS} (17,18,47) and the 70-kDa post-translational cleavage product of μ_{NS}, μ_{NSc} (67). μ_{NS} and μ_{NSc} are present in comparable and high concentrations within infected cells (67); their functions are not known.

The L1 genome segment specifies the 135-kDa minor inner capsid core polypeptide λ_3 (17). The function of λ_3 may relate to the virion-associated transcriptase as a combined genetic and biochemical analysis has established that the pH optimum of reovirus transcriptase activity is specified by the L1 genome segment (40).

The L2 genome segment specifies the 140-kDa major inner capsid core polypeptide λ_2 (17,39) and its 120-kDa post-translational cleavage product, $\lambda 2c$ (67). The core spikes are composed of pentamers of λ_2 and may represent the sites of extrusion of complete mRNA molecules (90,91). Pyridoxal-5'-phosphate reductive labeling of λ_2 (and λ_1) is accompanied by the inhibition of the core-associated transcriptase,

nucleotide phosphohydrolase, guanylyltransferase and methyltransferase activities (38), possibly because these λ polypeptides possess the above catalytic activities or becuase the λ_2 spike through which the modified mRNA is extruded becomes blocked. Genetic analysis has also identified the L2 segment as the gene which determines the level of shedding and thus efficiency of virus transmission between littermates of newborn mice in a controlled environment (92).

The L3 genome segment encodes the 155-kDa major inner capsid core polypeptide λ_1 (17,39). The function of λ_1 is not specifically known; it may be involved in the synthesis and modification of the reovirus mRNAs (38). Temperature sensitive mutants (group I) have been mapped to L3 (85), but they have not been extensively characterized in biochemical terms.

In summary, the combined genetic and biochemical analyses of orthoreoviruses have provided significant insight into the molecular mechanisms involved in the expression and replication of double-stranded RNA genomes of the reoviridae as well as the molecular basis of viral pathogenesis for viruses in general.

ROTAVIRUSES

Rotaviruses, comprising a genus of the family reoviridae (Table I), are a major cause of acute gastroenteritis in infants and in many species of young domestic animals (94-98). Rotavirus infections may result in diarrhea, dehydration, and death (94-98). The rotavirus genome consists of 11 segments of dsRNA (99). The dsRNA genome segments of rotaviruses, like those of other members of the reoviridae, possess short regions of terminal sequence which are conserved (100-106). The virion capsid consists of at least five polypeptides, three of which (vp1, vp2, vp6) make up an inner capsid shell while the other two (vp3 and vp7) form an outer capsid shell (107,108). Unlike the mammalian reoviruses, most mammalian rotaviruses display a restricted host

range and a remarkable degree of tissue tropism. They selectively infect the mature villus enterocytes of the small intestine (97,98,109).

Because rotaviruses are an important etiologic agent of gastroenteritis worldwide in infants and newborn animals of agricultural importance, significant effort has been devoted to their genetic and immunologic characterization. There are at least seven serotypes of rotavirus, four of which are found in humans (serotypes 1-4) and five of which (serotypes 3-7) are found in other animals (110). Analysis of reassortant reoviruses derived by coinfection in culture with two different rotavirus serotypes reveal that genome segments 4 and 9 cosegregate with the neutralization phenotype (111). Monoclonal antibodies directed against vp3 and vp7, the 82-kDa and 36-kDa outer capsid polypeptides, neutralize rotavirus infectivity and also inhibit viral hemagglutination (112). Genome segments 4 and 9 have been shown to encode the major outer capsid polypeptides vp3 and vp7, respectively, of rotaviruses (113,114). Functional diversity of the rotavirus genome segment 9 encoded products appears possible. Genome segment 9 gene is bicistronic; two glycoproteins differing in size by about 1.5-kDa due to distinct signal sequence processing are synthesized from two in-phase initiation codons (115).

The importance of serotypic differences defined by neutralizing antibodies induced by rotavirus polypeptide vp7 is unclear because in animals and in man cross-protection among strains belonging to different serotypes may occur (116). Furthermore, monoclonal antibodies to two distinct epitopes of simian rotavirus vp7 passively protect mice against challenge by the same virus strain, whereas a monoclonal antibody against porcine rotavirus vp3 passively protect mice against three different serotypes of rotavirus (117). The cDNA nucleotide sequence of the homologous genes encoding the 36-kDa serotype-specific glycoprotein vp7 have been determined for human rotavirus serotype 1 WA strain (101), human serotype 2 HU-5 strain (102), simian rotavirus

serotype 3 SA-11 strain (103,104), bovine rotavirus serotype 6 UK strain (105) and bovine rotavirus serotype 6 NCDV strain (106). Comparison of the deduced amino acid sequences of the vp7 neutralization glycoproteins of the rotavirus strains WA, HU-5, SA-11, UK and NCDV indicates about 75 to 85% homology among the vp7 glycoproteins (101-106). Several of the predicted hydrophilic regions of vp7 exhibit significant homology and may represent common antigenic determinants, however the hydrophilic domain specified by residues 83 to 102 shows significant sequence divergence and has been proposed to represent the determinant for serotype specificity (106).

Genome segment 4, which encodes vp3 (113,114), codes for the rotavirus hemagglutination activity and the protease-enhanced plaque formation activity (119) in addition to neutralizing activity (111,112,117,118). Rotavirus strains may exhibit markedly different patterns of gastrointestinal tract disease when inoculated into newborn mice; genetic analysis has indicated that genome segment 4 plays a major role in determining rotavirus virulence (119). The enhancement of infectivity by protease treatment commonly observed for rotaviruses is due to the specific cleavage of vp3 at two close arginine residues present within a sequence which is highly conserved among many rotaviruses (120).

The morphogenesis of rotaviruses appears to be unique among the reoviridae. Particles morphologically similar to virions, but devoid of the outer capsid shell, assemble in cytoplasmic inclusions at the periphery of the nucleus and then bud through the membranes of the rough endoplasmic reticulum (121,122). The envelope acquired in this process is subsequently lost, presumably as the particles move towards the interior of the endoplasmic reticulum. At late times after infection viral particles appear to be associated with both membranes and the cytoskeleton, although only virus particles with an outer capsid shell are found outside of infected cells (123). Exactly how the outer capsid shell polypeptides are incorporated to yield mature virions lacking

an envelope is unclear. The process of maturation, however, does appear to be calcium-dependent (124).

In vivo mixed infection can lead to reassortment of rotavirus genome segments at a very high frequency (125). Such efficient reassortment in infected animals may affect strategies for rotavirus immunization, as live, attenuated vaccines would have the potential to reassort with field strains and lead to a rescue of virulence. In addition to reassortant viruses with constellations of parental genome segments, reassortant rotavirus isolates have been obtained *in vivo* (126) and *in vitro* (127) which possess genome rearrangements.

ORBIVIRUSES

Orbiviruses, comprising a genus of the family reoviridae (Table I), are a group of viruses distinguishable from other members of the reoviridae in that they multiply both in insects and vertebrates (128,129). These arthropod-borne viruses were designated orbiviruses because they shared morphological and physicochemical properties distinct from other arthropod-borne viruses, properties which included a large doughnut-shaped structure (orbis, ring) when negatively stained particles were examined with the electron microscope (130). The orbiviruses differ from the orthoreoviruses and rotaviruses in their ecology; orbiviruses are not pathogens of the gastrointestinal tract (128,129).

Orbiviruses are divided into twelve serological groups (129,130). The genomes of viruses representing each of the recognized serogroups consist of 10 segments of dsRNA (131,132) except the Colorado tick fever serogroup whose members possess 12 segments of dsRNA (133). The terminal sequences of the genome segments of the orbiviruses with a given serogroup, for example the bluetongue serogroup, are conserved among all serotypes so far analyzed (134,135). In addition, the terminal sequences are highly conserved between some serogroups, for example the bluetongue and epizootic hemorrhagic disease serogroups, but absolute conservation is

not observed between all serogroups, for example bluetongue and Eubenangee serogroups (135).

Orbiviruses within each of the 12 serological groups share a common antigenic determinant, and the various serotypes within a given serogroup are distinguishable by serum neutralization tests. However, no common antigen exists which relates all of the orbivirus serogroups (128-130,132,136). Because of the importance of the bluetongue serogroup of orbiviruses in the livestock industry involving cattle and sheep (128,129,136), significant effort has been devoted to their characterization.

For the bluetongue virus serogroup, the ten dsRNA segments are surrounded by a double-capsid shell; the outer capsid shell consists of two polypeptides (P2, P5) and the inner core structure consists of five polypeptides, three minor (P1, P4, P6) and two major (P3, P7) polypeptides (136,137). Polypeptide P7 is the principal serogroup-specific antigen of the bluetongue viruses (138,139), whereas polypeptide P2 is the serotype-specific antigen as demonstrated by molecular and serological analyses (138,140). Monoclonal antibodies directed against polypeptide P2 are able to neutralize bluetongue virus infectivity and to provide passive protection of sheep against virus challenge (141,142). The polypeptide coding assignments of the genome segments of BTV serotypes 1 and 17 have been deduced by translation *in vitro* of the denatured dsRNA genome segments (143,144). Polypeptide P2, the serotype-specific neutralization antigen, is coded for by the L2 dsRNA genome segment (140,143,144).

The genetic diversity of bluetongue virus isolates is well established (145-147). Epidemiological studies of bluetongue virus in the United States have revealed that the genome segment electrophoretic patterns of some isolates from cattle, for example BTV-10, -11 and -13, are variable; by contrast, BTV-17 displays a consistent RNA pattern (147,149). Temporal distribution between 1982 and 1983 of two serotype 2 genome electrophoretic patterns observed in Florida indicated

a possible naturally occurring genetic shift (148). Genome segment diversity of BTV serotype 11 isolates from individual cattle in a California herd naturally infected with BTV-11 and BTV-17 has been reported (149). Oligonucleotide fingerprinting comparisons have also established that both genetic shift apparently resulting from segment reassortment and genetic drift resulting from the accumulation of point mutations in a genome segment may occur with bluetongue viruses in nature (150-152). Thus, vaccination strategies involving live, attentuated bluetongue virus strains would appear to have significant associated limitations and risks. Conceivably the use of either an inactive virus vaccine or a single polypeptide subunit vaccine would be advantageous in the case of the bluetongue.

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28

GENETIC VARIATION OF FOOT AND MOUTH DISEASE VIRUS

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ABSTRACT

Foot and mouth disease viruses occur worldwide in seven distinct serotypes which can be further subdivided into a great number of subtypes. This diversity is expressed mainly in the structural genes leading to more than 30% amino acid exchanges in the capsid proteins between serotypes, whereas the non-structural proteins differ by 2-7%. The viruses are subjected to a high genetic drift with a mutation rate of up to 3% base exchanges per year in the structural genes.

INTRODUCTION

Foot and mouth disease viruses (FMDV) belong to the family of Picornaviridae and are the most infectious and economically important causative agents affecting cloven-hoofed farm animals. As schematically shown in Fig. 1 the genome consists of a single-stranded RNA of approx. 8.4 kb with a small protein (VPg) covalently linked to the 5´-end, contains an internal homopolymeric tract of 100-200 cytidyl residues and a polyadenyl sequence at the 3´-end and can act directly as a messenger RNA. The primary translation product is a single polyprotein with a theoretical molecular mass of 260 K which is cleaved by host and virus-encoded proteases into the mature functional gene products.

The viruses are distinguished immunologically in seven serotypes (O, A, C, SAT1, SAT2, SAT3, and Asia1) which can be further subdivided into a steadily increasing number of subtypes. More than 60 different strains have been characterized by serological techniques. In recent years, sequence analysis of the major immunogenic regions of many of

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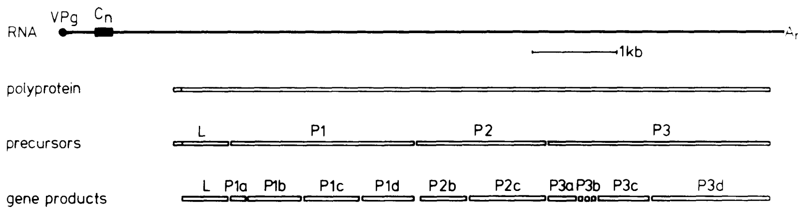

Fig. 1. Genetic map of FMDV. The upper line refers to the viral RNA. The genomic bound viral protein (VPg), the internal poly(C) tract (Cn) and the poly(A) tail (An) are indicated. The single translation product (polyprotein) is cleaved in statu nascendi into the precursors L, P1, P2, and P3 and by secondary cleavages into the mature proteins. The gene products are named according to the unified nomenlature for picornaviruses (1).

these viruses revealed that the real number of individual strains is probably much higher, although in some cases it is rather a question of semantics to differentiate between individual strains or variants of a given strain only. This variability, or genetic instability, is a major problem in the control of the disease by immunological means, e.g. in the practicability of the vaccine strains used and in constructing genetic engeneered vaccines.

The aim of this work is to provide an insight into the degree and the rate of genetic variation of FMDV. This is accomplished by analyzing some 40 individual FMDV strains for the variation of structural and non-structural genes and their corresponding gene products at the nucleotide level.

MATERIALS AND METHODS

Sources of FMDV nucleotide sequences

Most of the nucleotide sequences of the P1d coding region were derived directly from viral RNA by the extension of endlabelled oligonucleotide primers with reverse transcriptase. The cDNA of strain C1 was synthesized by oligo dT priming and was rendered double stranded and cloned in the PstI site of pBR322 via CG-tailing as described in (2). Four partially overlapping clones were found to cover most part of the P2 coding region and the P3a and P3b genes.

The nucleotide sequences were determined according to the method of Maxam and Gilbert (3). To analyse the P1d coding region of most of the field isolates we used a solid support for the cDNA during the chemical modification (4). This latter method is less time consuming and resulted in a higher resolution of the sequencing runs. The complete nucleotide sequences are published in ref. (5). Additional FMDV nucleotide sequences used in this work have been published by others as specified in the text and in the legends to Figs. 2 and 3. The sequences were processed using the computer programmes of Osterburg et al. (6).

RESULTS

Genetic diversity of FMDV strains

Individual FMDV strains differ from each other to a variable degree in their nucleotide sequence, especially amongst serotypes. Nucleotide exchanges are not randomly distributed over the whole genome, but are mainly concentrated in the structural protein coding region as shown schematically in Fig. 2 for the strains O1 Kaufbeuren (7) and A10 (8). A similar pattern of variation is also observed in comparison of these two serotypes with strain C1 Oberbayern (C1), a representative of the third European serotype, which is sequenced in most part of the polyprotein coding region (9 and unpublished results). In the most variable genomic region coding for the capsid protein P1d, up to 34% of the nucleotides are exchanged; such exchanges also include small deletions or insertions. In the non-structural genes on the other hand, variation between the serotypes is lower (approx. 8% in average).

Not all nucleotide exchanges affect the amino acid sequences. In the non-structural genes most changes are silent, i.e. they concern variable positions in the codons. The variations at the amino acid level in the corresponding proteins are in the range of 2 - 7 % (see Table 1). Most conserved gene products are the replicase (P3d), the proteins of the P2 region, P2b and P2c, and the smallest of the capsid

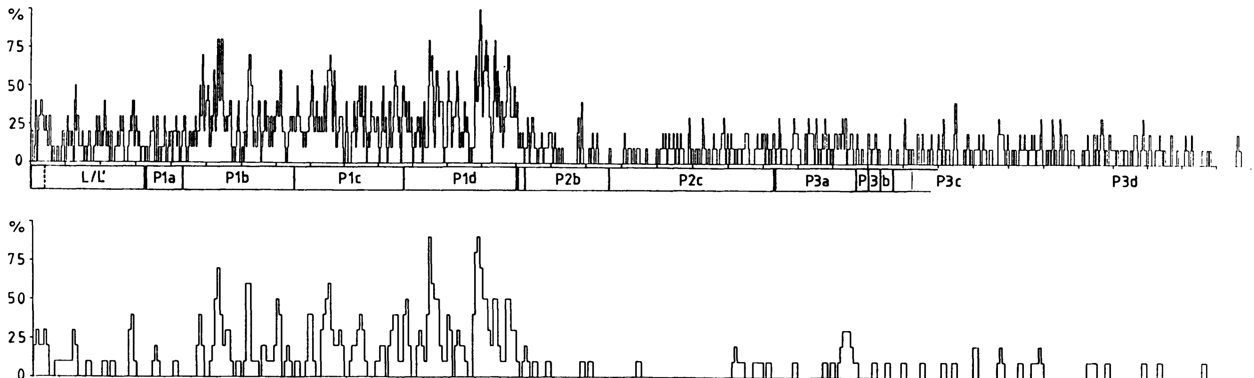

Fig. 2: Comparison of the polyprotein coding region of FMDV serotypes O1K and A10. The upper part of the Fig. shows the degree of variation at the nucleotide level. The nucleotide sequences of strains O1K (7) and A10 (8) were aligned and the number of changes per 10 nucleotides was plotted in steps of 3 nucleotides. The lower part shows a corresponding plot of the amino acid sequence (number of changes per 10 amino acid residues in steps of 3 residues). The positions of the genes are indicated.

proteins, P1a.

Table 1. Variation between FMDV strains O1K, C1 and A10

	L	P1a	P1b	P1c	P1d	P2b	P2c	P3a	P3b	P3c	P3d
O/C:	14 (6)	10 (0)	25 (17)	25 (16)	34 (30)	9 (6)	9 (5)	10 (4)	7 (3)	- -	- -
O/A:	13 (4)	13 (4)	26 (17)	27 (19)	34 (32)	6 (3)	8 (2)	14 (7)	7 (4)	8 (4)	7 (2)
A/C:	13 (6)	12 (4)	29 (23)	27 (18)	32 (29)	8 (7)	9 (6)	12 (7)	7 (6)	- -	- -

The polyprotein coding sequences of FMDV A10 (8), FMDV C1 (9 and unpublished results), and FMDV O1K (7) were compared. Differences in the individual genes are given in per cent. The numbers in parentheses refer to amino acid exchanges (in per cent).

This general rule does not hold for the genes of the structural proteins P1b, P1c, and P1d. Although the majority of base exchanges in some regions is also restricted to third positions in the codons, in other regions almost all of the exchanges result in amino acid changes. The most variable gene product is P1d carrying up to 34 % amino acid exchanges between two serotypes. Only 25 - 29 % of the amino acid residues are found to vary for P1b and P1c.

The genetic differences between subtypes of a given serotype are in principle similar to the situation of the serotypes, but base exchanges occur to a smaller degree. The only example for complete nucleotide sequences of the polyprotein coding region of two subtypes are the sequences of the strains A10 (8) and A12 (11). As shown in the latter reference the differences in the non structural proteins are in the range of 2 - 4 % of the amino acid residues, except for the VPg´s (P3b) and the L-gene product, whereas the structural proteins P1b, P1c and P1d differ by 4, 5 and 11 %, respectively.

Antigenic variation

The antigencity of FMDV is determined mainly by the

structural protein P1d (12). Two other capsid proteins , P1b and P1c, induce a lower immune response even though believed to be exposed at the surface of the virus to the same degree. The fourth structural protein, P1a, is localized inside of the viral capsid as demonstrated for poliovirus (13) and does not contribute to the antigenicity.

In order to study the relationships between FMD outbreaks in the past 20 years in Europe we have analysed the P1d sequence of some 30 individual isolated strains (5). The nucleotide exchanges between these and some additional strains sequenced in

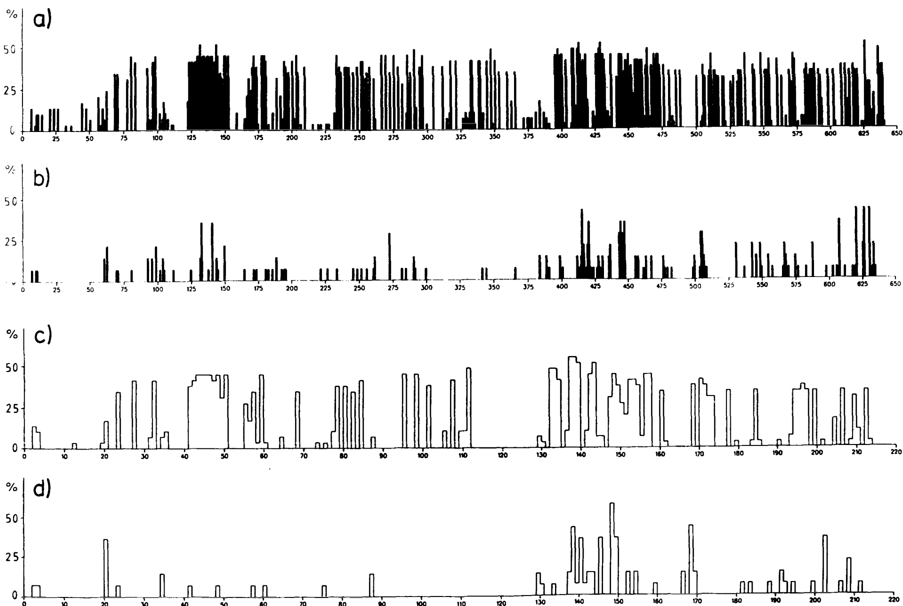

Fig. 3. Variation of the P1d gene. **a)** shows the degree of variation in single base positions between 16 A- and 15 O-strains (A5 Westerwald, A5 France, A5 Allier, A5 Parma, A Modena, A Salerno, A Bernbeuren, A Spain, A Murcia, A Valladolid, A Lerida, A Portugal, A, Morocco, A Aachen, A Ostdeutschland, (5) and A10 (10), O1 Kaufbeuren (9), O1 British Field Strain, O1 Austrian vaccine strain, O1 Lausanne, O1 Aulendorf, O Zusmarshausen, O Murchin I, O Murchin II, O Funen, O Wuppertal, O Thalheim, O Israel, O2 Normandie (5) and O1 Campos (14)); **b)** shows the comparison of the P1d nucleotide sequences between the A-subtypes only; **c)** shows the variation of the amino acid sequences between A- and O-strains and **d)** the variation of the A-type amino sequences only.

other laboratories are plotted in Fig. 3. Sixteen sequences have been compiled for the A-subtypes and 15 for the O-subtypes.

The majority of the codons is different between the two serotypes. At the amino acid level, however, some 60 % of the sequence are conserved. Most conserved are the N-terminus up to position 40, the middle part (position 60 to 130) and most part of the C-terminal quarter of the protein. These more constant parts are interrupted by several variable regions, the two most extended being located between positions 40 and 60 and positions 130 and 160. The latter site covers the major antigenic determinant of FMDV.

The variation of the P1d sequence amongst subtypes is not as pronounced as examplified for the A-subtypes in Fig. 3b and d. Variable regions amongst subtypes correpond to those also present in serotypes. Variations in the O- and C-subtypes display a similar pattern.

It is difficult to apply strict criteria for the definition of a specific subtype. None of our isolated strains analysed show exactly the same nucleotide sequence in the VP1 coding region, despite the fact that many of them have been derived from common origins. The strains are compared with each other regardless of their antigenic character, i.e. some of them may be discriminated by serological means and some may not. The variation between the individual isolates is due to the high genetic drift of this virus. The speed of this drifting will be discussed in the next chapter in more detail.

All virus neutralizing antibodies bind to P1d (12) in agreement with the finding that only P1d, or parts of it, can induce a protective immune response. The dominant immunogenic site in type O1K has been localized between position 144 and 159 in P1d (12). This site covers mostly a region which is highly variable between different strains. It however seems not to cover the immediately preceding most exchanged region from pos. 130 to 144. The highly variable anterior half of P1d is presumably also not implicated in the antigenicity of the virus.

Preliminary data suggest that P1b and P1c are involved in the presentation of antigenic determinants representing the

binding sites of neutralizing antibodies (unpublished results). Since no neutralizing antibodies could be found binding to P1b and P1c, the contribution of these two proteins to the antigenicitiy of the virus may be in maintaining a definite tertiary structure of the corresponding determinants rather than by a direct interaction with the antibodies.

The genetic drift

The number of base exchanges occuring in the genome of FMDV during the propagation in the field can be determined by nucleotide sequence analysis or with a certain accuracy also by RNase T1 oligonucleotide fingerprinting. Comparing the differences between strains isolated at different times during an epidemic in a defined geographic area, it may be possible to calculate the number of base exchanges for a certain time. This calculation is complicated, however, by the fact that in many cases the infectious viruses are genetically not homogenous but consist of a mixture of strains with different nucleotide sequences. This was demonstrated for strain A12 (15) and confirmed by analyses of C-type outbreaks in Spain (16).

In Table 2 three groups of FMDV strains are listed, each of which probably originating from common ancestors. Some of the strains were isolated at different times during an outbreak, others were collected at the same time after the onset of the epizootic. In any case a diversity of the P1d coding sequence has been observed.

The variability of the nucleotide sequence of the P1d coding region in individual isolates in comparison with the derived consensus sequence of a hypothetical original virus of each epidemic is between 0.2% and 0.8%. Only in one case (A Murcia) we found a 3.3% difference. This strain is presumably not derived from the same ancestor as the other isolates of this group but rather from a preexisting virus variant at the onset of the outbreak. The mean base exchange rate is 0.5 %. The strains were isolated on average two months after the onset of the epidemic. Therefore an exchange rate of approx. 3 % nucleotides per year can be calculated for the P1d coding region

as a rough measure for the genetic drift of the virus in the field.

Table 2. Nucleotide exchanges in field isolates

strain	place and date of of isolation	number of nucleotides determined	nucleotide exchanges[1)]		amino acid exchanges	
			total	in %	total	in %
A5 Parma[2)]	Italy 1962	600	1	0.2	-	-
A Modena	Italy 11.84	600	4	0.7	1	0.5
A Salerno	Italy 1.85	600	2	0.3	-	-
A Spain	Madrid 3.83	630	5	0.8	2	1.0
A Valladolid	Spain 3.83	400	2	0.5	1	0.8
A Lerida	Spain 3.83	500	1	0.2	1	0.8
A Murcia	Spain 3.83	240	8	3.3	5	6.2
O Murchin	GDR 3.82	600	3	0.5	-	-
O Funen I	Denmark 3.82	540	1	0.2	-	-
O Funen II	Denmark 1.83	500	3	0.6	1	0.6

1) compared to the consensus sequence of each group
2) Italian vaccine strain. Sequence as determined in 1985.

Similar results were obtained in a detailed study on the evolution of FMDV C1 in nature (16). Analyzing selected genomic regions of several Spanish C1 outbreak strains by T1 oligonucleotide fingerprinting, values ranging from 0.04 % to 4.5 % substitutions per nucleotide and year were measured, depending on the time period and the genomic segment considered. The exchange rates for structural protein genes were up to six fold higher than for non-structural protein genes.

DISCUSSION

FMDV circumvents the immune attack of its host mainly with help of two features: It multiplies extremely fast, so that

progeny viruses are already secreted before the immune system can respond adequately. In addition it varies its genetic information and thereby the structure of its surface antigens by base exchanges, evolving in this way with a certain, even low, probability in new subpopulations, which may not immediately be recognized by the immune system. Whether this latter property is the major reason for the ability to persist for a limited period of time in the host, or whether FMDV uses yet another unknown survival strategy, remains to be clarified.

The high degree of genetic drifting in FMDV is not unusual among RNA viruses and is explained by the lack of a proof reading mechanism during the replication. For influenza virus a similar degree of antigenic drifting has been reported (17) and for the small single stranded RNA phages an even higher genetic instability (18).

In terms of instability among the picornaviruses FMDV is probably exceeded by the rhinoviruses, which exist in many different serotypes and may even evolve new ones in short time. On the contrary poliovirus is one of the most conservative representatives of this family. The attenuated Sabin strains of poliovirus have been stable for more than ten years as live vaccines. It is known that in strain Sabin 3 a single base exchange is responsible in maintaining the avirulent character (19). In case of FMDV such a small genetic variation would certainly revert to virulence within a few passages in a permissive host.

This difference in genetic stability is correlated with the degree of virulence of the individual picornavirus species. It reflects the highly balanced parasite-host relationship, which forms the basis of their existence. The strategies used for the coexistence with the host differ from species to species and depend on many parameters like routes of transmission and infection, tissue tropism and modus of replication. Poliovirus is by far the most virulent member of the family. Due to its neurotropism this virus is able to kill its host with a certain probability. It is obvious that it cannot exist in many serotypes or change its antigenic properties frequently, since

otherwise it would have eradicated its host long time ago. In contrast rhinoviruses can infect repeatedly without drastically affecting the health conditions of their hosts. In this way these viruses can occur in many different serotypes and even evolve new ones in short time.

FMDV is for domestic animals, especially cattle, almost as virulent as poliovirus for men. This strong effect may be a consequence of cultivation and inbreeding since e.g. the African buffalo (Syncerus caffer) which is the natural host of the SAT-types is obviously not so hard affected by the disease (20). Other susceptible ungulates like sheep and goats show hardly any apparent signs of sickness if infected with FMDV. This fact may explain why this virus could evolve to the relative high antigenic diversity.

In view of the ability of FMDV to change speedily its antigenic character it is not expected that vaccination can be successful using only a limited number of types (usually not more than one or two strains per serotype) in the trivalent vaccines. The failure to protect against challenge with different strains was seen in the following example: In spring 1982 there was a serious outbreak of strain 01 in the German Democratic Republic, although cattle were vaccinated against this serotype, but with the different subtype 02. The normally observed protection of vaccinated animals is mainly due to the simple fact that almost all of the FMDV outbreaks in Middle Europe in the past ten years were identical with, or derived from, strains used for the production of the vaccines (5). In some cases these outbreaks were caused by inproperly inactivated vaccine charges, in other cases the viruses must have escaped from the vaccine producing plants. The only two outbreaks introduced from elsewhere into the Federal Republic of Germany in the past ten years fortunately remained local, due to the isolated breeding conditions of the infected animals.

It is evident that FMDV owns a remarkable genetic instability, which up to now has resulted in seven distinct serotypes and more than one hundred subtypes. Due to the permanent drifting in endemic areas, altered types with

different antigenic properties may evolve rapidly. This is a major challenge to vaccination strategies and demands a watchful eye on the occurence of new strains overcoming vaccine-mediated protection and leading to devasting epidemics.

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HANTAAN VIRUS

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ABSTRACT

Hantaan virus is the type species of the newly described Hantavirus genus of Bunyaviridae. Hantaviruses are endemic in rodents throughout most of the world and have been associated with a group of diseases termed hemorrhagic fever with renal syndrome (HFRS). Transmission of hantaviruses from rodents to humans has resulted in HFRS epidemics in rural, urban and laboratory settings. This review will focus on the antigenic and genetic properties of hantaviruses, experimental hantavirus infection of laboratory animals, and HFRS acquired in laboratory settings.

INTRODUCTION

Korean hemorrhagic fever

Hantaan virus is the etiologic agent of Korean hemorrhagic fever (KHF) (1). This disease first gained prominence in Korea as a serious health threat in 1951, when an explosive epidemic occurred among United Nations forces stationed in Korea. A mortality rate of 10 to 15% was reported for approximately 3,000 soldiers hospitalized with KHF during the Korean conflict (2, 3, 4). Since that time, KHF has remained endemic in Korea, although early diagnosis and improved patient care have reduced the mortality rate to less than 5% (3). Patients with classical KHF often manifest five clinical stages: 1) a febrile phase, of 3 to 7 days duration, sometimes characterized by development of petechial rash or conjunctival hemorrhage; 2) a hypotensive phase, which in moderate cases persists from 1 to 3 days, but in severe cases can result in irreversible shock and death; 3) an oliguric phase of 3 to 7 days, during which renal

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failure complications contribute to about 50% of total KHF-related deaths and during which hemorrhagic manifestations may appear; 4) a diuretic phase, which can last for days or weeks with daily urine outputs frequently in excess of 3 to 5 l; and 5) a convalescent phase of 2 to 3 mo duration. Not all phases occur in every patient, and often only flu-like symptoms are observed (3, 4, 5, 6, 7, 8, 9).

Hemorrhagic fever with renal syndrome

KHF is only one of many similar diseases which have been clinically diagnosed throughout Europe and Asia. KHF-like diseases have been reported in China, Japan, USSR, Czechoslovakia, Romania, Hungary, Bulgaria, Yugoslavia, Finland, Sweden, Norway, Denmark, Belgium, France, and Greece (10). In addition to KHF, at least 150 other synonyms for these diseases have been used; the most common are: epidemic hemorrhagic fever (EHF), a severe disease similar to KHF, which is found in Japan and China; hemorrhagic nephroso-nephritis, which occurs in the USSR both in classical and more mild forms; and nephropathia epidemica (NE), a less severe, nonhemorrhagic disease found in Scandinavia (10, 11). The World Health Organization (WHO) has recommended that all of these diseases be collectively termed HFRS (12).

Epidemiology and ecology

The epidemiology of HFRS and the ecology of its rodent hosts have been the subject of intense investigation in many areas of the world. Antibody reactive with Hantaan viral antigen has been detected in rodents trapped in North America, South American, Europe, Africa, Asia, and Australia (13). Rodent hosts identified include: *Apodemus*, *Rattus*, *Clethrionomys*, *Microtus*, *Peromyscus*, *Neotoma*, and *Bandicota* (13, 14). In addition to rodents, insectivores and cats have also been reported to possess antibodies to HFRS agents (14, 15, 16).

The rural reservoir of KHF in Korea is almost exclusively *Apodemus* field mice (4). In these areas, increased numbers of cases occur in two seasonal peaks during the spring and fall, closely following the two times each year the *Apodemus* leave their burrows to mate (4, 17, 18). Rural EHF in China similarly occurs twice each year and appears also to be related to the seasonal prevalence of *Apodemus* mating and rodent density (4, 19). The Scandinavian rural

reservoir of NE has been identified as the bank vole (*Clethrionomys*) (20). Both *Clethrionomys* and *Rattus* have been implicated in HFRS in rural areas of the Soviet Union (21). The appearance of HFRS patients who had never been outside of major cities and, thus, had no opportunity to come into contact with rural rodents, led to the discovery of house rats (*Rattus*) as the principal urban reservoir of hantavirus-associated disease. Cases of urban HFRS tend to occur more frequently in the fall and winter seasons, probably because of increased numbers of house rats invading houses during cold weather (21, 22).

Viruses serologically related to Hantaan have been detected in indigenous rodent populations world-wide (Fig. 1). Isolates which are nearly indistinguishable from one another by serological means have been obtained from both endemic disease regions and also in areas believed to be HFRS free (13, 24). Currently, it is unclear why some of these viruses are apparently innocuous for humans and others cause HFRS.

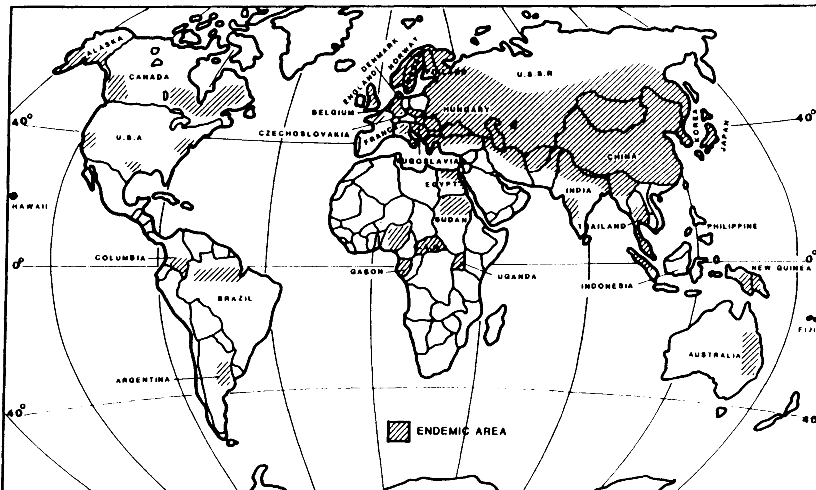

Fig. 1. Global regions where antibody to Hantaan virus has been detected in indigenous rodent populations.

Hantaviruses

Although HFRS had been clinically recognized for many years, the first documented isolation of one of the elusive causative

agents was reported in 1978 when Hantaan virus was identified in the lungs of Korean striped field mice (Apodemus agrarius coreae) (1). The virus derives its name from the Hantaan river which is located south of the demilitarized zone separating North and South Korea and flows through a hyperendemic foci of KHF (4, 25, 26). Propagation of Hantaan virus in cell culture provided the long-awaited opportunity to study a HFRS agent systematically (27).

The initial description of the physical structure of Hantaan virus came from electron microscopic observation of virion particles. The spherical, enveloped virions, with an average diameter of approximately 95 nm, were suggested to most closely resemble viruses in the Bunyaviridae family (28, 29, 30, 31). Biochemical characterization of Hantaan virus provided the first indisputable evidence that Hantaan is molecularly similar to members of the Bunyaviridae. Like other viruses in the family, Hantaan was found to possess a tripartite, single-stranded RNA genome of anti-message sense, enclosed in three ribonuclease-sensitive nucleocapsids surrounded by a lipid envelope containing two virus-specified glycoproteins (32, 33, 34, 35). No serological relationship, however, could be demonstrated between Hantaan virus and any other member of the Bunyaviridae (36). Only after the molecular characterization of several other viral isolates, serologically related to Hantaan yet antigenically unique, was it established that Hantaan and related viruses comprise a new and separate genus of Bunyaviridae, which has now been provisionally accepted by the International Committee on the Taxonomy of Viruses as the Hantavirus genus (24).

ANTIGENIC AND GENETIC PROPERTIES OF HANTAVIRUSES

Serological relationships

The Hantavirus genus contains at least four antigenically distinguishable virus groups (24, 37). Representative viruses of each group include: 1) Hantaan; 2) Seoul; 3) Puumala; and 4) Prospect Hill; which were isolated from Apodemus, Rattus, Clethrionomys, and Microtus rodents, respectively. Hantaan and Seoul viruses have been implicated in rural and urban KHF in Korea, respectively (1, 23), and Puumala in NE in Finland (20). Prospect

Hill, which was isolated in the United States (U.S.), is not known to be associated with any human disease (38).

In general, viruses isolated from the same rodent genus have been found to be antigenically more cross-reactive than isolates from different genera. Thus, two *Rattus* isolates originating from endemic and nonendemic HFRS regions (such as Korea and the U.S.) are more closely related than are *Apodemus* and *Rattus* isolates made in the same geographic region (24).

Numerous isolates of HFRS-associated viruses have now been obtained from a variety of rodents and also from HFRS patients. Many of these isolates have been deposited with Dr. Ho Wang Lee at the WHO Collaborating Center for Virus Reference and Research, Institute for Viral Disease, Korea University, Seoul, Korea. Once these viruses are antigenically characterized, it is quite possible that more than four serologically distinct groups will emerge. In fact, preliminary evidence already has been obtained which suggests that a fifth antigenic type may have been isolated from a Greek HFRS patient (39).

Viral proteins

Like other Bunyaviridae members, hantaviruses have three major structural proteins: a nucleocapsid protein (N), and two envelope glycoproteins (G1 and G2) (32, 33, 34, 35). The electrophoretic profile of hantavirus proteins, however, is different from other members of the family (Fig. 2). The hantavirus nucleocapsid proteins are larger than those of viruses in other genera of the Bunyaviridae, with relative molecular masses (M_r) of approximately 48,000 to 55,000 (48K to 55K). G1 and G2 vary in size among isolates, but for prototype Hantaan virus, have M_r of approximately 68K and 54K, respectively (32, 33, 35, 40, 41). In addition to N, G1, and G2, a large, 200K polypeptide (L) has been observed in purified virus preparations (35). This polypeptide is believed to represent the viral RNA polymerase found in association with virion particles (34). Unlike other viruses in the Bunyaviridae, no nonstructural polypeptides have been identified in hantavirus-infected cells (33, 35, 42).

Radiolabeling of Hantaan proteins with ^{3}H-mannose and ^{3}H-glucosamine provided evidence that G1 and G2 possess carbohydrate

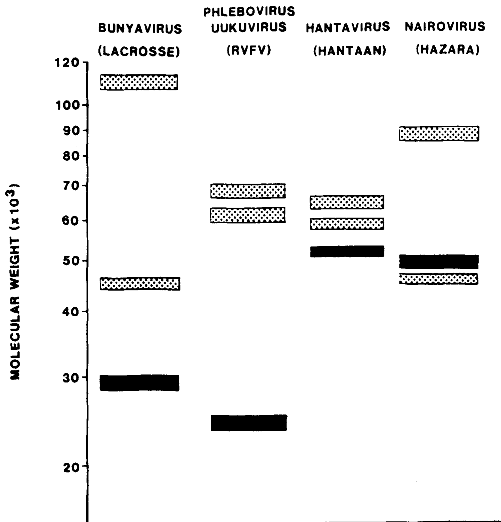

Fig. 2. Schematic illustration of the polyacrylamide gel electrophoretic migration of the structural proteins of viruses in the five genera of Bunyaviridae. Protein profiles of LaCrosse, Rift Valley fever (RVF), Hantaan, and Hazara viruses are representative of the Bunyavirus, Phlebovirus and Uukuvirus, Hantavirus, and Nairovirus genera, respectively. Solid bars symbolize the nucleocapsid protein and the dotted bars, the envelope glycoproteins of the representative viruses.

moieties (35, 40, 41). Reduced yields of Hantaan were observed when virus was propagated in the presence of glycosylation-inhibiting drugs, suggesting that the sugars may be necessary for production of mature, infectious viral particles (41). The nature and extent of glycosylation of G1 and G2 were determined by enzymatic cleavage with endoglycosidases H and F, with resultant electrophoretic mobility shifts corresponding to molecular weight reductions of approximately 7K for G1, and 3K for G2 (41). These data suggest that the carbohydrate component of Hantaan envelope proteins is mostly of the asparagine-linked, high mannose type.

The role of hantavirus proteins in viral infection and neutralization has not yet been completely defined. Immune precipitation of G1, G2, and N of Hantaan virus by antisera directed against other representative hantaviruses suggested the conservation of at least some antigenic sites on all three major structural

proteins (43). Preliminary epitope mapping with monoclonal antibodies revealed neutralizing sites on both G1 and G2 of a rat viral isolate (B1) and on G1 of Hantaan virus. Hemagglutinating activity was detected in association with G2 of both Hantaan and B1 viruses (44). More extensive mapping studies with a larger number of monoclonal antibodies will be required to identify conserved and variable polypeptide regions among hantaviruses.

Nucleic acid

The large (L), medium (M), and small (S) genome segments of Hantaan virus are each complexed with the N protein to form three separate nucleocapsids (32). Virion RNA is predominantly of negative polarity (anti-message sense), and is transcribed to yield messenger RNAs with the virion-associated polymerase (34). Strand-specific cDNA probes, however, have been used to demonstrate that message-sense RNA is also encapsidated in mature virion particles (42). Whether this indicates functional significance or is merely the result of inefficient packaging is not known.

The coding strategies of the M and S genome segments of Hantaan virus have recently been determined by molecular cloning and sequence analysis (45, 46). Partial restriction maps of M and S cDNA are displayed in Fig. 3. The viral M RNA was found to consist of 3616 nucleotides with a base composition of 29.9% A, 17.9% G,

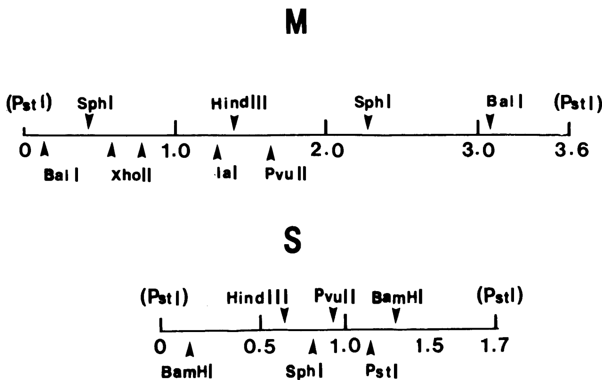

Fig. 3. Partial restriction maps of cDNA corresponding to Hantaan viral M and S genome segments. Gene lengths are shown as kilobase pairs.

21.4% C, and 30.8% U. Sequence complementarity was detected over the 18 terminal 3' and 5' nucleotides (Fig. 4). This feature, which is conserved among all members of the Bunyaviridae examined to date, is believed to allow formation of noncovalently closed circular nucleocapsid structures (47, 48, 49, 50, 51, 52).

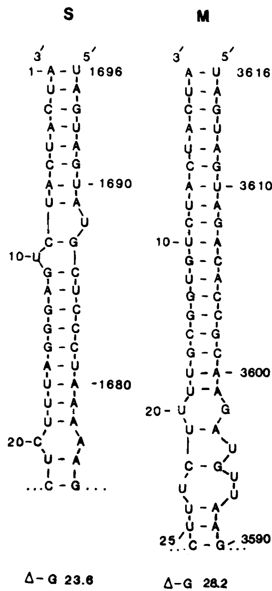

Fig. 4. Nucleotide sequence complementarity at the 3' and 5' termini of the S and M genome segments of Hantaan virus. Nucleotides are numbered with respect to the 3' terminus of viral-sense RNA and the free energy calculated for each structure are indicated.

A single, long, open reading frame in the viral complementary-sense M RNA has the potential to encode 1135 amino acids or a polypeptide of 126K daltons. Amino terminal sequence analysis of isolated G1 and G2 revealed a gene order with respect to message sense RNA of 5'-G1-G2-3'. Mature G1 was found to begin 18 amino acids beyond the first AUG of the open reading frame and was preceded by a short, hydrophobic leader sequence. The amino terminus of G2 was at the 649th amino acid of the open reading frame, also following a hydrophobic sequence. The carboxy termini of G1 and G2 were localized by using antisera generated to synthetic

peptides corresponding to predicted amino acids for immune-precipitation of authentic viral proteins. Based upon these results, molecular weights of approximately 64K and 53.7K were calculated for G1 and G2, respectively. Five potential asparagine-linked, glycosylation sites were contained within the G1 amino acid sequence and two within the G2 sequence (46).

The S RNA segment was determined to contain 1696 nucleotides with a base composition of 26.7% A, 19.2% G, 23.8% C, and 30.4% U. Terminal sequence complementarity extended over the 21 distal 3' and 5' nucleotides (Fig. 4). An open reading frame of sufficient size to encode N was detected in the cDNA corresponding to viral complementary-sense RNA. Examination of all six potential reading frames revealed no additional regions able to encode polypeptides in excess of 8K daltons. Direct evidence that the Hantaan S RNA encodes N was obtained by cell-free translation of RNA transcripts copied from the cDNA with SP6 polymerase. Only viral complementary-sense RNA transcripts served to program the reticulocyte lysate translation system. The major translation product was identified by specific immune-precipitation with monoclonal antibodies to be Hantaan N (42, 45). The apparent absence of coding potential for a second, nonstructural (NS_S) polypeptide in the S genome information suggested that the coding strategy of Hantaan differed from other viruses in the family, such as those in the *Bunya*- and *Phlebovirus* genera, which use elaborate overlapping reading frame and ambisense strategies, respectively, to encode NS_S proteins (49, 52). However, because a function has not yet been assigned to any Bunyaviridae NS_S protein, it is difficult to speculate on the significance of this difference (45).

The L RNA segment of Hantaan virus has not been well characterized. A molecular weight of 2.7×10^6 was calculated for L RNA based on its electrophoretic migration in denaturing agarose gels (32). It is presumed that the L segment encodes one or more proteins which function as the viral transcriptase.

INFECTION OF LABORATORY ANIMALS WITH HANTAVIRUSES

Pathogenesis

Infection of adult animals with hantaviruses is almost always

asymptomatic, a property which has greatly impeded development of a suitable animal model for HFRS. Experimental hantavirus infection has been accomplished with numerous species of colonized rodents, including mice, rats, hamsters, guinea pigs, gerbils, and rabbits (26, 53, 54). Although it is difficult to establish laboratory colonies of *Apodemus* mice, many studies have included these animals because of their sensitivity to Hantaan and because they are the natural hosts of this virus. Seronegative *Apodemus* trapped on Jeju Island, a nonendemic region of KHF in Korea, have served as the source of most laboratory colonies of these rodents (1). Intramuscular (IM), subcutaneous (SC), intraperitoneal (IP), oral, intrapulmonary, and intranasal (IN) routes of inoculation were all found to result in productive infections of *Apodemus* with Hantaan virus (55). Examination of tissues from animals injected by the IM route revealed a transient viremia from 7 to 12 days postinoculation (PI). Virus was excreted in saliva from 9 to 40 days PI, and in urine from 9 to at least 360 days PI with some samples containing in excess of 10^4 ID_{50}/ml. Small amounts of virus were detected in feces from 12 to 40 days after infection. The presence of infectious virus could first be demonstrated in *Apodemus* lungs at 12 days, and persisted up to 180 days PI. Viral antigen was observed in the lungs for at least 1 yr after infection. Virus was also recovered from kidneys and parotid glands from about 15 to 43 and 12 to 46 days PI, and antigen detected 60 and 260 days PI, respectively. Neutralizing antibody to Hantaan virus was demonstrable beginning at about 10 days and continued throughout the course of infection, although no clinical signs were observed. Inoculation of other colonized rodents produced far less evidence of viral infection, however, ICR mice, white rats (Sprague-Dawley and Wistar strains), Hartley and strain 13 guinea pigs, and New Zealand white rabbits all were shown to produce antibody detectable by indirect immunofluorescence assays. The native South American *Calomys* rodent not only develops antibody to Hantaan, but also viral antigen in lung tissues (53).

Adaptation of Hantaan virus for more efficient - but still asymptomatic - replication in common laboratory rats was accomplished by successive passages of lung tissue first from

infected Apodemus to rats and subsequently from rats to rats by IM inoculation. The distribution of viral antigen in rat tissues was similar to that in Apodemus except that rats exhibited antigen in their spleens as well (56).

Other serotypes of hantaviruses have now also been examined under experimental laboratory conditions. Puumala virus, the etiologic agent of Scandinavian HFRS (NE) was studied in laboratory-bred bank voles (Clethrionomys glareolus) and Mongolian gerbils (Meriones unguiculatus) (53, 54, 57). Intramuscular inoculation of voles with Puumala resulted in subclinical, chronic infections. Animals displayed viral antigen in their lungs, liver, spleen, pancreas, salivary glands, and small intestines. No antigen was observed in lymph nodes, adrenal glands, kidneys, and brains. Transient viremia from days 10 to 14 PI was observed. Infectious virus was present in the lungs from 14 to 270 days PI, and feces and urine from 35 to 130 days PI. Fluorescent antibodies against Puumala antigen could be detected from 18 days PI throughout the remainder of the 270-day observation period (57).

Experimental infection of laboratory rats with an HFRS agent which is naturally rat-borne (Seoul urban rat virus), revealed that, unlike Apodemus, rats excreted virus in their urine and feces for only 40 to 90 days PI. Infectious virus, however, could be detected in saliva from 18 to 100 days PI, and viral antigen was present in salivary glands and lungs up to 100 days PI (58).

Transmission

The most common natural route of hantavirus infection (of both rodents and humans) is believed to be *via* aerosolization of rodents' urine, feces, and saliva. Transmission of virus to cage mates, and also to animals kept in cages 1 to 4 m away from infected Apodemus has been demonstrated (55). Similarly, Clethrionomys infected with Puumala virus were able to infect other voles in the same cage, with highest transmission rates coincident with salivary shedding of Puumala virus (57).

Although vertical transmission has not been documented, transmission from infected mothers to offspring can occur (55). Ectoparasite vectors have not been conclusively demonstrated to play any role in viral spread, and transmission was found to occur even

when the presence of all such vectors could be ruled out (55). Transmission of HFRS to humans by rodent bite was reported in France (59) and may also be a factor in rodent/rodent transmissions.

Animal models of HFRS

Currently, there is no satisfactory animal model for HFRS. Although numerous species of nonhuman primates have been inoculated with hantaviruses and hantavirus-containing specimens from HFRS patients, the only detectable signs of infection were transient viremia, proteinuria, and the development of neutralizing antibodies (60). The only available laboratory model of disease is that of suckling rodents which develop fatal infections when injected with some HFRS viruses. Inoculation of suckling mice by the intracerebral (IC), IP, IM, SC, and IN routes with Hantaan virus all resulted in the development of lethal disseminated infection with antigen distributed in the brain, lungs, heart, liver, and kidneys of infected animals, irrespective of the route of inoculation. In some animals, antigen was also observed in salivary glands, trigeminal ganglia, adipose tissue, intestine, and muscle (61, 62, 63). Mice first became moribund 13 to 14 days PI and displayed symptoms such as ruffled fur, hunched posture, and hyperexcitability, followed by lethargy and coma. Animals generally died from 1 to 4 days after the first signs of illness, and virus could be recovered from their brains (63, 64). The clinical course and fatal outcome of disease were found to be age-dependent, with 100% of mice infected within 72 hr of birth susceptible, but only 50% of 7-day-old, and 0% of 2-wk-old animals, succumbing to viral infection (61). Suckling laboratory-bred bank voles and Mongolian gerbils, which are the only reported laboratory animals susceptible to infection with NE viruses, were found to develop only subclinical, persistent infections after IC inoculation (54, 57).

Although Hantaan viral infection is systemic in experimentally infected suckling animals, the clinical disease bears no resemblance to HFRS in humans. Therefore, it is essential that efforts be continued to develop an animal model for disease. Without such a model, testing of any vaccine or therapeutic agent will be quite difficult.

HFRS ACQUIRED FROM LABORATORY ANIMALS

Disease incidences

In addition to naturally acquired rural or urban HFRS, several disease outbreaks have occurred among research personnel, not only in laboratories engaged in HFRS studies, but also in laboratories where HFRS research had never been conducted. This third epidemiological type of HFRS, that is, disease acquired from laboratory animals experimentally or persistently infected with HFRS viruses, has been the subject of concern among animal handlers and breeders world-wide.

HFRS infections in laboratories engaged in hantavirus research. One of the earliest reported incidences of laboratory-acquired HFRS occurred in the Soviet Union in 1961, when an outbreak was observed among 113 workers in a research facility engaged in tick-borne encephalitis studies (65). The outbreak apparently originated when numerous wild-caught rodents were brought to the facility, quarantined for 2 wks, and then transferred to two separate animal rooms. Of the 63 permanent personnel, only five had a history of HFRS. None of these presumed immune individuals developed HFRS; however, of the 58 remaining susceptibles, 52 became ill. In addition, 11 out of 14 frequent visitors to the department, six out of 20 infrequent visitors, and 44 out of 94 chance visitors developed HFRS within a 6-wk period. Because many of the infected individuals had no contact with the rodents and, in most cases, did not even enter the animal rooms, airborne virus transmission was suspected (65).

Wild-caught rodents were also believed to be responsible for three cases of NE among laboratory workers at the University of Helsinki, who handled bank voles trapped in the NE-endemic Puumala region of Finland. The NE agent (Puumala virus) appeared not to be highly contagious, and several individuals who handled contaminated voles neither developed NE nor had any evidence of antibody titers to Puumala virus (20).

From 1976 to 1979, clinically apparent infections in nine laboratory personnel and visitors were reported at the Korea University Virus Institute (Seoul) (66). Seven cases occurred among employees who worked at the laboratory and included all six people

who worked in the area where wild-caught rodents and experimentally infected Wistar rats were housed. Only one of the six had also engaged in field collection of wild rodents. In addition to laboratory personnel, two visitors to the laboratory (one of whom was present in the animal room for less than 5 min) also developed KHF. In none of the cases was there evidence of any animal bite. A retrospective serosurvey of other laboratory personnel indicated that no inapparent infections had occurred. Because both wild- and laboratory-bred animals were contained in the same animal room, the source of KHF was not determined; however, it was of interest that five of the nine infections occurred within 4 mo of the initiation of experimental work with Wistar rats.

HFRS acquired in laboratories not engaged in hantavirus research. The threat of HFRS for laboratory workers who come into contact with persistently infected rats was first recognized when two outbreaks of EHF were reported to have occurred at Tohoku University, Sendai, Japan in 1975 and 1977 (67). In both outbreaks, people who developed EHF worked in one experimental animal room which was used exclusively for rats. Since then, 16 Japanese medical centers have reported a total of 195 cases of EHF, with one death, which were attributable to hantavirus-contaminated laboratory rats (4). Whether or not there was a common source of infection of the rats was not determined, but it was suggested that some of the inbred rat strains from common suppliers may have harbored hantavirus-persistent infections.

The origin of laboratory rats responsible for three cases of HFRS in a Belgian research institute in 1978 similarly could not be precisely determined. A retrospective study revealed that subclinical infection of approximately 50% of exposed staff had occurred. No human or animal infections with hantaviruses were recognized in Belgium before these incidences, and antibodies reactive with hantaviruses were found to be extremely rare in control human populations (68).

Belgian-bred rats, imported to the U.K., were identified as the source of an outbreak of HFRS among four staff members at the Institute of Cancer Research, Sutton, England. Hantaan antibody titers were demonstrated in the rats' sera and ascitic fluid by

immunofluorescence assays. Hantaan antibody titers were also detected in four of six remaining personnel who worked in the animal room with the suspect rats, and also in one of 14 technicians who worked with rat tissues in an area removed from the animal facility (69).

No HFRS infections have been associated with the handling of infected rodents in laboratory settings in the U.S. A serosurvey of 350 commercially-bred laboratory rats from three U.S. suppliers revealed no evidence of hantavirus infection. The practice of caesarian derivation and foster nursing, as well as rearing in areas maintained free of pathogens by barrier techniques, is probably responsible for the absence of hantaviruses among rat colonies. All certified rat cell lines and mouse-rat hybridoma lines maintained by the American Type Culture Collection are also proven to be free of hantaviruses (70). Despite these findings, the wide-spread distribution of hantaviruses in wild animals has raised concern that viruses may be introduced to some laboratory animal populations. Consequently, the following guidelines have been established by the National Institutes of Health for the surveillance, prevention, and control of Hantaan virus infection in laboratory animal colonies in the U.S. (71, 72).

Surveillance

1. The laboratory rat is the species of concern. Urban rats and certain other wild rodents carry Hantaan virus or similar agents.
2. Serological testing with noninfectious antigen is the only reliable surveillance method. Tests such as IFA, ELISA, and HAI may be used.
3. Sera should be tested from rats that are at least 6 mo old.
4. For established colonies, the frequency of testing and sampling size should be in accordance with recommendations contained in the Long-Term Holding of Laboratory Rodents, ILAR News XIX, 1976.
5. Infection is always asymptomatic, there are no pathological lesions noted in the infected animals.
6. A P3 facility is considered adequate for working with unconcentrated volumes of virus used for diagnostic tests.

7. Routine serum banking for laboratory personnel is recommended.

Prevention

1. Any rodents potentially harboring this agent should be tested routinely before introduction into the colony.
2. Any new biological material originating from rats, such as tumors and cell lines, should be tested for this agent before introduction into the laboratory.
3. A modified Rat Antibody Production (RAP) test should be used to test these biologicals.

Control

1. The only definitive, prudent method of control is eradication, disinfection, and restocking.
2. If valuable genetic rat strains are to be preserved, caesarian derivation using prescribed containment and surveillance methods should be instituted.

CONCLUSION

The recognition of hantaviruses as etiologic agents of HFRS, and the determination of their global prevalence among indigenous rodents has finally allowed a rational approach to disease control. Elucidation of the basic virological characteristics of hantaviruses has provided a basis for developing diagnostic methods and vaccines, either by conventional means or by utilization of recombinant DNA technology. The safety and efficacy of therapeutic drugs in the treatment of HFRS is also being explored. Preliminary work describing ribavirin inhibition of Hantaan viral replication in cell culture and in mice has led to a field trial, currently in progress, in which ribavirin is being used for treatment of Chinese EHF patients (72, 73). Until vaccines can be developed and diagnostic and therapeutic measures improved, however, limiting contact of humans with rodents and rodent excretions is obviously the most direct preventative measure for HFRS. Adoption of laboratory safeguards, such as those outlined, would further help to prevent infection of researchers using experimental rodents.

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