PERSISTENCE AND LATENCY OF HERPESVIRUSES IN EXPERIMENTALLY INFECTED TURKEYS

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Introduction

Herpesviruses have been isolated as the causal agents of disease in man and most species of animals (Nahmias, 1972). Because of their propensity to establish latent infections, herpesviruses have been extensively studied, since latent virus may provide the inoculum needed to establish a variety of acute recurring infections and perhaps initiate and maintain an oncogenic process. Significant accomplishments have included the demonstration that herpes simplex viruses induce latent infections in the spinal ganglia of mice and can be reactivated from the trigeminal ganglia of rabbits (Stevens and Cook, 1971; 1973; Stevens et al., 1972). In addition, Marek's disease virus of chickens has proven to be the only herpesvirus-induced neoplasm that can effectively be prevented by vaccination (Churchill et al., 1969; Okazaki et al., 1970a). One of the vaccine viruses is a cross-reacting herpesvirus of turkeys, which to date has not been associated with any pathology in chickens or turkeys (Witter et al., 1970b).

Recently, a cell-associated herpesvirus was isolated from male turkeys afflicted with yellow-colored semen containing high numbers of abnormal spermatids (Adldinger et al., 1974). This report represented the first evidence that a

virus may be responsible for abnormal spermatogenesis and concomitant yellow pigmented semen in male turkeys.

The proceeding literature review will discuss the following topics: 1) herpesviruses and latency; 2) properties and characteristics of turkey herpesviruses and their role in the etiology of yellow semen; and 3) normal and abnormal semen production in the domestic turkey.

Herpesviruses and Latency

Latent infections may be defined as persistent infections in which virus cannot be demonstrated by conventional means of viral cultivation between recurrent episodes of disease in the host. Herpesviruses, especially the herpes simplex viruses (HSV-1, HSV-2) have been extensively studied as classical examples of disease processes resulting from the recrudescence of latent virus. Within the last decade direct evidence has been obtained to support earlier theories that herpes simplex viruses reside latently in nervous tissue (Baringer, 1974; Stevens, 1975; 1978). Subsequently, this concept has been reiterated for other herpesviruses such as herpes zoster, cytomegalovirus, psuedorabies and infectious bovine rhinotracheitis but evidence has also accumulated that supports latency in epithelial or lymphoid tissues (Pagano, 1975; Stevens, 1978). This section will review the behavior of herpes simplex viruses in nervous tissue, since this aspect has been most thoroughly studied and has provided the groundwork for subsequent studies on latency in peripheral sites or organs. This latter concept

will also be briefly reviewed.

The indication that recurrent herpes simplex infection might be conditioned by residence of the virus in neural structures comes from a wealth of clinical and experimental observations. Interestingly, the earliest significant studies to influence the current concepts of the latent state of herpes simplex virus were reported by Goodpasture (1925; 1929). In an elegant series of experiments Goodpasture observed that inoculation of peripheral sites such as scarified cornea or masseter muscle with HSV (type unknown) resulted in intranuclear inclusion bodies in trigeminal ganglion and trigeminal motor nucleus cells, respectively. Goodpasture (1925) concluded from these experiments that HSV was capable of reaching the central nervous system via nerve trunks after inoculation at peripheral sites. This same investigator also concluded that since inclusions in the trigeminal motor nucleus preceded inclusions in the trigeminal nerve following inoculation of the masseter muscle, then virus must travel intraneurally within axons. Goodpasture (1929) suggested that herpesvirus does reside latently within nerve cells of the ganglia and that neural disturbances are frequently the basis of subsequent outbreaks. Apart from the early work of Goodpasture, clinical observations revealed that surgical manipulations of the trigeminal nerve, especially trigeminal root rhizotomy, frequently resulted in eruptions of oral herpetic vesicles (Carton, 1953; Ellison et al., 1959). In a series of 64 patients subjected to trigeminal root section

for treatment of trigeminal neuralgia, herpes simplex lesions were observed within the mouth of 60 in the period immediately following the surgery. Although these investigators further noted that herpetic vesicles were not found or induced in innervated skin, they concluded that virus latent within the skin was triggered to activity by section of the root. This conclusion, however, was inconsistent with earlier studies reported by Stadler and Zurukzoglu, 1936. These researchers suggested that latent HSV was harbored at sites other than the skin since transplantation of facial skin from a patient with recurrent herpes labialis to other sites on the body resulted in the recurrence of lesions at the site from which skin was removed and not at the transplant site. The possibility that virus might remain in the skin was also carefully explored by Rustigian et al., 1966, who maintained skin biopsies (removed between eruptions) from patients with recurrent herpetic lesions in vitro as explant cultures up to 5 months without isolating virus. These results are of special interest since the first direct evidence of latent herpes simplex infection in spinal ganglia was established by in vitro explantation of ganglia from mice previously infected into the rear footpad (Stevens and Cook, 1971).

Intradermal inoculation of HSV-1 or HSV-2 into the rear footpad of mice results in a paralysis of the ipsilateral leg at 7 days post-inoculation (Stevens and Cook, 1971; 1973). In the acute infection virus travels centripetally in the

nervous system of these mice from foot to sciatic nerve, sacrosciatic ganglia, dorsal roots, spinal cord and finally to the brain (Cook and Stevens, 1973). Infectious virus is readily recovered by conventional virus isolation from all these tissues during the acute period. In the sacrosciatic ganglia infectious virus first appeared at 2 days after infection, peaked at 4 days and disappeared by 7 to 8 days. The detection of the appearance of viral antigens by immunofluorescence methods and of viral particles by electron microscopy correlate closely with the assays for infectious virus. Virus-specific fluorescence was observed in nerve trunks (cell type unknown), neurons and satellite cells of sacrosciatic spinal ganglia and unidentified cells in the dorsal roots. Ultrastructurally, only the neurons appeared to support complete viral replication as exemplified by the presence of enveloped viral particles in the cytoplasm. Satellite cells and Schwann cells produced numerous nucleocapsids but few enveloped particles. Fibroblasts and lymphocytes were only rarely observed to contain herpesviruses. These results suggested that neurons are more likely to support viral replication than the supporting and satellite cells. Finally, Cook and Stevens (1973) reported that in all cases, infectious virus, viral antigens and viral particles were not detectable by conventional techniques of viral isolation, immunofluorescence or electron microscopy in sacrosciatic spinal ganglia beyond 8 days after infection.

All mice, which survive the acute infection and recover from the posterior paralysis, were found to harbor latent virus in the sciatic spinal ganglia (Stevens and Cook, 1971; 1973). The secret to demonstrating latent virus was to explant and maintain ganglia in vitro as organ cultures either on collagen-coated coverslips or monolayers of indicator cells. Reactivated virus was then detected either by plating supernatant fluid from coverslip cultures onto susceptible cells or by scoring cytopathic effects on the susceptible cell monolayers cocultivated with ganglia. Infectious virus or viral specific products were not, however, detected in the sciatic spinal ganglia at the time of explantation. Using the explantation technique, Stevens and Cook, 1971 observed that latent virus was reactivated in spinal ganglia between 5 to 11 days post-explantation (PE) and that mice harbored latent virus in the sciatic ganglia for at least 4 months after infection. In addition, viral antigens and ultrastructurally complete virus was detected in ganglion explants as early as 4 days PE.

These same investigators (Stevens et al., 1972) also demonstrated latent virus in the trigeminal ganglia of rabbits inoculated on the cornea with HSV-1. Rabbits were found to harbor latent herpesvirus for at least 6 months after infection. Supernatant fluids harvested from explant cultures revealed a requirement of 1 to 3 weeks PE before latent virus was reactivated. As in the mouse system, viral assays, immunofluorescence, and electron microscopy

failed to detect virus, viral antigens and viral particles at the time of explantation. This study was extended by Nesburn et al., 1972, who demonstrated latent HSV-1 in 70% of rabbit trigeminal ganglia up to 9.5 months after corneal infections. Thus, the initial experiments of Stevens and his collaborators (1971; 1972) have provided direct evidence that spinal ganglia harbor latent herpesviruses. They have also established an operational definition of herpesvirus latency: 1) at the time of explantation, infectious virus, viral antigens and virions should not be demonstrable by conventional viral isolation, immunofluoresence or electron microscopy techniques and 2) only after the establishment and maintenance of organ pieces in vitro for some period of time can infectious virus, viral antigens and virions be demonstated by the conventional virologic techniques. Experiments of this general design have been successfully repeated by several laboratories for sensory ganglia in many species. Thus, latent infections have been established in trigeminal ganglia of mice (Walz et al., 1974; Nesburn et al., 1977); the trigeminal, cervical, and lumbosacral ganglia of mice after lip, ear and vaginal inoculation, respectively (Walz et al., 1974); the lumbosacral spinal ganglia of guinea pigs (Scriba, 1975); the gasserian ganglia of pigs inoculated with pseudorabies virus (Sabo and Rajcani, 1976) and calves infected with infectious bovine rhinotraceitis (Davies and Duncan, 1974; Narita et al., 1978). A number of workers have also succeeded in recovering herpes

simplex viruses from the human trigeminal (Bastian et al., 1972; Baringer and Swoveland, 1973; Plummer, 1973) and lumbosacral ganglia (Baringer, 1974). Baringer (1975) has succeeded in reactivating latent HSV from approximately 40% of all trigeminal ganglia explanted from some 60 cadavers. In general, typing of virus isolates from the trigeminal ganglia have uniformly been of the type 1 strain, commonly associated with recurrent oral lesions. Latent herpes simplex virus, type 2 or the genital strain, has been recovered from the 3rd and 4th sacral ganglia which innervate the genitalia in man (Baringer, 1974). Thus, these results support the concept that sensory ganglia subserving the peripheral sites infected by HSV-1 or HSV-2 become latently infected. Latency with HSV-1 is not restricted to sensory ganglia, however, since several investigators have recovered latent HSV-1 from the superior cervical ganglia of the autonomic nervous system in mice (Price and Schmitz, 1979) and humans (Warren et al., 1978). Thus, latent HSV-1 in the superior cervical ganglia may be a potential source of infection for undetermined organ sites innervated by these nerves.

The range of tissues capable of supporting latent HSV infections appears to be limited to nervous tissue especially in the murine system described by Stevens and Cook (1971; 1973). In a comprehensive study, Cook and Stevens (1976) inoculated mice intravenously with HSV-1 and at intervals of 19 to 109 days after infection, tissues of interest were

explanted and maintained with monolayers of virus-susceptible cells. Tissues examined included the trigeminal ganglia, cervical, thoracic, lumbar and sacral spinal ganglia, bone marrow, lymph nodes, spleen, kidney, lung, liver, adrenal gland, spinal cord, posterior and anterior brain. Latent virus was most frequently found in the cervical and thoracic spinal ganglia, the other nervous tissue, except for the trigeminal ganglia, and the adrenal gland. Similarly, Nesburn et al., 1972 was unable to recover latent virus from explants of conjunctiva, lacrimal glands, cornea, iris, fifth nerve and brains of rabbits with latent virus in the trigeminal ganglia. In other systems latent herpesviruses, however, are not restricted to neural tissues. Thus, HSV-1 has been recovered from explants of guinea pig footpads (Scriba, 1977); tonsils, cervical lymph nodes and nasal mucosa of pigs infected with pseudorabies (Sabo and Rajcani, 1976); spleen, kidneys (Wise et al., 1979), salivary glands and prostate explant cultures (Cheung and Lang, 1977) from mice infected with cytomegalovirus and vaginouterine tissue of mice inoculated intravaginally with HSV-1 or HSV-2 (Walz et al., 1977). Although specific cell types which harbor the latent virus in the various foregoing tissues have not been identified it is possible that reactivated local virus may be responsible for symtomatic recurrences of disease. Whether latent virus located either in the peripheral organs and/or their associated ganglia is responsible for any of the manifestations of disease remains to be established.

One of the most perplexing questions which concern the pathogenesis of recurrent herpetic disease in man and animals is the manner in which virus may remain latent. Roizman (1965) has postulated that the virus may be restricted to a "static state" in which the virus exists in a nonreplicating form (extrachromosomal or integrated into cellular DNA) or may exist in a "dynamic state" in which virus is maintained by continued replication of a constant amount of virus by a few cells. Extension of the dynamic state infection would be inhibited by the humoral or cellular host defense mechanisms. Theoretically, it should be possible to discriminate between these 2 hypotheses, since the dynamic state would allow for demonstration of infectious virus whereas the static state would not. The current evidence obtained from the foregoing studies supports the static state hypothesis. This is based on the absence of infectious virus, viral antigens and virions in the tissue at the time of explantation as previously discussed. Cook et al., 1974 have also observed that neurons in ganglia explanted into syngeneic mice are found to contain viral DNA by in situ hybridization after 3 to 4 days post-explantation. The physical state of the viral genome in latently infected ganglia is unknown. Current evidence from lymphoblastoid cell lines of Epstein-Barr Virus (EBV) and Marek's Disease Virus (MDV) supports the concept that the viral DNA exists as an extrachromosomal plasmid, although linear segments of viral DNA covalently linked to host DNA have also been described (Pagano, 1975;

Kaschka-Dierich, 1979). Pagano (1975) has suggested that genomes which subsist as plasmids may be the basis for reactivation while those covalently linked to the host cell DNA may be responsible for oncogenesis. The existence of plasmid DNA in neurons seems logical since integration of viral DNA into host cell DNA usually requires DNA synthesis which is assumed not to occur in neurons (Stevens, 1978). Whether the herpesvirus DNA may be integrated into other tissues such as the vaginouterine tissue remains to be determined.

There is also evidence for the dynamic state hypothesis since HSV-1 can be isolated from oral, nasal and ocular secretions of clinically quiescent individuals (Docherty and Chopan, 1974). In addition virions have been identified in serial sections of a rare neuron examined at the time of explantation (Baringer, 1974; Rajcani et al., 1977). Stevens (1975; 1978), however, argues that these latter observations could equally denote in vivo reactivation of latent virus. Both of Roizman's hypotheses may be operative as exemplified by the results of Adldinger et al., 1974. These investigators concluded that their failure to detect viral antigens in testis cells coupled with a low rate of virus recovery from the semen would be consistent with the dynamic state hypothesis since only a few testes cells in vivo would have to be productively infected. However, the requirement for cocultivation of testis cells as a stimulus for plaque formation and the absence of infectious virus,

viral antigens and virions in the testes of males indicated that testes cells *in vivo* were latently infected. This latter observation supports the static state hypothesis although results from explantation experiments similar to those of Stevens and Cook (1971; 1973) are needed to confirm this.

The question now arises as to how the latent infection is maintained *in vivo*. One explanation is that an intracellular repressor exists and that such a repressor is subsequently lost when the tissue is explanted *in vitro* (Stevens, 1978). Current evidence, however, points to a role for anti-viral IgG; this concept is supported by evidence from Stevens and Cook (1974) that viral DNA synthesis is inhibited in latently infected ganglia transplanted into mice passively or actively immunized against HSV-1 but not in mice given nonimmune IgG. Similarly, *in vitro* studies have shown that explanted rabbit ganglia latently infected with HSV-1 had reduced HSV expression in the presence of antibody (Rajcani *et al.*, 1976). Babiuk and Rouse (1979) have postulated that IgG may bind to viral membrane antigen in latently infected cells resulting in repression of the viral genome or capping of viral antigens.

Turkey Herpesviruses

Several isolates of turkey herpesviruses (HVT) have been described (Kawamura *et al.*, 1969; Witter *et al.*, 1970b; Colwell *et al.*, 1973). During routine preparation of turkey kidney cell (TKC) cultures from apparently healthy turkeys,

Kawamura et al., 1969 isolated a virus, which produced small clear plaques on TKC cultures and formed pocks on the chorio-allantoic membrane of chick embryos. The properties of this virus (isolate WTHV-1) included a strongly cell-associated infectivity; syncytial type cytopathology; type A intranuclear inclusions, DNA virus as suggested by 5-bromo-2-deoxyuridine inhibition of plaque formation and the presence of herpes-virus type particles in the nucleus of infected TK cells as revealed by electron microscopy. Antibodies to the WTHV-1 isolate were also demonstrated in the serums of infected turkeys by the agar gel precipitin and indirect immunofluor-escence tests. Subsequently, the WTHV-1 isolate was found to be serologically identical to another HVT isolate, FC 126, which was isolated from the blood and kidneys of turkeys during investigations on the prevalence of Marek's disease virus (MDV) in turkeys (Witter et al., 1970a).

The FC 126 isolate of HVT was also found to be serolog-ically related to MDV by immunodiffusion, immunofluorescence and serum neutralization tests but unlike MDV, the FC 126 isolate was not pathogenic for either chickens or turkeys as determined by the lack of gross or microscopic lesions up to 8 weeks after inoculation (Witter, 1972). However, the recovery of virus and the detection of precipitating antibody indicated that both hosts were susceptible to infection. Recent studies have characterized a common antigen between HVT and MDV (Onuma et al., 1974). This antigen is detected by precipitation with antibody and is a glycoprotein, which

may be associated with the envelope of the virus. This is strongly supported by the observations that 1) the common antigen can be detected by immunofluorescence in the cytoplasm and on the cell membrane of HVT and MDV infected cells and 2) immunization of chickens with common antigens derived from HVT and MDV induces antibodies that neutralize both viruses (Onuma et al., 1975). Vaccination of chickens with the HVT isolate FC 126 offers excellent protection under both experimental and field conditions against tumor formation as a result of superinfection with MDV (Okazaki et al., 1970a). This represents the only system in which a vaccine is available for the prevention of tumors. At the time of its approval as a vaccine against MDV, the FC 126 isolate was believed to be apathogenic for both turkeys and chickens. However, FC 126 virus was recently found to induce transient lymphoproliferative lesions in the nerves and gonads (Witter et al., 1976) of chickens. These lesions were most prominent on the 12th day after inoculation and contain small lymphocytes and immature lymphoblastoid cells that appeared to be neoplastic. Thus, it appears that HVT has limited oncogenic potential at least in the chicken.

Although HVT is of extreme economic importance as an anti-MDV vaccine, the significance of this virus in its natural host remains unknown. Few comprehensive studies on the transmission and in vivo replication of HVT in turkeys have been reported probably because there is no known pathology induced by this virus in its natural host. Thus, much of

the information on the virus-host relationships of HVT in the turkey has been extrapolated from studies on the transmission and pathogenesis of MDV, since both viruses are closely related antigenically. Thus, a brief summary of the pathogenesis of MDV appears warranted.

Marek's disease virus can be isolated from chickens in infected flocks at 9 to 16 days after hatching (Witter et al., 1970a). There is no evidence for vertical transmission (Solomon et al., 1970) but the virus can be transmitted horizontally with materials derived from feathers, skin, dander and dust from poultry houses (Calnek et al., 1970; Beasley et al., 1970; Nazerian and Witter, 1970). Birds are infected by inhalation of infected feather dust and viral antigens can be detected in the lung as early as 24 hours post-exposure (Adldinger and Calnek, 1973). Within the first week after infection substantial virus replication occurs in the major lymphoid organs (spleen, bursa, thymus) resulting in a persistent viremia (Adldinger and Calnek, 1973; Payne and Rennie, 1973). Subsequently, the virus is disseminated to other tissues via bloodstream and MDV antigens or cell-associated infectivity can be demonstrated in nervous tissue, proventriculus, kidney and thyroid gland at 7 to 10 days post-exposure and in the skin, liver, gonads, skeletal muscle, heart and adrenal gland by 14-35 days post-exposure.

Mature, enveloped viral particles are observed ultrastructurally in the feather follice epithelium but only

rarely in tissues such as bursa, gonads or brachial nerve plexus in which naked or incomplete particles are found (Calnek et al., 1970). Relatively high titers of cell-free virus was present in the feather follicle epithelium within 2 to 3 weeks post-exposure and infectious virus is apparently shed into the environment via necrobiosis of feather follicle epithelium. This results in infection of susceptible birds by contact and inhalation of infectious feather dust and dander. Chickens have been found to shed infectious virus for at least 18 months PI (Witter et al., 1971).

Turkey herpesvirus is ubiquitous among commercial turkey flocks (Kawamura et al., 1969; Witter et al., 1970b; Witter and Solomon, 1971). Like MDV in chickens, there is no evidence of vertical transmission of HVT (Witter and Solomon, 1971; Paul et al., 1972; Witter, 1972) but virus is transmitted horizontally via infectious dander, feathers and dust as sentinel turkey poults placed in cages contaminated by HVT infected turkeys converted serologically and virus was isolated from buffy coat cells (Witter and Solomon, 1971). In commercial turkey flocks, virus can be detected as early as 5 weeks after hatching with the delay in the onset of viremia contributing to the presence of maternal antibody, which persists for approximately 3 weeks after hatching (Witter and Solomon, 1971). The virus then spreads rapidly throughout the flock as both virus and antibody were present in all birds examined 7 to 10 weeks after hatching and persisted in buffy coat cells of 90%, 93%, and 43% of the birds

in three flocks examined at 1 year after hatching (Witter and Solomon, 1971).

Presumably the respiratory tract is the natural portal of entry for HVT but studies on the early replication of HVT comparable to those described by Adldinger and Calnek, 1973 have not been done. Experimentally infected turkey poults have cell-associated infectivity in the buffy coat cells as early as 4 to 8 days after intra-abdominal inoculation of HVT (Witter and Solomon, 1972) and the buffy coat cells remain persistently infected for at least one year (Witter, 1972). There is only one published report on *in vivo* replication of HVT in the turkey. Witter *et al.*, 1972 were able to isolate cell-associated virus from 92% of the buffy coat, 70% spleen, 83% bursa and 40% of the kidney samples from turkey poults killed at 2 to 10 weeks post-inoculation. In contrast, cell-free HVT was demonstrated only in preparations of skin and the tips of feather quills. Quill tip preparations from 4/9 birds contained enveloped herpesvirus particles and preliminary evidence indicated that the virus was replicated in association with the feather follicle epithelium. Attempts to localize the site of viral replication in the tissues and in the feather follicle by immunofluorescence were unsuccessful. Antigens also could not be detected in quill tip preparations by use of immunoprecipitin tests. Virus is known to be shed by infected turkey poults prior to 16 days after infection (Witter, 1972). The foregoing data have pointed out substantial similarities in the mode

of transmission and *in vivo* replication of HVT and MDV in their homologous host. However, quantitative differences exist as exemplified by the inability to demonstrate viral antigens in the tissues and integument of HVT-infected turkeys. Likewise there is no evidence that HVT is pathogenic or oncogenic for the turkey (Witter, 1972).

Recently, Adldinger *et al.* (1974) reported the isolation of herpesviruses from the semen, buffy coat cells, testes and kidney of males afflicted with the so-called yellow semen syndrome which results in reduced infectivity. *In vitro* characteristics of these herpesvirus isolates were compatible with those previously described (Kawamura et al., 1969; Witter *et al.*, 1970b) and conformed to those characteristics of the cell-associated herpesviruses.

Normal and Abnormal Semen Production in the Domestic Turkey

Emphasis on the production of large-meat turkeys has reduced the ability of the birds to mate naturally. Therefore, most turkey hens in commercial breeder operations are artificially inseminated. The domestic turkey is a seasonal breeder with a reproductive cycle usually lasting 6 to 8 months. Turkey males begin producing semen from 132 to 291 days after hatching with the average of maturity (near 246 days) influenced by inherited traits and up to 50 days by the hatching date (Carson *et al.*, 1955a, b). Average volumes of semen produced and the concentration of spermatozoa can vary from less than 0.1 ml to greater than 0.5 ml and 5.2

to 16.7 x 10^6 spermatozoa/mm^3 (reviewed by Thurston, 1976). In general, the volumes and quality of semen are highest during the spring (March-May) and lowest during the winter months (Parker et al., 1946; Carson et al., 1955a, c). These seasonal changes in semen volume and quality were also noted by others (Law and Kosin, 1958; Payne et al., 1960; Harper and Arscott, 1969) and may result from variations in lighting, temperature, age of birds and undetermined environmental factors (Nestor and Brown, 1971).

Testicular development and spermatogenesis are most significantly influenced in avian species by the amount of environmental light. At least 14 hours of stimulatory lighting are required to induce maximum semen production in 7 to 9-month-old turkey toms (Parker, 1946; McCartney et al., 1961; Marsden et al., 1962) with semen volume and sperm concentration optimal at approximately 7 to 14 weeks post-lighting (McCartney, 1956; Marsden et al., 1962; Ogasawara et al., 1962; Nestor and Brown, 1971). Currently, most commercial producers initiate 28-week-old toms on a 10-hour dark, 14-hour light diurnal cycle at least 2 to 3 weeks prior to the time in which hens are exposed to similar lighting (Lorenz, 1970).

Changes in environmental temperature also influences semen production as indicated by the reduced productivity of males during the summer months (Carson et al., 1955a, c; Kamar and Rizik, 1972). Experimentally, turkeys yielded greater semen volumes and sperm concentrations when housed

at variable temperature ranges from -1 to 33 C or a constant 13 C than when subjected to a constant environmental temperature of 24 C (Jones et al., 1971).

Age of the bird and the frequency of semen collections also effect semen production. Semen production decreases after 5 months of sexual activity in 2-year-olds but not younger toms (Harper and Arscott, 1969). Semen production (volume and sperm concentrations) also decreases if birds are sampled at less than weekly intervals (Lorenz, 1956) or more often than 3 to 4-day intervals (Nestor and Brown, 1971).

In a January-to-June breeding season fertility remains constant through April, declining in May and June (Parker, 1947; Olsen and Marsden, 1956; Harper and Arscott, 1969). Artificial insemination has been shown to improve late season fertility but does not prevent seasonal fertility declines (Stotts and Darrow, 1955).

The cause of late season infertility and reduced hatchability of eggs remains an enigma. Strong evidence implicates poor semen quality, particularly yellow turkey semen observed to contain various abnormal cells, as the cause of reduced reproductive performance in turkeys (Saeki and Brown, 1962; Cherms, 1968; Thurston et al., 1975). Saeki and Brown (1962) were the first to associate yellow semen with the presence of semen abnormalities, which consisted of an increased number of crooked-necked spermatozoa in yellow compared to white semen. Similar findings were recently reported by Marquez

and Ogasawara (1975) but these investigators also observed increased numbers of macrophages in yellow semen samples. Fertility in hens bred to yellow semen males has been reported to range from 10 to 35% lower than fertility rates obtained with white semen (Saeki and Brown, 1962; Cherms, 1968; Marquez and Ogasawara, 1975).

The most complete and significantly different characterization of yellow semen abnormalities has been reported by Thurston et al., 1975. In a study of 210 males, these investigators found 28/210 (13.3%) males screened to have dark pigmented yellow semen. At the light microscope level normal seminal cells were identified as properly formed spermatozoa and large rounded macrophages, whereas abnormal cells were classified as spermatozoa with bent or bulbous heads, large rounded cells, and smaller ellipsoidal cells, which appeared flagellated. The significance of these results was that the predominant abnormal cell type in yellow semen was the large, rounded cell, rather than the previously described spermatozoon abnormalities. Macrophages were also present in 10-fold higher numbers in yellow as compared to white semen (Thurston et al., 1975).

Thurston et al., 1975 also described the ultrastructural macrophology of the large, rounded cells, the smaller ellipsoidal cells and macrophages. A summary of the ultrastructural findings are presented in Table 1. The Type I cells were the most numerous abnormal cell type observed in yellow semen, whereas the Type II cells were observed in samples

Table 1. Classification and Characteristics of Abnormal Cells in Turkey Semen (Thurston et al., 1975).

Type Classification	Light Microscope Morphology	Electron Microscopy Characteristics	
		Size	Morphology
I	Large, round	>8um	Pleomorphic; round. Organelles similar to those found in the early stages of domestic fowl spermatids. Cytoplasmic organelles randomly structured. Nuclear chromatin partly or completely condensed. Mitochondria cristae parallel to outer mitochondrial membrane.
II	Small, ellipsoidal	>6um	Ultrastructure of each cell unique. Elongated. Highly condensed nuclear chromatin. Complete 9+2 axial filaments typical of sperm flagellae. Other sperm structures such as formed acrosomes and midpieces. Mitochondria and other cellular organelles were randomly dispersed.
Macrophages	Large, rounded vacuolated Polymorphous nuclei	>10um	Pleomorphic. Polymorphous nuclei. Immense cytoplasmic vacuolation. Often contain sperm organelles and partly degraded Type II cells.

of both white and yellow semen. Seminal macrophages, which were differentiated on the basis of their immense cytoplasmic vacuolation, were present in increased numbers in yellow semen. On the basis of the ultrastructural morphology of these cells the authors concluded that the Type I and Type II abnormalities originated in the seminiferous tubules and were malformed early and late spermatids, respectively. This conclusion was verified in subsequent electron microscope studies of testicular tissues in which the Type I abnormal cells were observed in proximity to early spermatids, whereas the Type II abnormal cells were located with highly differentiated or late spermatids (Thurston, 1976). Thus, the abnormal seminal cells in yellow semen were indeed early and late spermatids, which had developed within the testes. On the basis of this ultrastructural evidence Thurston (1976) expanded the definition of the yellow semen syndrome to include the production of yellow pigmented semen which contains numerous abnormal spermatids and often macrophages in addition to abnormal spermatozoa.

The etiology of the spermatid aberrations found in yellow semen seemed unrelated to genetic or nutritional factors but could possibly be related to either hormonal imbalances, or virus, which somehow disrupts the mechanisms directing spermiogenesis. The first possibility was eliminated since yellow semen males showed no regression of secondary sexual characteristics but had the feather pattern and wattle color of sexually active males. Support for a

viral etiology of yellow semen was strengthened during the course of Thurston's studies, when Adldinger et al. (1974) isolated herpesviruses from the semen and testes of white and yellow semen males. These herpesviruses were isolated more frequently from the semen, buffy coat and kidneys of turkeys with the yellow semen syndrome. Testis tissue, however, was the most consistent source of herpesvirus and in 2 birds proved to be the only source of virus. Testicular tissues from turkeys with normal semen, however, also harbored herpesviruses and the unavailability of uninfected control birds in the study by Adldinger et al. (1974) made it impossible to evaluate differences in the virus isolation data on turkeys with and without the semen abnormality.

However, these same investigators observed that precipitating antibodies reactive with the yellow semen viruses were detected only in sera or plasma of turkeys with the yellow semen syndrome. Antibodies to antigens of the FC 126 virus were present in turkey sera regardless of the semen abnormality. Thus, the apparent absence of antibody in turkeys with normal semen to the yellow semen isolates strongly suggests that the yellow semen isolates represent a previously unidentified herpesvirus of turkeys. Serological cross immunoprecipitin tests with uncloned virus isolates could not distinguish between the possibilities that the yellow semen viruses were identical to FC 126; an unreported second herpesvirus of turkeys; mixed populations of FC 126

and other herpesviruses or some were FC 126 and others were not (Adldinger et al., 1974). Elucidation of the antigenic relationship of the FC 126 and yellow semen viruses will depend on immunoelectrophoresis or serum neutralization kinetics studies with cloned virus and hyperimmune sera. Currently one can only conclude that the FC 126 virus and the yellow semen viruses may share common antigens or a common contaminant was present in the viral preparations. Antigenic heterogeneity between the FC 126 and the yellow semen viruses does exist since indirect immunofluorescence titers between the yellow semen viruses and homologous antiserum were fourfold higher than reactions with heterologous antisera to FC 126 (Adldinger et al., 1974).

The relationship between testes cells and yellow semen viruses are of particular interest. Adldinger et al. (1974) established that viral antigens could not be localized in testes tissue by conventional means of immunofluorescence and immunodiffusion and that infectious virus could not be isolated from ground extracts of testis. Infectious virus was regularly isolated from testis cultures but cultures required a subcultivation as secondary testis cells to induce plaque formation. Subsequently Thurston et al. (1975) reported that germinal epithelial cells and macrophages in secondary testicular cultures, particularly those derived from testes of yellow semen males, contained numerous herpesvirus particles. Such particles were not, however, observed in abnormal seminal cells or in any epithelial cell type lining the seminiferous

tubules of testis from yellow or white semen males (Thurston, 1976). Thus, the inability to localize viral antigens in the testis cells and the need for subcultivation of testicular cells as a stimulus to induce plaque formation suggested that either testis cells were latently infected *in vivo* or that only a few testis cells *in vivo* were productively infected. Either possibility could explain the sporadic recovery of virus from the semen whereas latent herpesvirus may possess the capacity to alter normal spermatogenesis without the requirement for viral replication. The possibility that viral antigens are expressed in the abnormal seminal cells and the site at which virus persists in the testes has not been investigated.

Statement of the Problem

It would appear from the circumstantial evidence cited by Adldinger *et al.* (1974) that a cell-associated herpesvirus is the etiological agent responsible for yellow semen abnormalities and low fertility in male turkeys. In common with most other herpesviruses, such as Marek's disease and the FC 126 herpesvirus of turkeys, it would appear that the yellow semen viral isolates infect turkeys in the field at an early age. Infected turkeys develop a persistent viremia since virus can be readily isolated from buffy coat cells and establishes itself in various tissues, such as kidney, spleen, liver, testis, and probably ganglia as a result of transport there via the bloodstream. Once the virus has established itself in the testes, it apparently enters into a quiescent

or latent state. This assumption is supported by the apparent need to cocultivate testicular cells *in vitro* in order to reactivate the virus. Reactivation of the virus *in vitro* could be the result of increased rates of semen production as the bird reaches sexual maturity or as the breeding season approaches. Direct proof of a latent state *in vivo* will require an approach similar to that of Stevens and Cook (1971; 1973) since they have demonstrated that a most efficient way to detect the presence of latent herpesvirus is to culture *in vitro* the cells (in our case testicular and ganglion cells) containing repressed virus. Infectious virus is then induced and replicated. To summarize, our hypothesis is that the causal agent of yellow semen abnormalities and low fertility in male turkeys is a cell-associated herpesvirus which establishes residence in the testes of male turkeys at an early age; enters into a quiescent or latent state either in the testes or in spinal ganglia which innervates the testis and can be reactivated as males reach sexual maturity thereby interfering with normal spermatogenesis.

Specifically, the purpose of this study was 1) to demonstrate latent herpesvirus in gonadal cells and/or in spinal ganglia by establishing and maintaining explant cultures *in vitro*; 2) to examine sites of *in vitro* replication of turkey herpesviruses; and 3) to reproduce abnormal spermatogenesis and yellow semen in male turkeys infected with cloned viral isolates.

Chapter II

MATERIALS AND METHODS

Chickens

Sixteen to eighteen-day embryonated eggs were obtained from Ken-Roy Hatchery, Berger, Missouri and hatched in an isolated environment. Chickens were of white leghorn breed and two to twenty-four weeks of age when used for the preparation of primary chick kidney cells. Birds were housed in brooders and maintained on a standard commercial diet.

Turkeys

One hundred and twenty, 24-day embryonated eggs were obtained from Cargill Poultry Products, Inc., California, Missouri and hatched in isolation. A total of 100 poults of the Nicholas strain were hatched and individual groups of 25 poults each were maintained in brooders on a standard commercial diet until one week of age. Nine of the 100 poults died within one week after hatching and 6 weaker poults were used to assay buffy coat cells for virus and plasma for maternal antibody to herpesvirus of turkeys (HVT). All 6 poults were virus and antibody negative.

The remaining 84 poults were proportioned into four experimental groups and inoculated as described in the section entitled Experimental Design in this chapter. After inoculation each of the four groups of poults was housed and

maintained in different confinements at different phases of the experiments as follows:

1. Age 1 to 15 weeks. Each group of poults was housed separately in brooders set on raised wooden platform flooring in 6 ft x 8 ft steel buildings at the Sinclair Research Farm. Birds were fed a commercial turkey diet and water ad libitum.
2. Female turkeys 15 to 40 weeks. Each experimental group of females was transported to the Veterinary Medicine Research Farm on separate days to avoid cross contamination between groups. Each group was housed separately in 8 ft x 8 ft wooden houses. Straw was provided as bedding and birds were fed a commercial turkey ration and water ad libitum.
3. Male turkeys 15 to 40 weeks. Male turkeys were maintained in the steel buildings as described. Straw or wood shavings were provided as bedding over the wooden floor. All males were started on a 14-hr light, 10-hr dark diurnal cycle at 24 weeks of age (April 3, 1978). Males were maintained on artificial lighting until June 1, 1978 after which time they were exposed only to natural day-night cycles and allowed access outdoors to 6 ft x 8 ft wire runs.
4. Male turkeys 40 to 55 weeks. Each group of males were housed in separate 10 ft x 10 ft concrete floored pens at the Veterinary Medicine Research Farm. On September 1, 1978 artificial lighting on a 14-hr light, 10-hr dark

cycle was again initiated and birds were maintained on this cycle until the end of the experiments on November 11, 1978.

5. Rocheford Farm Turkeys. Four Large White breeder males, which were naturally afflicted with yellow semen abnormalities, were obtained at the end of a 6-month breeding season. Two males (#3118, 8019) were obtained in May, 1977 and in May, 1978 (#9028, 9071). All males were one year old when killed for virus isolation and latency studies.

Culture Media Components

Distilled, Deionized Water (DDW)

Incoming raw water was softened, glass distilled in a glass water still (Bellco Glass Inc., Vineland, NJ) and deionized by passage through a Barnstead High Capacity Filter (Sybron Corp., Boston, MA). This DDW was dispensed in 450 ml amounts in 500 ml serum bottles, autoclaved at 15 psi for 30 minutes and stored at ambient room temperature up to two weeks.

Media Stock

Minimum essential medium (MEM) (Microbiological Associates, Bethesda, MD) with either Earle's (MEM-E) or Hank's (MEM-H) balanced salt solution was prepared as 10x concentrated stocks in DDW. Media were filter-sterilized using a 0.22 um Millipore filter (Type GS) and a 47 mm all-glass filter apparatus (Millipore Corp., Bedford, MA). Sterile media were aseptically dispensed in 50 ml amounts into two-ounce

glass prescription bottles and stored at 4 C. Media were sterility tested by random selection of five 50 ml bottles and inoculation of 1 ml of the contents of each bottle into 4 ml of sterile thioglycollate broth (BBL, Cockeysville, MD). Sterility test tubes were incubated at 37 C for two weeks.

Fetal Bovine Serum, FBS

Fetal bovine serum was obtained in 500 ml amounts from Flow Laboratories, Rockville, MD and stored at -20 C. The stock FBS was thawed at 37 C, aseptically dispensed in 20 ml amounts into two-ounce glass prescription bottles, and sterility tested as described for the media stock. Serum was stored at -20 C until used.

Penicillin-Streptomycin, 5×10^4 units-ug/ml

One vial of 5×10^6 units of buffered sodium penicillin G (E.R. Squibb and Sons, Inc., NY) and one 5 gram vial of streptomycin sulfate (Pfizer Laboratories, NY) were dissolved per 100 ml of sterile DDW. The solution was then filtered through a 0.22 um disposable filter (#7103, Falcon Plastics, Oxnard, CA); aseptically dispensed in 2 ml aliquots into 12 x 75 mm plastic tubes with caps (#2054, Falcon Plastics, Oxnard, CA) and stored at -20 C for an indefinite period. Two ml of penicillin-streptomycin were added per 500 ml of media to give a concentration of 200 units-ug/ml.

Fungizone (Amphotericin B), 625 ug/ml

The contents of one vial of 50 mg Fungizone Intravenous (Amphotericin B, E.R. Squibb and Sons, Inc., NY) were dissolved in 80 ml of sterile DDW. The solution was then

filter sterilized, dispensed and stored as described for the penicillin-streptomycin mixture. Two ml of Amphotericin B were added per 500 ml of media to give a concentration of 2.5 ug/ml.

Sodium bicarbonate

A 10% stock of sodium bicarbonate $NaHCO_3$ was prepared by dissolving 30 grams of anhydrous powder in 300 ml of DDW. The solution was dispensed in 5 ml amounts into 13 x 100 mm glass tubes with screw caps and autoclaved at 15 psi for 30 minutes. Sterile solutions were sterility tested as per media stock and stored at ambient room temperature.

Complete Media

Growth media used for the growth of cell cultures in these studies was comprised of the following components:

10x MEM-H or MEM-E	50 ml
Fetal bovine serum (4% v/v)	20 ml
Sodium bicarbonate, 10%	
if MEM-H	1 ml
if MEM-E	2 ml
Penicillin-Streptomycin	2 ml
Fungizone	2 ml
Sterile DDW	450 ml

Maintenance media consisted of MEM-E prepared as above, except that FBS was reduced to a concentration of 2% (v/v) or 1% (v/v) per 500 ml of medium dependent on the confluency of cell monolayers. Complete media were stored at 4 C for up to two weeks.

Freezing medium for the cryopreservation of cell-associated virus inoculum was MEM-E supplemented with 15% (v/v) FBS and 10% (v/v) dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO). This medium was filter sterilized using a .45 um disposable filter (#7102, Falcon Plastics, Oxnard, CA) and stored at 4 C for indefinite periods.

Calcium-Magnesium Free Phosphate Buffered Saline, (CMF 10x)

Ten times concentrated stocks of CMF were prepared as outlined (USDA/ARS Agricultural Handbook N. 404, 1971):

NaCl	80.0 grams
KCl	2.0 grams
KH_2PO_4	2.0 grams
Na_2HPO_4	12.0 grams
DDW	1000.0 ml
Chloroform	0.01% (v/v) as preservative

Stock solutions were stored in 400 ml amounts in 16-ounce prescription bottles at 4 C. When CMF-1x was needed, CMF-10x was diluted tenfold with distilled water, dispensed in 400 ml amounts into 16-ounce prescription bottles, autoclaved and stored at 4 C. Sterility tests of CMF 1x were performed as described in the section on media stock.

Versene, 10% EDTA

A 10% stock of versene was prepared by dissolving 30 grams of disodium ethylenediaminetetraacetate (Fisher Scientific Co., Fair Lawn, NJ) in 300 ml of distilled water. The solution was aliquoted into 4 ml amounts in 16 x 100 mm screw cap tubes, autoclaved at 15 psi for 30 minutes and

stored at ambient room temperature.

Trypsin, 2.5%

A 2.5% (w/v) solution of trypsin was prepared by adding 25 grams of Difco or Gibco 1:250 trypsin to 1000 ml of CMF-1x. The mixture was allowed to stand overnight at 4 C with continuous mixing. After this time, the trypsin was filtered through a Seitz C-5 clarifying and ST3 sterilizing filter (Republic Filter Corp., Milldale, CT) into a sterile 2000 ml flask. The solution was aseptically dispensed in 15 ml amounts into sterile screw cap glass tubes and stored at -20 C. Sterility tests were performed as described in the section on media stock.

Trypsin-Versene, (TV)

A working solution of TV was prepared by mixing .20% (v/v) of 2.5% trypsin and .05% (v/v) of 10% EDTA and the desired volume of solution obtained by adding CMF-1x. The pH was then adjusted to 7.4-7.6 by adding 10% $NaHCO_3$. Solutions were stored at 4 C for up to one week.

Preparation of Primary Chick Kidney Cells (CKC)

Primary CKC were prepared essentially as described by Churchill (1965). Donor birds were killed by cervical dislocation and disinfected with a 70% solution of ethyl alcohol. All proceeding work was done in a laminar flow hood (Pure Air Corp. of America, Van Nuys, CA). Kidneys were aseptically removed using sterile forceps and scissors into a sterile 100 ml serum bottle containing 50 ml of cold

(4 C) CMF-1x. The kidneys were briskly shaken to remove blood and loose tissue. The wash fluids were then removed and the kidneys transferred into a sterile 15 x 100 mm petri dish. Cortical and medullary tissue were teased from the inner connective tissue of the kidney. The connective tissue was discarded and the remaining kidney tissue minced into 3-5 mm^3 pieces. Tissue pieces were placed into a 100 ml serum bottle containing 50 ml CMF-1x at room temperature, shaken briefly, and the fluid aspirated. The kidney pieces were then exposed to 25 ml of prewarmed (37 C) TV and manually shaken for 3 minutes at room temperature. The tissue pieces were allowed to settle, the free cells in the supernatant were withdrawn and passed through four layers of gauze into a 500 ml beaker, which contained 2 ml of calf serum per 25 ml of trypsin solution. The beaker was maintained on ice during the entire trypsinization procedure. The required number of trypsinization cycles varied with the amount of tissue to be digested (generally, 6-8 cycles). The gauze was then aseptically removed from the beaker and the cell suspension poured into two 125 ml conical centrifuge tubes. The cells were pelleted at 240 x g (International Centrifuge, Model PR-J) for 20 minutes at 4 C. The supernatant was aspirated and the cell pellet resuspended in 10 ml of MEM-H or MEM-E growth media per 1 ml of pelleted cells.

A viable cell count was obtained using a 1/20 dilution of the cell suspension in medium and 0.5% trypan blue. Cells in 32 squares along two diagonals of a Fuchs-Rosenthal

hemacytometer were counted and the average number of cells per 16 squares was used to calculate the number of cells/ml. Cells to be used within 48 hours were seeded as follows: 1 x 10^6/3 ml for 15 x 35 mm petri dishes; 3 x 10^6/5 ml for 15 x 60 mm petri dishes; 10 x 10^6/12 ml for 15 x 100 mm petri dishes and 200 x 10^6/75 ml for 210 mm (length) x 110 mm (diameter) glass roller bottles. Except for the roller bottles, cultures were maintained at 37 C in a humidified incubator in an atmosphere of 5% CO_2. Roller bottles were maintained at 37 C in a closed system on a Rolla Cell Apparatus (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 0.5 to 0.75 rpm. Monolayers were maintained on growth media (MEM-H or MEM-E) until confluent, usually 48-72 hours. Confluent monolayers of CKC were nourished with maintenance media containing 1% or 2% (v/v) of FBS depending on the degree of confluency. Medium was changed at 3 to 4-day intervals and pH was adjusted with 2.8% $NaHCO_3$ between media changes as needed. Cell cultures were observed on a Wild M-40 inverted microscope (Wild Heerbrugg Instruments, Inc., Heerbrugg, Switzerland).

Origin of Turkey Herpesvirus Isolates

Three isolates of herpesvirus of turkeys (FC 126, TT 197 and BC 87-527) were used in these studies. These isolates were provided by Dr. H.K. Adldinger (Dept. of Veterinary Microbiology, University of Missouri-Columbia, Columbia, MO). The FC 126 isolate, which was originally obtained from Dr. R.L. Witter (U.S. Dept. Agriculture, Agricultural Research

Service, Regional Poultry Research Lab, East Lansing, MI, Witter et al., 1970), was provided as cell-associated virus at the 25th passage level in CKC. The TT 197 and BC 87-527 isolates were originally isolated by Dr. Adldinger from the testis and buffy coat of male turkeys afflicted with yellow semen (Adldinger et al., 1974). The TT 197 isolate was obtained as cell-associated virus at the 20th passage level in CKC. The BC 87-527 isolate was the second passage level in turkeys of an original BC 1084 isolate and was provided as a cell-associated virus at the 10th passage level in CKC.

Due to the unavailability of stock BC, FC and TT isolates at identical and lower (<10) passage levels in vitro, reference virus was prepared from each isolate by passage in turkey poults and reisolation of each isolate in CKC. Six commercially derived one-week-old turkey poults were obtained for this purpose from Heart of Missouri Hatchery, Columbia, MO. Heparinized blood samples were obtained from each poult at the time of purchase and at 2 weeks of age. Buffy coat cells were assayed for virus and plasmas for antibody to antigen prepared from the BC, FC and TT isolates. All poults were negative on virus isolation and seronegative for antibody. Preparation of the reference virus stocks used in these studies is schematically outlined in Figure 1.

Cloning Procedure for HVT Isolates

The FC, BC and TT isolates were cloned two times on CKC using a procedure described by Purchase et al., 1971. Duplicate monolayers of CKC seeded in 15 x 60 mm petri dishes

Figure 1. Flow diagram on the origin of turkey herpesvirus isolates and cloning

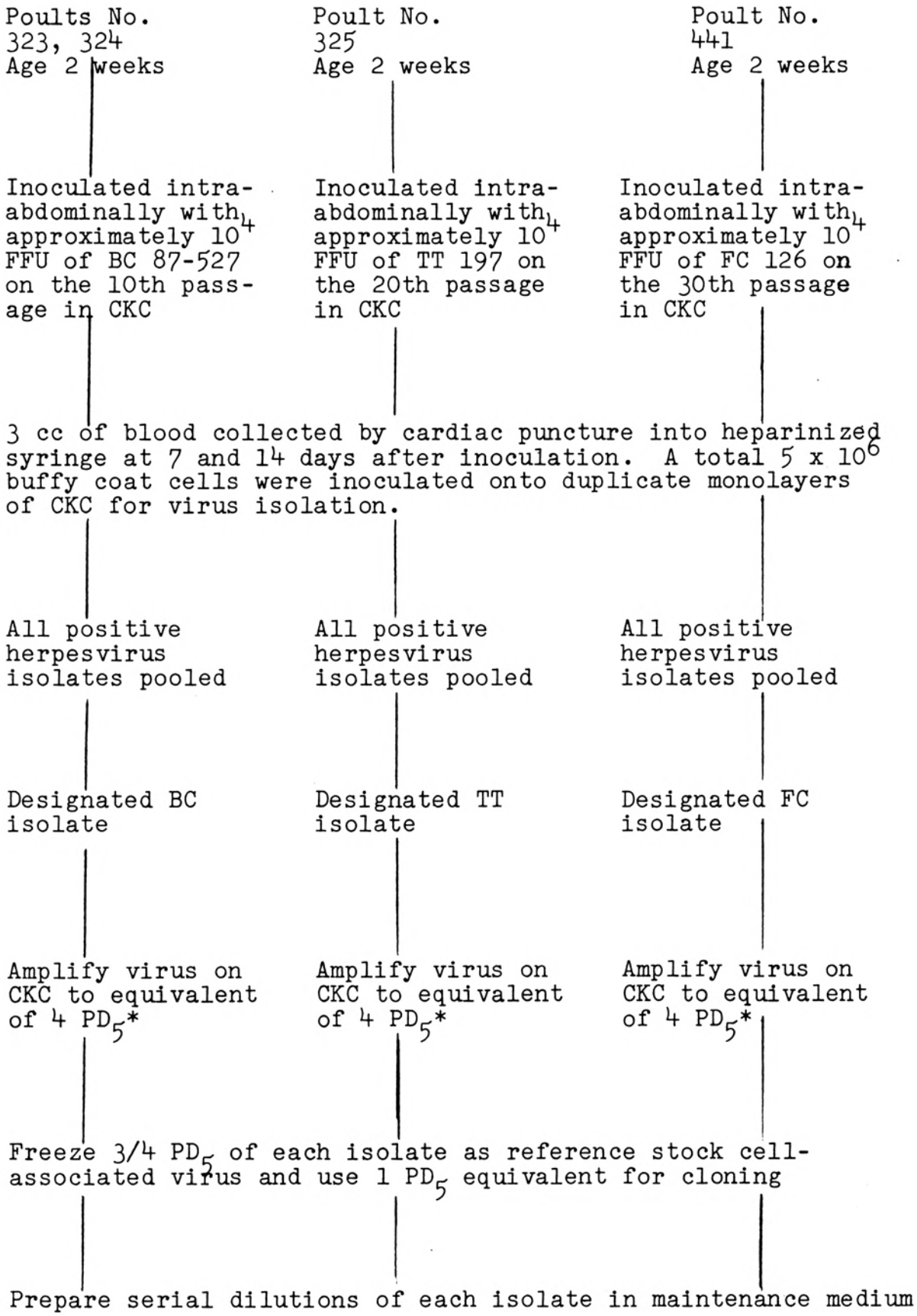
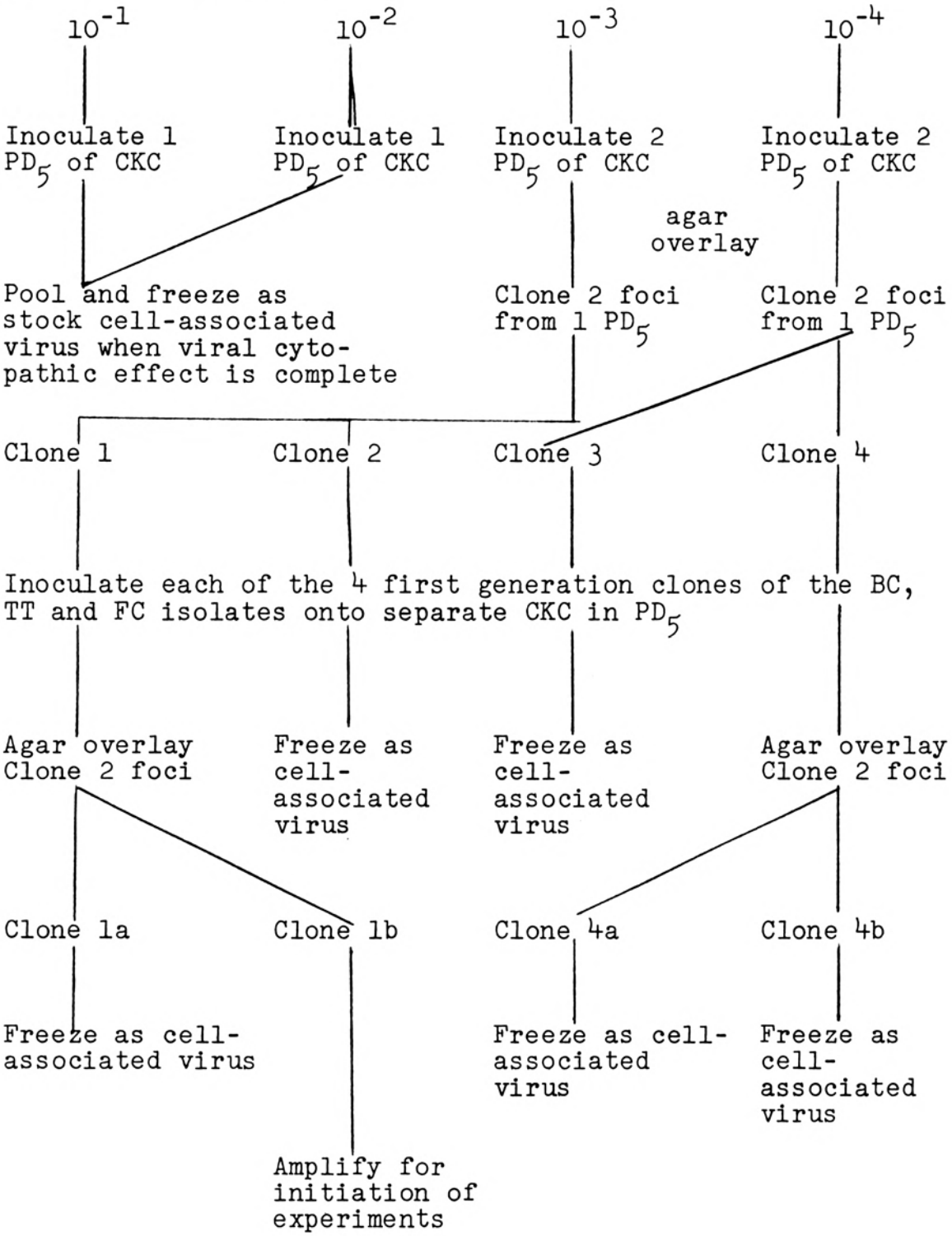

Poults No. 323, 324 Age 2 weeks
Poult No. 325 Age 2 weeks
Poult No. 441 Age 2 weeks
Inoculated intra-abdominally with approximately 10^4 FFU of BC 87-527 on the 10th passage in CKC
Inoculated intra-abdominally with approximately 10^4 FFU of TT 197 on the 20th passage in CKC
Inoculated intra-abdominally with approximately 10^4 FFU of FC 126 on the 30th passage in CKC
3 cc of blood collected by cardiac puncture into heparinized syringe at 7 and 14 days after inoculation. A total 5 x 10^6 buffy coat cells were inoculated onto duplicate monolayers of CKC for virus isolation.
All positive herpesvirus isolates pooled
All positive herpesvirus isolates pooled
All positive herpesvirus isolates pooled
Designated BC isolate
Designated TT isolate
Designated FC isolate
Amplify virus on CKC to equivalent of 4 PD_5*
Amplify virus on CKC to equivalent of 4 PD_5*
Amplify virus on CKC to equivalent of 4 PD_5*
Freeze 3/4 PD_5 of each isolate as reference stock cell-associated virus and use 1 PD_5 equivalent for cloning
Prepare serial dilutions of each isolate in maintenance medium

Figure 1 - continued



* PD_5 - a 15 x 60 mm plastic petri dish

were inoculated with 4 ml of a 10^{-3} and 10^{-4} dilution of a cell-associated inoculum of each HVT isolate in maintenance media. The inoculum was allowed to adsorb at 37 C for 24 hours. After this time, the inoculum was discarded, cultures were replenished with 4 ml of maintenance media and observed daily for viral cytopathic effects.

When foci containing 5 to 10 rounded, refractile cells appeared, the cultures were overlaid with maintenance media containing 1% agar (Difco Laboratories, Detroit, MI). Agar was allowed to solidify for at least two hours. Foci to be cloned were identified by marking the bottom of the petri dish below the focus with a felt tip marker while observing the culture with the inverted microscope. A hole approximately 5 mm in diameter was cut with a sterile scalpel and No. 11 blade through the agar above the focus. The area of the focus and a few surrounding cells were removed from the petri dish using approximately .05 ml of TV. The detached cells of each focus were aspirated into a sterile Pasteur pipette and transferred into 4 ml of cold (4 C) maintenance medium in a sterile crew-capped tube. Since some of the rounded, refractile cells adhered to the agar plug, the agar plug was also aseptically transferred to the tube containing the cells. This procedure was repeated for each focus to be cloned. Generally, two foci from the 10^{-3} and 10^{-4} dilutions of virus were cloned. Each of the four clones was inoculated onto monolayers of CKC in 15 x 60 mm petri dishes and the cloning procedure was repeated one additional time.

After the second cloning, each clone of the three isolates was propagated on CKC until a sufficient stock was obtained, usually 4 to 5 passages. The cloning procedure is schematically outlined in the latter portion of Figure 1. Stock virus clones were preserved as cell-associated inoculum in MEM-E supplemented with 10% (v/v) dimethylsulfoxide and 15% (v/v) FBS. All stock viruses were stored in sealed glass ampules in liquid nitrogen or at -120 C.

Viruses used for the propagation of cell-associated inoculum were recovered from the frozen state by rapidly thawing the contents of the ampule in a waterbath at 37 C. The cells were transferred to a conical centrifuge tube containing an equal volume of growth media and centrifuged at 240 x g for 10 minutes at 4 C. Media was aspirated and the cell pellet resuspended in the required amount of maintenance media for the amplification of cell-associated inocula.

Preparation of Cell-Associated Virus (CAV) Inocula

Cell-associated inoculum from one clone of each isolate (BC, FC and TT) was prepared by inoculation of a suspension of infected CKC onto drained, 48-hour monolayer cultures in 15 x 100 mm petri dishes. The inoculum was allowed to adsorb for 24 hours at 37 C and then replaced with 10 ml of maintenance media. Usually within 3 to 4 days after inoculation, extensive viral cytopathic effect consisting of rounded, refractile cells and polykaryocytes was observed to involve 75-90% of the inoculated monolayers. Culture fluids

containing detached, infected cells were removed and transferred into a conical centrifuge tube of appropriate volume held over ice. The remainder of the infected cells were detached from the petri dish with 5 ml of prewarmed (37 C) TV and pooled with the culture fluids. The plates were then washed with 5 ml of maintenance medium to remove additional cells and this wash medium was pooled with the cells in TV. Cell suspensions were centrifuged at 240 x g for 10 minutes at 4 C. The cell pellet was then resuspended in 10 ml of maintenance medium for each 15 x 100 mm petri dish to be inoculated. Each passage of CAV inoculum represented approximately a two to three-fold increase in the amount of virus inoculum i.e. 1 plate equals 2 or 3 plates.

An equivalent of eight, 15 x 100 mm petri dishes of CAV were used to inoculate one 110 x 210 mm roller bottle (LR) of CKC. Inoculation and maintenance of a LR was similar to that described for the propagation of CAV on 15 x 100 mm petri dishes. When CPE was complete, culture fluids were decanted into a centrifuge tube and 25 ml of prewarmed TV added to each LR. The dispersed cells in TV were pooled with the culture fluids and 25 ml of maintenance medium was added to each LR to rinse off residual cells. The wash medium was pooled with the fluid and cells. Cells were then pelleted at 240 x g for 20 minutes at 4 C. The supernatant was removed and the cell pellet was resuspended in an equal volume of freezing medium. A 1.0 ml amount of the cell suspension for titration of the CAV preparation was frozen

separately in a 2 ml glass ampule at -120 C. One-half of the volume of the cell suspension was frozen at -120 C in a 5 ml glass ampule and the remaining volume was retained for the preparation of cell-free virus inoculum. All CAV for the inoculation of turkey poults in these studies was on the 9th passage level in CKC.

Preparation of Cell-Free Virus (CFV) Inoculum

Cell-free virus inoculum was prepared using a procedure described by Calnek et al. (1970). The cell-associated inoculum remaining from the preparation of CAV was centrifuged at 240 x g for 10 minutes at 4 C. The supernatant was removed and the cell pellet was resuspended in 5 ml sucrose, sodium glutamate and bovine serum albumin (SPGA) in phosphate buffer. This solution has a protective effect on cell-free herpesvirus of turkeys (Calnek et al., 1970). The resultant cell suspension was then sonicated for 3 cycles at 30 seconds/cycle with an ultrasonic oscillator (Sonifier-Cell Disruptur, Heat Systems Ultrasonics, Inc., Plainview, NY) with the microtip at an intensity setting of 5.5. The sonicate was allowed to cool in an ice bath at least one minute between each cycle. The sonicates were then centrifuged for 10 minutes at 700 x g at 4 C. The supernatant fluids were carefully removed and filtered through a .45 um Millipore filter (Type HA) using a 47 um Swinney filter unit. The .45 um filters were previously sterilized using a UV light (General Electric Germicidal Lamp, 30 watt) at a

distance of 20 cm and then saturated overnight with FBS at 4 C to reduce the amount of virus adsorption to the filter.

The CFV was dispensed in 1 ml amounts in 1 ml glass ampules and stored at -120 C. All CFV for inoculation of turkey poults in these studies was prepared from CAV inoculum on the 9th passage in CKC.

Titration of CAV and CFV Inoculum

Ampules of cell-associated virus stocks were recovered from the frozen state by rapidly thawing at 37 C in a water-bath. The cell suspension was then pelleted at 240 x g for 10 minutes at 4 C. The pellet was resuspended in 1 ml of maintenance medium and then serially diluted in 10x increments from 10^{-1} to 10^{-5} in maintenance medium. Duplicate 48-hour monolayers of CKC in 15 x 60 mm plastic petri dishes were inoculated with 4 ml for each dilution. The CAV inoculum was allowed to adsorb 24 hours after which time, it was replaced by 4 ml of maintenance medium.

The number of foci were enumerated daily for 5 days. The titer was taken as the average number of focus forming units (FFU) at the highest dilution of virus expressing enumerable foci. CAV titers were expressed as (Average number of foci)/(Volume of inoculum x Dilution). Titers obtained for CAV stocks were 40,000 FFU/ml for the BC, 320,000 FFU/ml for the FC and 37,000 FFU/ml for the TT isolates. Cell-free virus was titrated using a procedure described by Adldinger and Calnek (1971). The CFV was thawed at 37 C and diluted in

10x serial dilutions ranging from 10^{-1} to 10^{-5} in SPGA containing 5mM EDTA. Duplicate 48-hour monolayers of CKC in 15 x 60 mm plastic petri dishes were inoculated with .5 ml of each virus dilution and allowed to adsorb for 30 minutes at 37 C. After 30 minutes, 3.5 ml of maintenance media was added to each culture and the inoculum allowed a secondary adsorption period of 24 hours. Media were changed after 24 hours and foci were enumerated for 5 days. Titers were expressed as described for the CAV inoculum. Cell-free virus titers were 31,000 FFU/ml, 106,000 FFU/ml and 32,000 FFU/ml for the BC, FC, and TT isolates, respectively.

Isolation of Cell-Associated Turkey Herpesvirus from Blood, Semen and Tissues

Buffy Coat Cells

Turkey poults, two months of age or younger, were bled by cardiac puncture. Approximately 3 cc of blood were withdrawn into a syringe containing .05 to .1 ml of heparin (1000 units/ml in PBS: Sigma Chemical Co., St. Louis, MO). At three months of age and later, 6 to 10 cc of blood was collected from the left or right brachial vein into a heparinized syringe. Blood was aseptically transferred into 17 x 100 mm plastic tubes (#2051, Falcon Plastics, Oxnard, CA) and centrifuged at a setting of 2 in a tabletop clinical centrifuge (International Clinical Centrifuge, Model CL, Needham Hts., MA) for 15 minutes. Plasma containing buffy coat cells was removed using a sterile Pasteur pipette and transferred into a 15 ml conical centrifuge tube on ice.

The whole blood was then resuspended, centrifuged and plasma harvested as previously described. This procedure was repeated until plasma was no longer separated from the red blood cells.

Samples of the plasma containing buffy coat cells were centrifuged at 700 x g for 10 minutes at 4 C. Plasma was removed using a sterile Pasteur pipette, transferred into 12 x 75 mm plastic tubes with caps and frozen at -20 C for future assay by immunodiffusion tests for precipitating antibody. Cell pellets were resuspended in maintenance medium and a viable cell count was obtained by trypan-blue dye exclusion. The cell suspension was then diluted with maintenance medium to a concentration of 10^6 buffy coat cells/ml. Duplicate monolayers of 48 to 72-hour CKC in 15 x 60 mm petri dishes were inoculated with 5 x 10^6 buffy coat cells and incubated at 37 C in a humidified 5% CO_2 atmosphere. The medium was replaced after 24 hours and subsequently every 3 to 4 days. Cultures were observed daily for herpesvirus cytopathic effect (CPE). Cultures negative or suspicious for viral CPE after 10 days of incubation were passaged onto fresh monolayers of CKC and observed an additional 11 days. Foci of infected cells were enumerated at 7 and 14 days post-inoculation and expressed as the average FFU/5 x 10^6 buffy coat cells. Controls were parallel cultures of uninoculated CKC passaged at the same intervals as the inoculated cultures.

Plasma

Plasmal samples obtained from male turkeys at the termination of the experiments were assayed for the presence of turkey herpesvirus. Approximately 0.20 ml of plasma was placed on drained 48 to 72-hour monolayers of CKC. After an absorption for 30 minutes at 37 C, maintenance medium was added and the cultures were maintained and examined as explained for the buffy coat cells.

Seminal Cells

Virus isolation from semen was done using a technique previously described (Adldinger et al., 1974; Thurston et al., 1975). Turkey males were placed in a restraining stand and semen was collected by the massage technique of Burrows and Quinn (1937) into a sterile tuberculin syringe. Control birds were always sampled first. Success in the procurement of semen samples is influenced by a variety of factors including 1) experience of caretaker in massage technique; 2) cooperation and response of the toms; 3) failure of birds to produce semen; 4) loss of semen in fecal material voided at the time of collection; and 5) inadequate sample volume (average volume obtained ranged from .1 to .2 ml but usually is .15 ml or less). The semen samples were transported to the lab and diluted 1:2 in MEM-E supplemented with 1000 units-ug/ml penicillin-streptomycin, 100 ug/ml Gentamicin (Schering Corp., Kenilworth, NJ), 7.5 ug/ml Amphotericin B and 2% (v/v) FBS. Approximately one-half of the diluted semen was added to 4 ml of maintenance medium and inoculated onto one 15 x

60 mm petri dish of CKC. Cultures were maintained and observed as described for buffy coat cells.

Spleen and Thymus

Cell suspensions were prepared by gently forcing 3-5 mm^3 tissue pieces through #80 stainless steel mesh. Loose cells were washed through the screen with maintenance media into a sterile 50 ml beaker. The cellular suspensions were then centrifuged at 700 x g for 10 minutes at 4 C and the pellets resuspended in 2 ml of maintenance media. Viable cells, as determined by trypan blue staining were counted. Duplicate 48 to 72-hour monolayer cultures of CKC were inoculated with 5 x 10^6 splenic or thymic cells and cultures were maintained and observed as described for the buffy coat cells.

Bursa and Kidney

Cell suspensions were prepared by trypsinization of organ pieces as described for the preparation of primary CKC. Duplicate 48 to 72-hour monolayers of CKC were inoculated with 5 x 10^6 bursal or kidney cells and cultures were maintained and observed as described for the buffy coat cells. Kidney cells were also directly cultivated by seeding duplicate 15 x 60 mm petri dishes with 8 x 10^6 cells in 5 ml of growth medium. Primary turkey kidney cells were maintained as described for CKC. Cultures of primary turkey kidney cells were observed for viral CPE for 10 days. Cultures negative after 10 days were subcultivated and observed an additional 11 days before being discarded as negative.

Testes

Studies on the prevalence of turkey herpesvirus in testicular tissue were done by direct culture of trypsinized testis cells and by cocultivation of testis cells with CKC. Portions of the left and right testis removed immediately post-mortem were transferred to a 15 x 100 mm glass petri dish. The soft core of the testis was scraped free of the tunica albuginea with a sterile scalpel and minced into 3 to 5 mm^3 pieces. The trypsinization procedure was identical to the procedure for the preparation of CKC except that a 0.2% (v/v) solution of trypsin in CMF-1x without versene was used. Testicular cultures were maintained and observed as described for the CKC.

Four 15 x 60 mm petri dishes were seeded at a concentration of 20 x 10^6 cells in 5 ml of growth medium per plate. The primary testicular cultures were subcultivated as secondary testis cells after 10 days in duplicate 15 x 60 mm petri dishes containing two 22 mm square glass coverslips and observed an additional 11 days. Foci typical of herpesvirus CPE were enumerated on 4, 7, 10, 14 and 21 days after cultures were initiated. Cultures negative for herpesvirus cytopathic effect (CPE) after 21 days were regarded as negative. Coverslips in secondary testicular cultures revealing CPE were fixed in acetone for immunofluorescence and in Bouin's solution for hematoxylin and eosin staining of viral inclusion bodies.

Cocultivation of testis cells with CKC was accomplished by seeding of 5 x 10^6 testis cells on duplicate 48 to 72-hour CKC monolayers in 15 x 60 mm petri dishes. The medium was changed after 24 hours and the co-cultures were maintained and examined as described for the buffy coat cells.

Ovary

Direct culture and cocultivation of primary ovarian cells with CKC was done as described for the primary testis cultures.

Isolation of Cell-Free Turkey Herpesvirus from Extracts of Cells and Tissues

Cell-free extracts were prepared from the cell suspensions of buffy coat, spleen, thymus, bursa, kidney, and gonads remaining after the assay for cell-associated virus. Cells were centrifuged at 700 x g for 10 minutes at 4 C and the cell pellets resuspended in 2 ml of SPGA in phosphate buffer. The suspensions were then sonicated for 60 seconds with an ultrasonic oscillator as previously described for the preparation of CFV inoculums. The disrupted cell suspensions were centrifuged at 700 x g for 20 minutes at 4 C. The clarified supernatant fluids were considered sufficiently free of cells and used as the inoculum for cell-free virus assays. Filtration of the clarified suspensions were not done due to the number of samples processed.

Portions of thoracic and lumbar spinal ganglia were pooled and ground in Ten Broeck grinders in 2 ml of maintenance media. The suspensions were not sonicated because the homogenization technique appeared sufficient to rupture

most viable cells. For the virus assays duplicate monolayer cultures of 48-hour CKC were inoculated with 0.5 ml of extract. Adsorption was for 30 minutes at 37 C, then maintenance media were added for a secondary adsorption period of 24 hours. Media were changed after 24 hours and the cultures were maintained and observed as described for the buffy coat cells. The remainders from each extract were transferred to 12 x 75 mm tubes and frozen at -120 C to be assayed later for herpesvirus antigens by immunodiffusion tests.

Explantation Techniques

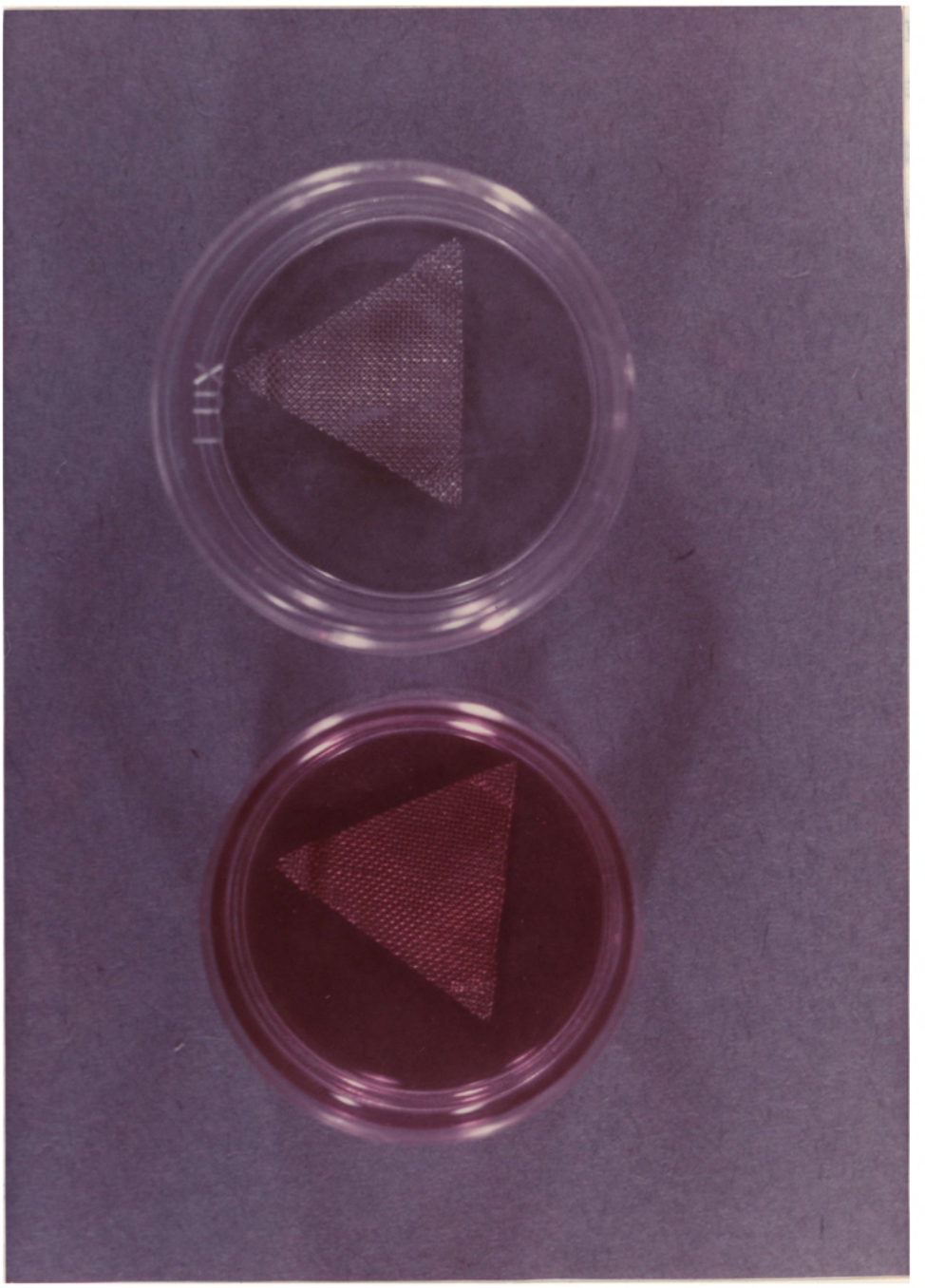
Efforts to demonstrate the presence of latent turkey herpesvirus in testis, ovary and spinal ganglia were made by explanting organ pieces *in vitro*. This was done either by directly establishing testis explants on wire screen supports or by placing organ pieces on monolayers of CKC (explantation-co-cultivation).

Testes

Explant cultures were established according to the protocol of Kalter *et al.*, 1973. Tissue portions reserved from the preparation of testicular cell cultures were minced into 2-3 pieces and transferred to a 100 ml serum bottle. The pieces were washed 3x with MEM-E containing antibiotics and poured into a 15 x 100 mm petri dish. For direct explantation 5 to 6 pieces were transferred using sterile curved forceps to a stainless steel wire grid (Falcon Plastics, Oxnard, CA) superimposed on a 22 mm square glass coverslip in 15 x 35 mm petri dishes as illustrated in PLATE I.

PLATE I. Culture Vessel for Maintenance of Explant Cultures

Culture vessel used for establishment and maintenance of gonadal and spinal ganglion explants.



Explants were maintained on 2 ml of MEM-E supplemented with 10% FBS and antibiotics. This 2 ml volume was sufficient to just wet the explants by capillary action. Explants were incubated at 37 C in a humidified incubator in an atmosphere of 5% CO_2 and air and medium was changed at 3 to 4-day intervals. Media from explant cultures were pooled and assayed on CKC for virus released into the extra-cellular environment at various days post-explantation (PE). Pooled media were allowed to adsorb for 24 hours on CKC monolayers and were then replaced with maintenance medium. Cultures of CKC were observed for 7 days, passaged onto fresh CKC monolayers after 7 days and observed for 7 additional days before being discarded as negative.

Outgrowths of testis, which originated from the explants and attached to the surface of the coverslips in the 15 x 35 mm plates, were observed with the inverted microscope at 2-day intervals for viral cytopathic effect (CPE). When viral CPE was observed in the outgrowths of the explants, the explants proper in these petri dishes were pooled into a 15 x 100 mm petri dish containing phosphate buffered saline, randomly divided into equal lots and treated as follows: 1) embedded in OCT compound (Miles Laboratories, Naperville, IL) and frozen at -120 C for immunofluorescence tests; 2) fixed in 3% glutaraldehyde for electron microscopy; and 3) ground in 5 ml of maintenance medium in a Ten Broeck grinder. This ground explant suspension was then inoculated onto one 15 x 60 mm petri dish of CKC and the cultures maintained and

observed as described for the cell-free extracts. Coverslips with testicular explant outgrowths were divided into equal numbers for immunofluorescence and hematoxylin and eosin staining for Cowdry type A inclusion bodies.

Explants with outgrowths negative for viral CPE were terminated after 30 days of maintenance in vitro and pieces and coverslips were processed as described for the explants with outgrowths positive for viral CPE.

Explantation-co-cultivation was done by transferring 6 to 8 testis pieces on duplicate 48-hour monolayers of CKC in 15 x 60 mm petri dishes. Cultures were maintained on 2 ml of maintenance medium for the first 48 hours to allow the explants time to adhere to the monolayer. After 48 hours and at subsequent 3 to 4-day intervals the cultures were maintained with 4 ml of maintenance medium. At 7-day intervals for 28 days, the explants were aseptically transferred to fresh CKC monolayers. Testicular explants proper from positive and negative cultures of CKC were treated as described for explants proper with positive outgrowth cells.

Ovary

Ovarian tissue portions were also established directly in vitro as explant cultures and used in the explantation-co-cultivation technique as described for the testis tissues.

Spinal ganglia

Thoracic and lumbosacral spinal ganglia were collected by removing the head, skin and breast musculature, viscera and extremeties of the bird so that only the backbone and

supporting structures remained. The backbone was packed on ice and dissection of the 1st to 7th paired thoracic ganglia and the first four paired ganglia in the lumbosacral region was done in a laminar flow hood. In order to guarantee an adequate quantity of samples for immunofluorescence, electron microscopy and explantation, thoracic ganglia 1 and 2 (T_1-T_2), 3 and 4 (T_3-T_4), 5 and 6 (T_5-T_6) and lumbar ganglia (L_1-L_4) and T_7 were pooled. Ganglia were held in cold (4 C) MEM-E supplemented with antibiotics and 5% FBS until all samples were collected. The ganglia were then given a brief wash and transferred to 15 x 60 mm petri dishes. The connective tissue surrounding the ganglia was teased away and explants were established and maintained as described for testicular tissue. Ganglion explants from male and female birds were also assayed by explantation-co-cultivation.

Immunodiffusion Tests

Plasmas and serum were collected at various intervals post-inoculation from birds in each experimental group and assayed for precipitating antibody to antigens prepared from the BC, FC and TT isolates using an immunodiffusion test described by Okazaki et al. (1970b). Antigens from each of the cloned isolates were prepared from infected monolayers of CKC maintained on serumless maintenance media 24 hours before harvesting the cells for viral antigens. When approximately 85% of the monolayers revealed CPE culture fluids were removed and pooled. Cells were scraped off the culture vessels with a rubber policeman and washed off the

vessels with the culture fluids. Cells and fluids were centrifuged 15 minutes at 700 x g at 4 C. The supernatant except for 2 ml was removed and the cell pellet resuspended. Cellular suspensions were transferred into 12 x 75 mm plastic tubes, freeze-thawed 3x and stored at -120 C. This cell lysate was used as crude viral antigen. All antigens used for immunodiffusion in these experiments were prepared from the 10th to 14th passage of the BC, FC and TT isolates in CKC.

The gel medium was composed of 8% (w/v) sodium chloride, 0.5% (w/v) sodium azide and 0.8% (w/v) agarose (Sea Plaque, Maine Colloids Inc., Brockland, MA) in 0.15 M phosphate buffer at a pH of 7.4. A gel depth of 4 mm was produced by plating 4.5 ml of molten gel medium in 15 x 60 mm plastic petri dishes (No. 1007, Falcon Plastics, Oxnard, CA) and plates were allowed to cool with the lids off to permit the escape of excess water vapor. After cooling, plates were immediately used or stored at 4 C in air tight plastic bags.

Wells were cut 5 mm in diameter using a Vari-Pattern Agar Gel Cutter (Bellco Glass Co., Vineland, NJ) and agar plugs were removed using a Pasteur pipette connected to a vacuum line. A nine-well pattern was used with 8 wells circumscribing one center well. The edge-to-edge distance from the center to peripheral wells was 8 mm while peripheral wells were 3 mm apart. Approximately 0.05 ml of antigen was added to the center well and an equivalent amount of serum or plasma to be tested was added to the peripheral wells.

A control positive turkey serum and a negative turkey serum were included with each group of plates tested. Sera from infected birds, which failed to develop specific lines on the initial test, were retested in an identical manner except that a control positive serum was placed on each side of the serum to be tested. A total of 4 serums were tested with each pattern. When cell or tissue preparations were to be tested for antigens then positive serum was added to the center well and the antigen samples were added to the peripheral wells. Antigens prepared from a crude extract of normal CKC was included as negative control antigen in each group of plates tested. The plates were incubated at ambient room temperature in moist chambers for 7 days and observed daily for development of precipitation lines.

Immunofluorescence Procedures

An indirect fluorescent antibody (IFA) procedure was used to detect antigens of turkey herpesviruses in buffy coat smears, semen smears, and frozen sections of tissue and explants proper.

Serum

Hyperimmune sera to each of the cloned isolates BC, FC and TT were prepared in 8-week-old isolation-reared chickens. Two chickens for each cloned isolate were inoculated intra-abdominally with 2-5 x 10^4 FFU of CAV inoculum at 2-week intervals for 6 weeks. Chickens were bled at the time of and two weeks after each inoculation and plasmal samples were assayed for antibody to the homologous inoculum on the

immunoprecipitin test. All birds were positive for precipitating antibody 2 weeks after the initial immunization and the intensity of the reaction and the number of specific precipitation lines increased after subsequent immunization. All birds were exsanguinated two weeks after the last inoculation and blood collected into 50 ml glass conical centrifuge tubes for separation of sera. After an overnight separation at 4 C, samples were centrifuged at 1500 x g for 20 minutes at 4 C and serum was removed and transferred to a 100 ml serum bottle. Sodium azide was added at a final concentration of 0.02% (w/v) as a preservative and sera were dispensed in 1 ml amounts in 12 x 75 mm tubes and stored at -20 C. All sera used was at a working dilution of 1:10 in PBS.

Conjugates

A lyophilized fluorescein-conjugated IgG fraction of anti-chicken serum prepared in rabbits (Cappel Laboratories, Cochranville, PA) was utilized as the secondary reagent in the immunofluorescence test. Conjugate was reconstituted in distilled water and dispensed in .1 ml amounts in 12 x 75 mm plastic tubes for storage at -20 C. Staining titers of the commercial conjugate were determined to be approximately 1:80 to 1:160. The conjugate was used at a working dilution of 1:30 in PBS (pH 7.4) and .1 ml of 0.1% solution of Evan's blue was added to each tube of diluted conjugate as a counterstain.

Staining of Frozen Sections

Frozen sections of tissue and explants embedded in OCT compound at -120 C were allowed to thaw overnight at -20 C. Blocks of OCT containing tissue were mounted on chucks in a cryostat and 6u sections were cut at -18 C. Sections were picked up using a warm slide technique, allowed to dry for 10 minutes at room temperature and then fixed for 30 minutes at -20 C in reagent grade acetone. After fixation slides were air-dried for 5 minutes at room temperature and either stained immediately or stored at -120 C in plastic slide boxes until used.

Sections were overlaid with anti-sera and incubated for 30 minutes at 37 C in a humidified incubator. The slides were then briefly dipped in distilled water, rinsed for 10 minutes in two changes of PBS (pH 7.4), dipped briefly in distilled water to remove the salt and air dried. Each section was then covered with the anti-chicken fluorescein conjugate, the slides were again incubated for 30 minutes and washed as described for the sera. Coverslips were then mounted with buffered glycerol (2 parts glycerol:1 part PBS, pH adjusted to 7.4). Controls included CKC coverslips infected with BC, FC and TT isolates, control tissues exposed to normal and immune serum, and infected tissue exposed to normal serum. Stained sections were examined with a Leitz Ortholux fluorescent microscope equipped with a dark-field condenser, high pressur mercury vapor lamp, a BG-12 excitation and K530 barrier filter. Photography

was done using a Leitz Ortholux fluorescent microscope with incident light fluorescence. Areas to be photographed were exposed 30-90 seconds on high speed (400 ASA) Ektachrome (Eastman Kodak Co., Rochester, NY) film.

Staining of Coverslips and Smears

Coverslips of CKC or outgrowths of explants with CPE typical of herpesviruses were also stained by the IFA procedure. Coverslips were removed from the culture vessel and washed for 5 minutes in prewarmed (37 C) PBS, dipped in distilled water to remove the salt and fixed in acetone at -20 C for 30 minutes. After fixation the coverslips were airdried and stained immediately or stored at -120 C in plastic specimen bags. Staining, observation and photography procedures are identical to those described in the preceeding section. Controls included BC, FC and TT infected CKC coverslip cultures and control CKC coverslip cultures or coverslip cultures of explant outgrowths from normal turkeys exposed to normal and immune serum. Smears of buffy coat and seminal cells from infected and control male turkeys were fixed, stained and observed as described for the coverslips.

Histological Staining Procedures

Tissues removed immediately post-mortem and various explant pieces of testis or ganglia were fixed in 10% phosphate buffered formalin. Tissues were parafin embedded and 7u sections were cut on a microtome, mounted on slides and stained with hematoxylin and eosin.

Coverslip cultures of infected CKC from virus isolation assays, testicular cultures and testis and ganglion cell outgrowths were examined for Cowdry type A inclusion bodies using a Harris hematoxylin and eosin stain. Briefly, coverslips were removed from the petri dish, washed for 5 minutes in prewarmed (37 C) PBS and fixed for 30 minutes in Bouin's fixative. After fixation, coverslips were decolorized in a graded series of ethyl alcohol and then stained according to a standard technique employing Harris hematoxylin and eosin (Manual of Histological Staining Methods of the Armed Forces Institute of Pathology, 1968). Light micrographs of hematoxylin and eosin stained sections and coverslips were exposed on Kodak Ektachrome (ASA 160) using a Leitz Ortholux microscope equipped with a bright field condensor and tungsten light source.

Electron Microscopy Procedures

Tissue samples (1-2 mm^3 pieces) were fixed with 3% gluaraldehyde in Millonig's buffer (Millonig, 1962), pH 7.3 for 4 hours at 4 C. After an overnight wash in Millonig's buffer, the specimens were post-fixed in 2% osmium tetroxide for 2 hours at 4 C, washed in Millonig's buffer, dehydrated in a graded ethanol series (20, 40, 60, 80, 95 and 100%) and embedded in Spurr's low viscosity resin (Spurr, 1969).

Testicular cultures and outgrowths of testis explants from approximately one-half of the birds used in these experiments were fixed for electron microscopy when viral cytopathic effect had developed. Cells were trypsinized

from the culture vessel, placed into a 15 ml conical centrifuge tube and pelleted at 700 x g for 10 minutes at 4 C. The supernantant was aspirated and 5 ml of 3% glutaraldehyde in Millonig's buffer was added. Pellets were fixed for one hour at 4 C. The wash, postfixation, dehydration and embedding procedures were identical to that described for tissue.

Ultrathin sections were cut with glass knives on an ultramicrotome (Ultratome III, LKB Stockholm-Bromma, Sweden) and placed on 200 or 300 mesh copper grids. Sections were stained 10 minutes with 1% ethanolic uranyl acetate and 20 minutes with Reynold's (1963) lead citrate. Samples were examined and micrographs taken with an RCA EMU-3G electron microscope (Camden, NJ) using an accelerating voltage of 100 KV.

Evaluation of Semen Smears by Light Microscopy

Semen was collected using a massage technique (Burrows and Quinn, 1937) and transferred into Beckman microfuge tubes (Beckman Instruments, Inc., Fullerton, CA). A drop of the fresh semen was mixed 1:2 with a 1% eosin and 2% migrosin stain (Friars and Chatterjee, 1969) and an approximation of the number of abnormal cells and macrophages was obtained by counting the appropriate cells in 32 squares of a Fuchs-Rosenthal hemacytometer. The samples were then diluted 1:2 in sterile phosphate buffered saline and a drop of semen was placed on each of 4 glass microscope slides

for the preparation of smears. Smears were allowed to airdry and 2 slides were fixed in Bouin's fixative for hematoxylin and eosin staining.

The remaining semen sample was centrifuged in a Beckman microfuge for 3 minutes. Seminal plasma was transferred to a 12 x 75 mm plastic tube and the plasma color was evaluated versus a blue background. The pellet of seminal cells was either used for virus isolation as previously described or aspirated into the tip of a Pasteur pipette and immersed into cold (4 C) Millonig's buffered 3% glutaraldehyde for electron microscopy (Thurston et al., 1975).

Twenty-five high-powered fields (95x) of the hematoxylin and eosin stained smears were evaluated for the number of abnormal cells and macrophages. The same number of high-powered fields were also observed for semen smears stained by indirect immunofluorescence.

Experimental Design

One-week-old turkey poults were randomly assigned to four experimental groups and inoculated with either a CFV or CAV inoculum as outlined in Table 2. Birds were inoculated intra-abdominally with .5 ml of a CAV inoculum containing approximately 6×10^6 viable cells or .2 ml of a CFV inoculum instilled through the nares. Uninfected control birds were inoculated intra-abdominally with 6×10^6 uninfected CKC in .5 ml of PBS or with .2 ml of PBS through the nares in lieu of CAV or CAV inoculum. Following

Table 2. Assigned Experimental Groups

	No. Birds Inoculated		Titers of Virus Inoculated		Approximate number viable CKC x 10^6 in .5 ml virus inoculum
	CFV	CAV	CFV[b] FFU/.2ml	CAV[c] FFU/.5ml	
BC	12	14	6,200	20,000	6.5
FC	7	8	5,300[d]	16,000[d]	5.7
TT	17	12	6,400	18,500	5.8
UC[a]	7	7	PBS[e]	CKC[f]	6.0

[a] UC = Uninfected controls

[b] CFV was inoculated intranasally

[c] CAV was inoculated intra-abdominally

[d] The CAV and CFV inocula for the FC group were diluted 10x prior to inoculation since the FC virus stocks titered approximately 10x higher than the BC and TT virus preparations.

[e] Controls received .2 ml of phosphate buffered saline in lieu of CFV inoculum.

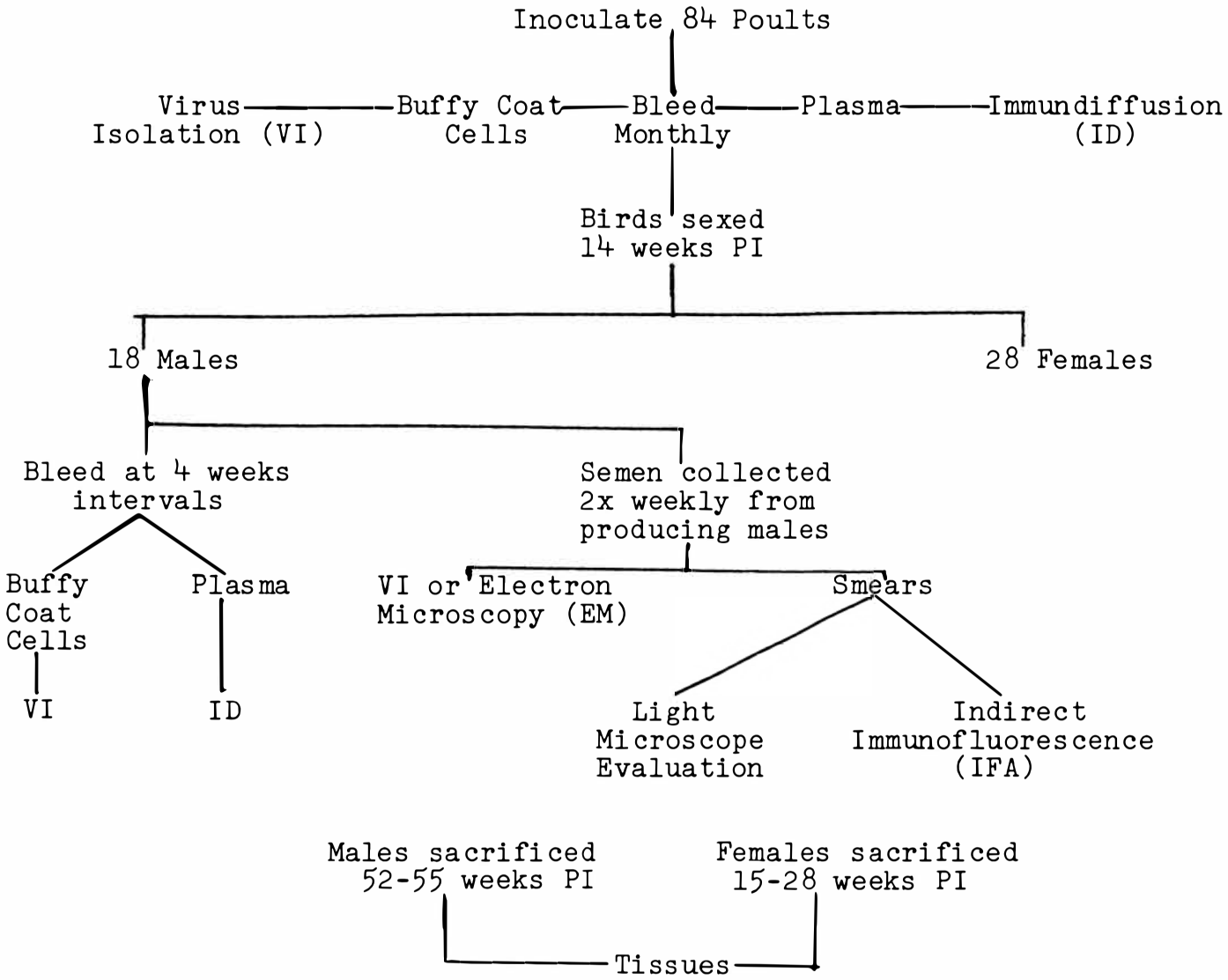
[f] Controls received uninfected CKC (6×10^6) to approximate the number of CKC in the CAV inoculum administered to the BC, FC and TT groups.

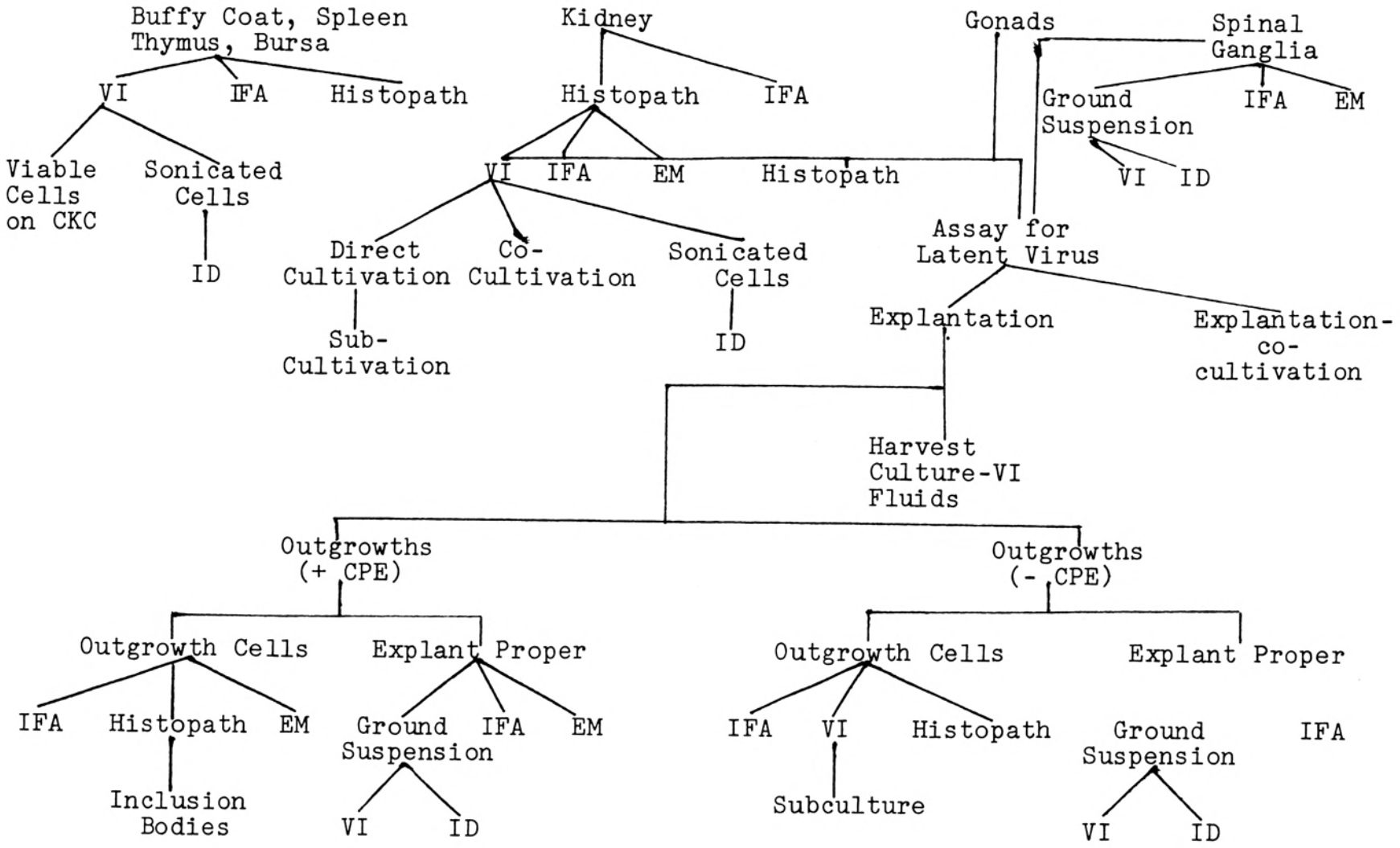
inoculation each experimental group was separately maintained as previously described in the section on Turkeys.

The entire experimental design is summarized in Figure 2. All inoculated turkeys in the BC, FC and TT groups and 2 control turkeys were bled at monthly intervals. Persistent infection of the birds in the BC, FC and TT groups was ascertained by assay of buffy coat cells for virus and plasma for precipitating antibody.

Birds were sexed at 14 weeks post-inoculation (15 weeks of age) and the females (BC, 5 females; FC, 4 females; TT, 11 females; UC, 8 females) were removed and transferred to the Veterinary Medicine Research Farm as previously described. Females were killed between 15 to 38 weeks PI by electrocution and exsanguination and the following tissues were obtained immediately post mortem, placed in growth media containing 1000 units/ml penicillin-streptomycin; 100 ug/ml gentamicin and 8.5 ug/ml Amphotericin B, and assayed for cell-associated and for cell-free virus: ovary, kidney, spleen, thymus and bursa. Ground extracts of spinal ganglia were also assayed for infectious virus. Portions of these same tissues were fixed in 10% neutral buffered formalin for histology or frozen in OCT compound at -120 C for immunofluorescence. Ovary and spinal ganglia were also fixed in 3% glutaraldehyde for electron microscopy and assayed for latent herpesvirus by explantation and explantation-cocultivation.

Figure 2. Flow Chart of
Experimental Design


Inoculate 84 Poults
Virus Isolation (VI)
Buffy Coat Cells
Bleed Monthly
Plasma
Immundiffusion (ID)
Birds sexed 14 weeks PI
18 Males
28 Females
Bleed at 4 weeks intervals
Semen collected 2x weekly from producing males
Buffy Coat Cells
Plasma
VI
ID
VI or Electron Microscopy (EM)
Smears
Light Microscope Evaluation
Indirect Immunofluorescence (IFA)
Males sacrificed 52-55 weeks PI
Females sacrificed 15-28 weeks PI
Tissues


Buffy Coat, Spleen Thymus, Bursa
VI
IFA
Histopath
Viable Cells on CKC
Sonicated Cells
ID
Kidney
Histopath
IFA
VI
IFA
EM
Histopath
Direct Cultivation
Co-Cultivation
Sonicated Cells
Sub-Cultivation
ID
Gonads
Spinal Ganglia
Ground Suspension
IFA
EM
VI
ID
Assay for Latent Virus
Explantation
Explantation-co-cultivation
Harvest Culture-VI Fluids
Outgrowths (+ CPE)
Outgrowths (- CPE)
Outgrowth Cells
Explant Proper
IFA
Histopath
EM
Inclusion Bodies
Ground Suspension
IFA
EM
VI
ID
Outgrowth Cells
Explant Proper
IFA
VI
Histopath
Subculture
Ground Suspension
IFA
VI
ID

Beginning at 15 weeks PI all male turkeys in each of the 4 experimental groups (BC, 2 males; FC, 3 males, TT, 11 males and UC, 2 males) were bled at 4-week intervals until the experiments were terminated at 55 weeks PI. Buffy coat cells and plasma from each male bird were assayed for virus and antibody, respectively. The males were initiated on a 14-hr light, 10-hr dark diurnal cycle at 23 weeks PI (24 weeks of age) to stimulate semen production. Males were sampled for semen twice weekly from 25 weeks PI to 36 weeks PI, once weekly from 37 weeks PI to 46 weeks PI and then twice weekly from 47 weeks PI to the end of the experiments. Semen samples were examined by light microscopy of smears for abnormal cells and macrophages, assayed on CKC for virus and fixed in 3% glutaraldehyde for electron microscopy. Semen smears were also examined by indirect immunofluorescence for the presence of viral antigens.

Male birds were killed between 52 to 55 weeks PI by electrocution and exsanguination. Immediately post-mortem the following tissues were aseptically removed and assayed for cell-free and cell-associated virus: testes, kidney and spleen. Semen was collected from the vas deferens and assayed for virus on CKC. Ground suspensions of ganglia were also assayed on CKC for infectious virus. Portions of the testes, kidney, spleen and vas deferens were also fixed in 10% buffered formalin for histopathology and frozen in OCT compound at -120 C for immunofluorescence. Additional portions of the testes and spinal ganglia were fixed in 3%

glutaraldehyde for electron microscopy or assayed for latent virus by explantation and explantation-co-cultivation. Similar samples were also procured from the 4 White Breeder males, which were naturally afflicted with yellow semen.

Chapter IV

RESULTS

General

From the starting numbers of 84 turkey poults, the actual experimental numbers were as follows: 1) BC - 3 males, 5 females; 2) FC - 3 males, 4 females; 3) TT - 11 males, 11 females; and 4) UC - 2 males, 8 females. The other poults died within 30 days of hatching with mortality related to non-specific factors and the stress of inoculation. One additional BC male (#324, not the same bird as the #324 listed in Figure 1) succumbed to airsacculitis at 12 weeks PI. Data obtained from birds inoculated with cell-associated and cell-free inocula were pooled since there were no apparent differences in the results.

Viremia and Precipitating Antibody Responses in Male Turkeys

Buffy coat cells and plasma, which were collected at 4-week intervals from inoculated (BC, FC and TT) and control (UC) birds, were assayed for virus and precipitating antibody, respectively, between 4 to 55 weeks PI to confirm persistent infection. As shown in Table 3, virus persisted in the buffy coat cells of most birds in each of the inoculated groups until the experiment was terminated at 55 weeks PI. Virus was not isolated from the BC birds at 12 and 16 weeks PI or from birds in any of the inoculated groups at 20 weeks PI. Virus was not isolated from the control birds at any time.

Table 3. Viremia and Precipitating Antibody Responses in Male Turkeys.

Weeks Post-Inoculation	Virus Isolation[a]			No. Positive Turkeys/No. Examined for:							
				Viremia				Antibody			
	BC	TT	FC	BC	TT	FC	UC	BC	TT	FC	UC
4	12.8	13.9	5.5	2/2	8/8	1/2	0/2	1/2	0/8	0/2	0/2
8	7.5	10.1	4.5	2/2	7/7	1/2	0/2	2/2	5/6	0/2	0/2
12	0	3.4	5.3	0/2	5/8	1/2	0/2	2/2	7/8	0/2	0/2
16	0	4.0	4.5	0/2	5/11	2/3	0/2	1/2	10/11	1/3	0/2
20	0	0	0	0/2	0/11	0/3	0/2	1/2	9/11	3/3	0/2
24	1.3	1.8	1.5	2/2	8/11	3/3	0/2	1/2	9/11	3/3	0/2
28	<1.0[b]	2.7[b]	1.7[b]	1/2	6/11	1/3	0/2	1/2	7/11	3/3	0/2
32	<1.0[b]	3.1[b]	11.3	1/2	4/10	1/2	0/2	1/2	6/10	3/3	0/2
36	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
40	<1.0[b]	6.8[b]	6.0	2/2	8/9	3/3	0/2	1/2	4/9	3/3	0/2
44	<1.0[b]	2.2	6.7	2/2	9/9	3/3	0/2	1/1	4/8	3/3	0/2
48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
52-55	2.0	1.6	14.8	1/1	8/8	3/3	0/2	1/1	5/8	3/3	0/2

[a] Average FFU from two replicate cultures of primary CKC inoculated per bird with 5×10^6 buffy coat cells.

[b] Average FFU/5×10^6 buffy coat cells enumerated at 14 days following a subculture onto fresh CKC at 10 days.

[c] ND = Not determined.

Viremia was maximal at 4 to 8 weeks PI in the BC and TT groups and decreased thereafter with increasing age of the bird. There is also an increased need for subcultivation in order to induce plaque formation in the BC and TT groups. The magnitude of viremia in the FC group was lower at 4 to 8 weeks PI when compared to the BC and TT groups.

Maternal antibody was not demonstrated in the plasma in 6 poults assayed by the immunoprecipitin test one week after hatching. Development of active antibody was first detected in most birds at 8 and 16 weeks PI and persisted throughout the experiment. No precipitating antibody was detected in the control birds.

Although the level of the antibody response was not quantitated, in general the plasma appeared to contain low titers. This was exemplified by the observation that positioning hyperimmune turkey sera between test serum wells increased the sensitivity of the test. In these experiments 0/27 (BC), 10/42 (FC), 18/141 (TT) and 0/30 (UC) of the serums tested positive against their homologous antigens without the hyperimmune sera in the alternate wells. If the test included the hyperimmune sera in the alternate wells then 19/27 (BC), 33/42 (FC), 93/141 (TT) and 0/30 (UC) of the sera tested positive when reacted versus their homologous antigens.

Distribution of Virus and Viral Antigens in Tissues of Female Turkeys

Twenty-six birds (5-BC, 4-FC, 11-TT and 6-UC) were killed between the 15th and 38th weeks post-inoculation

Samples of buffy coat, bursa, thymus, spleen, kidney and ovary were procured and examined for cell-associated and cell-free virus and viral antigens by immunofluorescence (except for buffy coat) and immunodiffusion tests.

Virus was isolated from most cellular inocula except the bursal cells of 2 BC birds (Table 4). Cell-associated virus (CAV) was most frequently isolated from the kidney (78%), buffy coat (74%), spleen (65%) and ovary (50%) of the three inoculated groups, whereas, bursa (33%) and thymus (28%) were inconsistent sources of CAV. In the individual groups, CAV was most readily isolated from cellular inocula of kidney and ovary in the BC, buffy coat and spleen in the FC and buffy coat and kidney in the TT group. Virus was not isolated from cellular inocula of similar tissues prepared from control turkeys.

Although the titer of virus in each cell-associated inoculum was not determined, Figure 3 revealed that 6/14 buffy coat, 3/4 bursal, 11/13 splenic, 5/5 thymic, 6/14 kidney and 7/9 ovarian samples required a subcultivation at 11 days onto fresh CKC before focus formation was induced. If the average time required for induction of focus formation on CKC is compared for all tissues, buffy coat and kidney require only 10 days, bursa and spleen 14 days, ovary 15 days, and thymus 17 days. Virus could not be recovered from cell-free preparations of the several tissues examined (Table 4).

Attempts to demonstrate immunofluorescence antigens in frozen sections of bursa, thymus, spleen, kidney and ovary

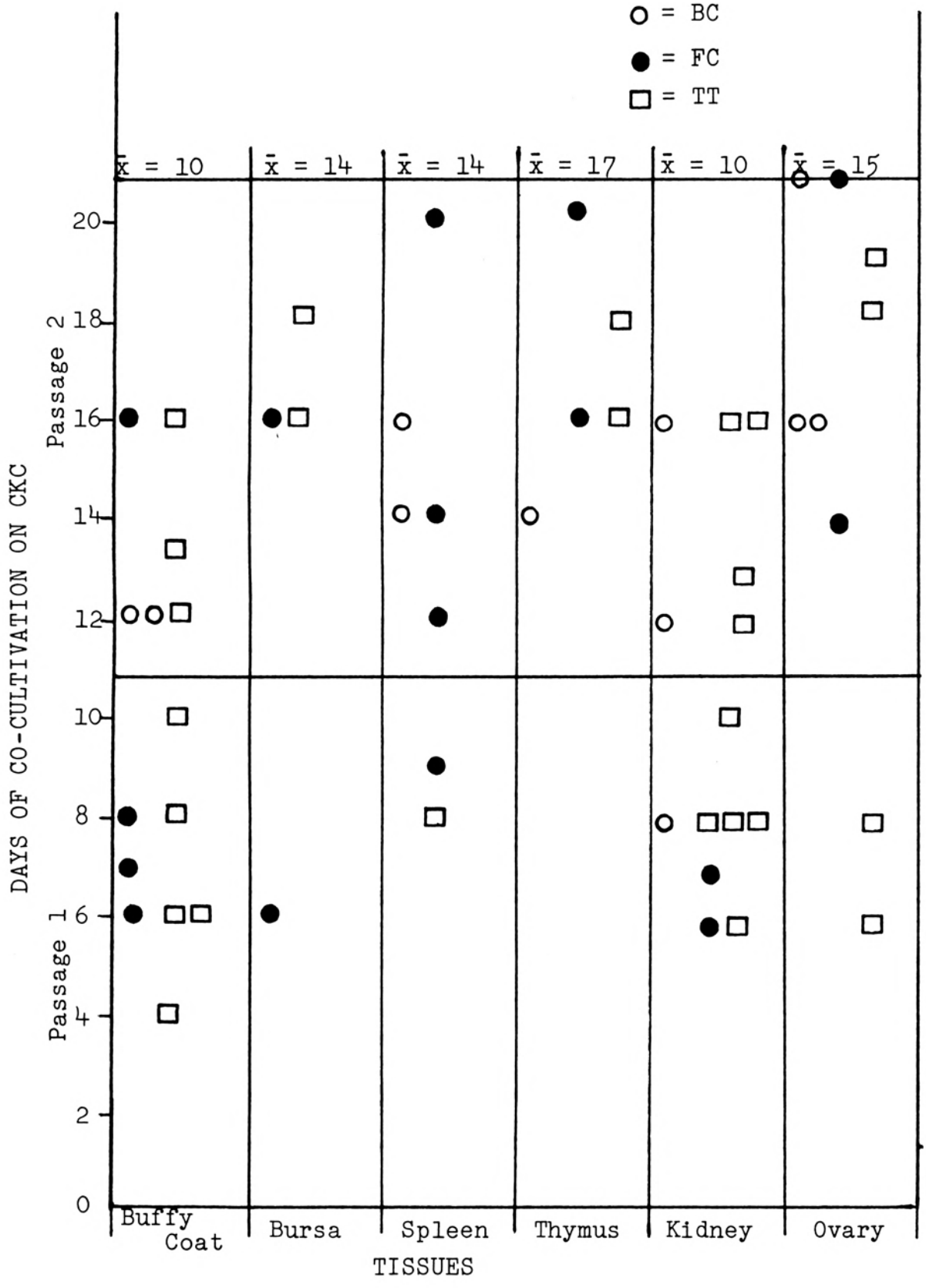
Table 4. Examination of Tissues from Female Turkeys for Viral Infectivity and Antigens.

	No. Turkeys Positive/No. Examined for the Following															
	Cell-Associated Virus[a]				Cell-Free Virus[a]				Immunofluorescence[b] Antigens				Immunodiffusion Antigens			
	BC	FC	TT	UC	BC	FC	TT	UC	BC	FC	TT	UC	BC	FC	TT	UC
Buffy Coat	2/5	4/4	8/10	0/6	0/3	0/2	0/9	0/1	ND	ND	ND	ND	0/3	0/2	0/8	0/6
Bursa	0/2	2/3	2/7	0/6	0/2	0/3	0/9	0/4	0/2	0/2	0/5	0/4	0/2	0/3	0/9	0/6
Thymus	1/5	2/4	2/9	0/4	0/5	0/4	0/8	0/4	0/3	0/3	0/7	0/4	0/5	0/4	0/8	0/4
Spleen	2/5	4/4	7/11	0/6	0/5	0/4	0/11	0/6	0/3	0/4	0/9	0/6	0/5	0/4	0/11	0/6
Kidney	3/5	2/4	9/10	0/6	0/5	0/4	0/10	0/6	0/5	0/4	0/11	0/6	0/5	0/4	0/10	0/6
Ovary	3/4	2/4	4/10	0/6	0/4	0/2	0/9	0/6	0/5	0/4	0/11	0/6	0/4	0/3	0/9	0/6

[a] One bird in each group was examined at the following weeks post-inoculation: BC (21, 26, 31, 36, and 38), FC (16, 27, 32, and 38), TT (15, 20, 24, 30, 31, 32, 35, 36, 37, and 2 at 38), and UC (15, 20, 25, 30, 35 and 38). Results from all sampling times in each experimental group are pooled.

[b] Six sections per tissue were examined.

Figure 3. Time required for plaque formation in primary chick kidney cells inoculated with cell suspension from tissues of female turkeys. $\bar{x}$ = Average time in days required for plaque formation to be induced.


○ = BC
● = FC
□ = TT
x̄ = 10
x̄ = 14
x̄ = 14
x̄ = 17
x̄ = 10
x̄ = 15
DAYS OF CO-CULTIVATION ON CKC
Passage 2
Passage 1
20
18
16
14
12
10
8
6
4
2
0
Buffy Coat
Bursa
Spleen
Thymus
Kidney
Ovary
TISSUES

were uniformly unsuccessful (Table 4). Likewise, soluble turkey herpesvirus antigens were not detected in sonicated cellular suspensions from each of the 6 tissues examined. Precipitating antibody was detected in 3/5 BC, 3/4 FC and 6/11 TT birds.

Herpesvirus Isolation by Direct Cultivation of Kidney and Ovary

Kidney and ovarian cells from the 26 female turkeys used in the above experiments were also assayed for cell-associated virus by direct cultivation. Complete monolayers formed in the cultures of primary kidney within 48 hours, while primary ovary cells were confluent within 48-72 hours. Primary kidney cells from 5 birds (#331, 332, 369, 379 and 382) and primary ovary from 2 birds (#331, 357) did not grow. Results are summarized in Table 5.

Foci were induced in the primary kidney cultures of 3/4 (75%) of the BC and 4/8 (50%) of the TT group. Subcultivation of the primary kidney cultures on day 10 of the assay induced focus formation within 2-4 days in one BC bird and 2 TT birds. Virus was not isolated from primary or secondary kidney cultures derived from 2 birds in the FC and TT groups.

In contrast to the results obtained from the primary kidney cultures, focus formation was induced in only 1/9 (11%) primary ovary cultures from TT birds. Combining all experimental groups, virus replication was observed in 7/14 (50%) primary kidney cultures compared to 1/17 (6%) primary ovary cultures. Subcultivation of primary ovary cells was

Table 5. Results of Virus Isolation by Direct Cultivation and Subcultivation of Kidney and Ovary.

Exptl. Group Bird No.	Weeks PI Killed	Primary Kidney 1-10 Days	Secondary Kidney 12-21 Days	Primary Ovary 1-10 Days	Secondary Ovary 12-21 Days
BC: 301	26	+[a](7)[b]	ND[c]	0	+ (21)
304	31	+ (10)	ND	0	+ (16)
307	21	ND	ND	0	+ (16)
308	36	+ (6)	ND	0	+ (16)
311	38	0	+ (14)	0	+ (21)
Ratio of Positive/Total		3/4(8)[e]	1/1 (14)	0/5	5/5 (18)
FC: 331	32	CFTG[d]	ND	CFTG	ND
332	27	CFTG	ND	0	+ (14)
338	16	0	0	0	0
340	38	0	0	0	+ (16)
Ratio of Positive/Total		0/2	0/2	0/3	2/3 (15)
TT: 357	20	0	0	CFTG	ND
361	24	+ (6)	ND	0	+ (12)
362	38	+ (6)	ND	ND	ND
367	38	0	+ (12)	0	0
368	30	+ (6)	ND	0	+ (12)
369	37	CFTG	ND	0	0
374	32	0	+ (14)	0	0
375	15	0	0	0	0
378	31	+ (8)	ND	+ (10)	ND
379	35	CFTG	ND	0	+ (18)
382	36	CFTG	ND	0	+ (18)
Ratio of Positive/Total		4/8 (7)	2/4 (13)	1/9 (10)	4/8 (15)

[a] + = positive for herpesvirus cytopathic effect, 0 = negative.

[b] Number in parentheses is time in days required for plaques to appear.

[c] ND = Not determined.

[d] CFTG = Cells failed to grow.

[e] Average time in days for viral plaques to appear.

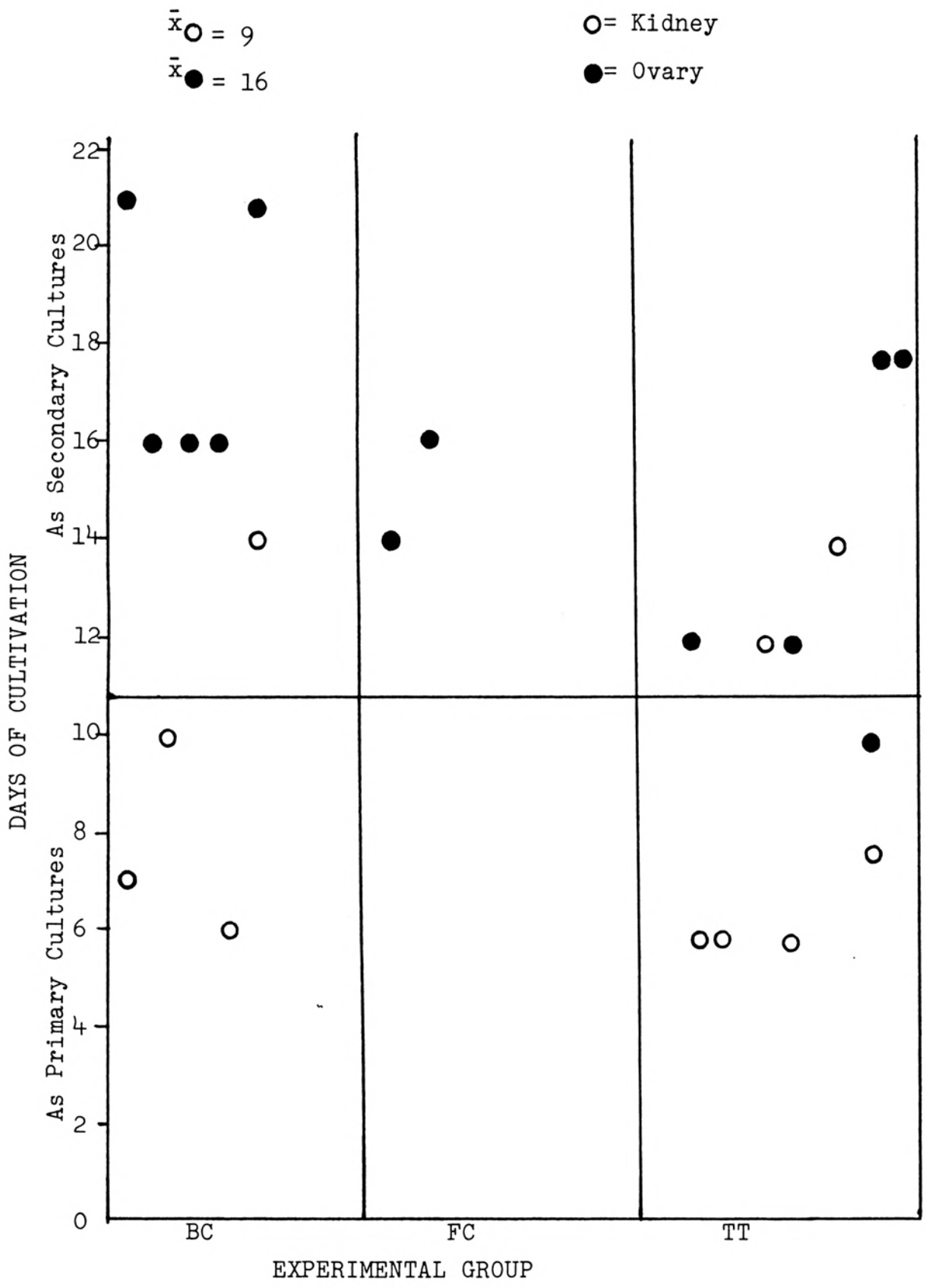
a necessary prerequisite to induce significant focus production. Subculturing induced herpesvirus foci within 2 to 11 days in 5/5, 2/3 and 4/8 birds in the BC, FC and TT groups, respectively. Virus was not isolated from the primary or secondary ovary cultures of 1 bird (#338) in the FC and 4 birds (#367, 369, 374, 375) in the TT group.

The time required for the induction of focus formation in primary and secondary cultures of kidney and ovary are summarized in Figure 4. This distribution revealed an average of 9 days of maintenance required to induce plaque formation in kidney cells, whereas, ovary cells required 16 days or an additional week to induce plaque formation.

Distribution of Virus and Viral Antigens in Tissues of Male Turkeys

Fourteen of 18 birds (1-BC, 3-FC, 8-TT and 2-UC) were killed between 51 and 55 weeks PI. Samples of buffy coat, spleen, kidney and testis were obtained and examined for cell-associated and cell-free virus as well as immunofluorescence and immunodiffusion antigens. Portions of the vas deferens were examined only for immunofluorescence antigens and plasma was assayed for cell-free virus. Two birds from the TT group (#376, 383) were killed at 29 and 44 weeks PI and tissues were examined as described above. Testis tissue salvaged from bird #366 (TT), which died of heat-stress at 35 weeks PI and 316 (BC), which succumbed to a bacterial septicemia at 47 weeks PI, was also examined as previously described.

Figure 4. Comparison of time required and need of subcultivation to achieve virus isolation by direct culture of kidney and ovary. $\bar{x}$ = average time in days required for plaque formation to be induced.


$\bar{x}_O$ = 9
$\bar{x}_●$ = 16
O= Kidney
●= Ovary
DAYS OF CULTIVATION
As Secondary Cultures
As Primary Cultures
22
20
18
16
14
12
10
8
6
4
2
0
BC
FC
TT
EXPERIMENTAL GROUP

The results on the distribution of virus and viral antigens are summarized in Table 6. Co-cultivation of similar numbers of cells (5×10^6) from buffy coat, spleen and kidney onto primary CKC consistently yielded virus from most all birds in the three inoculated groups. Cell-associated virus was not isolated from the buffy coat, spleen and kidney of #376 or the spleen of #370 and 383 in the TT group. Compared to the buffy coat, spleen, and kidney cell inocula, co-cultivation of testicular cells onto primary CKC induced focus formation in only bird #342 and bird #383 in the FC and TT groups, respectively. Virus was not isolated from cellular inocula of similar tissues assayed from the two UC birds.

Overall, cellular inocula from the tissues of the FC birds had the highest titers and the TT group the lowest titers at both the 7 and 14-day counts (Table 6). In each group the buffy coat or kidney cells usually yielded the highest titers with the exception of the spleen in the FC group. Testis tissues uniformly yielded the lowest titers of all 4 tissues examined. Subcultivation onto fresh CKC at 10 days PI increased the average FFU/5×10^6 cells for all tissues examined but it did not increase the number of positive isolations from the cellular inocula of the 4 tissues examined (Figure 5). Comparing the average time required for induction of focus formation on CKC, buffy coat required 8 days, kidney 9 days, spleen 10 days and testis 11 days.

Table 6. Examination of Tissues from Male Turkeys for Viral Infectivity and Antigens.

	No. Turkeys Positive/No. Sampled for the Following															
	Cell-Associated Virus[a] Average FFU/5 x 10^6 Cells				Cell-Free Virus[a]				Immunofluorescence[g]				Immunodiffusion			
	BC	FC	TT	UC	BC	FC	TT	UC	BC	FC	TT	UC	BC	FC	TT	UC
Buffy Coat	1/1	3/3	9/10	0/2	0/1	0/2	0/9	0/2	0/1	0/3	0/9	0/2	0/1	0/2	0/9	0/2
	2.0[b]	11.3	1.0	0												
	10.0[c]	62.5	43.6	0												
Spleen	1/1	3/3	7/10	0/2	0/1	0/3	0/10	0/2	0/1	0/3	0/9	0/2	0/1	0/3	0/10	0/2
	0	1.2	1.0	0												
	9.0	74.7	8.1	0												
Kidney	1/1	3/3	9/10	0/2	ND	0/3	0/10	0/2	0/1	0/3	0/9	0/2	ND	0/3	0/10	0/2
	2.0	2.5	1.0	0												
	60.0	57.3	24.2	0												
Testis	0/2[d]	1/3	1/11[f]	0/2	0/1	0/3	0/11	0/2	0/2	0/3	0/9	0/2	0/1	0/3	0/11	0/2
	0	0	0	0												
	0	3.3	5.3	0												
Vas Deferens	ND[e]	ND	ND	ND	ND	ND	ND	ND	0/1	0/3	0/9	0/2	ND	ND	ND	ND
Plasma	ND	ND	ND	ND	0/1	0/3	4/10	0/2	ND	ND	ND	ND	ND	ND	ND	ND
					0[b]	0	0	0								
					0[c]	0	17.8	0								

Table 6 - continued

[a] Birds in each experimental group were killed and their tissues examined for cell-associated and cell-free virus at the following weeks post-inoculation: BC (51-1 bird), FC (53-2, 54-1), TT (29-1, 44-1, 52-2, 55-4), UC (54-2).

[b] Average FFU/5 x 10^6 cells enumerated 7 days after inoculation of primary CKC.

[c] Average FFU/5 x 10^6 cells enumerated 14 days after cultivation onto primary CKC or 4 days following a subculture at 10 days after the initial inoculation of primary CKC.

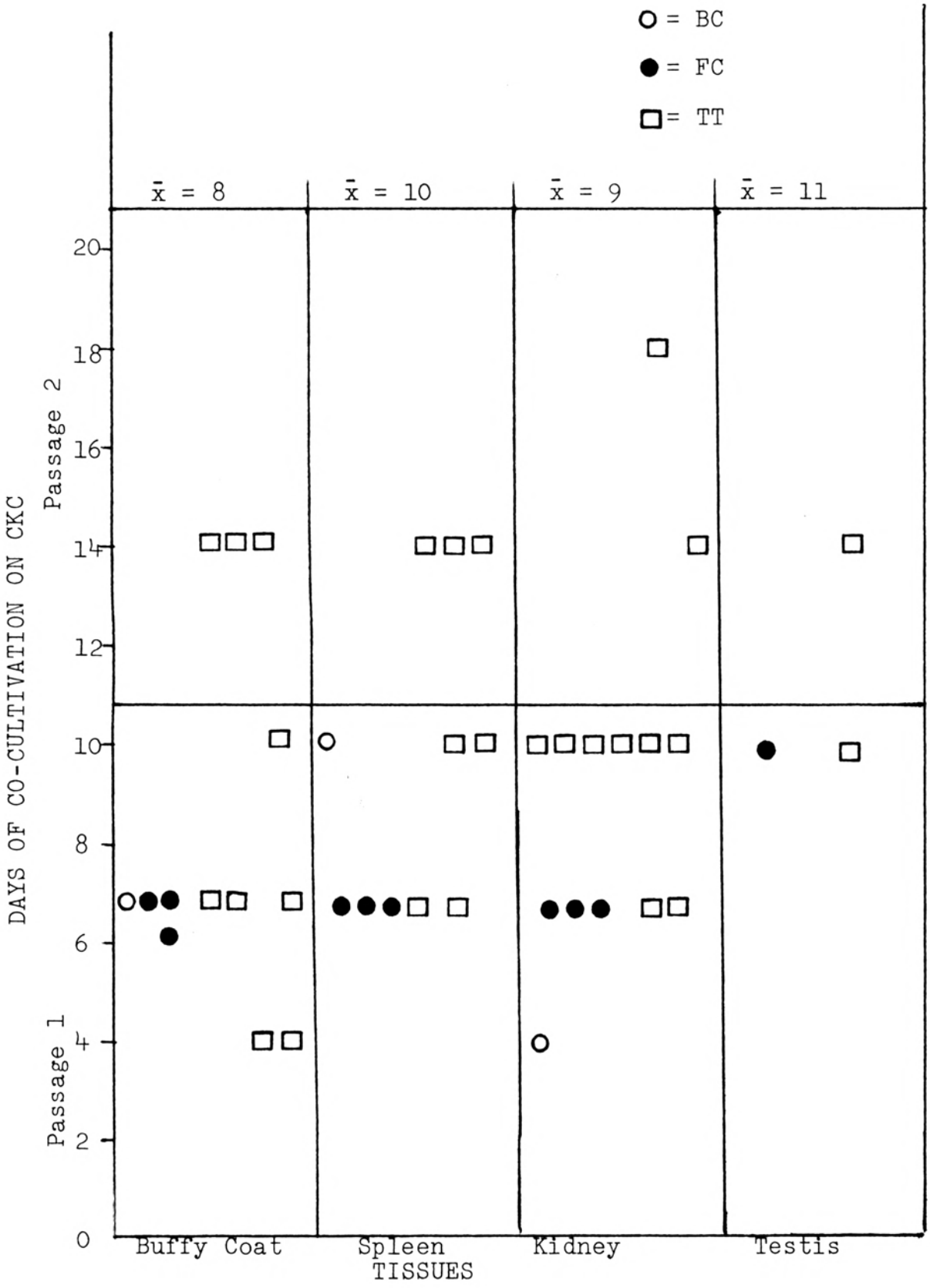
[d] Includes testis salvaged from one bird which died at 47 weeks post-inoculation.

[e] ND = not determined.

[f] Includes testis salvaged from one bird which died at 35 weeks post-inoculation.

[g] Six sections per tissue were examined except for the buffy coat cells of which two air-dried smears on glass microscope slides were examined for turkey herpesvirus antigens by indirect immunofluorescence. Nine sections of testis for each bird were examined.

Figure 5. Time required for plaque formation in primary chick kidney cells inoculated with cell suspensions from tissues of male turkeys.


○ = BC
● = FC
□ = TT
x̄ = 8
x̄ = 10
x̄ = 9
x̄ = 11
DAYS OF CO-CULTIVATION ON CKC
Passage 2
Passage 1
20
18
16
14
12
10
8
6
4
2
0
Buffy Coat
Spleen
Kidney
Testis
TISSUES

Virus was not recovered from cell-free preparations of the several tissues examined. Plasma, however, was considered predominantly free of cells and yielded virus on subcultivation in 4/10 (40%) of the TT males requiring 14 days maintenance for focus formation.

Attempts to demonstrate immunofluorescent antigens in frozen sections of spleen, kidney, testis and vas deferens and in smears of buffy coat cells were unsuccessful.

Similar data on the distribution of virus and viral antigens was obtained from 4 males with a history of yellow semen (Table 7). The results obtained were identical to those of the males in the BC, FC and TT groups in that virus was regularly isolated from all buffy coat, spleen, and kidney cells assayed for cell-associated virus. Again testis were the only tissue from which CAV was not regularly isolated by co-cultivation onto CKC. Quantitatively, kidney yielded the highest titers of virus, followed by spleen, buffy coat and finally as in the experimentally inoculated groups the testis had the lowest titer. Again, subcultivation, although increasing the average FFU, did not increase the number of positive birds except for the testis which required 14 days to induce plaque formation compared to 9 days for the buffy coat and 7 days for the spleen and kidney. Viral antigens were not demonstrated by immunofluorescence or by immunodiffusion. Precipitating antibody against BC, FC and TT antigens was detected in 2/3 birds (#8019, 3118). Bird #9028 also possessed precipitating antibody to the TT isolate only.

Table 7. Examination of Tissues from Males with History of Yellow Semen for Viral Infectivity and Antigens.

Tissue Sample	No. Turkeys Positive/No. Examined for the Following			
	Cell-Associated Virus[a]	Cell-Free Virus	Immunofluorescence[e]	Immunodiffusion
Buffy Coat	4/4 1.8[b] 15.8[c]	ND[d]	0/4	0/4
Spleen	2/2 2.8 29.0	ND	0/2	0/2
Kidney	4/4 4.6 41.1	ND	0/4	0/4
Testis	1/4 0 4.0	ND	0/4	0/4

[a] Results obtained from birds with history of yellow semen. These birds were killed at one year of age and their tissues examined for cell-associated virus infectivity (Average FFU/ 5 x 10^6 cells).

[b] Average FFU/5 x 10^6 cells enumerated 7 days after inoculation of primary CKC.

[c] Average FFU/5 x 10^6 cells enumerated 14 days after cultivation onto primary CKC or 4 days following a subculture at 10 days after the initial inoculation of primary CKC.

[d] ND = not determined.

[e] Three sections of spleen and kidney and six sections of testis from each bird were examined.

Herpesvirus Isolation by Direct Cultivation of Kidney and Testis

Kidney and testicular cells from the 18 experimental and 4 naturally afflicted turkeys used in the preceding section were also assayed for cell-associated virus by direct cultivation and subcultivation. Primary kidney cell monolayers were confluent within 48-72 hours after plating whereas primary testis cells required 72-96 hours to attain confluency. Within 48 hours small and often rounded polykaryocytes were observed in primary testis cultures from the experimental and naturally infected birds. A similar phenomenon was also observed in primary testis cultures from uninoculated control birds, but polykaryocytes were fewer in number compared to the experimentally infected groups. Ensuing plaques did not develop except in the primary testis cultures of bird #309 at 10 days after plating. In contrast, plaque induction occurred within 4 to 10 days after plating of primary kidney cells from the same birds (Table 8). Virus titers were highest in primary kidney cells of the FC, intermediate in the NC and lowest in the BC and TT groups.

Subcultivation of primary kidney cells at 10 days after plating was of no consequence except to increase the average viral titer from each bird. However, subcultivation of primary testis cells induced plaque formation within 4 to 8 days after passage. As shown in Table 8 all secondary testis cultures except for TT birds #376, 377 and 381 and NC bird #8019 yielded herpesvirus plaques. Three TT birds (#371,

Table 8. Results of Virus Isolation by Direct Cultivation and Sub-cultivation of Kidney and Testis.

Exptl. Group / Bird No.	Weeks PI Killed	Primary Kidney	Secondary Kidney Subculture	Primary Testis	Secondary Testis Subculture
BC: 309	51	4.0[a](7)[b]	19.0[d]	2.0 (10)	54.0 (14)
316	47	ND[c]	ND	0	25.5 (14)
Group Average FFU		4.0(7)	19.0	2.0 (10)	39.8 (14)
FC: 342	54	4.0 (7)	40.0	0	40.5 (14)
345	53	12.0 (4)	112.0	0	7.0 (18)
349	53	25.0 (4)	123.0	0	10.0 (16)
Group Average FFU		13.7(5)	91.7	0	19.2 (16)
TT: 356	55	19.0 (4)	30.0	0	1.0 (14)
364	55	3.5 (10)	14.5	0	18.5 (16)
366	35	ND	ND	0	29.5 (14)
370	55	1.0 (7)	19.0	0	12.0 (18)
371	53	4.0 (7)	29.5	0	+ (28)[e]
372	55	4.5 (4)	37.5	0	2.0 (30)[e]
373	53	3.5 (4)	28.0	0	+ (28)[e]
376	29	0	0	0	0
377	52	4.0 (7)	55.5	0	0
381	52	1.5 (7)	18.0	0	0
383	44	3.0 (10)	18.0	0	9.0 (18)
Group Average FFU		4.4 (7)	25.0	0	11.1 (16)
UC: 341	54	0	0	0	0
350	54	0	0	0	0
NC: 3118		6.0 (7)	35.5	0	23.5 (14)
8019		12.5 (7)	36.5	0	0
9028		10.5 (7)	40.0	0	17.5 (14)
9071		12.0 (7)	52.5	0	15.5 (14)
Group Average FFU		10.3(7)	41.1	0	18.9 (15)

Table 8 - continued

[a] Average FFU expressed from replicate cultures in 60 mm petri dishes.

[b] Days after plating that herpesvirus foci were observed.

[c] Not determined.

[d] Average FFU expressed from replicate cultures of secondary kidney at 14 days after subcultivation.

[e] Secondary testis required a third passage at 11 days after subculture to induce plaque formation. Monolayers of birds #371 and 373 were too sparse to enumerate plaques.

372,373) even required a third passage at 11 days after subcultivation before plaques were finally induced 8 to 9 days later. In general, virus titers were higher in secondary kidney as compared to secondary testis cultures. Virus titers were highest in secondary testis cultures of the BC turkeys while titers of the FC, TT and NC birds were of equal magnitude. Virus was not isolated from primary or secondary cultures of either kidney or testis of the control birds.

That subcultivation was a definite requirement for plaque formation in testis versus kidney cells is clearly illustrated in Figure 6. The distribution of data revealed that testis cells distinctly require an average of 15 days cultivation to induce plaques compared to only 7 days for the kidney.

The cytopathic effect observed in secondary testis cultures was of two types: 1) typical rosettes of rounded, refractile cells, which eventually develop into plaques with rounded syncytia (Plate II, 1 and 2); and 2) small clusters of rounded, dark and often granular cells which conferred a "dirty" appearance to the monolayer (Plate II, 3 and 4). Hematoxylin and eosin staining revealed rounded, intensely stained cells often containing Cowdry type A nuclear inclusions (Plate III, 1, 2 and 4). Secondary testis cultures from control birds revealed the cells to be vacuolated with fused cellular membranes but no type A inclusions or plaques as observed in the infected cells (Plate III, 3).

Figure 6. Comparison of time required and need of subcultivation to achieve virus isolation by direct culture of kidney and testis.

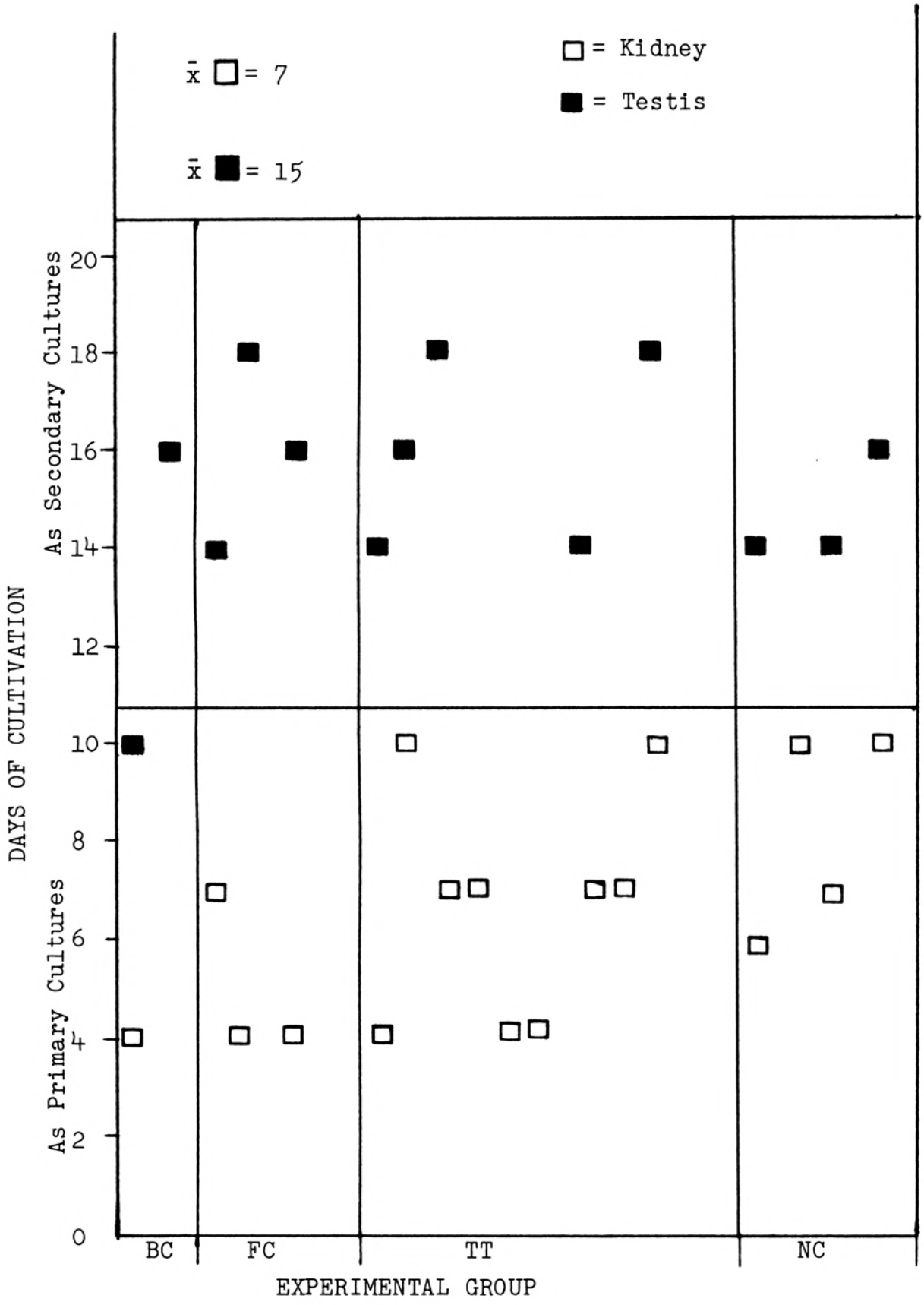

x̄ □ = 7
x̄ ■ = 15
□ = Kidney
■ = Testis
DAYS OF CULTIVATION
As Secondary Cultures
As Primary Cultures
20
18
16
14
12
10
8
6
4
2
0
BC
FC
TT
NC
EXPERIMENTAL GROUP

PLATE II. Cytopathic Effect of Turkey Herpesvirus in Secondary Testis Cell Cultures

1. Large focus of rounded cells (RC) observed in a secondary testicular cell culture from a TT bird at 4 days after a subculture at 10 days. Cytopathic effect is typical of that observed for turkey herpesvirus. Unstained; X112.

2. Virus plaque (VP) observed on primary testicular cell culture at 10 days after seeding from a bird of the BC group. Center of plaque contains large rounded cell (LR) with adjoining cytoplasmic strands. Unstained; X112.

3. Extensive cytopathic effect seen on secondary testicular cell culture at 8 days after a subculture at 10 days. Several areas of dark, rounded cells (DR) are seen, which give the monolayer a characteristic "dirty" appearance. Unstained; X112.

4. Herpesvirus cytopathic effect on secondary testicular cells at 5 days after a subculture at 10 days. Large, rounded and dark cells (DC) and smaller rounded cells (arrows) are observed. Unstained; X196.

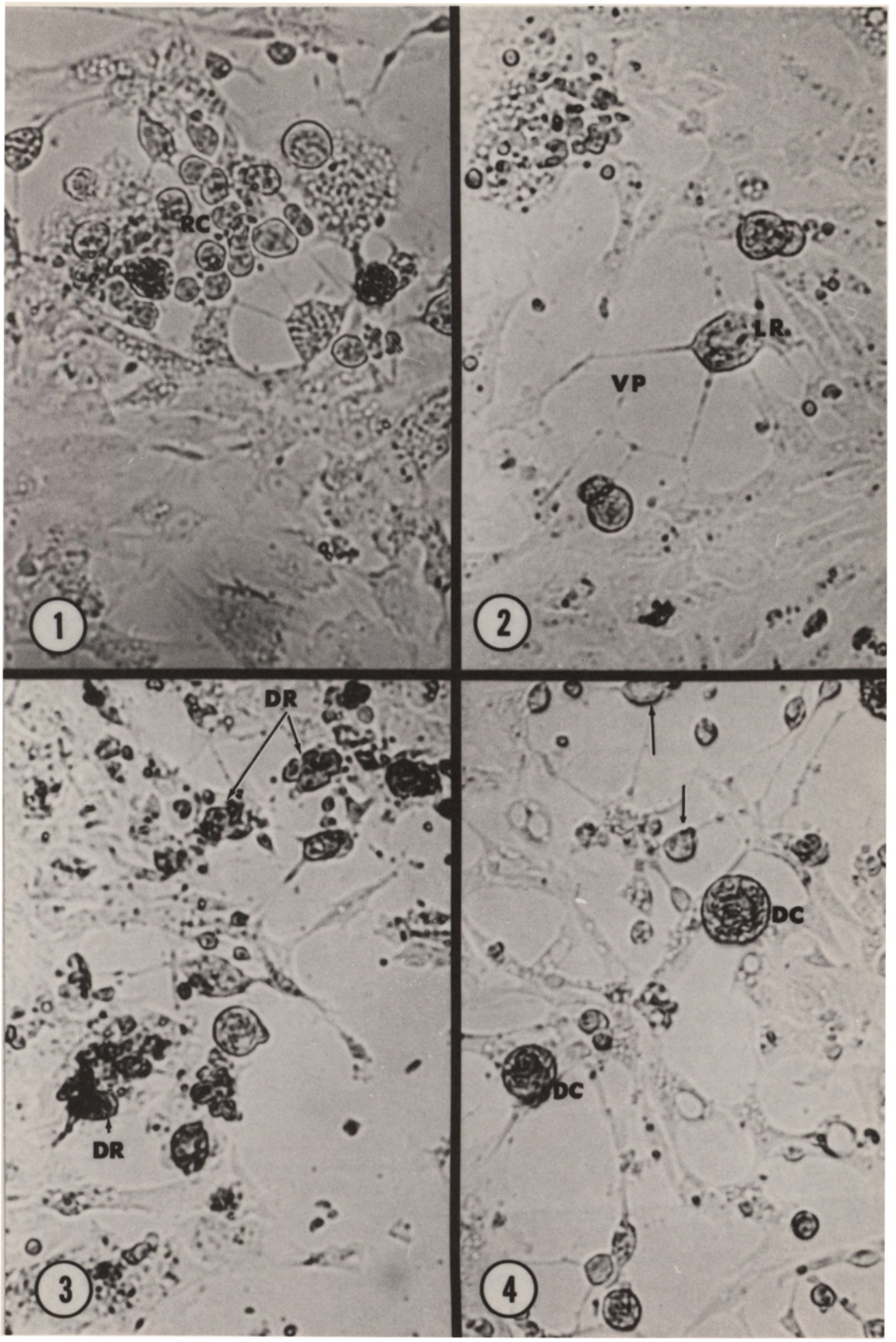

RC
1
LR
VP
2
DR
DR
3
DC
DC
4

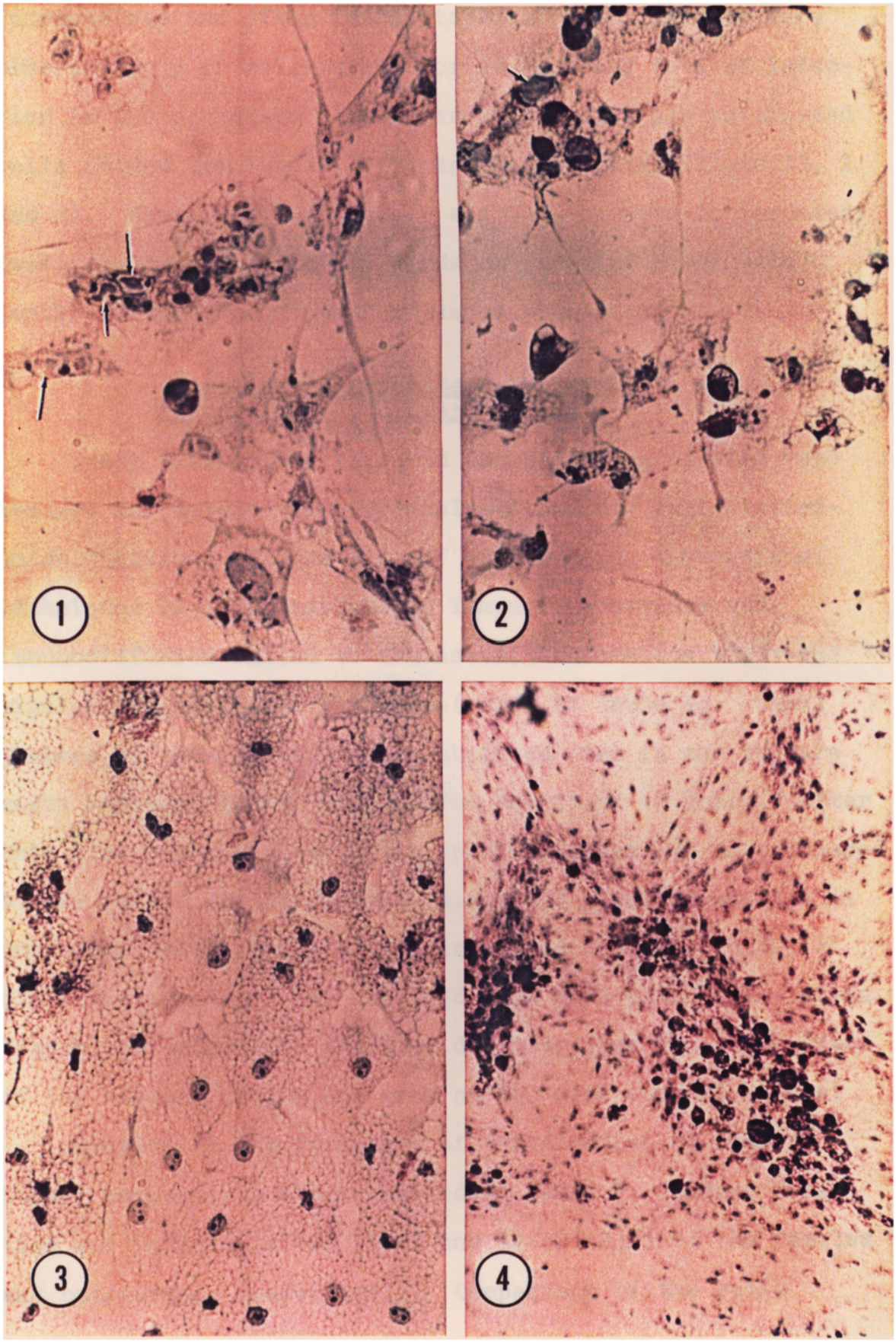
PLATE III. Hematoxylin and Eosin Staining of Normal and Infected Secondary Testis Cultures

1. Cytopathic effect on secondary testis from a FC bird at 4 days after subcultivation. Several type A intranuclear inclusions are observed (arrows). H & E stain; X328.

2. Cytopathic effect on secondary testis from a TT bird at 8 days after subcultivation. Most cells are rounded, intensely stained with pyknotic nuclei. One cell (arrow) contains intranuclear inclusion. H & E stain; X328.

3. Culture of secondary testis cells from control bird showing vacuolation and fusion of cell membranes 14 days after subculture. H & E stain; X328.

4. Focus of infection on secondary testis cells of a TT bird at 6 days after subcultivation. Infected cells are rounded, more intensely stained and contain pyknotic nuclei as compared to surrounding uninfected cells. H & E stain; X127.


1
2
3
4

Intranuclear and often intracytoplasmic fluorescence were observed only in cells within or surrounding foci of infection in the secondary testicular cultures, while uninfected cells showed low levels of background staining (Plate IV, 1 and 2). Specific fluorescence was not observed in secondary testis cultures of control birds or infected birds with testis cultures negative for plaque formation.

Reactivation of Latent Turkey Herpesvirus from Ovary Explants

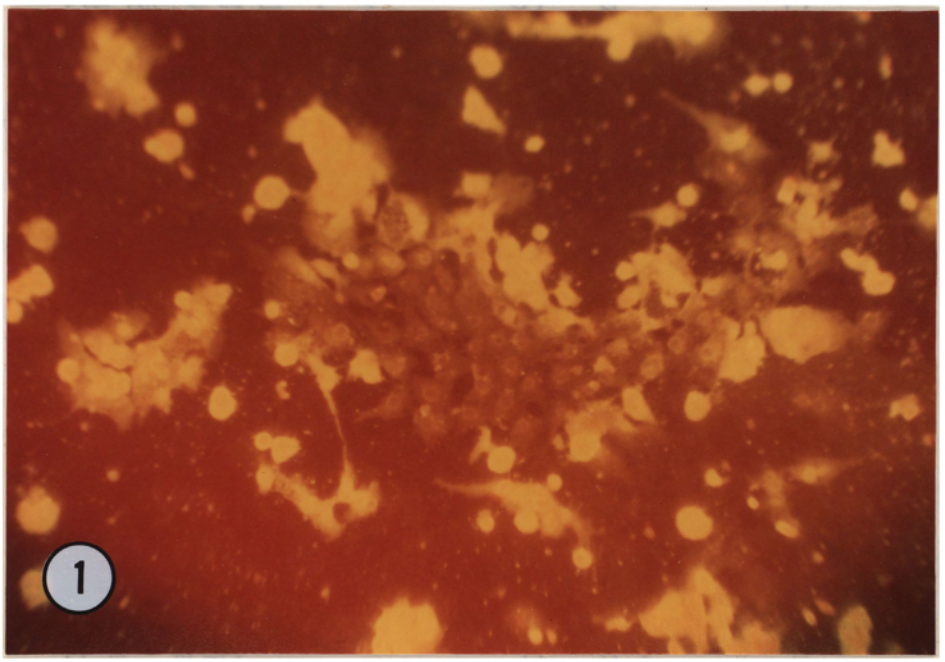
Ovary tissue from birds in the preceding studies were assayed for latent virus by explantation and explantation-co-cultivation. At the time of explantation conventional techniques of virus isolation, immunofluorescence and immunodiffusion failed to demonstrate infectious virus and viral antigens in ovary tissue (refer to Table 4). Small numbers of rounded cells were observed free in the culture media surrounding the explant for the first 48 hours. After this time most of these cells apparently settled and attached to the bottom of the culture vessel and glass coverslips as outgrowth cells, which slowly increased in number until a confluent monolayer was attained 8-10 days post-explantation. Fibroblast outgrowths migrated directly from the cut surface of the explant proper and grew on the wire support screen.

Results on the frequency of reactivation of latent turkey herpesvirus are presented in Table 9. Plaque formation was induced in 4/9 (44%) and 12/52 (23%) of the females and outgrowths, respectively. Comparison of the ratio

PLATE IV. Immunofluorescent Staining of Infected Secondary Testicular Cultures

1. Intense intranuclear and intracytoplasmic fluorescence in focus of infection from secondary testis of a TT bird 6 days after subcultivation. Stained with TT hyperimmune serum and commercial conjugate by the indirect fluorescent antibody technique. X127.

2. Intranuclear and intracytoplasmic fluorescence in secondary testis of an FC bird 4 days after subcultivation. Stained as in #1 above. X127.


1

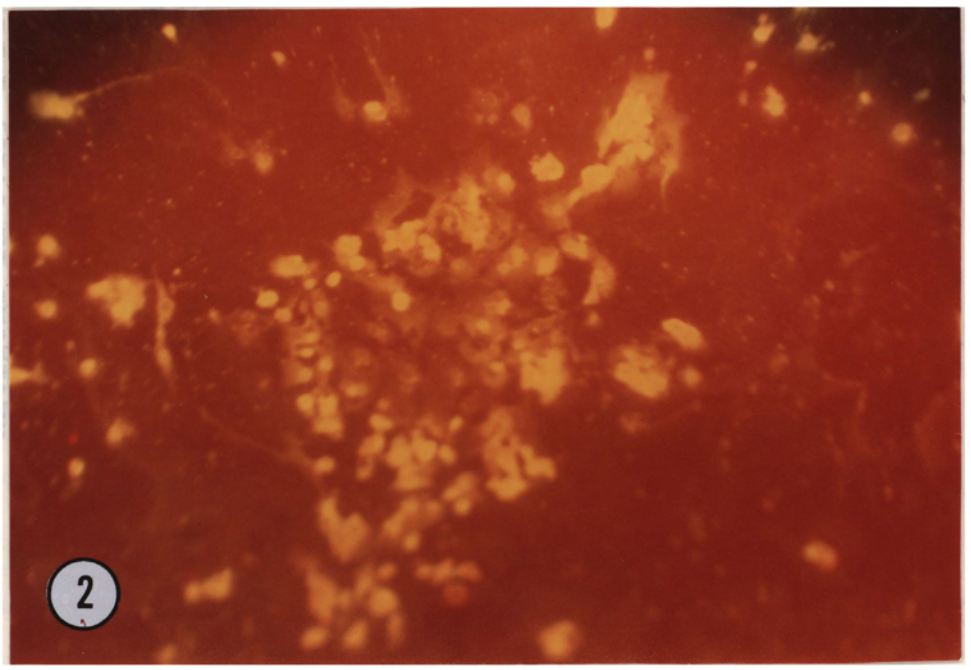

2

Table 9. Frequency of Reactivation of Latent Turkey Herpesvirus and Demonstration of Viral Antigens in Ovary Explant Outgrowths.

Exptl. Group	No. Positive Turkeys/No. Turkeys Examined[a]	Petri Dishes with Outgrowths Yielding Virus/No. Petri Dishes with Explants	Mean Time for Reactivation in Days	No. Turkeys with Positive[b] Results/No. Turkeys Exam.		
				Immuno-fluor-escence	Inclusion Bodies	Pooled Culture Media[c]
BC	1/1 (100%)	1/6 (17%)	28	1/1	1/1	ND
FC	-----	-----	--	---	---	--
TT	3/8 (38%)	11/46 (24%)	26 (20-28)[b]	3/8	3/8	0/5
Combined BC, TT	4/9 (44%)	12/52 (23%)	27 (20-28)	4/9 (44%)	4/9 (44%)	
UC	0/4 (0%)	0/16 (0%)	--	0/3	0/3	0/3

[a] Additional birds were done in each group but results from 3 birds in BC, 4 birds in FC and 5 birds in TT were lost due to contamination.

[b] Range

[c] Represents results on assay of culture media removed from the explants at 7, 14, 21 and 21 days PE.

positive/total to outgrowths (12/52) inferred that at least 4 petri dishes of explants must be established per bird to allow detection of plaque formation in at least one culture of ovarian explant outgrowths. An average time of 27 days PE was required for plaque formation in ovarian outgrowth cells suggesting that the virus is latent or present in small quantities.

Cytopathic effects of herpesvirus in ovarian outgrowth cells were identical to those described for secondary testicular cultures. Hematoxylin and eosin staining revealed type A intranuclear inclusion and infected cells within viral plaques showed intranuclear and intracytoplasmic fluorescence. Media fluids did not contain infectious virus. Plaque induction, inclusion bodies and specific fluorescence were not observed in ovarian outgrowths of control birds (Table 9).

Explants proper from all 9 birds were examined for infectious virus and immunofluorescence antigens at 28 days PE. Infectious virus was isolated only from homogenates prepared from explants proper of the 4 birds with virus positive outgrowths. Although the titers of virus in each homogenate was not determined, the CKC had to be subcultivated at 10 days PE before plaque formation was induced 4 days later. No viral antigens could be demonstrated in the explants proper by immunofluorescence. Plaques were not induced on the CKC monolayers of 0/2 petri dishes of explants in the BC, 0/16 in the TT and 0/2 in the UC group by explantation-co-cultivation.

Reactivation of Latent Turkey Herpesvirus from Testis Explants

Testis tissue from male birds used in the foregoing studies were assayed for latent virus by explantation and explantation-co-cultivation. Within 24 hours after explantation, large numbers of rounded cells were observed free in the culture media bathing the explant proper. After 48 hours most of these cells had attached to the surface of the culture vessel as small colonies or outgrowths of testicular cells and expanded until confluent monolayers of testicular cells were established 8 to 10 days post-explantation (PE). Few outgrowths, however, originated directly from the cut surfaces of the explant proper, although some outgrowth did occur to anchor the explants to the wire screen.

Data on the frequency of virus reactivation in testis explant outgrowths is presented in Table 10. Plaque formation was induced in 88% of all experimentally-inoculated males (BC, FC and TT) and 75% of the naturally-afflicted (NC) males. Combining these results, latent herpesvirus was harbored in 85% (17/20) of all infected males examined. Virus was not reactivated in explant outgrowth cells of NC bird #8019 and TT birds #376 and 381. Previously, direct cultivation of testicular cells from these latter two birds also failed to induce plaque formation. Whereas 85% of all infected males harbored latent herpesvirus, plaque formation was observed in only 43% (45/105) of the outgrowths. Comparison of the ratio of positive outgrowths/total (45/105)

Table 10. Frequency of Reactivation of Latent Turkey Herpesvirus and Demonstration of Viral Antigens and Particles in Testis Explant Outgrowths.

Exptl. Group	No. Positive Turkeys/No. Turkeys Examined	Petri Dishes with Outgrowths Yielding Virus/ No. Petri Dishes with the Explants	Mean Time for Reactivation in Days	No. Turkeys with Positive Results/ No. Turkeys Examined[b]			
				Immuno-fluor-escence	Inclusion Bodies	Viral Particles	Pooled Culture Media[c]
BC	2/2 (100%)	9/12 (75%)	16 (10-28)[a]	2/2	1/1	ND	0/2
FC	3/3 (100%)	8/15 (53%)	16 (10-28)	3/3	2/2	1/1	1/3[d]
TT	9/11 (82%)	22/64 (34%)	18 (10-28)	9/11	7/7	5/5	2/6[e]
Combined BC,FC,TT	14/16(88%)	39/91 (43%)	17 (10-28)	14/16 (88%)	10/10 (100%)	6/6 (100%)	3/11 (27%)
NC	3/4 (75%)	6/14 (43%)	28 (14-35)	3/4	2/2	ND	0/3
UC	0/2 (0%)	0/10 (0%)	-----	0/2	0/2	0/1	0/2

[a] Range

[b] Results based on examination of outgrowth cells on glass coverslips for immunofluorescence antigens and inclusion bodies. Cell pellets from outgrowths with herpesvirus CPE were used to demonstrate viral particles by electron microscopy.

[c] Represents results on assay of culture media removed from the explants at 7, 14, 21 and 28 days PE.

[d] One pooled sample of media removed at 21 days PE from one bird was positive for virus.

[e] Two pooled samples of media from 2 different birds at 21 days PE were positive for virus.

revealed that a minimum of 3 petri dishes of explants/bird must be set to detect plaque formation in one outgrowth culture.

The frequency of reactivation of latent herpesvirus was highest in explant outgrowths of the BC group, intermediate in the FC and lowest in the NC and TT groups. Quantitatively the amount of virus reactivated in the positive outgrowths (not shown in Table 10) was low. The average FFU/number of positive outgrowths was 3.9, 3.5, 3.0 and 2.8 for the BC, NC, FC and TT groups, respectively. Induction of viral plaques required at least 10 days with the average time PE essentially the same in the BC, FC and TT groups. Although there is a wide range of variation within each group, the combined average time of 17 days PE needed for the induction of viral plaques in testis outgrowth cells corroborated closely with the average 15 days required to effect plaque formation in secondary testicular cultures. Testis explant outgrowths of the NC group, however, required an additional 11 days PE for plaque formation compared to the BC, FC and TT groups.

The frequency of plaque formation in explant outgrowths as a function of the time PE is shown in Figure 7. Viral plaques were observed in 17/45 (37%) and 25/45 (55%) of the explant outgrowths at 2 weeks and 3-4 weeks PI, respectively. Explant outgrowths from NC #3118 were able to be maintained for 6 weeks with 1/3 and 2/3 explant outgrowths yielding virus at 5 and 6 weeks PE, respectively. No virus was

observed in explant outgrowths of testis from control birds maintained for 28 days PE.

Characteristics of turkey herpesvirus cytopathic effect on testis explant outgrowths were identical to those described for the secondary testicular cultures. Both the characteristic plaques with a central clear area surrounded by rosettes of rounded cells and the rounded, dark granular cells were observed (Plate V, 1 and 2, Plate VI, 1 and 2). Testis explant outgrowths from control birds were predominantly epithelial type cells with large vacuoles (Plate VI, 3) as previously observed in secondary testicular cultures. Hematoxylin and eosin staining of infected outgrowths showed type A intranuclear inclusions (Plate VII, 1-4). Intranuclear and intracytoplasmic fluorescence was observed in areas of specific cytopathic effect while neighboring cells had low levels of background fluorescence (Plate VIII, 1-3).

Viral particles were found in all 6 of the infected outgrowth cells observed. Outgrowths consisted of predominantly gonial cells, which could not be subclassified morphologically, and fibroblasts. Macrophages were rarely observed in the 6 explant outgrowths examined by electron microscopy. Several cells presumed to be gonadocytes contained numerous intranuclear viral particles having morphological characteristics of herpesviruses; nucleocapsids were hexagonal, measured approximately 100nm in diameter and were either empty, containing a dense 35-40 nm core or having the characteristic translucent cross-shaped

PLATE V. Herpesvirus Cytopathic Effect in Outgrowths of Testis Explants

1. Herpesvirus plaque on outgrowths of cells from testis explant 21 days post-explantation from bird in TT group. Plaque has characteristic clear area (CA) with large and small rounded cells on the periphery. Unstained; X112.

2. Cytopathic effect observed 14 days post-explantation in outgrowths of testis explant cells from a BC inoculated bird. Several isolated (arrows) and clusters of round, granular cells giving a "dirty" appearance are observed. Unstained; X196.

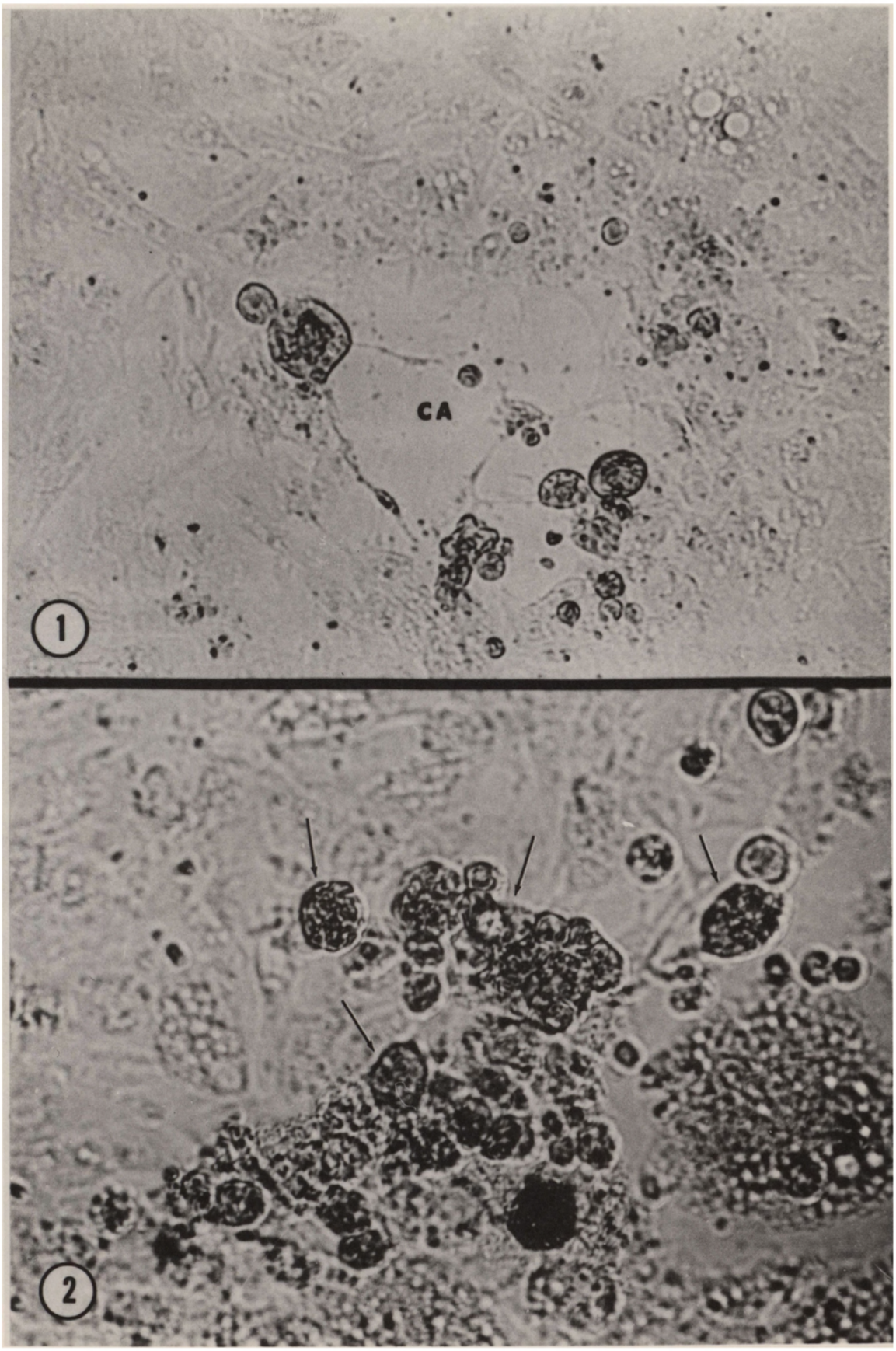

CA
1
2

PLATE VI. Cytopathic Effect in Outgrowths of Testis Explants

1. Cytopathic effect observed 21 days post-explantation in outgrowths of testis explants from a TT bird as viewed through the wire support screen. Large and small rounded cells (small arrow) can be observed. Dark areas surrounding infected cells is a portion of the wire support screen. Unstained; X112.

2. Different area from same outgrowths of testis explants as described in A. Note cluster of large rounded and granular cells (RC). Unstained; X196.

3. Testis explant outgrowths at 23 days PE from an uninfected control bird. Cells are predominantly of the epithelial type (and contain numerous vacuoles (v)). Unstained; X196.

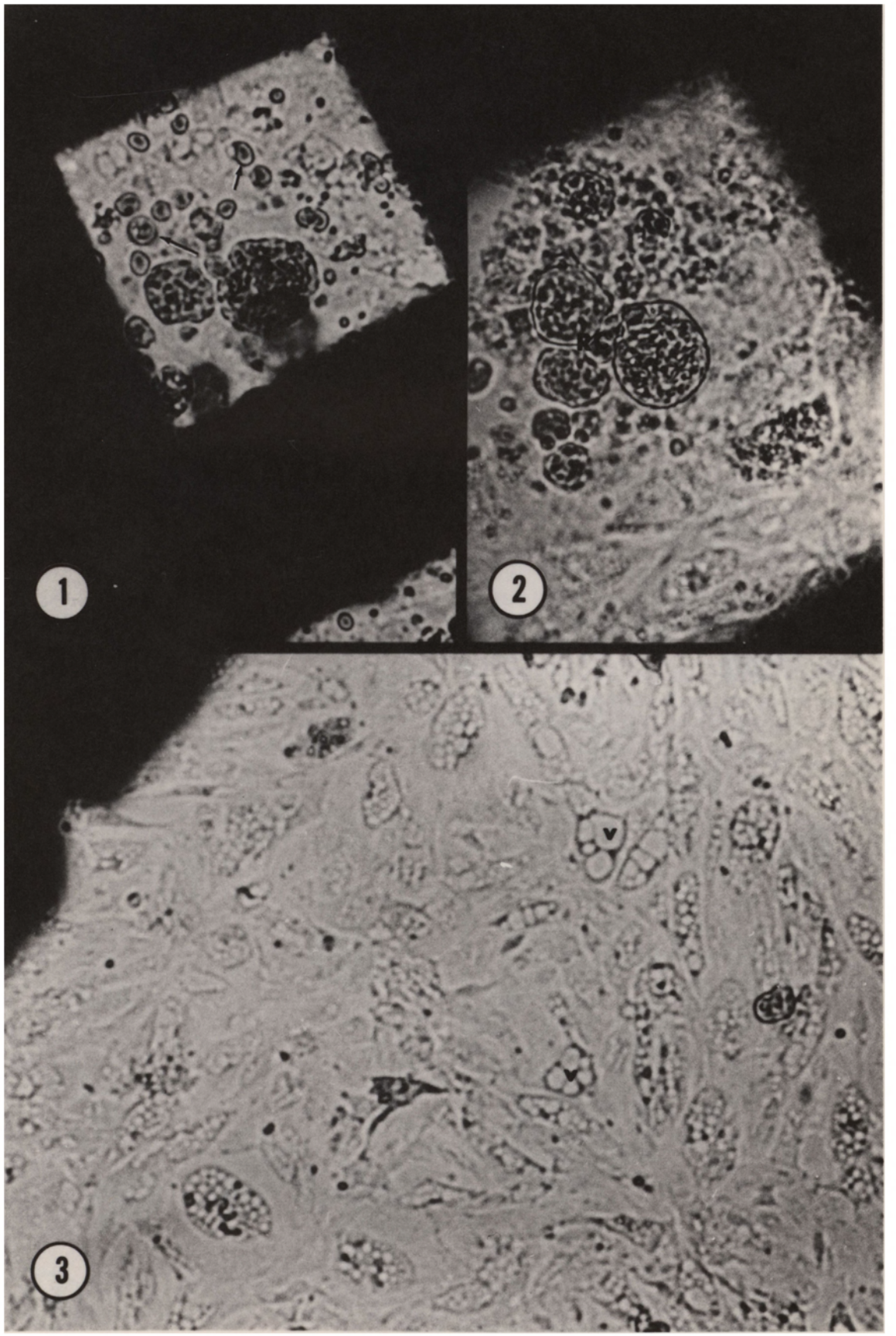

1
2
3
V

PLATE VII. Hematoxylin and Eosin Staining of Infected Testis Explant Outgrowth Cells

1. Syncytium (s) observed in outgrowth cells from testis explant of TT bird at 28 days PE. Several cells show type A intranuclear inclusion (arrows) and margination of chromatin. Hematoxylin and eosin; X328.

2. Multinucleated cell in outgrowth from testis explant of a TT bird at 14 days PE. Type A intranuclear inclusions (arrows) are shown. Hematoxylin and eosin; X530.

3. High magnification of syncytium (s) in testis explant outgrowths of an FC bird at 28 days PE showing intranuclear inclusions (i) and margination of chromatin. Hematoxylin and eosin; X1300.

4. High magnification of syncytium (s) in testis explant of a TT bird at 28 days PE showing intranuclear inclusions (i) and margination of chromatin. Hematoxylin and eosin; X1300.

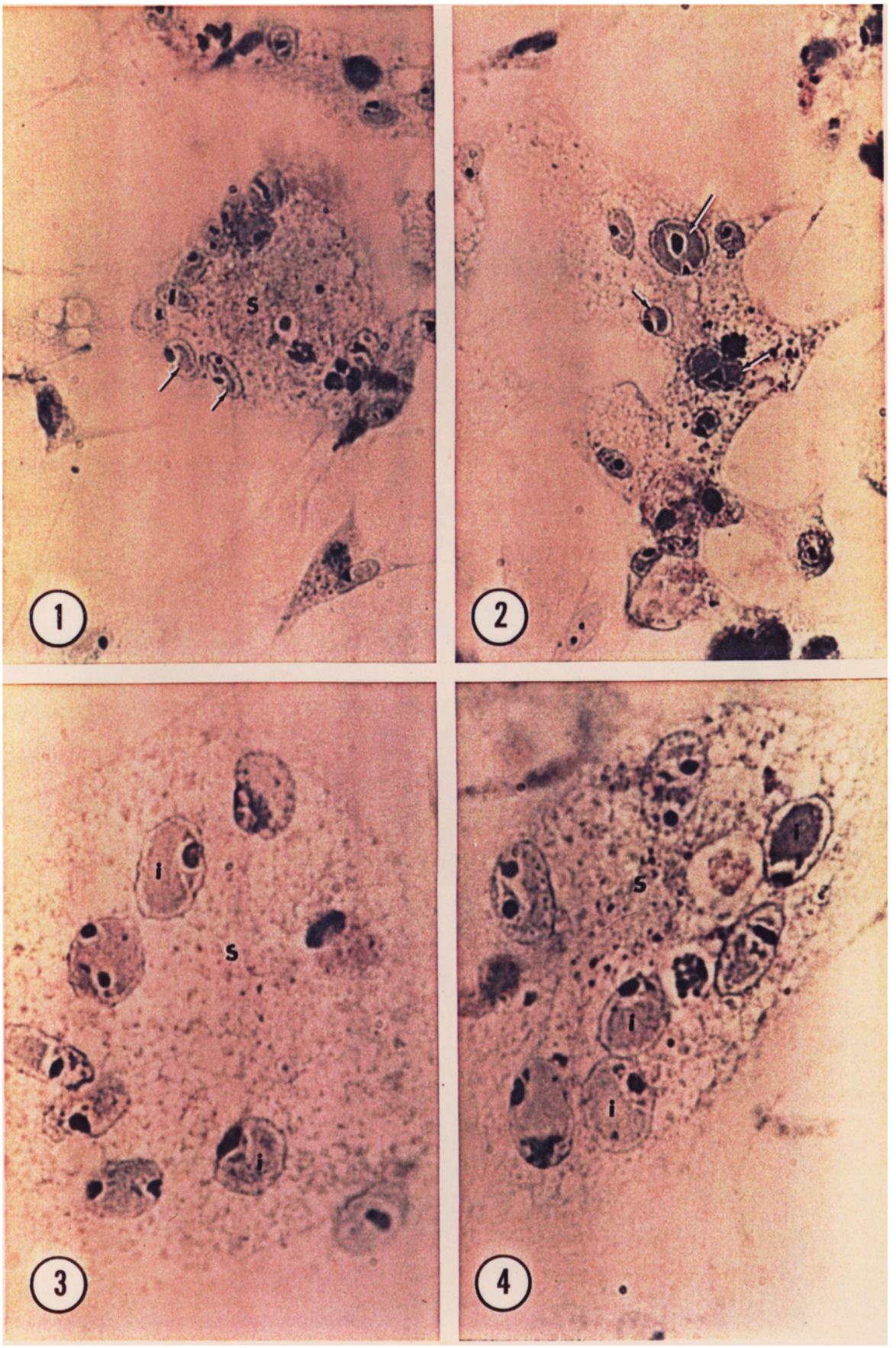
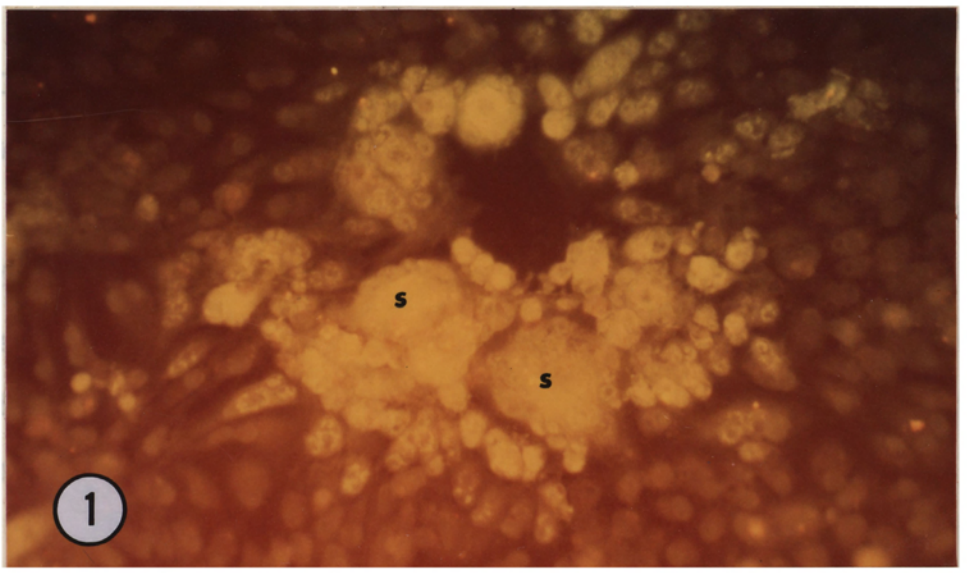

1
i
s
2
3
i
s
i
4
i
s
i
i

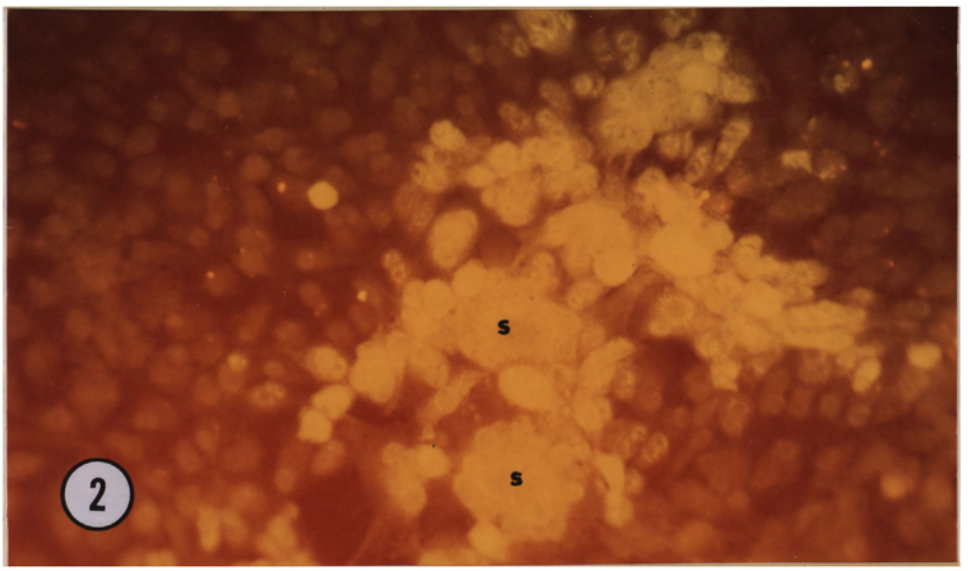
PLATE VIII. Indirect Immunofluorescence Staining of Testis Explant Outgrowths

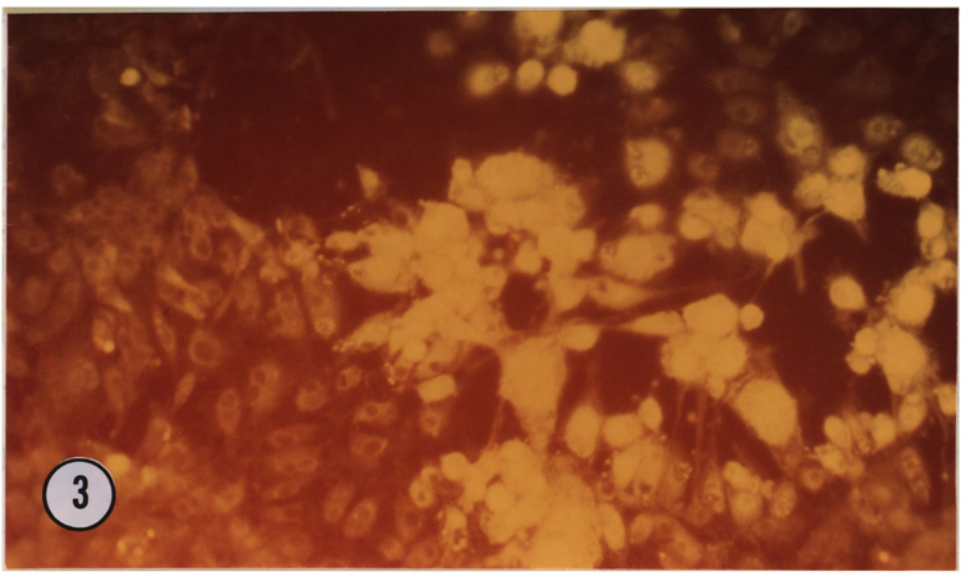
1. Outgrowth cells from testis of a TT bird at 28 days PE showing intense intranuclear and intracytoplasmic staining in syncytia (s) and surrounding cells. Indirect immunofluorescence stain with hyperimmune sera to the TT isolate and commercial conjugate; X328.

2. Area of cytopathic effect in outgrowth cells from testis of a TT bird at 18 days PE showing intense intranuclear and intracytoplasmic staining in syncytia (s) and surrounding cells. Surrounding uninfected cells show low level of background fluorescence. Indirect immunofluorescence stain with hyperimmune sera to the TT isolate and commercial conjugate; X328.

3. Intranuclear and intracytoplasmic staining in outgrowths from testis of an FC bird at 10 days PE. Cells show various stages of virus replication. Indirect immunofluorescence stain with hyperimmune sera to FC isolate and commercial conjugate; X328.


S
S
1


S
S
2


3

core (Plates IX, X, XI, XII). Enveloped particles approximately 180-250 nm were observed only in 1/6 outgrowth cultures and were a rare event (Plate XII).

Virus was also isolated from the media of 1/3 FC and 2/6 TT birds at 21 days PE from outgrowths with cytopathic effect. Media sampled from outgrowths of control birds and those in which plaques were not induced remained negative for virus after 2 blind passages in CKC cells.

When plaque formation was first observed in the testis explant outgrowths, the corresponding explants proper were then removed and examined for the presence of infectious virus, viral antigens and viral particles. The results are presented in Table 11. Virus was isolated from homogenates prepared from the explant proper of at least 50% of all birds examined in each group. On the average, the titer of virus isolated from the homogenates was of equal magnitude for the BC, TT and NC group, whereas the FC group titer was 7x higher. Again at least 15-20 days of cultivation on CKC was required to induce plaque formation. However, the immunofluorescence, immunodiffusion and electron microscopy results did not correlate with those of virus isolation as viral antigens or viral particles could not be demonstrated in the explant proper. Inclusion bodies were also not demonstrated by light microscopy.

Interestingly, testis explant proper from bird #324, which died at 12 weeks PI revealed viral particles in the explant proper after 10 days PE (Plate XIII, 1 and 2).

PLATE IX. Ultrastructure of Herpesvirus Particles in Testes Explant Outgrowths

An infected cell from outgrowths of testis explants 24 days post-explantation of a bird inoculated with the TT isolate. Nucleus (N) contains several immature naked herpesviruses (IV) including a characteristic cross-shaped particle indicated by the small arrow. Cell shows loss of cytoplasmic (Cy) organization and free ribosomes. X20,450.

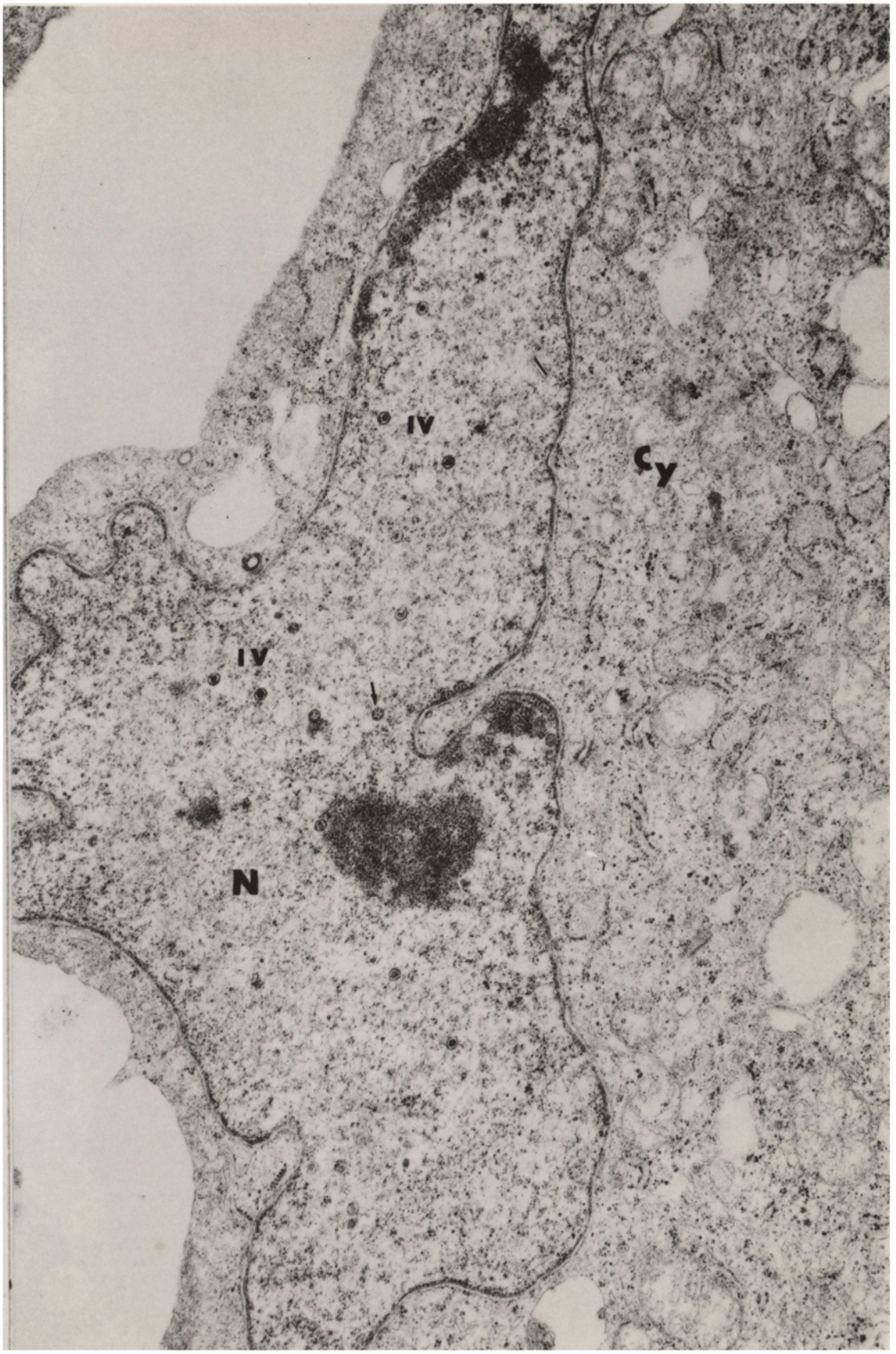

IV
Cy
IV
N

PLATE X. Ultrastructure of Herpesvirus Particles in Testes Explant Outgrowths

1. Portion of a nucleus (N) of a cell from outgrowths of testis explants 18 days post-explantation. Nucleus contains mature enveloped (E) and immature naked (IV) herpesviruses. Chromatin also appears to have clumped around the nuclear membrane. X31,350.

2. Area of nucleus outlined by the square in A showing enveloped herpesviruses (E) contained within an invagination (Ne) of the nuclear membrane. X58,800.

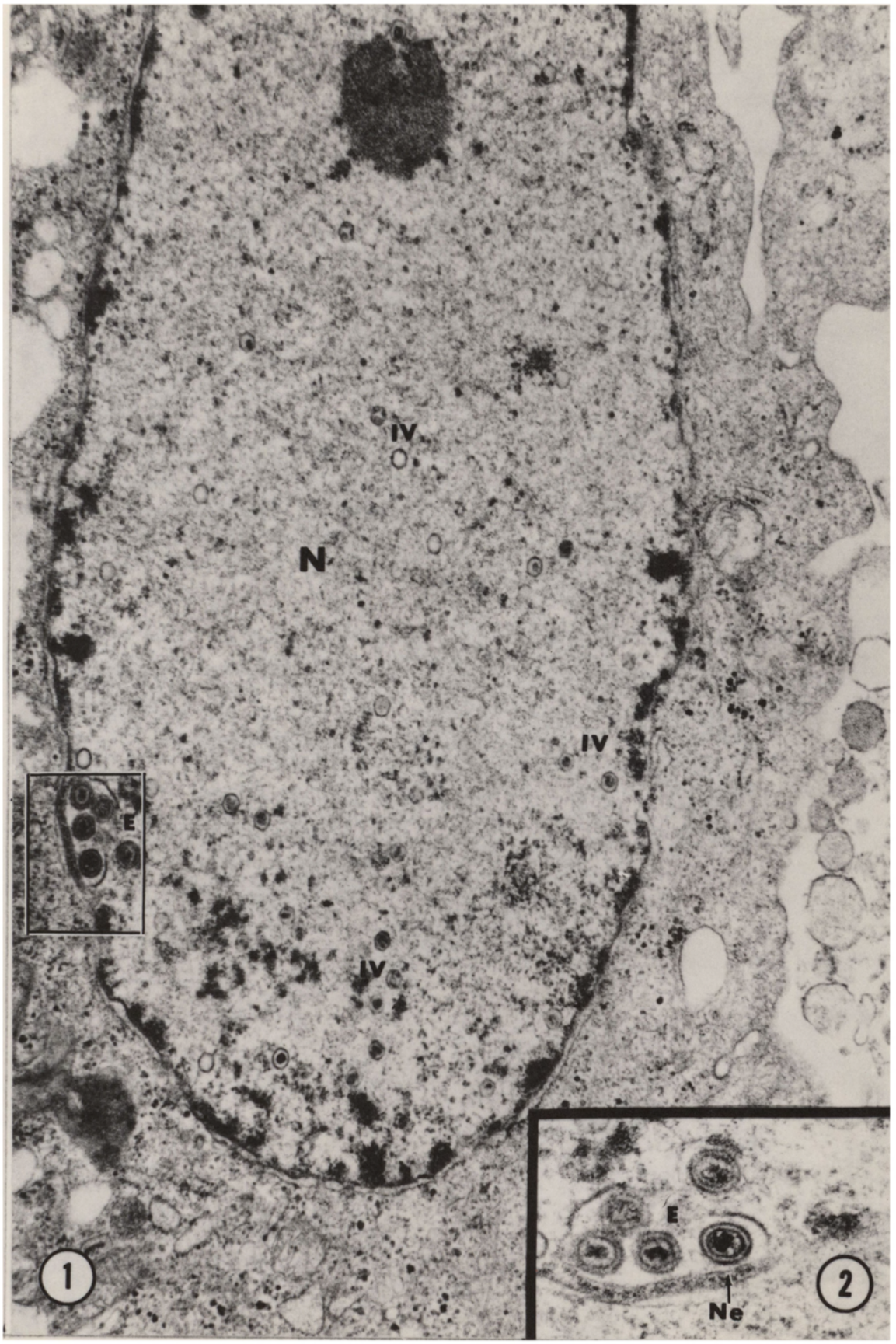

IV
N
IV
IV
E
E
Ne
1
2

PLATE XI. Ultrastructure of Herpesvirus Particles in Testes Explant Outgrowths

1. Binucleated or dividing cell from testis explant outgrowths of a TT bird at 24 days post-explantation. Immature, naked herpesvirus particles (IV) can be observed in both nuclei (N). Cytoplasm (Cy) reveals some loss of structure but a Golgi apparatus (GA) and mitochondri (M) are still discernible. X15,100.

2. Herpesvirus particles present within the nucleoplasm of cell from testis explant outgrowths. Two particles contain dense core material (D), one a lighter core (L), one is an empty capsid (C) and the remaining two are variations of particles with cross-shaped cores (X). X89,600.

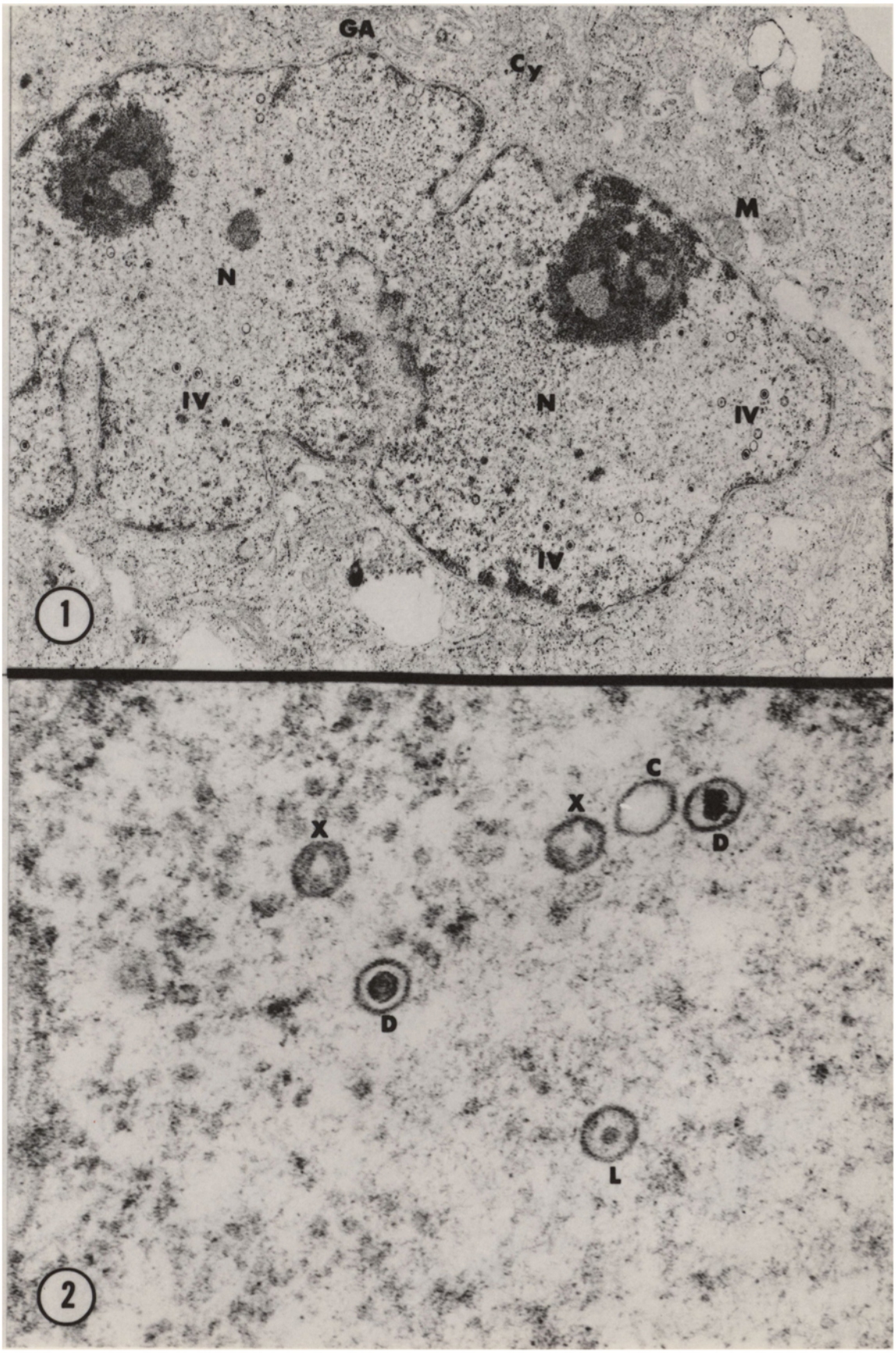

GA
Cy
M
N
IV
N
IV
IV
1
X
X
C
D
D
L
2

PLATE XII. Ultrastructure of Herpesvirus Particles in Testic Explant Outgrowths

Portions of an infected cell from outgrowths of testis explants at 16 days post-explantation. Nucleus (N) contains numerous types of immature herpesvirus particles (D = dense core, C = empty capsid and X = cross configuration) and clumping of chromatin (Cr). Cytoplasm contains a large vesicle enclosing mature enveloped herpesviruses (E). These enveloped particles contain a translucent core surrounded by a dense envelope. X45,350.

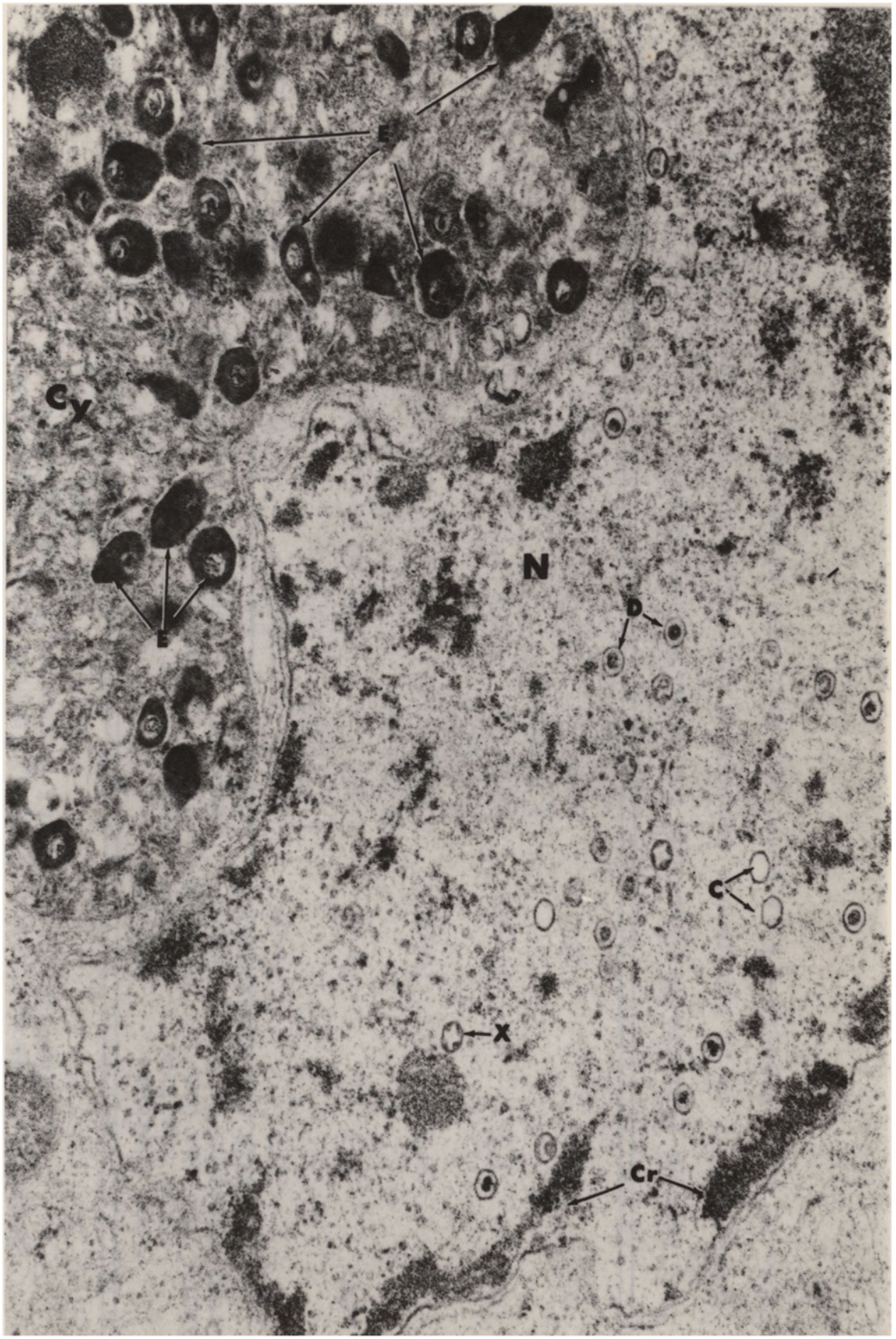

Cy
N
D
C
X
Cr

Table 11. Demonstration of Viral Infectivity, Antigens and Particles in Explant Proper of Testis Explants

Exptl. Group	No. Turkeys with Positive Explant Outgrowths/No. Turkeys Examined	Time PE (Days) Explant Proper Examined	Homogenate of Explant Proper	Immunofluorescence	Immunodiffusion	Viral Particles	Inclusion Bodies
BC	2/2	14-26	2/2[a] 3.5[b](20)[c]	0/2	0/2	ND	0/1
FC	3/3	14-28	3/3 22.0 (15)	0/3	0/3	0/2	0/2
TT	9/11	14-28	6/11 3.0 (17)	0/11	0/11	0/6	0/5
NC	3/4	28-42	2/4 3.5 (17)	0/4	0/4	ND	ND
UC	0/2	28	0/2 0 (0)	0/2	0/2	0/1	0/1

[a]No. Turkeys Positive/No. Examined for viral infectivity.

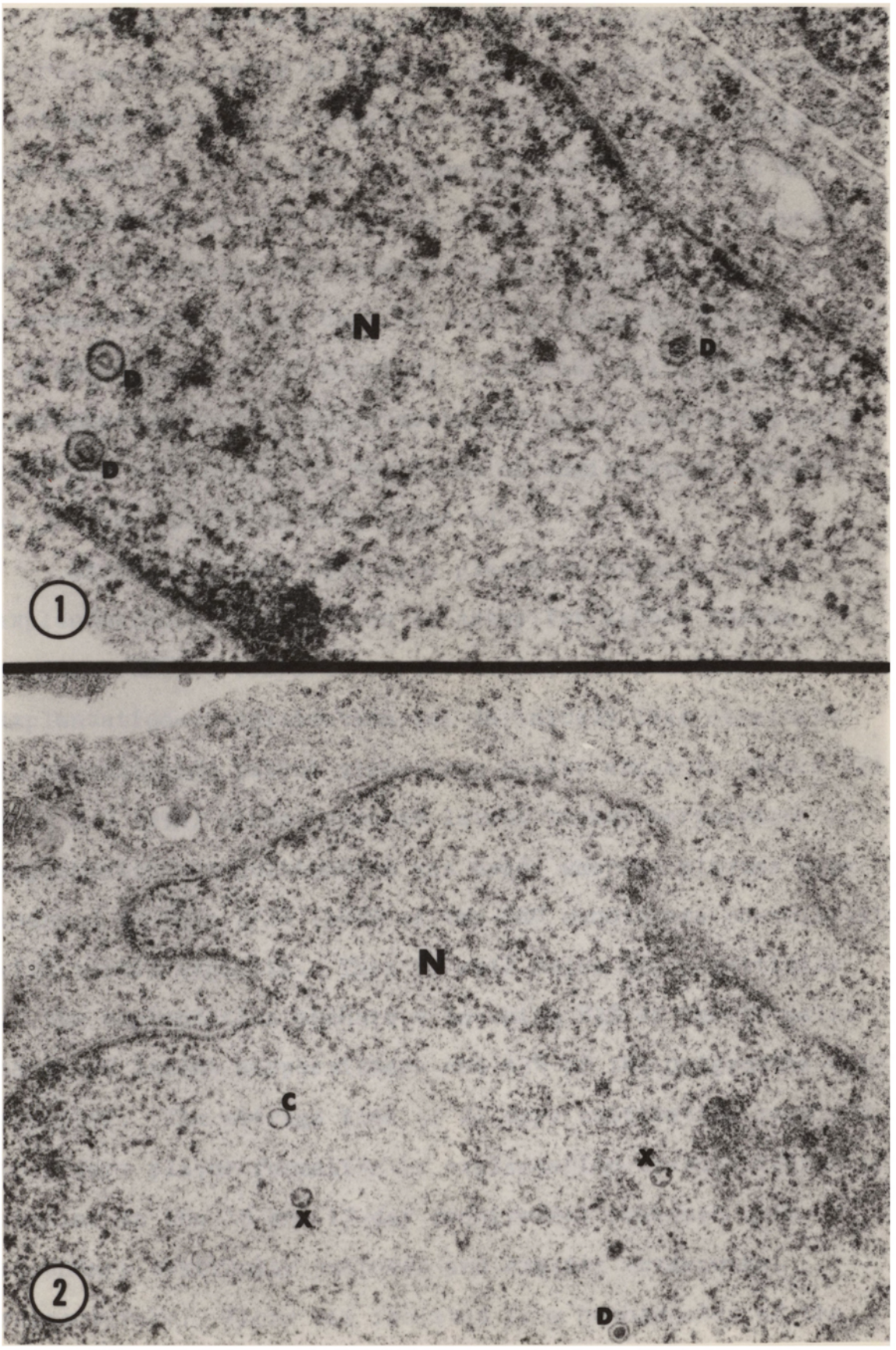
[b]Average FFU/2 ml of homogenate inoculated onto duplicate monolayers of primary CKC.

[c]Time in days at which CPE appeared and foci were enumerated.

PLATE XIII. Herpesvirus Particles in Explant Proper of Testis

1. Portion of a nucleus (N) from the testis explant proper of bird number 324 showing three herpesvirus particles with dense core material (D). X89,600.

2. Nucleus (N) of a germinal epithelial cell from the testis explant proper of bird number 324. Three types of particles (D = dense core, C = empty capsid and X = cross configuration) are observed. X31,350.


N
D
D
D
1
N
C
X
X
D
2

At 7 days PE the structure of the seminiferous tubules contain spermatagonial cells in various stages of differentiation including spermatozoa present in the lumen of the seminiferous tubules. At 14 days PE some necrosis is evident as characterized by pale staining areas but most cells appear to be intact although few spermatozoa are present. Necrosis becomes evident at 21 days PE with an increase in pale staining areas and loss of germinal epithelial cells lining the seminiferous tubules which continued until most cells were necrotic and present in the lumen of seminiferous tubules at 30 days PE (Plate XIV, 3 and 4).

Explants proper were also placed on primary CKC explantation-co-cultivation to assay for latent turkey herpesviruses. Compared to the explantation method, explantation-co-cultivation was definitely less sensitive in reactivating latent virus from testis explants (Table 12). Only 2/7 (29%) birds harbored latent turkey herpesvirus if explantation-co-cultivation was used, whereas plaques were induced in only 2/14 (14%) of the CKC monolayers co-cultivated with testis explants. This is well below the sensitivity of the explantation technique in which 83% of the TT males harbored latent herpesviruses and 43% of the outgrowths were positive. The average time of 21 days cultivation required to effect plaque formation in explantation-co-cultivation is similar to the 15 and 17-day requirement observed with secondary testicular cultures and explant outgrowths, respectively. Virus was isolated from homogenates of the

PLATE XIV. Histological Appearance of Testis Explant Proper of a TT Bird at Various Time Post-Explantation.

1. Outline of a seminiferous tubule (ST) of explant proper at 7 days PE. Note presence of various populations of spermatogonial cells including spermatozoa in the lumen. Few if any necrotic areas are noticed at this time. Hematoxylin and eosin stain; X328.

2. Low magnification of explant proper at 14 days PE showing pale staining areas which apparently are necrotic. Outlines of seminiferous tubules are not easily discernible although most of the cell types appeared to stain well. Hematoxylin and eosin stain. X127.

3. Portion of explant proper at 21 days PE showing three adjacent seminiferous tubules (ST) bordered by interstitial tissue (I). Most of the cells are in the lumen of the seminiferous tubule and many are necrotic as evidenced by the pale staining. Hematoxylin and eosin stain; X530.

4. Seminiferous tubule (ST) of explant proper at 30 days PE showing necrotic cells in lumen. Cells in interstium (I) also fail to stain. Hematoxylin and eosin stain; X530.

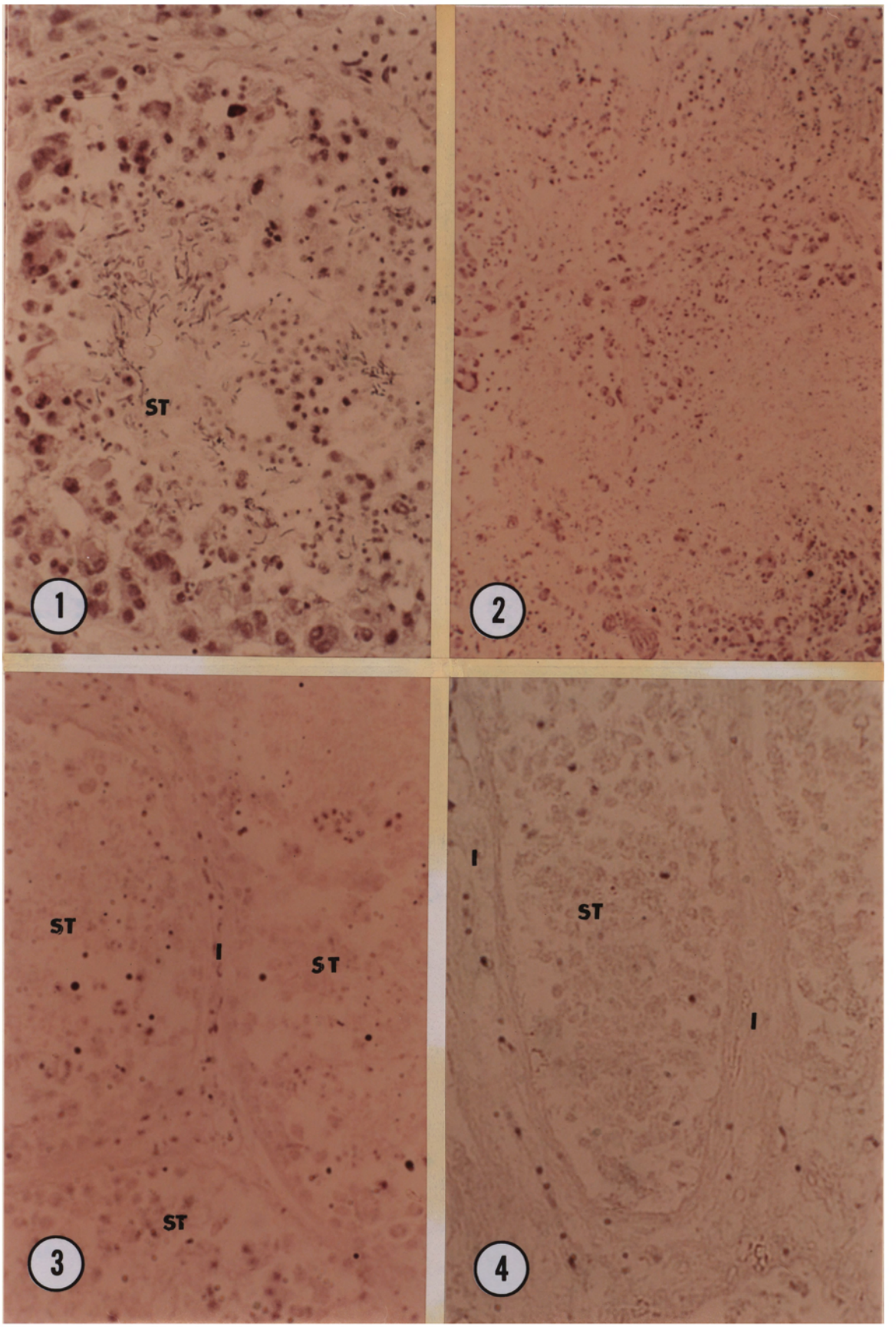

ST
1
2
ST
I
ST
ST
3
I
ST
I
4

Table 12. Frequency of Reactivation of Latent Turkey Herpesvirus from Testis Explants by Explantation-Co-Cultivation

Exptl. Group	No. Positive Turkeys/ No. Turkeys Examined	No. Petri Dishes with Virus Yielding Explants/No. Petri Dishes with the Explants	Mean Time for Reactivation in Days
BC	0/2 (0%)	0/4 (0%)	-----
FC	0/3 (0%)	0/6 (0%)	-----
TT	2/7 (29%)	2/14 (14%)	21 (20-24)[a]
NC	0/4 (0%)	0/8 (0%)	-----
UC	0/2 (0%)	0/4 (0%)	-----

[a] Range

explants proper from the 2 petri dishes with virus yielding explants but viral antigens were not demonstrated by immunofluorescence or immunodiffusion and viral particles were not observed with electron microscopy.

A comparison of all methods used in the foregoing studies to demonstrate latent turkey herpesviruses in testis is presented in Table 13. Essentially all efforts to demonstrate infectious virus, viral antigens and viral particles at the time of explantation were unsuccessful. After several weeks as explants, however, plaques were induced in testis explant outgrowths and virus could be isolated from homogenates of the explant proper of most birds. Subsequently, viral antigens, viral particles and viral inclusion bodies were demonstrated in outgrowth cells by immunofluorescence, electron and light microscopy, respectively. Similar results, however, could not be obtained with the explants proper.

Co-cultivation of testicular cells onto CKC and explantation-co-cultivation proved to be insensitive methods for the demonstration of latent turkey herpesviruses. Direct cultivation of testis cells also failed to induce plaque formation, whereas subcultivation of primary testicular cells readily induced plaque formation thereby supporting the explantation data that testicular cells harbor latent herpesvirus of turkeys.

Kinetics of Reactivation of Latent Turkey Herpesvirus in Testis Explants Proper

The influence of *in vitro* explantation on the reactivation of HVT was evaluated using testis explants from birds

Table 13. Comparison of Methods Used to Demonstrate Latent Virus, Viral Antigens and Particles in Testis

Methods	Groups		
	Exptl.[a]	Natural	Control
Before Explantation			
Testis Sonicates	0/15[b]	0/4	0/2
Immunofluorescence	0/4	0/4	0/2
Immundiffusion	0/15	0/4	0/2
Electron Microscopy	0/6	ND[c]	0/1
Histopathology (Type A inclusions)	0/15	ND	0/1
Cultivation of Testis Cells			
Primary Testis Culture	1/16	0/4	0/2
Secondary Testis Culture	13/16	3/4	0/2
Testis/CKC Co-culture	2/16	1/4	0/2
After Explantation			
Explant Proper:			
Homogenates	11/16	2/4	0/2
Immunofluorescence	0/16	0/4	0/2
Immunodiffusion	0/16	0/4	0/2
Electron Microscopy	0/8	ND	0/1
Viral Inclusions	0/8	ND	0/1
Explant Outgrowths:			
Outgrowth Cells	14/16	3/4	0/2
Immunofluorescence	14/16	3/4	0/2
Immunodiffusion	ND	ND	ND
Electron Microscopy	6/6	ND	0/1
Viral Inclusions	10/10	2/2	0/2
Explantation-co-cultivation	2/12	0/4	0/2

[a] Combined totals for BC, FC and TT experimental groups.

[b] No. Turkeys Positive/No. Turkeys Examined.

[c] Not determined.

of the TT group. To minimize environmental differences which might arise by establishing the explants in several 35 mm petri dishes, a large pool of explants from each of the three birds were seeded on large wire grids in 15 x 60 mm petri dishes. At various days post-explantation, approximately 4 to 5 explant pieces were harvested for virus isolation, immunofluorescence and immunodiffusion. Virus isolation was accomplished by grinding the explant pieces in 5 ml of maintenance media in a Ten Broeck grinder. Four ml were then inoculated onto a 60 mm petri dish of CKC and allowed to adsorb for 24 hours. Media was changed after 24 hours and the cultures were then observed daily for virus cytopathic effect and transferred at 7-day intervals to maintain cell viability. The remaining one ml of ground explant was used to test soluble antigens using an immundiffusion test. A similar number of explant pieces were also frozen in OCT compound for indirect immunofluorescence.

The results revealed that at least 16 days of explantation were required before virus could be isolated from the explants proper of one bird #370 (Table 14). Birds #356 and 364 required an additional 6-9 days of explantation before viral plaques were induced at 22 and 25 days PE, respectively. Subsequent sampling times in all three birds yielded virus. It is of note that a subculture of the CKC from bird #370 was necessary to effect plaque production at 16 days PE, while in all other cases plaques were formed within 4-10 days after inoculation of the CKC. With the

Table 14. Kinetics of Activation of Latent Turkey Herpesvirus in Testis Explants Proper

Time Post Explantation (Days)	TT Experimental Group Turkey No. 356 VI[a]	IFA[b]	ID[c]	364 VI	IFA	ID	370 VI	IFA	ID
0	0	NEG	NEG	0	NEG	NEG	0	NEG	NEG
2	0	NEG	ND	0	NEG	ND	0	NEG	ND
4	0	NEG	NEG	0	NEG	NEG	0	NEG	NEG
6	0	NEG	ND	0	NEG	ND	0	NEG	ND
9	0	NEG	NEG	0	NEG	NEG	0	NEG	NEG
12	0	NEG	ND	0	NEG	ND	0	NEG	ND
14	0	NEG	NEG	0	NEG	NEG	0	NEG	NEG
16	0	NEG	ND	0	NEG	ND	28.0(14)	NEG	NEG
19	0	NEG	NEG	0	NEG	NEG	10.0(4)	NEG	ND
22	3.0(10)[d]	NEG	ND	0	NEG	ND	2.0(4)	NEG	NEG
25	3.0(4)	NEG	NEG	1.0(7)	NEG	NEG	27.0(4)	NEG	ND
28	3.0(10)	NEG	NEG	3.0(4)	NEG	ND	27.0(4)	NEG	NEG
30	ND[e]	ND	ND	1.0(4)	NEG	NEG	ND	ND	ND

Table 14 - continued

[a] VI = Virus isolation.

[b] IFA = Indirect fluorescent antibody tests (9 sections of 4-5 explants proper)

[c] ID = Immunodiffusion

[d] Average number of FFU obtained from ground suspension of explants proper (time in days for viral plaques to appear.

[e] ND = Not determined.

exception of bird #370 the titer of virus was low, rarely exceeding 3.0 FFU/4.0 ml homogenate. Viral antigen formation in explant proper did not, however, precede the isolation of infectious virus as immunofluorescence and immunodiffusion assays were negative throughout the sampling period.

Reactivation of Latent Turkey Herpesvirus from Spinal Ganglia of Male and Female Turkeys

Thoracic and lumbar spinal ganglia were assayed for latent virus by explantation and explantation-co-cultivation. At the time of explantation infectious virus, viral antigens or viral particles were not demonstrated by virus isolation, immunofluorescence and electron microscopy, respectively (Table 15).

Within 3 to 4 days PE fibroblastic outgrowths originated from the cut surfaces of the ganglion explant proper and grew on the wire support screen. Fibroblasts rarely attached to the bottom of the culture vessel. The results of virus reactivation in these outgrowths are presented in Table 16. The data obtained from males and females was pooled since no sex related differences were observed.

Turkey herpesvirus was harbored latently in only 35% (8/23) of the birds examined and 9% (17/180) of the ganglion outgrowths observed. Comparison of the latter ratio (17/180) revealed that plaques were induced in only one of 10 petri dishes examined. Latent virus was also reactivated with equal frequency from outgrowths of the thoracic or lumbar spinal ganglion indicating that virus probably reached the ganglion via blood.

Table 15. Recovery of Latent Turkey Herpesvirus from Spinal Ganglia of Male and Female Turkeys

Methods	Females[a]		Males[a]		
	Exptl	Control	Exptl	Natural	Control
Before Explantation					
Homogenates	0/21	0/2	0/14	0/3	0/2
Immunofluorescence	0/21	0/2	0/14	0/3	0/2
Electron Microscopy	0/4	0/1	0/6	ND[b]	0/1
After Explantation					
Explant Proper:					
Homogenates	3/9	0/2	6/14	0/3	0/2
Immunofluorescence	0/9	0/2	0/14	0/3	0/2
Electron Microscopy	ND	ND	0/6	ND	0/1
Explant Outgrowths:					
Outgrowth Cells	3/9	0/2	5/14	0/3	0/2
Immunofluorescence	3/3[c]	ND	5/5[c]	ND	ND
Electron Microscopy	ND	ND	ND	ND	ND

[a] Results expressed as total number turkeys positive/number examined. Results from thoracic and lumbar ganglia are pooled.

[b] ND = Not determined.

[c] Represents specific fluorescence in CKC monolayers inoculated with virus positive outgrowth cells.

Table 16. Recovery of Latent Herpesvirus of Turkeys in Outgrowth Cells from Spinal Ganglion Explants

Exptl. Group	No. Petri Dishes Positive for Virus/ No. Petri Dishes with Explants[a]	Mean Time for Reactivation in Days (range)	No. Turkeys Positive/No. Examined[a]
BC			
T	0/10 (0%)	-----	0/2 (0%)
L	1/5 (20%)	28	1/2 (50%)
FC			
T	4/17 (24%)	19 (18-21)	2/3[b] (67%)
L	4/10 (40%)	19 (18-21)	2/3[b] (67%)
TT			
T	5/73 (7%)	28 (20-30)	3/18 (17%)
L	3/65 (5%)	20 (14-24)	2/16 (13%)
Combined BC,FC,TT			
T	9/100 (9%)	24 (18-30)	5/23 (22%)
L	8/80 (10%)	22 (16-28)	5/21 (24%)
Total	17/180 (9%)	23 (14-30)	8[c]/23 (35%)
NC			
T	0/9 (0%)	-----	0/3 (0%)
L	0/9 (0%)	-----	0/3 (0%)
UC			
T	0/26 (0%)	-----	0/4 (0%)
L	0/10 (0%)	-----	0/4 (0%)

[a] Data from females and males for all groups except the FC and NC are pooled. The FC and NC groups are data from males only. Additional ganglia were explanted from each group of females but results from 3-BC, 4-FC and 5-TT birds were lost due to contamination.

[b] Homogenized suspension of thoracic and lumbar ganglia at 28 days PE yielded virus after 12 days of cultivation on CKC.

[c] Virus was recovered from both the thoracic and lumbar ganglia of two FC birds.

The frequency of reactivation of latent virus as a function of time PE is shown in Figure 8. Most outgrowths required at least 3 to 5 weeks PE for plaques to be induced. The highest frequency of reactivation occurred at 3 weeks with the average time for reactivation of virus from all groups at 23 days PE.

Characteristics of the cytopathic effect on spinal ganglion outgrowths consisted predominantly of rounded, refractile cells and syncytia (Plate XV, 1-3). Initial plaque formation was always observed next to the outgrowth cells adjacent to the explant proper. Hematoxylin and eosin staining revealed densely staining cells and type A intranuclear inclusions (Plate XVI, 1-3). Since the ganglion outgrowths rarely attached to the glass coverslips in the culture vessel, outgrowths with viral cytopathic effect were transferred onto primary CKC monolayers grown on coverslips. Subsequently, intranuclear and intracytoplasmic fluorescence were observed within the plaques found on CKC (Plate XVII, 1-2).

When plaque formation was first observed in the ganglion explant outgrowths, the corresponding explants proper were removed and examined for infectious virus, viral antigens and viral particles. The results are presented in Table 15. Virus was reisolated from homogenates of the explants proper with virus positive outgrowth cells after an average 14 days cultivation on CKC. In one FC bird, virus was isolated from a homogenate of explant proper with virus negative outgrowth

Figure 8. Frequency and time required for reactivation of latent turkey herpesvirus in explant outgrowths of spinal ganglia from male and female birds. [a] = larger bars circumscribing smaller bars gives total number of positives for the combined experimental groups.

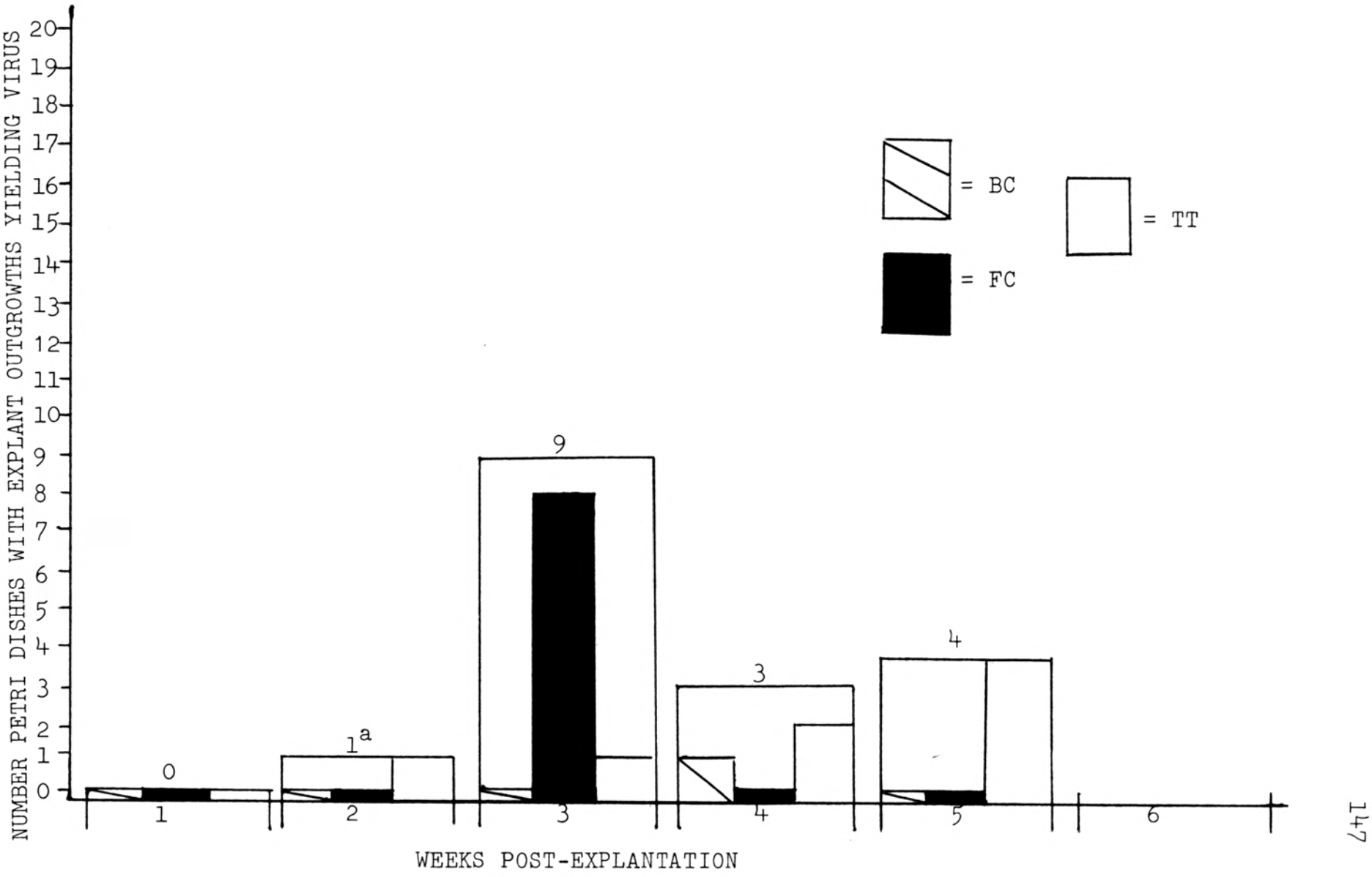

NUMBER PETRI DISHES WITH EXPLANT OUTGROWTHS YIELDING VIRUS
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
= BC
= TT
= FC
0
1a
9
3
4
1
2
3
4
5
6
WEEKS POST-EXPLANTATION

PLATE XV. Herpesvirus Cytopathic Effect in Outgrowths of Spinal Ganglia

1. Viral cytopathic effect in outgrowths of thoracic ganglia explants at 23 days post-explantation. Small rounded cells (arrows) are the most prominent effect noticed. Ganglion explants were derived from a bird inoculated with the TT isolate. Unstained; X196.

2. Outgrowths of spinal ganglion explants established from an uninfected control bird. Cells are predominantly fibroblastic at 21 days post-explantation. Unstained; X112.

3. Extensive viral cytopathic effect observed at 21 days post-explantation in explant outgrowths from lumbosacral ganglia in a bird inoculated with the TT isolate. Unstained; X112.

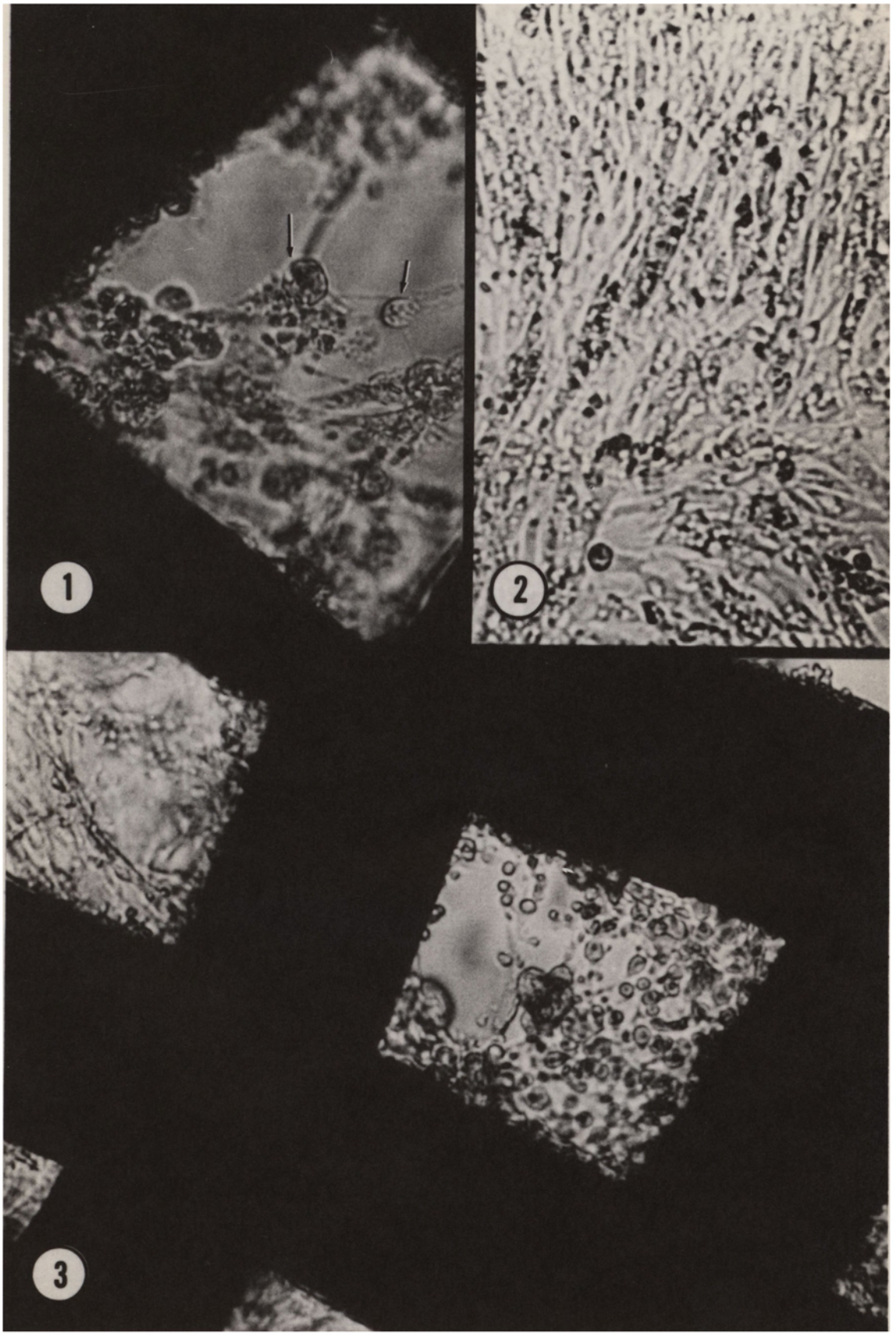

1
2
3

PLATE XVI. Hematoxylin and Eosin Staining of Herpesvirus Cytopathic Effect on Spinal Ganglion Outgrowths

1. Ganglion outgrowth cells from control birds at 24 days PE. Cells are predominantly fibroblastic. Hematoxylin and eosin stain; X328.

2. Extensive cytopathic effect in ganglion outgrowth cells from an FC bird at 21 days PE or 3 days after plaque formation initially appeared. Hematoxylin and eosin stain; X127.

3. Syncytia (s) containing type A intranuclear inclusions (arrows) and clumping of chromatin in CKC cells 4 days after being inoculated with outgrowth cells from TT bird spinal ganglia at 21 days PE. Hematoxylin and eosin stain; X530.

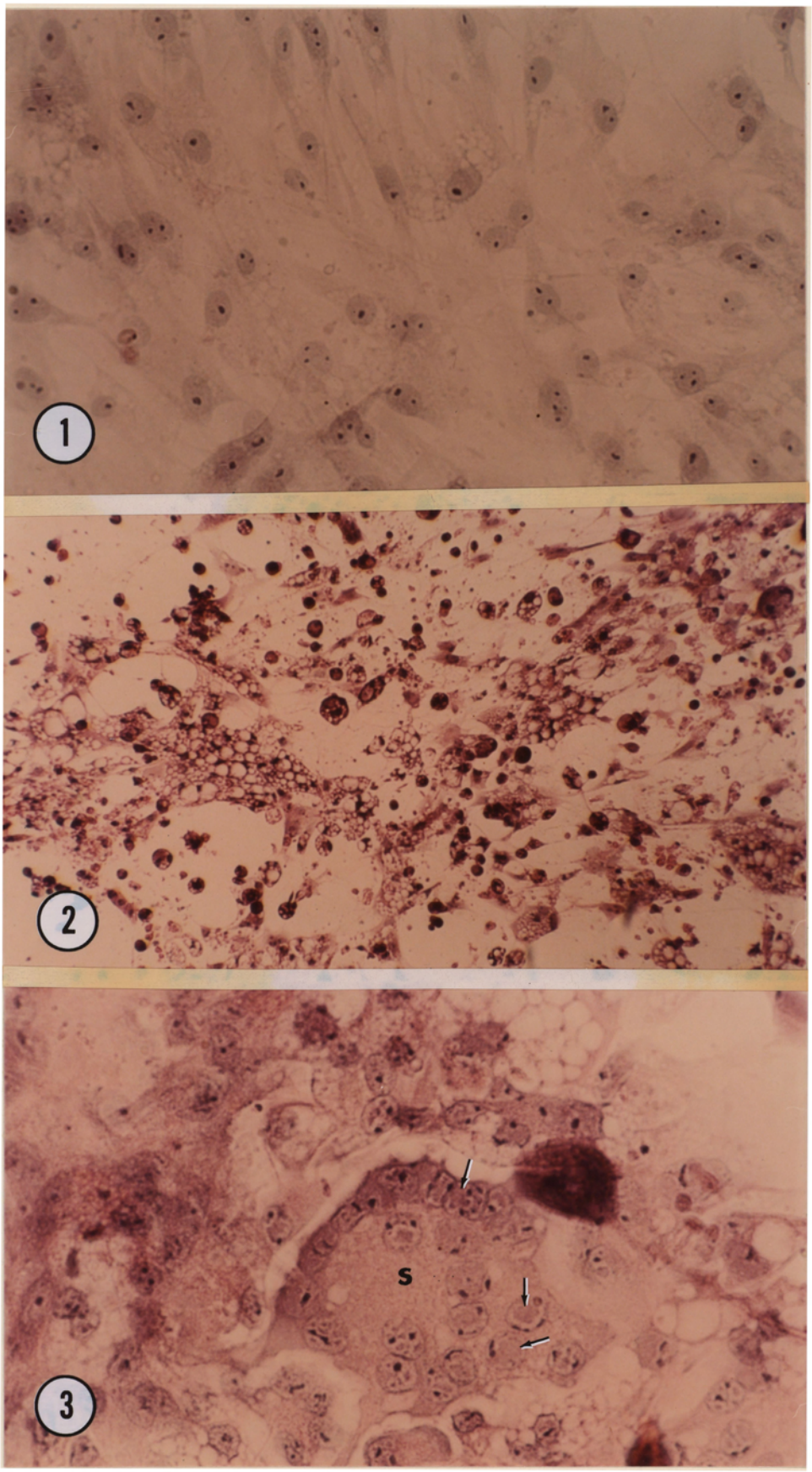
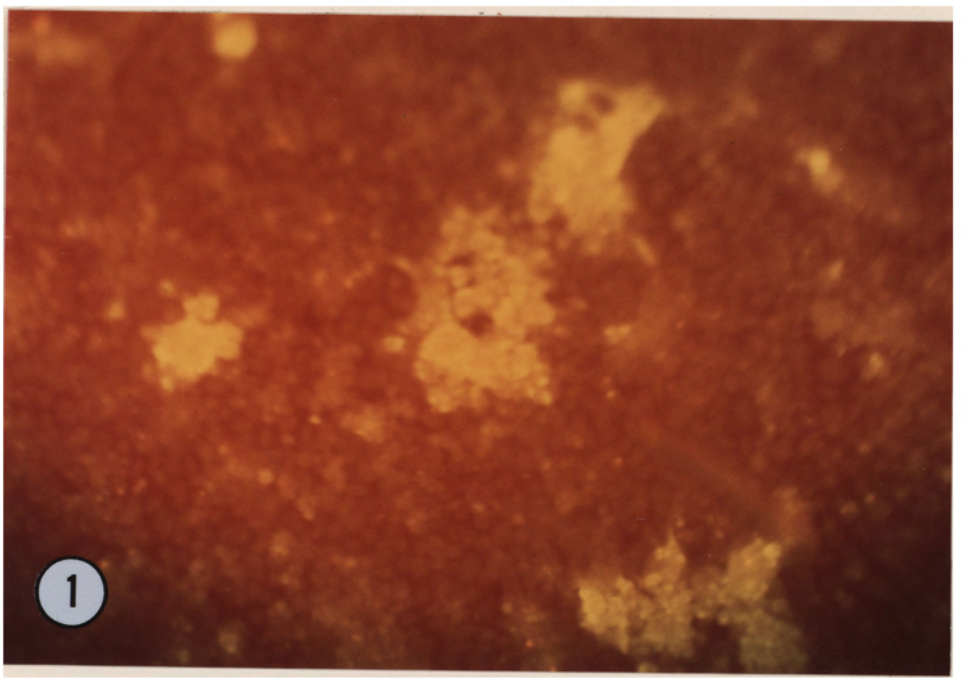
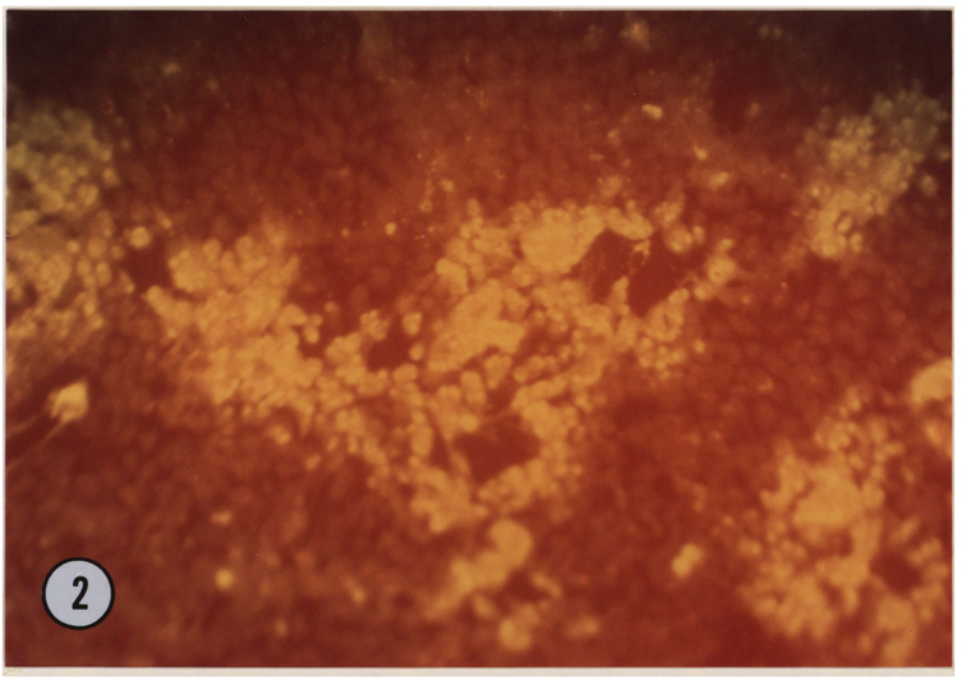

1
2
S
3

PLATE XVII. Indirect Immunofluorescence in Chick Kidney Cells Inoculated with Spinal Ganglion Outgrowth Cells

1-2. Both photographs show intranuclear and intracytoplasmic fluorescence in primary CKC 3 days after inoculation with spinal ganglion outgrowth cells from a TT bird at 21 days PE. Indirect immunofluorescence staining with hyperimmune sera to TT isolate and commercial conjugate; X127.


1


2

cells (refer to Table 16). Fluorescence was observed in the supporting tissues and neurons of ganglion explants proper. The staining, however, was dull, included very few cells and a similar staining pattern was observed in the explant proper of control birds. Electron microscopy also failed to detect viral particles in the explants proper examined.

Characteristics of Seminal Cells and Presence of Virus or Viral Antigens in Semen

Males in each of the four experimental groups (2-BC, 3-FC, 10-TT and 2-UC) were photostimulated at 24 weeks PI with 14 hours of light per day. Sampling for semen was attempted 2x weekly from 25 to 36 weeks PI. During this interval semen production failed to commence in all birds. One TT bird #376 was killed at 29 weeks PI and the testes were examined for evidence of spermatogenesis. The testes were approximately one half the 7.0 cm length (Hess et al., 1976) expected for sexually mature males and the vas deferens contained only clear seminal plasma. Histologically, the testes had some sperm heads in the epididymis but most of the seminiferous tubules showed very little evidence of active spermatogenesis. The gross and histological evidence in conjunction with the fact that the birds were from an out-of-season hatch suggested that these males could be late producers as described by Carson et al. (1955a, c). At 44 weeks PI, TT bird #383 was killed and the testes examined as described for TT bird #376. Gross and histological

observations on bird #383 were identical to those reported for bird #376 except that there appeared to be an increased number of seminiferous tubules supporting active spermatogenesis. One BC male #316, which died at 47 weeks PI, was also not producing semen, possessed testes approximately 1/3 normal size and failed to demonstrate active spermatogenesis in any of the seminiferous tubules examined histologically. After 36 weeks PI the males in the 4 experimental groups were sampled weekly until the first measurable samples of semen were obtained from 2 UC and 8 TT birds at 46 weeks PI.

Examination of semen quality twice weekly in the 2 UC males and the 8 TT males began at 46 weeks and ended at 52-55 weeks PI. During this time, ejaculate volume, estimates of seminal plasma color, number of macrophages and abnormal Type I spermatids were evaluated. The second sample collected each week was also used for virus isolation and preparation of semen smears for examination by immunofluorescence. Absolute counts of abnormal cells and macrophages were attempted but later abandoned because the motility of the spermatozoa made counting of cells in avian semen with a hemacytometer extremely difficult. Thus, the number of abnormal cells and macrophages were qualitatively estimated by counting the number present in 25 high-powered fields (95x).

The results are summarized in Table 17. Semen production was consistent through the 10-week sampling period in both control birds (341, 350) and in 4/8 TT birds (356, 364, 370, 371). The remaining 4 TT birds produced semen inconsistently

Table 17. Characteristics of Seminal Cells in Experimentally Inoculated Turkeys 46-55 Weeks After Inoculation

Group Bird No.	No. Positive/Total No. Sampled for: Abnormal Cells	Macrophages
UC: 341	1/10	3/10
350	2/10	2/10
TT: 356	2/10	1/10
364	0/10	4/10
370	0/10	1/10
371	0/10	2/10
372	2/7	3/7
373	0/5	2/5

whereas the BC and FC males failed to produce measurable semen samples. There appeared to be only small variations in the average weekly volumes of semen or the color and no differences between samples obtained from the control and TT birds were found. Semen from TT birds #356 and 370 was found to contain rounded, abnormal cells at 46-47 and 47-48 weeks after inoculation, respectively. Similar but somewhat smaller cells were observed at 48 and 48-49 weeks in control birds #341 and 350, respectively. Examination of hematoxylin and eosin stained semen smears revealed the cytoplasm of the rounded, abnormal cells to be devoid of structure except for an occasional densely staining crescent of chromatin near the cell membrane. On the basis of the light microscopic characteristics the rounded, abnormal cells appeared to resemble Type I cells, which have been previously described (Thurston et al., 1975; Adldinger, personal communication). The total number of Type I cells observed in the 25 high-powered fields were extremely low, TT birds contained approximately 2-3x more Type I cells (range 4 to 10) than the control birds (2 to 4). Electron microscopy was attempted in an effort to further identify the abnormal Type I cells but due to their relatively small numbers was abandoned for lack of results. Macrophages were observed at various sampling times in TT and UC birds but appeared most numerous in the TT birds.

Virus was isolated from semen of 1/8 TT birds (#364) at 55 weeks PI and 1/3 FC birds (#342), which was from

semen expressed from the vas deferens at 54 weeks PI. The amount of infectious virus in the semen was extremely small as a subcultivation was required to express FFU of 3.0 for the TT birds after 16 days and 15.0 for the FC bird after 14 days. Virus was not isolated from semen of the control birds. Likewise, attempts to demonstrate viral antigens in semen smears by immunofluorescence were uniformly unsuccessful.

Chapter IV

DISCUSSION

The studies reported within this thesis have demonstrated that turkey herpesviruses established persistent infections in buffy coat cells, spleen, bursa, thymus, kidney, gonads and spinal ganglia. Most importantly, the findings reported herein provide the first direct evidence that gonads, especially the testes, and the spinal ganglia of turkeys were latently infected with turkey herpesviruses. This finding was of particular interest since a turkey herpesvirus has been implicated as the etiological agent of abnormal spermatogenesis and yellow semen observed in turkey breeder flocks (Adldinger et al., 1974). Experimental findings on the persistence and latency of turkey herpesviruses as well as limited experimental attempts to reproduce the so-called "yellow semen syndrome" in experimentally inoculated turkeys and the role of latent turkey herpesviruses in the etiology of abnormal spermatogenesis and yellow semen abnormalities will be discussed.

Male turkeys inoculated at one week with the BC, FC or TT isolate of turkey herpesvirus (HVT) were shown to be persistently infected, except at a few sampling periods where virus was not isolated from buffy coat cells, by repeated isolation of virus from buffy coat cells assayed at monthly intervals. Sporadic failures to isolate virus, such as those

encountered at 12 and 16 weeks after inoculation in the BC group, are not unusual with cell-associated herpesviruses and probably reflect the presence of only small quantities of virus in the tissue examined. The inability to isolate virus from the buffy coat cells of all birds at 20 weeks after inoculation is conspicuous since it is highly improbable that all birds sampled would be negative for viremia at one specific time. Thus, the results at 20 weeks were considered artificial and possibly related to technical errors in sample collection and processing, or to an insufficiency in the assay system.

The incidence of viremia was 92% in all males bled at the earliest sampling time of 4 weeks post-inoculation (PI). Although viral assays on buffy coat samples were not done earlier than 4 weeks PI in the experimental males, results obtained from the turkey poults (refer to Figure 1) used for the propagation of the original virus inocula in conjunction with previously reported data (Witter and Solomon, 1972) indicated that viremia typically developed within one week PI. Thus, it is likely that the experimental males were viremic earlier than 4 weeks PI and that this early appearance of viremia in the blood would lend support for the hematogenous spread of virus in buffy coat cells to other tissues, a situation analogous to that of Marek's disease virus in chickens (Biggs, 1973; Adldinger and Calnek, 1973).

Although virus is known to persist in turkeys over 1 year of age (Witter and Solomon, 1971; Witter, 1972), the

duration of viremia has not been extensively studied and no quantitative studies have been reported. The infection of the blood persisted in most birds throughout the experimental period. Quantitatively, the results in the present study revealed peak viral titers in the buffy coat cells of the BC and TT males at 4 weeks PI. Subsequently, the level of viremia appeared to decrease with the increasing age of the bird to a low but persistent level as reflected in the lower titer of virus and the increased need for subcultivation to detect plaque formation. In Marek's disease, the incidence and levels of viremia have been shown to be higher in day-old compared to age-resistant (>3 weeks of age) chickens (Calnek, 1973). Whether the decrease in the quantity of blood-borne virus in the turkey was due to age resistance and/or an immune-mediated reduction is a matter of conjecture but may perhaps be related to both i.e. age resistance possibly related to immunological maturity; however, direct evidence for either mechanism is lacking. In general, virus was most readily isolated from the buffy coat cells of the FC males and persisted in titers higher than those obtained from buffy coats from BC and TT males. The significance of this is not known but may relate either to a potential difference in the degree of productive infection induced in buffy coat cells by the FC compared to the BC and TT isolates or it may be a coincidence due to the small number of birds examined.

Maternal antibody to the BC, FC and TT isolates was not demonstrated in any of the plasmal samples from 6 poults

sampled one week after hatching. In addition, antibody could only be detected in 1/14 males sampled at 4 weeks after inoculation, a time when maternal antibody should still be present. These results were particularly surprising in view of the ubiquitous nature of the FC 126 turkey herpesviruses and of the results of others in which the incidence of maternal antibody in newly hatched poults is usually 100% (Witter and Solomon, 1972; Witter, 1972). This discrepancy can perhaps best be explained by the unusual cleanliness and the extraordinary sanitary measures practiced by the industrial breeder from which the eggs were obtained to initiate the studies reported herein. In this particular enterprise, hatcheries and breeder operations were located on separate premises and maintained by separate personnel. Thus, newly hatched and highly susceptible poults were less likely to be infected with infectious litter and dander that could have existed in the mature breeder flocks and it is conceivable that these birds may avoid infection altogether.

Active antibody was not detected in most birds until 8 weeks after inoculation. This is in contrast to a previous report in which antibody was detected by indirect immunofluorescence within 3 to 4 weeks after inoculation of experimental turkeys (Witter and Solomon, 1972). The differences may be related to the increased sensitivity of the indirect immunofluorescence test as compared to the immunoprecipitin test for the detection of antibody. Alternatively, antibody levels may have been low at 4 weeks or possibly

complexed with viral antigens thereby rendering antibody undetectable. Precipitating antibody persisted in most birds throughout the 55-week experimental period. Presumably, the persistence of precipitating antibody was associated with that of viral antigen as described for Marek's disease virus (Witter et al., 1971).

Several investigators have reported success in using the immunoprecipitin test to detect precipitating antibody in turkey sera (Kawamura et al., 1969; Witter et al., 1970b; 1972; Adldinger et al., 1974) but some have concluded that turkey antisera contain small amounts of precipitating antibody and are therefore unsuitable for immunprecipitin tests (Witter et al., 1970; 1972). Results in the present studies support these latter observations, since the placement of hyperimmune sera in wells adjacent to test sera increased the percentage of positive samples. Similar techniques are common practice for the detection of precipitating antibody to other viruses such as equine infectious anemia (Coggins and Norcross, 1970) and pseudorabies (Gutekunst et al., 1978), but have not been previously reported for the turkey herpesviruses.

Studies on the distribution of turkey herpesviruses and associated antigens in various tissues was done using experimental female and male turkeys from the three inoculated groups and 4 males with a history of yellow semen. Since experimental male turkeys were retained throughout the 55-week experimental period for studies on abnormal spermatogenesis, the females killed between 15 to 38 weeks PI provided the

opportunity to gain information on the early distribution and persistence of turkey herpesviruses in various tissues.

Cell-associated HVT was regularly isolated from the buffy coat, spleen and kidney cells, whereas bursa, thymus and gonads, particularly the testes were less frequent sources of cell-associated virus. From these observations, it appeared likely that cell-associated virus was persistent in most, and perhaps all, tissues of infected turkeys. This closely resembles the results of Witter et al. (1972), who isolated cell-associated HVT (FC 126) most frequently from buffy coat, bursa, spleen, and kidney but not the gonads, and is analogous to the virus distribution in chickens infected with Marek's disease virus (Witter et al., 1969; Adldinger and Calnek, 1973).

The source of cell-associated HVT in the spleen and thymus was probably infected lymphocytes, since the technique used in the preparation of these cell suspensions selected predominantly for lymphocytes. As previously discussed, blood borne virus in the buffy coat cells was the likely route of viral dissemination into the major organ sites. Thus, the CAV recovered from the bursa, kidney and gonads may have been generated from blood cells, which were not segregated from the trypsinized cellular suspensions. However, the isolation of virus from the bursa, kidney and ovary of 2 females with negative buffy coat cultures suggests that the kidney, bursal and ovarian cells per se were the source of herpesviruses.

A similar analogy could not be made for tissues from male turkeys since all males examined were viremic. Specific cell types supportive of viral replication, however, could not be localized within the various tissues by immunofluorescence. This observation is in direct contrast to the situation reported for MDV replication in chickens (Calnek and Hitchner, 1969; Calnek et al., 1970; Adldinger and Calnek, 1973) but in agreement with the results of Witter et al. (1972), who were unable to detect immunofluorescent antigens in similar tissues of FC 126 inoculated turkeys at 2 to 10 weeks PI. Possible explanations for the absence of immunofluorescent antigens are: 1) susceptibility of various tissues decreased with age; 2) antibody impeded the spread or access of virus in tissues; or 3) cells supportive of virus replication occurred too infrequently to be detected. The specificity of the immunofluorescence test was unquestionable since hyperimmune sera reacted strongly with the corresponding homologous tissue culture antigen in both immunofluorescence and immunodiffusion tests.

The first possibility seemed unlikely in view of the report of Witter et al. (1972) in which viral antigens could not be detected in tissues of FC 126 infected turkeys beginning at 2 weeks PI. It is tempting to speculate that antibody may have interfered with the extent of viral replication and spread within infected tissues or possibly masked antigen within the tissue by forming virus-antibody complexes. However, precipitating antibody was not

demonstrated in all birds at the time that tissues were procured for immunofluorescence. Therefore, the third possibility appeared most likely as exemplified by the increased need to subcultivate samples of tissue from the females in order to induce plaque formation and by the low cell-associated viral titers (7-day reading) obtained from most male tissues. These results suggest that few cells in vivo were productively infected and therefore were below the detectable limits of the immunofluorescence test. These same observations also can be advanced to explain the failure to demonstrate immunoprecipitin antigens. The lack of correlation between the virus isolation, immunofluorescence and immunoprecipitin data is conspicuous. However, the ability to more readily identify cell-associated infectivity in tissues as compared to antigens by immunofluorescence and immunodiffusion could perhaps reflect the stage of viral replication attained. This may be analogous to the situation in Marek's disease (MDV) where lymphoid cell infiltrations show a scantiness of immunofluorescence staining and no particles by electron microscopy but infectious virus is isolated by cocultivation onto chick kidney cells (Calnek and Hitchner, 1969). Thus, in the studies reported here the inoculation of cellular suspensions onto primary chick kidney cells constituted a short term co-cultivation and may have been sufficient to detect small amounts of virus in productively infected cells or in cells containing only the turkey herpesvirus genome.

Cell-free virus was isolated from the plasma of 4/14 experimental males, whereas the other tissues (buffy coat, spleen, thymus, bursa, kidney and gonads) were negative. However, it was conceivable that a few cells were present, since the samples were not filtered. Witter et al. (1972) were not able to detect cell-free virus in the plasma as well as the tissues previously mentioned, whereas cell-free virus was demonstrated only in the feather follicles of turkeys, which were not examined in this study.

Cell-associated virus was recovered in only 1/3 FC, 1/11 TT and 1/4 naturally afflicted males by inoculation of primary chick kidney cells with testicular cells. Moreover, the time required for the development of culture positivity was generally longer than that required for the buffy coat, spleen or kidney cells and viral titers were the lowest of all tissues examined. Similar results were obtained with ovarian cell suspensions, which like the testes required prolonged cultivation and often subcultivation to effect plaque formation. Thus, only a few gonadal cells appeared to be productively infected in vivo.

In contrast to the cocultivation techniques, cell-associated virus was consistently isolated by the direct cultivation of testicular and ovarian cells. However, development of plaque formation required at least one subcultivation of the primary gonadal cell cultures whereas primary kidney cultures prepared from the same birds demonstrated no such requirement. Similar results have previously

been reported by Adldinger et al. (1974). Ovarian cells required an average of 16 days cultivation i.e. one subculture at 10 days and 6 additional days of maintenance before plaque formation was detected, whereas kidney cells from the same birds required only an average of 9 days maintenance before plaque formation was evident. Similarly, virus plaques developed in the primary testicular cultures of only 1/20 birds after 10 days of cultivation, whereas the primary kidney cells of 17/20 birds exhibited culture positivity (plaques) between 4 to 10 days after plating. However, plaque formation was induced in the secondary testicular cultures of 13/19 birds within 4 to 8 days after subcultivation. Interestingly a second subculture was required for three of the TT birds before plaque formation was induced. The average time required for development of viral plaques was 7 days for the primary kidney cultures compared to 15 days (one subculture at 10 days and 5 days maintenance thereafter) for the testes. This latter observation is in marked contrast to the finding that turkey testes cultures, which were prepared from uninfected control birds, inoculated with a cell-associated inoculum in vitro produces viral plaques within 3 days (Benfield, unpublished data). Thus, the virus appears to persist in testis tissue in a nonreplicating form. Cytopathic changes observed on secondary testicular cultures were typical for herpesviruses on the basis of the morphology of the infected cells, the presence of Cowdry type A inclusions and the demonstration

of nuclear and cytoplasmic fluorescence using hyperimmune sera specific for the cloned BC, FC or TT isolates. Thus, the results presented here confirm the earlier observation (Adldinger et al., 1974) that testes tissue is a consistent source of turkey herpesviruses and that the requirement of prolonged cultivation or subcultivation of testicular cells was necessary to allow for the reactivation of latent turkey herpesviruses and subsequent plaque formation in vitro. This same conclusion can also be expanded to include the similar results obtained with ovarian cell cultures. The greater success in isolating virus by direct cultivation of testes and ovary as compared to cocultivation suggested that the activation of latent viruses is promoted by the enhanced cellular proliferation associated with the in vitro survival and growth of the gonadal cells.

The results of the explantation experiments reported herein provide the first direct evidence that turkey herpesviruses are harbored latently within gonadal cells. In all instances, the recovery of virus from gonadal tissue by explantation techniques closely resembled that observed in latent infections of sensory ganglia (Stevens and Cook, 1971; 1973; Stevens, 1975; 1978). At the time of explantation infectious virus, viral antigens or virions could not be demonstrated in testes or ovary tissue by conventional techniques of virus isolation, immunofluorescence and immunodiffusion and electron microscopy. Turkey herpesvirus was reactivated most frequently in explant cultures derived

from testis as compared to ovary. Testis cells were particularly supportive of the latent infection as virus was reactivated in the testis explant outgrowths of 85% of all males examined compared to ovary explant outgrowths in which only 44% of the females were positive. The explantation technique although being sensitive and reliable for the demonstration of latent turkey herpesvirus had certain limitations. This is exemplified by the observation that the ratio of virus positive explant outgrowths/total explant outgrowths was only 45/105 (43%) and 12/52 (23%) in the males and females, respectively. Thus, it is apparent that a minimum of 3 to 4 petri dishes with explants proper and viable outgrowth cells must be established to guarantee at least one positive observation. In general, the time required for the development of plaques in the explant outgrowths of testes and ovary was 2 to 4 weeks. Plaques were observed to develop between 10 to 45 days after explantation but a majority of the explant outgrowths were positive within 2 weeks after explantation. These findings are most interesting when compared to data in the studies reported here on direct cultivation of testicular and ovarian cells, which required an average of 15 and 16 days of cultivation to induce plaque formation. Intranuclear inclusions, immunofluorescent antigens and virions were regularly identified in explant outgrowths in which plaque formation was induced. As assessed by the ultrastructural techniques employed here, viral replication in the testis explant outgrowths was similar to

turkey herpesvirus infections studied in various permissive cell cultures (Nazerian et al., 1971) and those observed in secondary testicular cultures derived from yellow semen males (Thurston et al., 1974). Thus, the results reported here directly confirm the original hypothesis of Adldinger et al. (1974) that testis cells harbor latent turkey herpesviruses in vivo and are consistent with a latent infection of the "static state" mechanism described by Roizman (1965). A similar situation exists in the ovary. In both cases the observed absence of virus and associated antigens at the time of explantation and subsequent activation of virus replication in explant outgrowths after prolonged cultivation in vitro fulfill the criteria for demonstrating latent viruses as originally described by Stevens and Cook (1971; 1973).

Explantation-co-cultivation was also utilized to detect latent virus. It was thought that this technique would be more sensitive since the chick kidney cell monolayers would allow for a continuous sampling of the environment and thus perhaps detect viral replication that occurred earlier than that observed in the explant outgrowths which require several days to grow out. This was not the case, however, as explantation-co-cultivation was less successful than explantation in reactivating latent virus, although the time required for reactivation was 3 weeks and therefore similar to that obtained for plaque formation on the explant outgrowths. Virus was not released into the media as sequential samples

of media bathing testis explants were negative on virus isolation. In summary, the subcultivation of primary testicular cultures and prolonged cultivation of explant outgrowths are the most sensitive techniques for reactivating latent turkey herpesviruses with subsequent demonstration of viral plaques, inclusions, antigens and virions (refer to Table 17). This is perhaps related to the enhanced cellular proliferation associated with these culture conditions as previously discussed.

The findings presented above raise the question as to which cell type in the testis or ovary serves as the reservoir for latent virus. The cell type containing herpesvirus nucleocapsids in the testis explant outgrowth cells are predominantly germinal epithelial cells similar to those previously described in cells from secondary testicular cultures (Thurston et al., 1975). It was anticipated that the cell type harboring latent virus could be localized by immunofluorescence and electron microscopy in the corresponding explants proper from cultures with positive outgrowth cells. Small amounts of infectious virus were isolated from homogenates of explants proper of most birds after cultivation for 15-20 days on primary chick kidney cells. However, intranuclear inclusion bodies, viral antigens and viral particles were not found within any cell type in the explants proper of ovary or testis with the one exception of bird #324, which died 12 weeks PI. The explant outgrowths of this bird were positive within

10 days after explantation. The presence of viral particles in this explant proper and the relatively early time the virus was detected in vitro may indicate that the testes of this bird was productively infected. However, it is equally likely that this may have represented a spontaneous reactivation of latent virus in vivo. Based on the morphology of the cells from the testis explant outgrowths and those of the explants proper of bird #324, it appeared that latent virus could reside within the germinal epithelial cells of the testes. Buffy coat cells seemed to be an unlikely source of virus despite a persistent viremia in the females and males due to the subcultivation requirement observed for ovarian and testicular cultures and the prolonged cultivation time necessary for the induction of plaques in ovary and testes explant outgrowths as compared to the relatively short period of time necessary for plaque formation when chick kidney cells are inoculated with buffy coat cells.

The induction of latent turkey herpesvirus in testis explants proper from three TT birds were sequentially examined for infectious virus and viral antigens by immunofluorescence and immunodiffusion. The results revealed that the explants proper required 16 to 25 days of explantation in vitro before infectious virus could be isolated. Viral antigens however, could not be shown by either immunofluorescence or immunodiffusion either before or after virus had apparently been induced within the explant proper. These findings are in contrast to the kinetics of the reactivation

of latent HSV-1 in sensory ganglia in which the appearance of viral antigens as determined by immunofluorescence preceded release of infectious virus into the media (Rajcani et al., 1977). However, latent pseudorabies virus has been reactivated from explants of lymph nodes, tonsils, nasal mucosa and gasserian ganglia but corresponding viral antigens cannot be demonstrated by immunofluorescence except in the gasserian ganglia (Sabo and Rajcani, 1976). These authors observed differences in the quantity of viral antigens in nerve as compared to epithelial cells. This could mean that fewer epithelial cells than neurons harbor latent virus. The observations on the testes explant proper as exemplified (with the exception of bird #370) by the detection of small quantities of infectious virus support this contention because latent virus appeared to be reactivated within the testis explants proper in relatively few cells and these cells were too infrequent to allow detection of viral antigens by immunofluorescent and immunodiffusion techniques. An alternative explanation may be related to the observation that at the time latent virus appeared to be reactivated (16-25 days PE), necrosis was evident in stained sections of explants proper examined by light microscopy. The reduction in the number of viable cells within the explants proper may have precluded subsequent spread of the infection and thus no foci of antigen containing cells would be found by immunofluorescence. Regardless of the mechanism of reactivation and subsequent replication of latent virus in the testis

explants proper, the results of the sequential study support the concept that some virus is latent in testes, since a prolonged period of in vitro explantation is required before infectious virus is isolated. However, the exact cellular localization and mechanism of reactivation merits further experimentation.

These studies also represent the first report that turkey herpesviruses latently infect thoracic and lumbosacral spinal ganglia of experimentally inoculated turkeys. The results reported are similar to those for the gonads in that at the time of explantation conventional techniques of virus isolation, immunofluorescence, immunodiffusion and electron microscopy were negative. Explantation revealed that 5/23 or 22% of all birds harbored latent herpesvirus in the thoracic and 5/21 or 24% harbor latent herpesvirus in the lumbar ganglia for a combined total of 8/23 (34%) of all birds harbor latent turkey herpesviruses in the spinal ganglia compared to 85% in the testes. As in the testes explants, virus plaques were observed in explant outgrowths and viral antigens and inclusion bodies could be demonstrated in primary chick kidney cells inoculated with infected ganglion outgrowth cells. The spinal ganglion explants required at least 3-5 weeks of maintenance as explants before latent virus was reactivated as compared to the 2-4 weeks period for testes explants. These differences in time required to demonstrate plaques in outgrowth cells could be related to different mechanisms of viral reactivation in the two types

of tissues. Although infectious virus could be isolated from homogenates of explants proper (with corresponding positive outgrowth cells), viral antigens and particles could not be detected. This failure to demonstrate viral antigens and particles within the corresponding ganglion is conspicuous since viral antigens and particles have been universally observed in all other systems from which latent herpesviruses have been reactivated from sensory ganglia (Stevens, 1975; 1978). This discrepancy at first was thought to represent an inadequacy in the indirect immunofluorescence test employed. However, using the same technique and reagents readily demonstrated viral antigens in chick kidney cells inoculated with viral positive outgrowths from the ganglia. Thus, it would appear that only very few cells within the ganglia support replication of reactivated virus within the ganglion explants proper as discussed for the testes. It has been reported that HSV-1 gains access to the spinal ganglia via either neural or hematogenous pathways depending on whether the virus is inoculated into peripheral sites or administered intravenously (Cook and Stevens, 1976; Price and Schmitz, 1979). However, intravenous injection of HSV-1 led to a lower prevalence of HSV-1 in the superior cervical ganglion of mice (Price and Schmitz, 1979). Thus, the lower rate of recovery from the spinal ganglion as opposed to the testes, the persistent viremia and the apparent equal distribution of latent virus in thoracic and lumbosacral ganglia suggests that the turkey

herpesvirus reached the ganglia via bloodstream. On the other hand the infrequent recovery of latent virus from spinal ganglia may have been related to technical problems in removal of the ganglia from the birds. Since at least 30-45 minutes was required to remove the ganglia from the bird, there is no doubt that cell death occurred within some ganglia thereby reducing the chances of recovering virus.

The finding that spinal ganglia harbored latent turkey herpesviruses raises the question as to whether such virus is responsible for seeding epithelial cells of the testes since lumbosacral ganglia may innervate into the genital area. Certainly, in view of what is known in other systems with HSV-1 and other herpesviruses such as herpes zoster this is a possibility (Docherty and Chopan, 1976). However, if the frequency of reactivation of latent virus is related to the number of testis or ganglionic cells infected, then the higher frequency of positive explant outgrowths from the testes suggests that reactivated virus from the ganglia is not the primary source of virus in the testes.

Attempts to reproduce the yellow semen syndrome and abnormal spermatogenesis were of limited success. Two TT birds did produce small numbers of abnormal cells similar to the Type I cell described by Thurston et al. (1975). The occurrence of the abnormal cells, however, was transient and no correlation could be made with semen color. Thus, although abnormal cells did exist, the lack of yellow-colored semen made it difficult to satisfy the definition of the yellow

semen syndrome as described by Thurston, 1976. The Type I abnormal cells were also found in the two control birds but their relative numbers were 2 to 3x lower than those found in the TT birds. Other investigators have also observed on occasions Type I cells in normal semen (Thurston *et al.*, 1975; Adldinger, personal communication). Considering the rapid turnover of germinal epithelial cells of the testis that is necessary to supply the billions of spermatozoa in semen, however, it should not be surprising that sperm abnormalities would be present in the semen from the uninoculated control birds. Further studies using *in situ* hybridization will be necessary in order to distinguish which abnormal cell types carry the latent turkey herpesvirus genome. Interestingly, virus was isolated from semen of one TT and one FC bird at the end of the experiment. The amount of virus recovered was small and required 14-16 days of cultivation *in vitro* before plaques were formed. Adldinger (personal communication) has had success in reproducing yellow semen and abnormal spermatogenesis in experimentally infected 33-week-old specific pathogen free males, but the syndrome produced was also transitory.

The difficulties experienced in reproducing the yellow semen syndrome are related in part to the inability to sample a sufficient number of birds producing semen and to the inconsistent semen production by males used in this study. Only the TT and UC males produced measurable quantities of semen. It was later learned that the breeder experienced

similar problems in that some mature toms failed to produce semen. Thus, due to these unanticipated problems the data on the semen studies is at best preliminary.

Although these studies did not satisfy a cause and effect relationship between a turkey herpesvirus and yellow semen abnormalities, the present studies are of significance in consideration of evidence of previous studies (Adldinger et al., 1974; Thurston et al., 1975; Adldinger, personal communication) that circumstantially suggest an etiological link between a turkey herpesvirus and the abnormal spermatogenesis with concomitant yellow semen. Based on the epidemiology of other avian herpesviruses, especially the FC 126 turkey herpesvirus and Marek's disease virus, it was postulated that poults were infected in the natural environment at an early age or shortly after hatching. Although this was not established in our studies, it was found that poults inoculated at one week of age with either the FC or the TT and BC isolates were persistently infected throughout the entire experimental period as determined by assaying repeated samples of buffy coat cells. The virus is thus disseminated throughout most major tissues, including the gonads and spinal ganglia and persists there probably throughout the commercial life of the bird (about 1-1.5 years).

The question arises as to how the virus could alter the normal course of differentiation of germinal epithelial cells to produce the abnormal Type I and possibly Type II spermatids observed in yellow semen males. The explanation seems to be

in the fact that the virus is latent within germinal epithelial cells where it could be either integrated into the cellular genome or exist as an extrachromosomal element. This is entirely feasible based on the results reported here on the latency and on the demonstration that other herpesviruses such as Epstein-Barr Virus and Marek's Disease Virus persist with genomes integrated or as plasmids in lymphoblastoid cell lines (Adams and Lindahl, 1975; Tanaka et al., 1978). As the germinal epithelial cells in the testes divide, one generation will remain as a daughter cell while others undergo a variety of biochemical and morphological changes to become mature spermatids. The presence of the viral genome may alter the normal course of events either by deleting a portion of the host cell genome or by substituting specific viral genetic sequences into the host genome. An alternative hypothesis is that a specific viral product is capable of altering normal differentiation of gonial cells.

While the studies reported here show the importance of latent herpesvirus in the testis as a possible cause for altering normal spermatogenesis, the significance of its latent presence in spinal ganglia remains to be found. Further substantiation of the etiology of abnormal spermatogenesis will require the demonstration of the viral genome in testis and/or abnormal seminal cells by in situ hybridization.

Chapter V

SUMMARY

Turkey poults were experimentally inoculated at one week of age with either of two cloned herpesviruses isolated previously (TT and BC) from male birds with semen abnormalities or a prototype herpesvirus of turkeys (FC 126). Studies were conducted to 1) demonstrate latent turkey herpesviruses in gonadal and spinal ganglia by explantation techniques; 2) examine sites of *in vivo* replication of turkey herpesviruses; and 3) to reproduce abnormal spermatogenesis and yellow semen in male turkeys. Female birds were killed between 15-38 weeks after inoculation while males were retained for semen collection and studies on abnormal spermatogenesis before being killed at 52-55 weeks after inoculation. Various lymphoid (buffy coat, spleen, thymus and bursa) and epithelial cells (kidney and gonads) were examined for cell-associated and cell-free virus and viral antigens. In addition, gonads and spinal ganglia were explanted *in vitro* to determine if these tissues harbored latent turkey herpesviruses.

Male turkeys were confirmed to be persistently infected throughout the 55-week experimental period by repeated isolation of virus from buffy coat cells and the detection of precipitating antibody. Turkeys were found to produce low levels of precipitating antibodies, which could best be

detected on the immunoprecipitin tests if a hyperimmune serum was positioned in reaction wells adjacent to the test sera.

Detection of latent virus in gonadal cells was done either by prolonged cultivation and subcultivation of gonadal cell cultures or explantation of ovary and testis organ pieces in vitro. Direct culture of testicular and ovarian cells revealed that gonadal cells required at least one subcultivation and 2 weeks of maintenance to induce virus plaque formation as compared to kidney cultures which have no requirement for subcultivation and need only 1 week of maintenance before viral plaques are detected. The apparent requirement for subcultivation of gonadal cultures to induce plaque formation substantiated earlier studies that testis cells were latently infected in vivo. This evidence supported the contention that increased cellular proliferation and viability associated with the growth of secondary gonadal cultures function as a stimulus to induce latent turkey herpesviruses to replicate.

Studies using explantation techniques have provided direct evidence that turkey herpesviruses induce latent infections in gonads. At the time of explantation infectious virus and viral antigens could not be demonstrated in biopsies of gonads by conventional techniques of virus isolation and immunofluorescence and immunodiffusion. In addition, herpesvirus particles were not observed in testis biopsies examined by electron microscopy. Explantation of gonads resulted in

outgrowths of epithelial cells from the corresponding organ pieces (explants proper). After 1 to 4 weeks of *in vitro* cultivation as explants, viral plaque formation was observed in gonadal explant outgrowths of 4/9 (44%) females and 17/20 (85%) of the males. The morphology of the viral plaques was typical of herpesviruses with syncytia and Cowdry type A intranuclear inclusions. Viral antigens were also detected in outgrowth cells by immunofluorescence and naked and intranuclear viral particles were observed by electron microscopy in testis explant outgrowth cells.

Attempts to localize the cell type harboring latent virus in testis and ovary were unsuccessful. At least 16 days of *in vitro* maintenance was required before infectious virus could be isolated from homogenates of testis explants proper. Viral antigens were not, however, detected by immunofluorescence or immunodiffusion prior to or after infectious virus was isolated. In one case viral particles were detected in a testis explant proper derived from a bird which died 12 weeks after inoculation. This latter case may have represented a productive infection of the testis or latent herpesvirus may have been reactivated *in vivo*. The cell type containing the latent virus could not be identified in either the explant outgrowths or the one explant proper but were presumed to represent germinal epithelial cells because this was the predominant cell type observed by light microscopy of hematoxylin and eosin stained preparations of testes explant outgrowths. In spite of the inability to localize

the cell type latently infected, these results directly demonstrated that gonads are latently infected.

These studies also report new evidence that turkey herpesviruses induced latent infections in thoracic and lumbosacral spinal ganglia. As described for the gonads, conventional virologic techniques of virus isolation, immunofluorescence, and immunodiffusion failed to detect infectious virus and viral antigens and electron microscopy also failed to detect viral particles in biopsies of spinal ganglia taken at the time of explantation. After an average of 3 weeks of in vitro cultivation as explants, homogenates of spinal ganglia explants proper yielded infectious virus and viral antigens were detected in explant outgrowth cells. Only 8/23 birds (35%) were found to harbor latent virus in spinal ganglia and latent virus was reactivated with equal frequency from both the thoracic and lumbosacral ganglia. Thus, turkey herpesviruses are capable of latent infections of spinal ganglia as has been described for other herpesviruses such as herpes simplex virus. It is not known if latently infected spinal ganglia serve as a reservoir of virus which may infect gonadal cells. This appears to be insignificant in view of the fact that virus is latent within gonadal cells also.

Cell-associated virus was found to be widespread and persistent among turkeys infected with turkey herpesvirus. Spleen, kidney and buffy coat were the most consistent sources, whereas the bursa, thymus, gonads (particularly the testis) were inconsistent sources of cell-associated virus. Attempts

to localize the site of viral replication in these same tissues by immunofluorescence were unsuccessful and antigens could not be demonstrated by immunodiffusion tests. However, cell-free virus was not demonstrated in the buffy coat, spleen, thymus, bursa, kidney or gonads. It was concluded that virus-replicating cells occurred too infrequently to be localized by immunofluorescence and immunodiffusion procedures.

Attempts to reproduce abnormal spermatogenesis and yellow semen were of limited success. There was a transitory appearance of abnormal seminal cells in 2 turkeys infected with the TT isolate but problems were encountered in obtaining a sufficient number of males to produce measurable semen samples. The abnormal cells observed were large, rounded, few in number and presumably Type I cells. However, the small number of abnormal cells made electron microscopy impractical, therefore the morphology of the abnormal cells could not be ascertained. Thus, a direct role of a turkey herpesvirus in the etiology of abnormal spermatogenesis and yellow semen could not be verified in the present studies.

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VITA

The author was born on October 13, 1951 in Rushville, Indiana. He attended public schools in Rushville, graduating from Rushville Consolidated High School in May, 1969. In September, 1969 he enrolled in Purdue University and from that institution received his Bachelors of Science degree in microbiology in May, 1973. He was employed as a graduate assistant in the Department of Microbiology, Pathology and Public Health, Purdue University from July, 1973 to July, 1976 and during that time completed the requirements for the degree Master of Science in animal virology. In August, 1976 he began studies toward the Doctor of Philosophy degree in the Department of Veterinary Microbiology, University of Missouri-Columbia and was employed as a research assistant from August, 1976 to August, 1979.

The author married the former Marylin Anne Plant on June 1, 1974 and during the course of this study was blessed with the birth of their first child, Melissa Ann, born January 16, 1977.

During the course of these studies, two papers presented on the work reported in this thesis received special recognition being awarded the Area of Microbiology, Student Award for Excellence in Research in April, 1978 and receiving a similar award for a paper presented at the Biomedical Student Research Symposium sponsored by the Missouri Academy of Science on April 30, 1979.

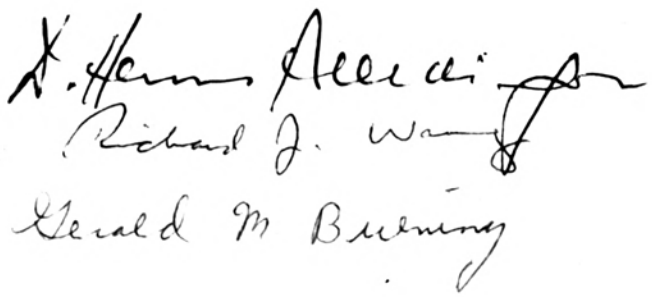
The undersigned, appointed by the Dean of the Graduate Faculty, have examined a thesis entitled

PERSISTENCE AND LATENCY OF HERPESVIRUSES
IN EXPERIMENTALLY INFECTED TURKEYS

presented by David A. Benfield

a candidate for the degree of Doctor of Philosppphy

and hereby certify that in their opinion it is worthy of acceptance.



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