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NOTE

Sensitivity of the transmissible green turtle fibropapillomatosis agent to chloroform and ultracentrifugation conditions

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ABSTRACT: Transmission experiments were conducted to further characterize the filterable transmissible agent that causes GTFP, fibropapillomatosis of green turtles *Chelonia mydas*. Cell-free homogenates (unfiltered or 0.45 µm filtered), prepared from fibropapillomas of free-ranging green turtles (donors) and inoculated into 18 captive-reared recipients, induced tumors in recipients with an overall success rate of 83.3% (range: 0 to 100%). Chloroform treatment prior to inoculation destroyed tumorigenic activity of these homogenates. These data are consistent with the hypothesis that the GTFP agent contains a lipid component, such as a viral envelope, necessary for tumorigenicity. Ultracentrifugation of tumor homogenates for 2 h at 100 000 × *g* cleared the supernatant of tumorigenic activity. Tumorigenicity was recovered in the ultracentrifuge pellet. The pellet, however,was less effective at inducing tumors than the starting material, indicating that the GTFP agent was damaged by these ultracentrifugation conditions. The possibility that the GTFP-associated herpesvirus is the etiologic agent of GTFP was further supported by the observation of eosinophilic intranuclear inclusions in 24.3% of experimentally induced tumors in this study. Tumorigenic activity survived storage for several months at –80°C and at least 1 yr at –180°C, making it feasible to isolate the GTFP agent and its genome from frozen archived material once methods have been optimized.

KEY WORDS: Sea turtles · *Chelonia mydas* · Fibropapilloma · Herpesvirus · Tumorigenicity

Green turtle fibropapillomatosis (GTFP) was first reported in 1938 in green turtle *Chelonia mydas* originating from Florida (USA) waters (Smith & Coates 1938). Since then, GTFP has been reported with increasing prevalence from around the world and has become a significant threat to populations of this endangered species (Balazs & Pooley 1991, Herbst 1994). The exact cause of GTFP is unknown, but controlled transmission experiments have recently demonstrated that the disease is induced by a filterable transmissible agent (Herbst et al. 1995). Herpesvirus-like particles measuring approximately 110 to 125 nm diameter have been observed in some spontaneous tumors as well as in experimentally induced tumors (Jacobson et al. 1991, Herbst et al. 1995). Attempts to isolate this virus in culture have been unsuccessful, however, and until Koch's postulates can be fulfilled using this virus, it remains but one of several possible viral etiologies (Herbst 1994, 1995, Herbst et al. 1995).

In lieu of purified virus with which to conduct transmission experiments, further characterization of the GTFP agent was attempted using cell-free tumor homogenates. This paper provides some basic information about the characteristics of the GTFP agent that are necessary for infectivity (tumorigenicity), thereby providing additional clues to its identity.

We tested the hypothesis that the GTFP agent has a lipid component (viral envelope), that is required for infectivity and that is destroyed by organic solvent extraction. Additional experiments determined whether the GTFP agent survived long-term freezer storage and whether it could be sedimented by ultracentrifugation. This information will be useful in developing methods for purifying the agent from tumor homogenates for examination by electron microscopy and for extraction of viral nucleic acid sequences.

Materials and methods. ***Cell-free tumor homogenates:*** Cell-free twice-frozen and thawed 33% (w/v) tumor homogenates were prepared from 39.0 to 84.0 g of cutaneous fibropapillomas that were surgically removed from each of 7 free-ranging juvenile green turtles (donors) following a published protocol (Herbst et al. 1995). Two tumor homogenates were prepared and used on the same day that fibropapillomas were

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removed from donors. The remaining tumor pools were stored at –80°C for 14 to 88 d prior to processing and in these cases were prepared 24 to 48 h before inoculation and stored in liquid nitrogen. In addition, frozen aliquots of tumor homogenates from 2 turtles, 'Flamingo' (donor 1) and 'Everglades' (donor 2), used in a previous transmission study (Herbst et al. 1995) were pooled and used. This material had been stored for just over 1 yr at –180°C prior to use. Before further processing, a portion of each cell-free preparation was filtered through a 0.45 µm syringe tip filter to remove contaminating bacteria and any remaining intact cells.

Chloroform extraction: Aliquots (5 to 6 ml) of tumor homogenate or filtered extract were diluted with an equal volume of ice-cold chloroform and vortexed for approximately 5 min. After centrifugation at 2000 rpm (1000 × *g*) in a Beckman (Palo Alto, CA, USA) centrifuge for 10 min at 4°C, the aqueous phase was decanted and used for inoculation.

Ultracentrifugation: Samples of filtered tumor homogenate (9 ml total volume) were centrifuged at 34 000 rpm (100 000 × *g*) for 2 h at 4°C in a SW50.1 rotor (Beckman) onto a glycerin cushion. The top supernatant was removed and saved for inoculation, leaving the 1 to 2 ml of liquid immediately above the pellet. The pellet was resuspended in 0.5 ml of bottom supernatant and used for inoculation. Assuming that the GTFP agent was completely cleared from the supernatant, the resuspended pellet had an approximately 5-fold higher concentration of agent than the homogenate.

Transmission experiments: Twenty-one captive-reared green turtles were used as recipients in this study. These turtles were raised from eggs that were collected from each of 4 green turtle nests deposited by different females within a 1 wk period in August 1993 on Hutchinson Island, Martin County, Florida. The protocols for egg collection and incubation, raising of hatchlings, and husbandry of experimental turtles were the same as previously described (Herbst et al. 1995). Turtles (5 or 6 from each clutch) were between 9 and 13 mo old when used in experiments.

The tumor preparations from each donor were used to treat 1 to 3 recipient turtles each. Each recipient turtle was injected with various tumor preparations from only a single donor. Treatments included unfiltered homogenate, 0.45 µm filtered homogenate, chloroform extracted homogenate, ultracentrifuge pellet, ultracentrifuge supernatant, and saline (control), administered as 200 µl intradermal injections at various anatomic sites. Treatments were given in a fixed sequence starting at a different injection site on each turtle so that treatments were somewhat evenly distributed with respect to anatomic location. Injection sites included the proximal margin of a large scale on the palmar surface of the front flipper, the inguinal area, proximal margin of a large scale on the dorsal surface of the rear flipper, dorsal neck, ventral neck, and shoulders. Three turtles received no injections but were housed with their treated clutch-mates to act as sentinels for spontaneous disease.

Turtles were held for up to 1 yr and observed weekly for tumor growth. Blood samples for health monitoring and plasma banking were collected prior to experiments and at 1 to 3 mo intervals during the monitoring period. Experimentally induced tumors were biopsied and the samples processed for light and electron microscopic examination.

Results and discussion. Table 1 summarizes information for the 7 free-ranging green turtles used as tumor donors in this study. These turtles were juveniles (34 to 56 cm straight carapace length, SCL) that stranded or were collected in Florida between August and December 1994. Each had multiple cutaneous fibropapillomas ranging in size from a few millimeters to over 22 cm in diameter involving the axillary and inguinal regions, flippers, neck, and eyes. Data for 'Everglades' and 'Flamingo' (donors 1 and 2) have been published previously (Herbst et al. 1995).

Transmission experiments were conducted on the following dates: 30 August 1994 (using homogenates from donors 'N5218', 'N5219', and pooled material from 'Everglades' and 'Flamingo'), and 5–6 December 1994 (using homogenates from the remaining donors). Results reported here were tabulated after 12 and 9 mo of monitoring, respectively (Table 2). During this period, the 3 control (sentinel) turtles did not develop spontaneous tumors. Tumors developed at one or more anatomic sites in 15 (83.3%) of the 18 turtles that were inoculated with various tumor preparations. Tumors did not develop at saline inoculated sites or at any uninoculated sites. Both unfiltered and 0.45 µm filtered cell-free tumor homogenates were tumorigenic but transmission success varied among individual donors, as has been shown previously (Herbst et al. 1995).

Table 1. Free-ranging green turtles used as fibropapilloma donors. SCL: straight carapace length

Identity	Recovery date (1994)[a]	Location (Florida, USA)	SCL (cm)
'N5218'	09 Aug	Indian River Co.	34.0
'N5219'	16 Aug	Indian River Co.	39.0
'Billy'	01 Sep	Monroe Co.	55.8
'Sunshine'(QQR465)	08 Sep	Citrus Co.	41.0
'Mate'(QQR466)	12 Sep	Monroe Co.	53.6
'Muddy'	29 Nov	Monroe Co.	49.2
'Carrie'(QQJ255)	02 Dec	St. Lucie Co.	45.2

[a]Date of capture or stranding

Table 2. Tumorigenicity of tumor homogenates from various donor turtles. nt: not tested. No tumors developed at uninoculated sites

Donor	Storage duration (d)[a]	No. of recipients	Material inoculated		
			Saline[b]	Untreated homogenate[c]	Chloroform extract
'N5218'	21	3	0	3	0
'N5219'	14	3	0	2	0
'Billy'	82	2	0	1	0
'Sunshine'	88	1	0	0	0
'Mate'	83	2	0	2	0
'Muddy'	0	2	0	2	0
'Carrie'	0	2	0	2	0
Donors 1&2	>365	3	0	3	0
Controls	–	3	nt	nt	nt

[a]All materials stored at –80°C, except donors 1 and 2 which were stored at –180°C
[b]Data are the number of turtles that developed tumors in at least one inoculation site in each treatment group
[c]Untreated preparations were either 0.45 µm filtrates or unfiltered homogenates

Except for the tumor homogenate prepared from 'Sunshine', which was not tumorigenic in the one recipient tested, unfiltered and 0.45 µm filtered homogenates successfully induced tumors in 50 to 100% of the recipients (Table 2).

Experimentally induced fibropapillomas were first identified as slightly raised epidermal swellings ranging from 0.5 to 5.0 mm maximum diameter as early as 7 wk post inoculation in some turtles. Most tumors, however, became apparent 20 to 28 wk post inoculation. Tumors were biopsied between 24 and 40 wk post inoculation. Histopathologic examination confirmed that these tumors were fibropapillomas. Eosinophilic intranuclear inclusions were found in 9 (24.3%) of the tumor biopsies from 7 (46.7%) of the 15 tumor positive turtles. These intranuclear inclusions were similar to those found in a previous transmission study (Herbst et al. 1995).

Chloroform sensitivity: Among enveloped viruses, such as herpesviruses, poxviruses, and retroviruses, the viral envelope is important for virion stability in the environment and for efficient attachment and entry into cells (early infection), although cells may also become infected via pinocytosis of nucleocapsids (Roizman & Furlong 1974, Howe et al. 1980). If an enveloped virus, such as the GTFP-associated herpesvirus, causes GTFP, one would expect transmission success (tumorigenicity) to be greatly reduced by treatment of GTFP homogenates with detergents or organic solvents, such as chloroform, that disrupt the viral envelope (Fenner et al. 1974). On the other hand, if an unenveloped virus, such as a papillomavirus, caused GTFP, transmission success should not be reduced. None of the 18 recipient turtles developed tumors at any site that was inoculated with chloroform extracted tumor preparations. These results indicate that the GTFP agent is chloroform sensitive and are consistent with the hypothesis that GTFP is caused by an enveloped virus.

Ultracentrifugation: Tumor homogenates from only 4 donors were processed by ultracentrifugation. Table 3 compares the frequencies of tumor induction in 10 recipient turtles that were inoculated with 0.45 µm filtered tumor homogenate, ultracentrifuge pellets, and ultracentrifuge supernatants. While treatment with filtered tumor homogenates resulted in tumor induction in 8 (80%) of the recipients, the ultracentrifuge pellets induced tumors in only 2 (20%), and supernatants did not induce tumor formation in any turtles. There was a statistically significant difference in the frequency of success among these preparations ($\chi^2 = 15.7$, $p < 0.001$).

Ultracentrifugation was used to partition the agent (tumorigenic activity) between pellet and supernatant based on the agent's sedimentation coefficient. According to the manufacturer's specifications for the SW50.1 rotor (Beckman), a 2 h run at 34 000 rpm should effectively pellet all particles with sedimentation coefficients >64 Svedberg Units (S). Sedimentation coefficients for viruses range between 40 and several thousand S. The largest DNA particles have sedimentation coefficients ≤100 S. Although particles with lower sedimentation coefficients may have been

Table 3. Partitioning of tumorigenicity of GTFP homogenates by ultracentrifugation

Donor	Treatment		
	0.45 µm filtered homogenate	Ultracentrifuge pellet	Ultracentrifuge supernatant
'N5218'	3/3[a]	0/3	0/3
'N5219'	2/3	0/3	0/3
'Billy'	1/2	0/2	0/2
'Mate'	2/2	2/2	0/2
Total	8/10	2/10	0/10

[a]Number of turtles that developed tumors in at least one inoculation site out of number of turtles that were treated

bound to and sedimented with cellular debris, this experiment demonstrated that the tumorigenic activity could be concentrated by pelleting in an ultracentrifuge, which can be used as a preliminary step in a scheme to purify the agent or its genome. However, although the pellets should have contained higher concentrations of virus particles than the starting tumor homogenates, pellets were less successful at inducing experimental tumors, suggesting that the ultracentrifugation conditions (speed, time, buffer) may have damaged virus particles. This apparent sensitivity to conditions provides another clue that the GTFP agent may be a large enveloped virus, such as the GTFP-associated herpesvirus.

Storage (freezer) sensitivity: The infectivity of enveloped viruses may also be destroyed by frequent freezing and thawing and prolonged storage, although most viruses are stable at –70°C or below (Fenner et al. 1974). Five tumor pools used in this study had been stored for 14 to 88 d at –80°C. Four of these preparations successfully induced tumors. The unsuccessful preparation had been stored the longest (88 d) but 2 successful preparations had been stored nearly as long (82 and 83 d). Similarly, the pooled aliquots of tumor homogenate from 'Everglades' and 'Flamingo' (donors 1 and 2) that had been stored for 1 yr at –150°C, thawed, and refrozen, also remained tumorigenic.

The fact that the GTFP agent remained tumorigenic after being held for nearly 3 mo at –80°C or for a year at –150°C shows that it is not extremely sensitive to long-term storage and thawing, and that, potentially, it can be purified in fully infectious form from archived frozen tumors and tumor homogenates. This opens the possibility for future experiments using tumor homogenates, whose effective (tumorigenic) dose has been titrated, to test the sensitivity of the GTFP agent to physical and chemical factors such as temperature, desiccation, pH, osmolarity, disinfectants, and detergents.

This study began to describe the characteristics of the GTFP agent that are associated with tumorigenicity. Until this agent can be isolated and propagated in pure culture, further characterization will be dependent on the availability of donor material and additional turtles for experimentation.

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