THE DETECTION OF A VIRAL INTERFERING SUBSTANCE IN THE SHOPE PAPILLOMA AND THE Vx7 AND Vx2 CARCINOMAS*

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Fluorescent antibody studies have revealed Shope papilloma viral antigens in the nuclei of the keratinized and keratohyalinized layers of both the cottontail and domestic rabbit papillomas (1, 2). High levels of antibodies to Shope papilloma virus were found in the blood of animals bearing these tumors. Rabbits bearing the Vx7 carcinoma, originally derived from Shope papilloma, also had high serum titers of neutralizing antibodies against the Shope virus, but the tumor itself stained poorly with fluorescent antibody preparations and in only a few cells. Animals bearing the Vx2 carcinoma, a tumor also derived from the papillomas, had neither serum antibodies against the papilloma virus nor demonstrable viral antigens in the carcinoma (3–5).

Three theories have been offered to explain the above phenomena: (*a*) the virus did not keep up with the growth rate it had induced in the cells and was, as a result, diluted out; (*b*) the virus had been incorporated into a genome of the cell in a manner analogous to the lysogenic state of bacteriophage; and (*c*) the virus had entered a prolonged eclipse period within the cells and was in a form incapable of reacting with the viral antibody. The last two propositions encompass the phenomenon of viral masking, i.e., the presence of a protein-deficient virus in cells which fail to yield the infectious agent under ordinary laboratory conditions (2).

Several mechanisms have been found which are operative in the viral carrier state (6). Significant among these is the induction of interferon synthesis by the masked virus, with the consequent establishment of a "chronic low-grade infection." The resistance to further viral invasion is increased in such infected cells and in those not yet infected but in the same interferon milieu. The demonstration of resistance to superinfection has become an accepted means of detecting the viral carrier state, particularly in rubella studies (7). Henle and Henle have recently reported the release of low titers of interferon into the

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tissue culture medium overlying Burkitt lymphoma lines EB-1 and EB-2. On superinfection with Newcastle disease virus, much higher titers of interferon were produced (8).

This paper reports the presence of viral interfering substance, probably an interferon, in the protein extracts of Shope papilloma, Vx7 and Vx2 carcinomas grown in vivo.

Materials and Methods

Tumors[1].—

Shope papilloma: 1 g of stock Shope papilloma, originally taken from a wild cottontail rabbit, was macerated in 10 cc of phosphate-buffered sterile saline (PBS), *p*H 7, and centrifuged at 2000 rpm for 10 min to remove debris. The flanks of four New Zealand rabbits were scarified and coated with the virus-containing supernatant. Papillomas appeared on about the 12th day and were harvested at the end of 4 wk when the tumors were large, but not necrotic or blood encrusted.

Vx7 and Vx2 carcinomas: 1 cc of cell suspension of Vx7 at a concentration of 1×10^6 cells/cc was injected into the anterior and posterior thighs of three New Zealand rabbits. Three rabbits were similarly inoculated with Vx2. These tumors were allowed to grow for 1 month before harvesting, at which time they were firm, 4–5 cm in diameter, and slightly necrotic at the center.

Muscle control: The anterior and posterior thigh muscles of one healthy New Zealand rabbit were taken for preparation of control extracts.

Tissue Extracts.—All three tumors and the muscle were similarly prepared after aseptic harvesting, washing, and removing any necrotic tissue. 100 g of each tissue was cut into pieces of about 1 cc and homogenized in a Waring Blendor with enough sterile Earle's balanced salt solution (BSS), *p*H 7.3, to keep the suspension of broken cells fluid (150–200 cc). The homogenates were then centrifuged at 2000 rpm at 8°C for 30 min until a compact pellet of cellular debris had been deposited at the bottom of the tubes. The supernatants were transferred to new centrifuge bottles and sterile ammonium sulfate $(NH_4)_2SO_4$ added to 100% saturation as calculated by volume. The solutions were allowed to precipitate over a 24 hr period at 5°C. They were then centrifuged for 3 hr at 3000 rpm at 5°C until a discrete pellet of precipitated protein was formed.

The supernatants were discarded and the pellets transferred to 18/32 Visking dialysis bags and dialyzed against six 1000 cc changes of Earle's BSS over an 8 hr period, thereby removing the $(NH_4)_2SO_4$. The extracts were then dialyzed for 12 hr against 0.1 M KCl-HCl acid buffer (*p*H 2.1) at 5°C to inactivate any bacteria or virus present. Four final dialyses over a 24 hr period were then carried out against Earle's BSS. As a final precaution against viral or bacterial contaminants, all solutions were passed through size F Seitz filters (100 μ) and centrifuged at 100,000 *g* for 60 min at 8°C. The resulting supernatants were lyophilized and the powdered extracts made up in sterile Earle's BSS such that 1 cc contained 50 mg of extract.

Viruses.—

Fibroma virus: 0.5 g of Shope fibroma was macerated in a mortar in 5 cc of sterile Ringer's solution. The suspension was centrifuged for 15 min at 2000 rpm and the supernatant taken for assay with fibroma virus.

Vaccinia virus: For each assay a stock lyophilized pellet of live vaccinia virus obtained from Wyeth Laboratories, Philadelphia, Pa., (Dryvax Smallpox Vaccine) was suspended in 1 cc of PBS and then diluted to either 10^{-3} or 10^{-4}. The virus suspension was titered for primary

[1] Obtained from Dr. John G. Kidd.

plaque-forming units on rabbit kidney monolayer cultures simultaneously with the running of each assay.

In Vivo Assay.—The papilloma protein extract was first tested in vivo for viral interfering activity against Shope fibroma virus. The flanks of two New Zealand rabbits were shaved and seven paired subcutaneous injections of fibroma virus and protein extract were given in various combinations.

In Vitro Assay.—1 cc (50 mg) of protein extract was incubated with primary rabbit kidney monolayers for 1 hr at 37°C. Maintenance (Melnick's) medium was added and the cultures then challenged with vaccinia virus suspension at 10^{-4} dilution. The monolayers were placed in a vibration-free incubator at 37°C for 48 hr at which time the medium was decanted and the cell layers stained with gentian violet (9).

TABLE I

Inhibition of Fibroma Virus In Vivo by Shope Papilloma Extract

	Site of injection						
	A	B	C	D	E	F	G
Fibroma virus (*cc*)	1	1	1	1	1	0	0
Papilloma extract (*mg*)	50	37.5	25	12.5	0	50	100
Fibroma development	0	0	0	0	++++	0	0

The first assays on each extract were run as separate controlled experiments with vaccinia virus of varying titers from different stock pellets. Three types of flasks were set up: those containing only virus; those with both virus and protein extract, either tumor or control; and those with protein extract alone. These last flasks were observed for signs of cellular toxicity.

In tests to obtain information on comparative potency another group of rabbits was inoculated as previously described with Shope papilloma virus, Vx7 or Vx2 cells and a new set of extracts were prepared. All three extracts were then assayed for viral interfering activity in vitro simultaneously using the same stock vaccinia virus suspension at 10^{-4} dilution. The Shope papilloma extract was also challenged with a lower virus dilution of 10^{-3}.

RESULTS

In Vivo Assay.—The combinations of fibroma virus, papilloma extract, and the results are shown in Table I. There was an initial mild inflammatory reaction at all sites where protein extract had been injected, regardless of the presence or absence of virus. This disappeared by the 7th day. On the 5th day firm subcutaneous nodules, typical of fibromas, appeared at all four sites where virus alone had been injected (Table I, E). These reached maximum growth of 1.5 cm diameter by day 11 and began to regress by day 14. Ulcers occurred in all. At no time did any nodules appear at sites where either virus and protein (Table I, A–D), or protein extract alone (Table I, F–G), had been injected.

In Vitro Assay.—Although the in vivo assay indicates that the papilloma extract did interfere with the action of the virus, the method itself was not satisfactorily quantitative. For this reason, an in vitro assay was undertaken

to test the effects of tumor extracts on viral growth. The combinations of vaccinia virus, protein extract, and the results are shown in Tables II, III, and IV. Figures 1, 2, and 3 present the results graphically. It is evident from Table II and Fig. 1 that the presence of the protein extract of Shope papilloma caused almost complete suppression of plaque formation. The extracts of Vx7 and Vx2 carcinomas (Tables III and IV; Figs. 2 and 3) caused partial inhibition of

TABLE II

Inhibition of Plaque Formation by Shope Papilloma Extract

Flask No.	Vaccinia virus	Muscle extract	Papilloma extract	No. of plaques	
				Average	Range
		mg	*mg*		
1–2	10^4	0	0	117	111–124
3–5	10^4	50	0	194	180–208
6–8	10^4	0	50	1	0–3
9–10	0	50	0	0	0
11–12	0	0	50	0	0
13–14	0	0	0	0	0

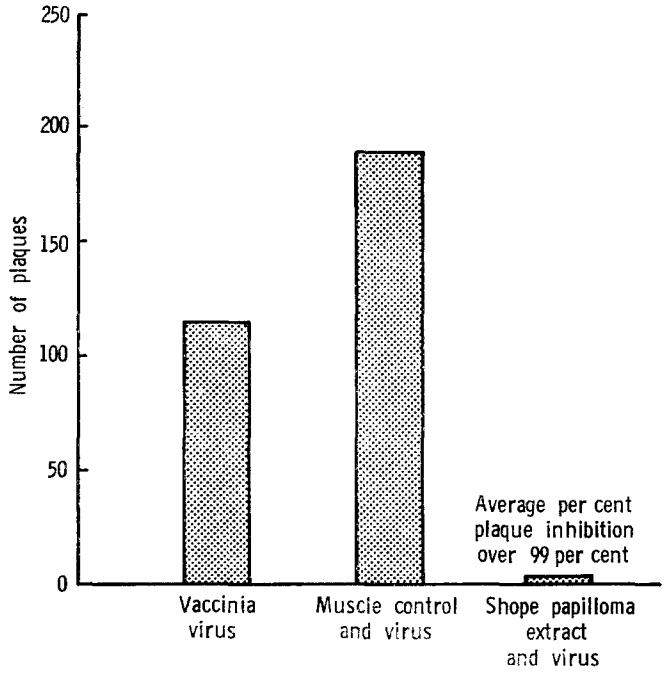

FIG. 1. Inhibition of plaque formation by Shope papilloma extract.

TABLE III

Inhibition of Plaque Formation by Vx7 Extract

Flask No.	Vaccinia virus	Muscle extract	Vx7 extract	No. of plaques	
				Average	Range
		mg	*mg*		
1–2	10^4	0	0	135	128–143
3–5	10^4	50	0	224	210–238
6–8	10^4	0	50	78	66–88
9–10	0	50	0	0	0
11–12	0	0	50	0	0
13–14	0	0	0	0	0

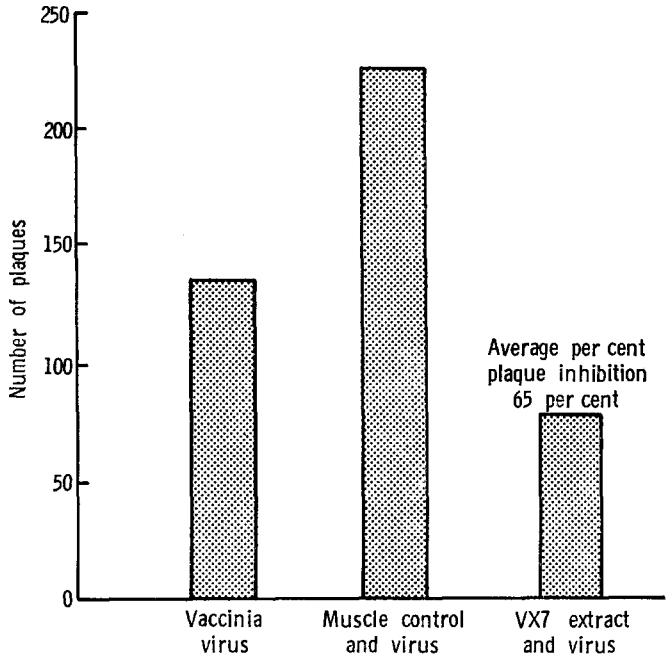

FIG. 2. Inhibition of plaque formation by Vx7 carcinoma extract.

plaque formation. The number of plaques produced in the presence of the Vx7 extract was 65% lower than the number produced in flasks containing control muscle extract. The Vx2 extract caused a 64% reduction in plaques. Vx2 had, however, been challenged with a higher virus titer than had the Vx7 extract. Monolayers exposed to protein extract alone did not differ morphologically from untreated controls and showed no evidence of toxicity. It was noted that

TABLE IV

Inhibition of Plaque Formation by Vx2 Extract

Flask No.	Vaccinia virus	Muscle extract	Vx2 extract	No. of plaques	
				Average	Range
		mg	*mg*		
1–2	10^4	0	0	296	288–305
3–5	10^4	50	0	363	347–374
6–8	10^4	0	50	127	109–140
9–10	0	50	0	0	0
11–12	0	0	50	0	0
13–14	0	0	0	0	0

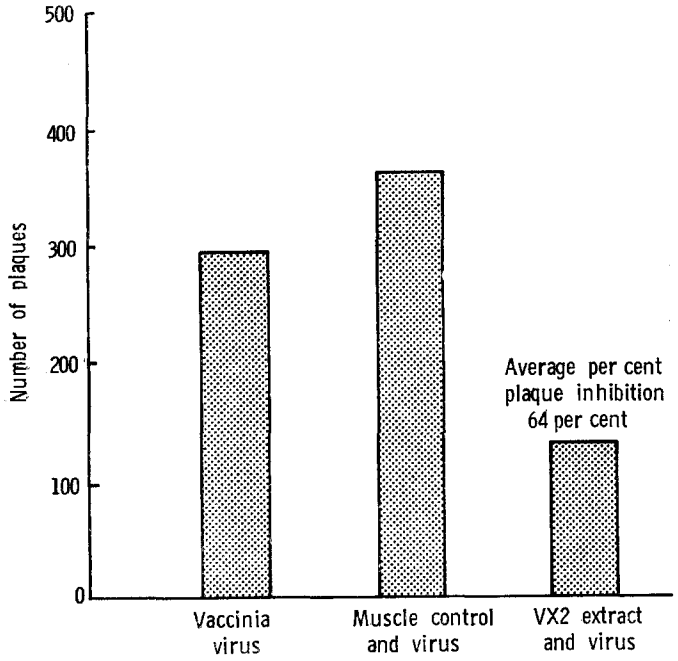

FIG. 3. Inhibition of plaque formation by Vx2 carcinoma extract.

the plaques in the tumor extract treated bottles were not only fewer in number than the controls, but also approximately one-half to one-third smaller in diameter. This observation is in accordance with that made by Gifford et al. in their work with vaccinia virus and chick embryo interferon (10).

The per cent of virus inhibition by an extract was calculated using muscle for control values as it was believed that these monolayers more closely re-

sembled the flasks with tumor extract in that both had increased amounts of protein in the medium. Comparison of the vaccinia virus plaque count (flasks

TABLE V

Simultaneous Assay of Shope Papilloma, Vx7 and Vx2 Extracts for Plaque Inhibition

Flask No.	Vaccinia virus	Muscle extract	Papilloma extract	Vx7 extract	Vx2 extract	No. of plaques		Per cent inhibition
						Average	Range	
		mg	*mg*	*mg*	*mg*			
1–5	10^{4}	0	0	0	0	66	58–73	
6–10	10^{4}	50	0	0	0	76	72–82	
11–14	10^{4}	0	50	0	0	1	0–1	99
15–18	10^{4}	0	0	50	0	45	41–51	40
19–22	10^{-4}	0	0	0	50	50	48–53	34
23–24	0	50	0	0	0	0	0	
25–26	0	0	50	0	0	0	0	
27–28	0	0	0	50	0	0	0	
29–30	0	0	0	0	50	0	0	
31–32	0	0	0	0	0	0	0	

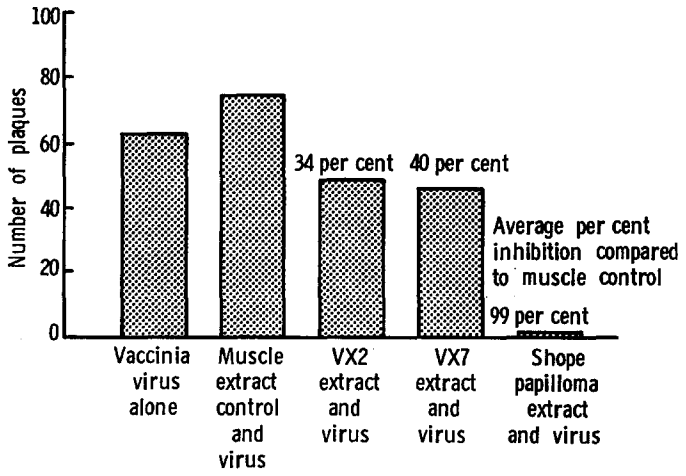

FIG. 4. Simultaneous assay of Shope papilloma and Vx7 and Vx2 extracts for plaque inhibition.

1 and 2), with the virus-muscle extract plaque count (flasks 3–5), clearly reveals the enhancing effect of the richer medium on virus action.

The results of the individual assays indicated the presence of a viral-inhibitory substance in all three tumor extracts. Because vaccinia virus from different stock pellets and of different titers had been used, it was not possible to com-

pare the relative potency of the extracts. To obtain this comparative information, new extracts were made up and assayed simultaneously with the same stock vaccinia virus at dilution 10^{-4}. The results are shown in Table V and Fig. 4. There was virtually complete suppression of plaque formation in flasks containing the extract of Shope papilloma. The extracts from the Vx7 and Vx2

TABLE VI

Incomplete Plaque Suppression by Shope Papilloma Extract Using High Virus Titer

Flask No.	Vaccinia virus	Muscle extract	Papilloma extract	No. of plaques	
				Average	Range
		mg	*mg*		
1–3	10^{-3}	0	0	370	360–382
4–5	10^{-3}	50	0	671	661–682
6–8	10^{-3}	0	50	246	224–282

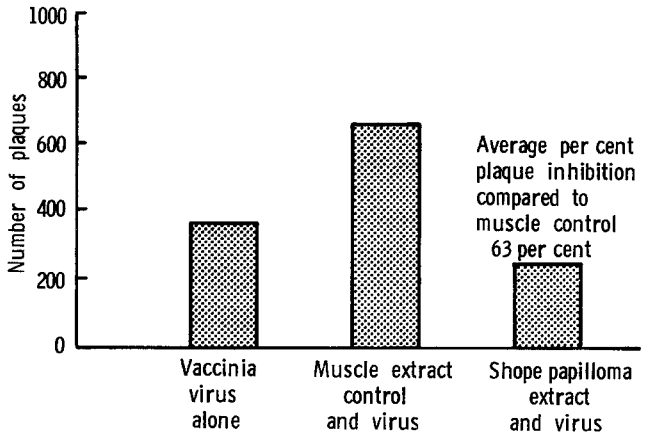

FIG. 5. Incomplete plaque suppression by papilloma extract using high virus titer.

carcinomas produced 40% and 34% inhibition, respectively, compared to the muscle control.

As in the individual assays, the plaques in tumor extract–containing bottles were smaller than those in control bottles. Flasks containing protein extract alone did not differ morphologically from untreated controls when examined microscopically, and showed no signs of toxicity.

Because one characteristic of interferon is that its effect may be overcome if viral titers are sufficiently high, another assay was done using 10^{-3} virus dilution with papilloma protein extract which had given almost 100% suppression at dilution of 10^{-4}. The results of this experiment are shown in Table VI and Fig. 5.

These reveal that, at a higher titer of virus, partial (63%) rather than complete suppression of plaque development occurs with the Shope papilloma extract. The plaque-enhancing effect of the muscle extract is again seen.

DISCUSSION

These experiments indicate the presence of a viral interfering substance in the protein extracts from Shope papilloma, Vx7 and Vx2 carcinomas. The following characteristics indicate that the substance is most likely an interferon: (*a*) precipitated out with the protein fraction; (*b*) nondialyzable; (*c*) biologically active after being maintained at pH 2.1, and 5°C for several hours; (*d*) morphologically nontoxic to tissue cultures on microscopic examination; (*e*) able to reduce the size and number of virus plaques produced; (*f*) overcome by high virus titers; and (*g*) not virus specific. Because the substance was active against the unrelated vaccinia and fibroma viruses, it is unlikely that this effect is produced by the presence of antibody.

The greatest amount of interfering material is produced by the papilloma, the only growth from which good yields of infectious virus may be easily obtained. The Vx7 and the Vx2, however, appear to produce less but approximately equal amounts of interfering material. The finding of a viral inhibiting substance in the Vx7 is not unexpected as this tumor has not only been shown to contain small amounts of capsid antigen, but also to yield nucleic acid capable of inducing typical papillomatous growths on rabbit hosts (11). Evans et al. have noted that growth of Vx7 carcinomas may be suppressed by previous immunization with homologous papilloma virus (12). There is, however, no significant suppression of infection by papilloma viral nucleic acid in carcinoma-bearing animals (13), implying that the amount of interfering substance produced is not sufficient to prevent further invasion by homologous viral DNA.

The extraction of an interferonlike substance from the Vx2, a carcinoma which no longer contains detectable viral antigen and elicits no viral antibody production, is an unexpected finding and implies the presence of viral nucleic acid within the cells. The negative results of fluorescent staining on the Vx2 indicate only that there is no viral protein coat present and are in no way related to the presence or absence of viral nucleic acid. This finding of interferonlike activity contradicts the proposition that loss of antigenicity in the Vx2 is secondary to the virus' being "diluted out" of the system via rapid tumor growth. Whether this nucleic acid is established in lysogenic form or in a prolonged eclipse state cannot be determined by the present experiments. There have been no reported studies on the existence of viral specific tumor antigens in the Vx2 carcinoma. Such studies would, of course, further clarify the question of persistance of masked virus in this tumor which, somewhere between its 22nd and 46th passage, lost all trace of detectable virus (14).

Although fluorescent antibody studies reveal only rare viral protein antigen in the Vx7, the production of approximately equal amounts of viral-inhibiting substance by the Vx7 and Vx2 indicate that viral nucleic acid is present in the nonfluorescing cells as well. The extraction of equal amounts of the substance from the Vx2 may imply that the comparatively small amount of fluorescing antigen in the Vx7 has little bearing on the amount of interferonlike substance produced. The production of considerably more interferonlike material by the papilloma, however, may indicate that the virus, in appreciably greater amounts in its complete form, is a greater stimulus to the production of the substance than is the agent in incomplete form; a situation somewhat analogous to the increased interferon production by lymphoma cells in the Henles' study (8).

SUMMARY

In vivo assay of Shope papilloma protein extract and in vitro assay of extracts from Shope papilloma, Vx7 and Vx2 carcinomas showed strong interferon-like activity in the papilloma and moderate activity in the carcinomas. The interpretation is that the presence of viral nucleic acid in all three tumors stimulated the production of this substance even though fluorescent antibody studies reveal the protein coat only in the papilloma and Vx7.

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