

A Quantitative Micro-Complement Fixation Method in Studies of Human Wart Viruses

(Accepted 10 August 1977)

SUMMARY

This report offers a description of a quantitative micro-complement fixation method (Cikes, 1975) for detecting human wart virus antigens and their specific antibodies, and proof of the specificity of the reactions being detected. The increased sensitivity demonstrated by chromium-release measurement is compared to the results of visual interpretation of complement fixation.

The humoral response to human wart virus has been studied by standard complement fixation by Almeida *et al.* (1969), Ogilvie (1970) and by Pyrhonen & Johansson (1975). If, however, complement fixation is to be used for quantification of wart virus antigen in tumour tissue containing less virus antigen, or in cells infected *in vitro* (Eisinger *et al.* 1975), a more sensitive quantitative method is needed. In our studies, therefore, we have used a micro-complement test (Cikes, 1975) in which the quantification of complement fixation involves correlating the amount of unbound complement with the haemolytic release of radioactivity from Chromium 51 (^{51}Cr)-labelled sheep erythrocytes. Cikes' modification of the standard complement fixation method also includes the use of a small number of erythrocytes per reaction volume to increase the sensitivity.

Guinea pig complement, sheep red blood cells and anti-sheep erythrocyte serum (amboceptor) were all obtained from Flow Laboratories, and Chromium 51, from New England Nuclear (Boston, Massachusetts 02118), was used at a specific activity of 400 mCi/mg. The dilution and storage of materials, the radioactive labelling procedure, and the sensitization of sheep red blood cells is identical with the procedures described by Cikes. Our testing routinely employed 100 λ of sheep red blood cells with 0.3 mCi (in 0.3 ml) of ^{51}Cr . This results in approx. 9.000 ct/min/ 10^6 red blood cells. Complement-fixing antigens were prepared from pooled wart tissues and uninfected skin samples. The tissues were collected, washed and incubated (24 h) in tissue culture medium (minimum essential medium with Earle's salts) with penicillin (1000 units/ml), streptomycin (1000 mcg/ml) and Fungizone (0.25 mcg/ml). The samples were then minced, ground with glass powder and resuspended to 10% (w/v) in medium. This suspension was sonicated twice for 30 s (Biosonic sonicator at maximum setting for micro-tip) and clarified by centrifugation at 10000 g for 30 min. The supernatant was then considered to be a 10% suspension (viral or normal skin), with a protein concentration of approx. 0.7 mg/ml. Monospecific rabbit antisera against human hand wart virus purified on caesium chloride isopycnic gradients were prepared as described by Eisinger *et al.* (1975). Sera employed in the test were heat inactivated at 56 °C for 30 min.

The micro-test was performed in a final reaction volume of 100 λ . This total was composed of equal volumes of antigen, antibody, complement and sensitized radio-labelled sheep red blood cells. The antigen, antibody and complement were allowed to react at 4 °C for 16 to 18 h before the addition of the sensitized radio-labelled sheep red blood cells. The test was performed in microplates with V-shaped wells, the materials being added with 25- λ

calibrated pipette droppers (test supplies from Cooke Engineering Co., Alexandria, Virginia). Within the test plate the complement was added to dilutions so that one free CH_{80} unit remained per reactant well. These values were determined preliminary to the test, and the anti-complementary activity of each dilution of antigen and antiserum to be included was measured. These procedures duplicate the conditions of the test proper, and are described in detail by Cikes.

Results of the micro-test may be read visually, but greater sensitivity is provided by the comparison of the amount of released radioactivity in the reactant supernatant to the amount in the control supernatant. Equal volumes of 75 λ were collected from each test well and counted in a gamma scintillation counter. Antigen and antiserum controls were run for each material and every dilution of this material was titred. The titre was obtained by comparing the ct/min of the test wells with the ct/min of the control wells. The ct/min of the control samples indicate approx. 80% haemolysis. The titre of the test material was read as the dilution at which the ct/min of the reactant samples rise to this control ct/min level. Data obtained from these measurements of radioactivity were analysed by the Student *t* test to insure that the chosen end-point (titre) is significantly different from the control values at a probability factor of 95%. The logarithms of the control ratio values were used to determine the mean (\bar{x}) and the standard deviation (*s*). The control value is expressed as the mean less the standard deviation times the *t* factor for 95% probability [$\bar{x} - t(s)$]. The ratio of each experimental ct/min to the ct/min of its control is determined. Experimental ratios less than the control value are considered significantly different. The application of this to the results reported in Table 2 resulted in a control value of 92% [$\bar{x} - t(s) = -1.98940 - 2.353(0.012) = -1.96116$].

The micro-complement fixation test was used to detect the reaction between human hand wart virus antigens and monospecific rabbit antiserum directed against human wart virions. Typical reactions, including the detection of wart virus in 10% suspensions and tissue culture materials, are listed in Table 1. The Table lists six different 10% suspensions of pooled hand wart virus, two similar preparations from human plantar warts and three samples of infected tissue culture cell suspensions. Comparison of the antigen titres listed revealed those obtained from a visual interpretation of the micro-test red blood cell pellets (scored at 80% haemolysis) to be constantly four- to eightfold lower than the titres obtained from the count analysis of the micro-test. The majority of wart virus 10% suspensions, including those prepared from plantar warts, reacted to high titre even when the results were interpreted visually; however, antigen titres of tissue culture material reacted only minimally according to visual readings, but revealed titres signifying specific reaction when analysed by ct/min evaluation. Repeated micro-testing of uninfected tissue culture cells never gave any indication of a positive reaction.

To insure the specificity of this reaction we performed cross-absorption studies. Equal volumes of wart virus antiserum (WVAS), undiluted or diluted 1:10 or 1:100 with Veronal-buffered diluent (VBD), were mixed with 10% wart virus suspension or 10% normal skin suspension. After 2 h incubation at 37 °C the mixtures were centrifuged under conditions sufficient to pellet wart virus (40000 rev/min for 90 min in an SW 50.1 rotor).

Unabsorbed and undiluted WVAS reacted with wart virus up to 1:700 dilution, and with normal skin to 1:40. Because of the high titre of the specific antibodies their removal by absorption with the virus antigen contained in a 10% suspension could not be achieved. Absorption with skin antigens could remove any antibodies directed against normal skin components

The same WVAS diluted 1:10 had no reduction of detectable titre to wart virus or normal

Table 1. *Titres of wart virus suspensions reacted with monospecific antisera: comparison of visual interpretation with radioactive analysis.*

Virus preparation*	Visual titre†	Ct/min-determined titre‡
I	1:64	1:1024
II	1:256	1:2048
III	1:256	1:2048
IV	1:640	1:2560
V	1:2560	1:20480
VI	1:5120	1:20480
VII (Plantar)	1:1280	1:10240
VIII (Plantar)	1:128	1:1024
Tissue culture 4‡	1:8	1:128
Tissue culture 8	1:8	1:64
Tissue culture 10	1:64	1:512

* I to VI = various 10% suspensions of pooled human hand warts; VII, VIII = 10% suspensions of pooled human plantar warts.

† Reciprocals of greatest antigen dilution showing positive reaction. Testing performed using 6 units of antiserum with 10% wart suspensions; 5 units of antiserum with tissue culture suspension. 1 unit of antiserum = the amount of antibody, contained in 0.0251 ml, required to fix 80% of the complement in the presence of 17.5 µg of virus protein.

‡ Tissue culture 4, 8, 10 = infected-cell suspensions from successive *in vitro* passages of human hand wart virus.

Table 2. *Complement-fixation titres of wart virus antiserum (WVAS) absorbed with human wart virus (HWV) and normal skin antigens (NSA)*

Antisera	Antigen	Dilution								Diluent	Titre
		1:200	1:250	1:300	1:350	1:400	1:450	1:500	1:600		
WVAS: unabsorbed	HWV	3478*	5837	6196	5509	5539	5939†	6404	6123	7380	1:450
	Diluent	6230	6379	6901	6414	6405	6550	6583	6471	7057	
	NSA	6518	6584	6390	6667	5877	6317	6566	6402	7086	0
WVAS: absorbed with HWV	HWV	5555	5971	4085	5796	6094	6131	6415	5826	7804	0
	Diluent	5838	6175	4583	5159	5905	6754	6022	6886	6810	
	NSA	5438	5742	5027	5673	6387	6524	6719	6649	6642	0
WVAS: absorbed with NSA	HWV	5593	5592	5918	6280†	6402	6250	7173	6258	6947	1:350
	Diluent	7018	7217	7156	7251	6578	6633	7584	6606	7589	
	NSA	6375	7427	6868	7058	6944	6164	7239	6629	7438	0

* Ct/min for analysis of released radioactivity.

† End-point titre of reaction.

skin. Absorption with wart virus antigen of this 1:10 dilution decreased the specific titre to 1:500.

WVAS diluted 1:100 reacted with wart virus to a titre of 1:450, but revealed no titre to normal skin antigen. Table 2 details the ct/min values of the micro-complement fixation testing of WVAS. Absorption of 1:100 diluted WVAS was wart virus removed all activity against virus antigens, and the same serum absorbed with normal skin showed only a small reduction of its original titre to the wart virus.

Since our monospecific antisera to human wart virus did not react in Cikes' micro-complement fixation with other Papova viruses, such as SV40, BK or JC virus and all the reactivity could be removed by absorption with human wart virus and not with normal skin, it can be concluded that the reactions observed are specifically between human hand wart viruses and antibodies directed against them. Human papilloma viruses are known to

be the inducers of benign human epithelial tumours (Rowson & Mahy, 1967). Recent evidence indicates some of these viruses to be involved in the induction of human neoplasia (zur Hausen, 1976). Since data concerning the immunological response to these viruses are scarce (Ogilvie, 1976; Pyrhonen, 1976), and the need to learn about it is pertinent, we hope that the use of this more sensitive and specific complement-fixation will be of general use.

We wish to thank Dr N. Orentreich and Dr H. Katz for the supply of wart material. The normal skin samples were kindly provided by the Tumor Procurement Center of Memorial Hospital, headed by Dr Y. Hirshaut. The authors are indebted to Dr M. Cikes for his personal introduction of this technique. This work was supported by a contract No. 1-CP-61022 from the Virus Cancer Program of the National Cancer Institute. Additional support was received from the Fund for Advanced Study of Cancer, the Zelda R. Weintraub Foundation and the American Cancer Society.

Laboratory of Human Tumor Virology
Memorial Sloan-Kettering Cancer Center
New York, New York 10021, U.S.A.

J. M. HEFTON
 M. EISINGER

REFERENCES

- ALMEIDA, J. D., ORIEL, J. D. & STANHARD, L. M. (1969). Characterization of the virus found in human genital warts. *Microbios* **1**, 225-232.
- CIKES, M. (1975). An improved quantitative micro-complement fixation test. *Journal of Immunological Methods* **8**, 89-100.
- EISINGER, M., KUCAROVA, O., SARKAR, N. H. & GOOD, R. A. (1975). Propagation of human wart virus in tissue culture. *Nature, London* **256**, 432-434.
- OGILVIE, M. M. (1970). Serological studies with human papova (wart) virus. *Journal of Hygiene, Cambridge* **68**, 479-492.
- OGILVIE, M. M. (1976). Basics in human wart immunology. In *Biomedical Aspects of Human Wart Virus Infection*, Proceedings of a Fondation Merieux Seminar held in Lyon on December 15-16, 1975, pp. 183-190.
- PYRHONEN, S. & JOHANSSON, E. (1975). Regression of warts. An immunological study. *Lancet* **i**, 592-595.
- PYRHONEN, S. (1976). Serological aspects of the immunology of human warts. In *Biomedical Aspects of Human Wart Virus Infection*, Proceedings of a Fondation Merieux Seminar held in Lyon on December 15-16, 1975, pp. 191-197.
- ROWSON, K. E. & MAHY, B. W. J. (1967). Human papova (wart) virus. *Bacteriological Reviews* **11**, 110-131.
- ZUR HAUSEN, H. (1976). Condylomata acuminata and human genital cancer. *Cancer Research* **36**, 794.

(Received 20 May 1977)