IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS

II. Properties of the Complement-Binding Antigen Present in Extracts of the Growths: Its Relation to the Virus

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The sera of rabbits bearing virus-induced papillomas will fix complement in the presence of antigens consisting of saline extracts of the growths, as the experiments of Paper I have shown. The nature of the complement-binding antigen and its relation to the papilloma virus will now be considered.

Tests with Extracts of Normal Skin

Since the papillomas consist of epidermal cells infected with the virus it was necessary to learn first whether the antigen exists only in papillomatous tissue or whether it can be extracted from normal epidermal tissue also. In an experiment to decide the point, portions of the skin and of the naturally occurring papillomas of 3 cottontail rabbits were extracted and tested for capacity to bind complement in the presence of known immune sera, according to the technique described in Paper I.

Experiment 12.—Using aseptic technique, a disk of skin weighing approximately 500 mg. was removed from the flank of each of the 3 cottontails, and similar amounts of the naturally occurring papillomas of the same animals were also procured. The materials were weighed and ground separately, and saline added to them to make 1:20 suspensions. They were left overnight in the refrigerator and then centrifugalized twice, first at 3500 R.P.M. for 5 minutes, after which the supernatant fluids were again spun at 3500 R.P.M. for 10 minutes. The water-clear extracts were then heated at 56°C. for 30 minutes as usual and tested for capacity to bind complement. Sera were used from 4 cottontails bearing experimentally induced growths, 2 of which had been "hyperimmunized" by means of

repeated injections of Berkefeld filtrates of the growths. One of the hyperimmune sera (W.R. 8-S) had a complement fixation titer of 1:128 when tested with potent antigens, the other 1:64. The sera of 2 normal cottontails were included for comparison.

In this experiment (Table XII) none of the specific complement-binding antigen could be extracted from the normal skin of cottontail rabbits though it was obtained in large amount from the virus-induced papillomas of the same animals. The objection might be raised that the skin of domestic rabbits is a tissue consisting mostly of dermis, with

TABLE XII

Complement Fixation Tests with Extracts of Normal Skin and of the Naturally Occurring Papillomas of Three Cottontail Rabbits

Antigens		Sera						
W.R. No.	Tissue	Immune		Hyperimmune		Normal		Antigen controls (no serum)
		15-S	19-S	8-S	12-S	30-T	36-T	
53	Skin	0	0	0	0	0	0	0
56	"	0	0	0	0	0	0	0
55	"	0	0	0	0	0	0	0
53	Papilloma	++++	++++	++++	++++	0	0	0
56	"	++++	++++	++++	++++	0	0	0
55	"	++	+++	++++	++±	0	0	0
Serum controls (no antigen).....		0	0	0	0	0	0	

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigens, 1:20 extracts of fresh tissues.

a relatively small proportion of epidermal cells, a material notably difficult to extract. But these objections do not hold in the case of cottontails, for their skin is quite thin, consisting almost wholly of epidermis with but a shallow web-like corium underneath, and it can be readily ground in mortar.

Filtration of the Complement-Binding Antigen

An experiment was next undertaken to find whether the antigen is held back by filters that retain the virus. The latter is known to

pass readily through Berkefeld V filters, but in our experience, most of it has been retained on filtration through Berkefeld W candles or Seitz pads.

Experiment 13.—5 per cent suspensions of the glycerolated, infectious papillomas of W.R. 8-N and W.R. Tx were made as usual, and allowed to stand 7 and 20 days respectively in the refrigerator, to give time for the dissociation of any

TABLE XIII

Pathogenicity and Complement Fixation Tests with Berkefeld V and W and Seitz Filtrates of Cottontail Papillomas

Papilloma extracts		Pathogenicity tests*			Complement fixation tests		
		Test rabbits			Dilution of extracts		
Source	Preparation	A	B	C	1:20	1:100	1:500
W.R. 8-N	Unfiltered	++++	++++	++++	++†	++++	0
	V filtrate	+++±	++++	++++	++++	++	0
	W "	±	+	+	±	0	0
	S "	+	±	+±	0	0	0
W.R. Tx	Unfiltered	+++	+++	+++	++++	+	0
	V filtrate	+++	++±	+±	++	0	0
	W "	+	+	+	±	0	0
	S "	0	±	0	0	0	0

Complement, 2 units in all tubes.

Immune serum D.R. 7 diluted 1:4.

Papilloma extracts diluted 1:100 in pathogenicity tests,—as indicated in complement fixation tests.

The immune serum and papilloma extracts showed no anticomplementary effect when tested in double amount in control tests.

* Readings made on the 42nd day after inoculation, according to the standard scale.

++++ = confluent papillomatosis.
+++ = semiconfluent "
++ = many discrete papillomas.
+ = a few papillomas.
± = 2, 3, or 4 discrete papillomas.
± = 1 papilloma.
† Prozone effect.

soluble constituents (1). The extracts were then centrifugalized twice as usual and the supernatant fluids removed. 5 cc. aliquots of these were then filtered through Berkefeld V and W filters (size 5) under negative pressure of about 30 cm.

of water, and through single Seitz EK disks under a positive pressure of 5 pounds. The filtrates were water-clear. About 4 cc. was obtained by filtration through the Seitz pads, while only 1.5 to 2.0 cc. came through the Berkefeld candles.

The unfiltered extracts as well as the filtrates were tested for pathogenicity according to the routine method (2), and for complement-binding capacity in the presence of a known immune serum. Table XIII shows the findings. Both of the unfiltered extracts were highly pathogenic, and they bound complement completely, that of W.R. 8-N being the more active in both respects. The Berkefeld V filtrates of both materials showed a slight loss in pathogenicity and complement fixation titer, while the Berkefeld W and Seitz filtrates showed a marked or complete loss of both.

The findings (Table XIII) show clearly that the complement-binding antigen was retained by the filters in almost precisely the same proportions as the virus. There was no indication that a "soluble antigen" existed more readily filterable than the virus, nor that aggregates too large to pass through Berkefeld V filters played any significant part in the findings.

Centrifugation of the Complement-Binding Antigen

In the next experiment the findings were extended by a test with the centrifuge, a papilloma extract known to contain much active virus being centrifugalized at widely various speeds, and the supernatant fluids and sediments tested thereafter both for pathogenicity and complement-binding capacity.

Experiment 14.—A 5 per cent extract of the glycerolated, naturally occurring papillomas of W.R. 54 was prepared as usual and let stand overnight in the refrigerator. It was then mixed and spun in the horizontal centrifuge at about 940 R.P.M. for 5 minutes. This amount of centrifugation sufficed to throw down the sand, but little more, and the supernatant fluid was quite turbid. A portion of this was set aside for testing, and 12 cc. of the remainder was centrifugalized in the angle head centrifuge at 4000 R.P.M. for 20 minutes in a round-bottom tube with an internal diameter of 12 mm. and an overall length of 13 cm. This procedure rendered the supernatant fluids almost water-clear, and packed a considerable amount of fine sediment in the bottom of the tube. The clear supernatant fluid was removed (to within 0.5 cm. of the packed sediment, which left less than 0.4 cc. of fluid), and the sediment was resuspended in the original volume of saline, with result in a suspension quite as turbid as before centrifugation. 8 cc. of the clear supernatant fluid was next centrifugalized in a lusteroid tube (internal diameter 1.25 cm., overall length 7.5 cm.) at about 18,000 R.P.M. for 6 hours, in an International centrifuge with high speed conical head attach-

TABLE XIV

Complement Fixation and Pathogenicity Tests with a Centrifugalized Extract of Infectious Papillomas

Papilloma extract			Pathogenicity tests*						Complement fixation tests†				
			5 per cent suspensions Test rabbits			1 per cent suspensions Test rabbits			Dilution of extract				
Centrifugation	Material used	Gross character	A	B	C	A	B	C	1:20	1:40	1:80	1:160	1:320
(*a*) 940 R.P.M., 5 min.	Supernatant	Turbid	++++	++++	++++	++±	++++	++±	++++	++++	++++	++++	+++±
(*b*) 4000 R.P.M., 20 min.	Supernatant	Faintly opalescent	++++	++++	++++	++±	+++	+++±	++++	++++	++++	++++	+++±
	Sediment resuspended	Turbid, like (*a*)	+±	+±	+±	+	±	0	0	0	0	0	0
(*c*) 18,000 R.P.M., 360 min. (portion of the supernatant of (*b*))	Supernatant	Water-clear	0	0	0	0	0	0	0	0	0	0	0
	Sediment resuspended	Faintly opalescent	++++	+++±	++±	+++	+++	+	++++	++++	0	0	0

Immune serum W.R. 55, diluted 1:4.

Serum and antigens showed no anticomplementary effect when tested in double amount.

* Readings made on the 20th day after inoculation.

† 2 units of complement in all tubes.

ment, used through the courtesy of Dr. Albert Claude. The final high speed centrifugation deposited a considerable amount of brownish sediment, the supernatant fluid becoming absolutely water-clear. The latter was removed, down to within 0.5 cm. of the sediment, and sufficient saline added to resuspend the sediment in 8 cc. Much of the brownish deposit remained caked and could not be resuspended, but some of it was resuspended readily, causing a barely perceptible opalescent sheen.

The various materials thus procured were tested for pathogenicity and complement-binding capacity as usual. Table XIV shows the results. The turbid fluid obtained after centrifugation at 940 R.P.M. was highly pathogenic, and it bound complement in high titer. The clear supernatant fluid obtained after centrifugation at 4000 R.P.M. for 20 minutes was likewise highly pathogenic, and it bound complement practically as well as the crude, turbid extract. The cloudy suspension resulting from the resuspension of the sediment deposited at 4000 R.P.M. showed no complement-binding capacity in the test, although there was a small amount of the virus in it, as the pathogenicity tests showed. The water-clear supernatant fluid obtained after centrifugation at 18,000 R.P.M. for 6 hours was likewise devoid of complement-binding capacity, and it was practically free from virus, no lesions appearing by the 20th day after it was inoculated into 3 rabbits, though a few discrete lesions made their appearance on the 27th day. The resuspended sediment contained virus in large amount and it bound complement in dilutions equivalent to 1:20 and 1:40 of the original extracts. It is apparent that the virus was sedimented, though not completely, by the high speed centrifugation.

The experiment shows plainly (Table XIV) that the complement-binding antigen was present in the crude and centrifugalized extracts of the papillomas, and in much the same proportions as the virus. It was sedimented by high speed centrifugation, as was the virus, the supernatant fluids being thus rendered practically free from the latter and having no detectable capacity to bind complement. The findings yield no hint that aggregated material played any significant part in the findings, for the supernatant fluid, rendered practically water-clear by centrifugation at 4000 R.P.M. in the angle head centrifuge, contained as much virus as the turbid, crude extract, and it bound complement as well; whereas the crude sediment, resuspended in saline, showed no complement-binding capacity and very little pathogenicity.

Effect of Heat on the Complement-Binding Antigen and the Virus

Shope has demonstrated that the papilloma virus is notably resistant to heat (3). Does the complement-binding antigen share this attribute?

Experiment 15.—5 per cent suspensions of the highly infectious, glycerolated warts of W.R. 8-N and W.R. Tx were centrifugalized twice and filtered through Berkefeld V candles, after having stood overnight in the refrigerator. Portions were put into the bottom of a series of test tubes without touching the sides. The tubes were then corked tightly and submerged almost completely in water baths at 56°, 60°, 63°, 66°, and 69°C. for 30 minutes. The heated filtrates, and the unheated portions, were then compared for pathogenicity and complement-binding capacity.

TABLE XV

The Effect of Heat on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests*			Complement fixation tests
Source	Temperature (30 min.)	Test rabbits			Immune serum D.R. 7
		A	B	C	
	°C.				
W.R. 8-N	Unheated	++++	++++	+++	
	56				+++
	60	++++	++++	+++	++++
	63	0	0	0	0
	66	±	0	0	0
	69	0	0	0	0
W.R. Tx	Unheated	++++	++++	+++	
	56				++++
	60	++++	++++	+	++++
	63	0	0	0	0
	66	0	0	0	0
	69	0	0	0	0

Serum diluted 1:4.

Papilloma extracts, 1:20.

None of the extracts was anticomplementary when tested concurrently in double amount, nor was the immune serum.

* Readings made on 36th day after inoculation, according to the standard scale.

In Shope's experiments, the papilloma virus withstood temperatures of 65–67°C. when heated in crude suspension for 30 minutes. The filtrates of the present experiment, however, were inactivated at 63°C. (Table XV), with a single exception,—a discrete growth arising in test rabbit A, where the W.R. 8-N filtrate heated at 66° had been inoculated.

The findings (Table XV) show that heating at 56° and 60° had no noteworthy effect on either the virus or the complement-binding antigen, whereas 63°, 66°, and 69°C. rendered both inactive.

Effect of Ultraviolet Light

Ultraviolet light is known to inactivate viruses generally, some of them soon, others only after considerable exposure. Comparative tests were now made of its effects on the papilloma virus and the complement-binding antigen. Extracts of the papillomas were exposed to ultraviolet light for various periods, and their infectious and complement-binding capacities later determined.

An unreported experiment done several years ago for other purposes had shown the papilloma virus to be notably resistant to ultraviolet light, a potent 1 per cent Berkefeld V filtrate of W.R. 1240 virus withstanding irradiation for 20 to 30 minutes under conditions inactivating the Shope fibroma virus in 5 minutes.

Experiment 16.—5 per cent extracts of the glycerolated, naturally occurring papillomas of 2 cottontails were prepared and centrifugalized at 3500 R.P.M. for 15 minutes. 3 cc. aliquots of the slightly opalescent, supernatant fluids were then irradiated for various periods in open Petri dishes, in which they formed a layer about 2 mm. deep. The Petri dishes were placed directly beneath a mercury vapor lamp[1] at a distance of 48 cm., and each was shaken and turned round at frequent intervals throughout the irradiation. The temperature of the air about the irradiated fluids did not rise above 28°C. There was some evaporation (up to 1.5 cc.) from the fluids irradiated for the longer periods, and sufficient saline was added immediately after the irradiation to restore their bulk.

Complement fixation and pathogenicity tests with the irradiated and control fluids were carried out in the usual ways. The results are recorded in Table XVI. One of the 3 test rabbits died 2 days after inoculation, and the 2 remaining showed a rather pronounced difference in susceptibility to the virus. From their lesions (recorded in the table on the 42nd day) it is obvious that the pathogenicity of the extracts was not notably diminished by irradiation for 10 minutes, but was progressively reduced by longer exposures and completely abolished by irradiation for from 60 to 90 minutes. The single discrete growth, arising on the most susceptible rabbit where the material of W.R. 56 irradiated for 120 minutes had been inoculated, was most probably due to the chance survival of a single infectious entity that had not received its full quota of irradiation. In contrast to the loss of pathogenicity, the complement-binding capacity of the irradiated materials was not reduced a whit, even after irradiation for 120 minutes.

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[1] Alpine sun lamp, Hanovia Chemical Company.

The findings show (Table XVI) that ultraviolet light can render papilloma extracts non-pathogenic without diminishing their capacity

TABLE XVI

The Effect of Ultraviolet Light on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests		Complement fixation tests						
Source	Irradiation with ultraviolet light, 48 cm.	Test rabbits		Immune sera		Hyperimmune sera		Normal sera		Antigen controls (no serum)
		A	B	15	55	8-S	12-S	30	36	
	min.									
W.R. 54	Nil	++++	+++	++++	++++	++++	++++	0	0	0
	10	++++	++±	++++	++++	++++	++++	0	0	0
	20	++++	±	++++	++++	++++	++++	0	0	0
	30	++++	±	++++	++++	++++	++++	0	0	0
	45	++±	±	++++	++++	++++	++++	0	0	0
	60	+++	+	++++	++++	++++	++++	0	0	0
	90	+	0	++++	++++	++++	++++	0	0	0
	120	0	0	++++	++++	++++	++++	0	0	0
W.R. 56	Nil	++++	+++	++++	++++	++++	++++	0	0	0
	10	++++	+++	++++	++++	++++	++++	0	0	0
	20	++++	++±	++++	++++	++++	++++	0	0	0
	30	++++	+	++++	++++	++++	++++	0	0	0
	45	+++	±	++++	++++	++++	++++	0	0	0
	60	+++	+	++++	++++	++++	++++	0	0	0
	90	++	0	++++	++++	++++	++++	0	0	0
	120	±	0	++++	++++	++++	++++	0	0	0
Serum controls (no antigen).....				0	0	0	0	0	0	

Complement, 2 units in all tubes.
Sera diluted 1:4.
Papilloma extracts, 1:20.

to bind complement,—a finding that parallels the work of others with different virus materials (4).

Effect of Changes in Hydrogen Ion Concentration

The papilloma virus is known to be inactivated rapidly at certain pH levels, slowly at others (5). In the next experiment the effect of

TABLE XVII

The Effect of Hydrogen Ion Concentration on the Pathogenicity and Complement-Binding Capacity of Papilloma Extracts

Papilloma extracts		Pathogenicity tests*			Complement fixation tests						
Source	pH	Test rabbits			Immune sera		Hyperimmune sera		Normal sera		Antigen controls (no serum)
		A	B	C	55	56	8	12	30	36	
W.R. 54	1.8	0	0	0	0	0	0	0	0	0	0
	2.8	0	0	0	0	0	0	0	0	0	0
	4.4	++++	+++±	+++±	++++	++++	++++	++++	0	0	0
	6.6	++++	++++	+++±	++++	++++	++++	++++	0	0	0
	9.0	+++	+++	+++	++++	++++	++++	++++	0	0	0
	9.8	0	0	0	++++	++++	++++	++++	0	0	0
	10.5	0	0	0	0	0	±	0	0	0	0
W.R. 56	1.8	0	0	0	0	0	±	0	0	0	0
	2.8	0	0	0	0	0	±	0	0	0	0
	4.4	++++	+++	+++	++++	++++	++++	++++	0	0	0
	6.8	++++	+++±	++++	++++	++++	++++	++++	0	0	0
	9.0	++++	+++	+++	++++	++++	++++	++++	0	0	0
	9.8	+±	0	0	++++	++++	++++	++++	0	0	0
	10.5	0	0	0	0	0	±	0	0	0	0
Serum controls (no antigen)					0	0	0	0	0	0	

Complement, 2 units in all tubes.

Sera diluted 1:4 in tests with antigen, 1:2 in controls.

Papilloma extracts, 1:20. They were kept at the stated pH levels for 14 hours at 37°C., then brought back to pH levels of 6.6 and 6.8 and tested.

* Lesions recorded on the 28th day.

changes in hydrogen ion concentration on the complement-binding capacity and the pathogenicity of papilloma extracts was determined.

Experiment 17.—The pH of 10 per cent centrifugalized extracts prepared in saline as usual of the glycerolated, naturally occurring papillomas of 2 cottontails (54 and 56), was found to be respectively 6.6 and 6.8. 2 cc. aliquots of these extracts were brought to pH levels of 1.8, 2.8, 4.4, 9.0, 9.8, and 10.5,—as determined by indicator dyes,—by the addition of 0.1 to 0.2 cc. of appropriate dilutions of normal HCl or NaOH. These were incubated, along with portions of the untreated extracts, for 14 hours at 37°C., and then all were adjusted to pH 6.6 to 6.8. Sufficient saline was added next to bring the final dilution of the extracts to 1:40. The fluids were then tested for pathogenicity and complement-binding capacity (Table XVII).

At pH levels of 1.8, 2.8, and 10.5 the pathogenicity and complement-binding capacity of the materials were abolished (Table XVII), while both remained unaltered at pH 4.4 and 9.0, as also at 6.6 and 6.8. At pH 9.8, however, the extracts had lost completely or almost completely their infectious properties, while retaining undiminished their capacity to bind complement. It is of interest to compare these findings with those of Svedberg and his associates (6), who showed that certain proteins tend to become unstable at the extremes of their pH stability ranges, and with the findings of Wyckoff and Beard (5), who found that the active material that can be isolated from extracts of infectious papillomas by centrifugation is fragmented at pH levels below about 3.0 and above about 10.2, while it remains intact at hydrogen ion concentrations in-between. In view of their findings and ours it is reasonable to suppose that the infectivity and the antigenicity of the active material in extracts of the papillomas depends upon its integrity, and that this is destroyed by heating to 63–67°C., or by treating with acid or alkali in pH ranges below about 3.0 or above about 10.0. In this relation the fact assumes importance that the pathogenicity of a virus material can be abolished by irradiation with ultraviolet light and by treatment with weak alkali without destroying its antigenicity.

SUMMARY AND COMMENT

The antigen that binds complement in the presence of sera neutralizing the Shope papilloma virus can be readily extracted from

papillomas yielding infectious virus, but not from the normal skin of rabbits bearing the growths. The virus and the complement-binding antigen appear to have the same particle size, as determined by filtration, and they are thrown down together in the centrifuge. They are destroyed by the same amounts of heating and, in general, by the same changes in pH. It is possible, nevertheless, by irradiation with ultraviolet light, or by treatment with weak alkali, to render papilloma extracts non-pathogenic without diminishing their capacity to bind complement when mixed with immune serum.

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