THE *IN VITRO* AND *IN VIVO* ANALYSIS OF MAMMALIAN TUMOUR VIRUSES

THE HAEMAGGLUTINATING SYSTEM OF THE POLYOMA VIRUS

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Received for publication April 23, 1959

SINCE the classical work of Ellerman and Bang (1908) on chicken leukaemia and of Rous (1911) on chicken sarcoma first demonstrated the viral origin of certain tumours, such studies have been extended to various species including mammals (Rous, 1935; Oberling and Guerin, 1954; Gross, 1958). It was shown in our previous investigations (Sachs, Fogel and Winocour, 1959a; Sachs *et al.*, 1959b, Winocour and Sachs, 1959), that basic questions relating to the role of viruses in the origin of mammalian tumours can best be answered by combining an *in vivo* with an *in vitro* analysis, and that by using such a combined approach it is possible to establish particularly favourable experimental systems. One of the viruses used in these studies was the polyoma virus (Eddy *et al.*, 1958a) originally isolated from AK mice with lymphatic leukaemia (Gross, 1958; Stewart, Eddy and Borgese, 1958). This virus can agglutinate red blood cells from various species at refrigerator (4° C.) temperature (Eddy *et al.*, 1958a; Sachs, Fogel and Winocour, 1959a), and it was shown that this ability for haemagglutination can be used both for an *in vitro* virus identification, and to determine various aspects of virus behaviour (Sachs, Fogel and Winocour, 1959a; Sachs *et al.*, 1959b).

In order to establish more fully the characteristics of this virus, and to compare these with non-tumour forming viruses, a further investigation was carried out on its haemagglutinating system. The present studies are therefore concerned with the properties of the haemagglutinating particles, the formation of haemagglutination inhibition antibodies after virus inoculation in relation to tumour formation, and the existence of such antibodies in AKR mice.

MATERIALS AND METHODS

Animals

The Swiss mice used for the tissue cultures and virus inoculations into mice, are derived from a line that has been brother × sister mated for at least 20 generations. The AKR mice are derived from breeding pairs originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, and the golden hamsters from a randomly bred colony originating in this country.

Virus

The polyoma virus stocks used for the present experiments were from lines IL 1-6; and from a line isolated (Sachs *et al.*, 1959b) from leukaemic C3HGr

mice kindly supplied by Dr. Ludwick Gross and inoculated by him with his strain A leukaemic virus (Gross, 1957). The IL lines, which were obtained after culturing of tumour cells (Sachs *et al.*, 1959b), consist of isolations 1 and 4 from mouse parotid tumours, 2 and 5 from mouse mammary adenomas, 3 from a mouse mammary adenocarcinoma, and 6 from a rat kidney sarcoma. The tumours used for these isolations were induced with virus derived from strain 3919 (Steward, Eddy and Borgese, 1958) kindly supplied by Dr. Sarah E. Stewart and Dr. Bernice E. Eddy. All virus stocks were made and used in a medium consisting of 0.5 per cent lactalbumin hydrolysate in Earle's saline and 20 per cent horse serum.

Haemagglutination

Haemagglutination tests were routinely made with guinea-pig red blood cells, using 0.15 c.c. of a 1 per cent suspension in saline added to 0.5 c.c. of each dilution of virus. The saline was adjusted with NaOH to pH 6.8–7.2. Virus dilutions were made in saline. Blood was taken from the heart in Alsever's solution, and used on the same day. Sedimentation of the controls was found to be inadequate, if the red blood cells were kept more than 4 days after collection. In order to standardise the 1 per cent suspension, 1 c.c. of the cell suspension was mixed with 4 c.c. of double distilled water and after complete haemolysis the concentration of haemoglobin measured in a Coleman Junior spectrophotometer at a wavelength of 545 m μ . An optical density of 0.26 was taken to be equivalent to a 1 per cent suspension of red blood cells. The pattern method of agglutination (Salk, 1944) was used, and the tests read after 3 hours or overnight at 4° C. Since the agglutination is destroyed at room temperature, the tests were read immediately after the tubes were taken from the refrigerator.

Haemagglutination inhibition

Inhibition tests were made using 0.25 c.c. of antiserum, previously heated at 56° C. for 30 minutes, and 0.25 c.c. of virus suspension containing 16 haemagglutinating units of virus. Complete inhibition was used as the end point. Before adding the red blood cells, the virus antiserum mixture was incubated for 30 minutes at 37° C. Serum from normal Swiss mice gave inhibition titres up to 1 : 40.

EXPERIMENTAL

The haemagglutination spectrum

The polyoma has a specific haemagglutination spectrum when tested with red blood cells from various species (Sachs, Fogel and Winocour, 1959a). The results with two lines of this virus, and a comparison with the spectrum of some other haemagglutinating viruses (data from Smadel (1948) and Burnet and Stone (1946)) are shown in Table I. The two lines of polyoma used were line IL-3, and the line isolated from a C3HGr leukaemic mouse (Sachs *et al.*, 1959b). Although we have so far found no haemagglutination with cells from our hamsters, this species has been reported as positive by Eddy *et al.* (1958a). This may be due to variation within the species or the existence of virus mutants. The most rapid reading was given by cells from the frog *Hyla arborea*, where there was complete sedimentation after 30 minutes at 4° C. in contrast to the usual 3 hours with cells from other species. The cells from this frog also gave the highest titre (Sachs, Fogel and

TABLE I.—*Haemagglutination Spectrum of Polyoma Compared to some other Haemagglutinating Viruses*

Red blood cells	Poly- oma	Virus from Gross	Influ- enza A	Influ- enza B	New- castle disease	Vaccinia	Pneu- monia virus of mice	Ectro- melia
Guinea-pig . . .	+	+	+	+	+	+	-	-
Rat . . .	-	-	±	±	+	±	-	-
Mouse . . .	+	+	±	±	+	±	+	+
Hamster . . .	*	-	N.D.	N.D.	N.D.	N.D.	+	N.D.
Dog . . .	+	+	+	N.D.	+	N.D.	-	N.D.
Cat . . .	-	-	-	+	-	+	-	N.D.
Human . . .	+	+	+	+	+	±	-	-
Monkey . . .	+	+	-	N.D.	-	-	N.D.	N.D.
Horse . . .	-	-	-	±	-	-	N.D.	N.D.
Cow . . .	-	-	-	+	+	-	N.D.	-
Sheep . . .	+	+	±	±	±	-	-	-
Goat . . .	-	-	±	+	+	-	N.D.	N.D.
Chicken . . .	+	+	+	+	+	+	-	+
Turkey . . .	+	+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Goose . . .	-	-	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Pigeon . . .	-	-	N.D.	N.D.	N.D.	+	N.D.	+
Duck <i>Anas moscant</i>	+	+	}	+	+	+	N.D.	-
<i>Anas boschas</i>	-	-						
Frog <i>Hyla arborea</i>	+	+	}	+	+	-	N.D.	N.D.
<i>Bufo viridis</i>	-	-						

+ = positive. - = negative. N.D. = No data. * = reported as + by Eddy *et al.* (1958a).

Winocour, 1959a), and if available in quantity would be the most suitable for haemagglutination. Because of availability, guinea-pig red blood cells have been used in all the following experiments.

The effect of pH

In order to test the effect of pH, haemagglutination was carried out in 0.15 M phosphate buffer at different pH from 5.4–8.4. At the higher (7.7–8.4) and lower pH (5.4 and 6.0), there was not a complete sedimentation of cells in the buffer control. It is clear (Table II), that the virus can agglutinate over a wide range of pH, although there is a lower titre at the lower pH. These tests and all the following experiments on the properties of the haemagglutinins were made with a virus stock of line IL-3, obtained by harvesting the fluid from cultures of tumour cells (Sachs *et al.*, 1959b).

Adsorption and elution from red blood cells

Adsorption and elution experiments were carried out to determine the possession by the virus of receptor destroying enzyme, and the speed of the reactions. A final concentration of 2 per cent red blood cells was used with the stock virus fluid diluted 1 : 8. Adsorption was carried out at 4° C. and elution at 37° C. For the experiments shown in Table III, after adsorption of the virus, the cells were sedimented by centrifugation in a clinical centrifuge at 1500 r.p.m. at 4° C., and then washed twice by resuspending in the same amount of saline and centrifugation at 4° C. Each elution was for 1 hour at 37° C, followed by centrifugation at 37° C. to sediment the red blood cells. The cells were then resuspended in the same amount of saline for the next elution. After 4 such elutions the cells were tested for their ability to again adsorb virus.

TABLE II.—*Effect of Differences in pH on Haemagglutination*

[illegible]

TABLE III.—*Virus Adsorption and Elution from Red Blood Cells*

[illegible]

t.c.f. = tissue culture fluid. r.b.c. = red blood cells.

TABLE IV.—*Haemagglutination Tires After Centrifugation at 105,000 g*

[illegible]

The results given in Table III show that saline washing does not remove the haemagglutinins from the red blood cells, and that even after 4 elutions at 37° C. there are still some agglutinins attached to the cells. They further show that the cells used for elution can again adsorb virus, so that polyoma does not seem to have a receptor destroying enzyme such as is found in influenza, mumps and NDV (Hirst, 1952).

To determine the speed of the reactions, the suspension was sampled at various intervals after the beginning of adsorption and elution. Each test was made in a different tube, the suspension centrifuged at the appropriate times, and the

FIG. 1.—Adsorption and elution curves for polyoma haemagglutinins on guinea-pig red blood cells.

haemagglutination titre assayed. The results (Fig. 1) show a rapid adsorption and a rapid elution. The titre dropped from the original 2048 to 64 after an adsorption time of 10 minutes, and during elution, the titre rose to 512 after 1 minute and to 1024 after 10 minutes. A rapid adsorption was also found by Hirst (1942) for influenza virus, although elution with influenza was not as rapid as with polyoma. The polyoma did not show spontaneous elution at 4° C.

Sedimentation at 105,000 g.

It was found that supernatant fluid after centrifugation for 1 hour at 105,000 *g* produced tumours when inoculated into newborn Swiss mice. Experiments were therefore carried out on the sedimentation of the virus at this speed. Stock virus was first centrifuged at 9000 *g* for 10 minutes in a Servall Angle Centrifuge, and the supernatant then centrifuged in a Spinco Model L Preparative Ultracentrifuge

with a No. 40 Rotor at 40,000 r.p.m. average = 105,000 *g*. The supernatant and sediment were sampled for haemagglutination titre from 12 c.c. of virus stock after 1 hour, and from another 12 c.c. after 3 hours centrifugation in the Spinco. The sediment was resuspended in 5 c.c. of medium without serum.

The results, Table IV, show that after 1 hour haemagglutinins have sedimented. Samples of 1 c.c. from supernatant after 1 hour, and from the top 2 c.c., the bottom 10 c.c. and the resuspended sediment after 3 hours centrifugation, were inoculated intraperitoneally into adult Swiss mice. Haemagglutination inhibition antibodies in the pooled serum from 2 mice per group were determined 14 days after inoculation and these gave titres of 1 : 1280, 1280, 1280 and 2560 respectively. The supernatants thus still contain some virus particles. The size of the particles and possible existence of aggregates is being checked by electron microscope observations. Filtration of stock virus through a Selas 02 candle filter did not decrease the haemagglutinin titre. The production of tumours by the supernatant after 35 minutes at 105,000 *g* has also been reported by Buffett *et al.* (1958).

Ultraviolet inactivation

The inactivation of polyoma by ultraviolet was examined in order to determine the degree of inactivation of the haemagglutinating particles and to compare this with effects on infectivity. Infectivity was measured on tissue cultures and in animals. Stock virus was centrifuged in a Servall Angle Centrifuge at 9000 *g* for 10 minutes, and for each test, 1 c.c. of the supernatant was irradiated in a watch glass with a 15 watt Philips TUV germicidal lamp placed at a distance of 24 cm. from the virus containing fluid. This gives a radiation intensity of 30 ergs/mm.²/second. The watch glasses were agitated every 10 minutes, and before titration any medium loss by evaporation was added to make the original 1 c.c. The haemagglutination titres obtained after various exposures (Table V) show that there was a decrease of one dilution after 60 minutes, a further decrease by one dilution after 100 minutes, and that there was still some agglutination after 150 minutes.

To compare the effect on haemagglutination with effects on infectivity, virus after various periods of irradiation was inoculated onto mouse embryo monolayers. The monolayers were grown in petri dishes in a medium of 0.5 per cent lactalbumin hydrolysate in Earle's saline and 20 per cent horse serum, in an incubator with a humidified atmosphere containing 10 per cent CO₂. Each test was made on a pool from two petri dishes, and each petri dish containing 4 c.c. of medium. 0.3 c.c. of virus was adsorbed for 3 hours, without washing, and the medium changed at 2, 5, 8 and 11 days later. The haemagglutination titres per 0.5 c.c. were determined on days, 5, 8 and 11, and the cultures were also examined for a clear cytopathogenic effect. It can be seen (Table VI) that virus irradiated for 30 minutes before inoculation on to monolayers gave after 11 days growth a haemagglutination titre of 256 (in contrast to a titre of 2048 for non-irradiated virus) and that after 60 minutes irradiation the titre was only 32 in comparison to 16 for normal tissue culture fluid. After an exposure of 90 minutes or longer there were no haemagglutination titres higher than those of the normal tissue culture fluid. Since the yield of haemagglutinins in tissue cultures is correlated with the amount of infective virus in the inoculum, these results show that, as in the case of influenza

(Henle and Henle, 1947), infectivity of the polyoma virus is more sensitive to ultraviolet than haemagglutination. There was also a correlation between haemagglutinin yield and the cytopathogenic effect on the culture (Table VI).

The infectivity of the irradiated virus was further measured by determining the haemagglutination inhibition antibody titre at 2 and at 4 weeks after inoculation of 0.05 c.c. subcutaneously into 2-day-old Swiss mice. Each test was made with the pooled serum from 2 or 3 mice, and the results are shown in Table VI. The increase in titre between 2 and 4 weeks indicates that there may still have been some infective virus after 150 minutes' irradiation.

Temperature inactivation

In order to determine stability at different temperatures, experiments were carried out on the temperature inactivation of the haemagglutinins and infectivity. The tests for infectivity and cytopathogenic effect were made in the same way as for the ultraviolet experiments, with samples from the same virus stock. There was no decrease in haemagglutination titre (Table VII) after 30 minutes at 56° C., a decrease at higher temperatures, and no significant titre with the method used after 30 minutes at 66° C. or 15 minutes at 70° C. The infectivity tests on the cultures (Table VIII) showed no decrease in yield of haemagglutinins after 30 minutes at 56° C., a decrease at higher temperatures, and that there was still some infectious virus after 30 minutes at 66° C. and 15 minutes at 70° C. The existence of some haemagglutination titres lower than the control of 32 in column 2 of Table VIII can be explained by the inactivation at these temperatures of the agglutinating antibody in the horse serum of the medium.

The haemagglutination inhibition titres after inoculation into mice (Table VIII) also show the high temperature stability of the virus under the present conditions, and that there may still have been some infective virus even after 30 minutes at 70° C. The existence of some infective virus after 30 minutes at 70° C. has also been reported by Eddy, Stewart and Grubbs (1958b). In tests on the stability of the haemagglutinin at 37° C., it was found that there was no loss of titre after 8 weeks at this temperature.

It can be seen from the above that whereas in the ultraviolet inactivation tests infectivity was clearly more sensitive than haemagglutination and this has also been found with inactivation by formalin (Sachs and Fogel, unpublished), such a clear distinction between these two properties was not found in the temperature tests.

The haemagglutinins from different tumours

Virus has been isolated from different polyoma induced tumours, and the antigenic similarity of the haemagglutinins determined by cross inhibition tests. The lines used were IL 1-6, and these consist of isolations from mouse parotid tumours, mouse mammary adenomas, a mouse mammary adenocarcinoma, and a rat kidney sarcoma. It was found that these virus isolates all gave cross haemagglutination inhibition, so that the haemagglutinins from different tumours have common antigens. Cross inhibition was also obtained with virus isolated from a C3HGr leukaemic mouse (Sachs *et al.*, 1959b). These results provide further evidence that more than one type of tumour may be produced by the same virus.

TABLE V.—*Ultraviolet Inactivation of Haemagglutinating Particles*

Time of irradiation (minutes)	Virus dilution 1 :													Saline control	
	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192		
None (control)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
70	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
120	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

TABLE VI.—*The Effect of Ultraviolet Irradiation on Haemagglutination and Infectivity*

Time of irradiation (minutes)	Original haemagglutination titre per 0.5 c.c. 1 :	Haemagglutination titre** after growth in tissue culture 1 :			Cytopathogenic effect on tissue culture			Haemagglutination inhibition titre after inoculation into 2-day-old mice.		
		Period of growth (days)			Period of growth (days)			Time after inoculation (weeks)		
		5	8	11	5	8	11	2	4	
None (control)	2048	1024	2048	2048	+	+	+	320	2,560	
5	2048	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	320	10,240	
15	2048	256	1024	2048	-	+	+	80	10,240	
30	2048	32	512	256	-	-	+	160	10,240	
60	1024	16	16	32	-	-	-	160	5,120	
90	1024	32	16	8	-	-	-	80	10,240	
120	512	32	16	16	-	-	-	160	5,120	
150	128*	32	16	8	-	-	-	80	5,120	
Normal tissue culture fluid	32	32	16	16	-	-	-	N.T.	N.T.	

N.T. = Not tested. + = Positive. - = Negative. * Agglutination of + at dilution 1 : 2, 1 : 16 and 1 : 32. ** Titre per 0.5 c.c. in 4 c.c. of tissue culture fluid. End point for haemagglutination titres taken at +.

TABLE VII.—*Heat Inactivation of Haemagglutinating Particles*

Treatment	Virus dilution 1 :										Saline control
	2	4	8	16	32	64	128	256	512	1024	
None (control) .	+	+	+	+	+	+	+	+	+	+	—
56° C. for 30 minutes.	+	+	+	+	+	+	+	+	+	+	—
62° C. " 30 "	+	+	+	+	+	+	+	+	+	+	—
64° C. " 15 "	+	+	+	+	+	+	+	+	+	+	—
64° C. " 30 "	+	+	+	+	+	+	+	+	+	+	—
64° C. " 45 "	+	+	+	+	+	+	+	+	+	+	—
64° C. " 60 "	+	+	+	+	+	+	+	+	+	+	—
66° C. " 10 "	+	+	+	+	+	+	+	+	+	+	—
66° C. " 15 "	+	+	+	+	+	+	+	+	+	+	—
66° C. " 30 "	+	+	+	+	+	+	+	+	+	+	—
66° C. " 40 "	+	+	+	+	+	+	+	+	+	+	—
70° C. " 15 "	+	+	+	+	+	+	+	+	+	+	—
70° C. " 30 "	+	+	+	+	+	+	+	+	+	+	—

TABLE VIII.—*The Effect of Temperature on Haemagglutination and Infectivity*

Treatment	Original haemagglutination titre per 0.5 c.c. 1 :	Haemagglutination titre** after growth in tissue culture 1 :			Cytopathogenic effect on tissue culture			Haemagglutination inhibition. titre after inoculation into 2-day-old mice 1 :		
		Period of growth (days)			Period of growth (days)			Time after inoculation (weeks)		
		5	8	11	5	8	11	2	4	
None (control) .	2048	1024	2048	2048	+	+	+	320	2560	
56° C. for 30 minutes	2048	2048	2048	1024	+	+	+	320	2560	
62° C. " 30 "	256	32	1024	1024	+	+	+	80	1280	
64° C. " 15 "	256	64	512	1024	+	+	+	320	2560	
64° C. " 30 "	128	32	64	512	?	—	—	320	1280	
66° C. " 15 "	128	32	128	256	—	—	—	320	2560	
66° C. " 30 "	16*	32	32	128	—	—	—	160	5120	
70° C. " 15 "	4	16	32	64	—	—	—	80	5120	
70° C. " 30 "	0	16	16	16	—	—	—	80	5120	
Normal tissue culture fluid	32	32	16	16	—	—	—	N.T.	N.T.	

* Agglutination of — at 1:2 and 1:4, and + at 1:8. + = Positive. — = Negative. N.T. = Not tested. ** Titre per 0.5 c.c. in 4 c.c. tissue culture fluid. End point for haemagglutination titres taken at ++.

Haemagglutination inhibitors in extracts of normal and tumour bearing animals

It has previously been found that extracts of tumours taken straight from the animal did not give haemagglutination, whereas high haemagglutination titres could be obtained from tumour cells in culture (Sachs *et al.*, 1959b). The absence of haemagglutination by extracts of tumours from the animal may be due either to a low haemagglutinin content or the existence of inhibitors. In the present study the latter possibility has been examined. Organs from normal mice, tumour bearing mice, and the tumours themselves, were homogenized with a motor driven homogenizer, centrifuged for 10 minutes at 9000 *g* in a Servall Angle Centrifuge, and the supernatant tested for haemagglutination inhibition. The tests were made in duplicate using supernatant unheated and heated for 30 minutes at 56° C., and the results compared with the effects of normal and immune serum. Three series of tests gave similar results and the data from one of these are given in Table IX.

The results show that inhibitors to a titre of 1 : 80 exist in organs from normal mice and that these are destroyed by heating at 56° C. for 30 minutes. Antibodies in immune serum, and the inhibitors (up to 1 : 40) in normal serum are not destroyed by this heating. In addition to the heat labile inhibitors, the extracts of organs from tumour bearing mice and from the tumours themselves also have high titres of antibodies, and both these, particularly the latter, could explain why extracts from tumours do not give haemagglutination. The presence of these inhibitors is also of significance in the actual isolation of viruses from tumours.

Haemagglutination inhibition antibodies and tumour formation in mice and hamsters

It has previously been shown that newborn mice inoculated with virus develop haemagglutination inhibition antibodies, and that there are high inhibition titres when palpable tumours are formed (Sachs *et al.*, 1959b). Further experiments were therefore carried out on haemagglutination inhibition antibodies and tumour formation in mice and hamsters. The newborn animals used were less than 24 hours old. Mice were inoculated subcutaneously with 0.05 c.c. and hamsters subcutaneously with 0.1 c.c. of virus suspension. Curves for inhibition antibody in three separate mice, tested at various intervals after virus inoculation into newborn, are shown in Fig. 2. In two of these with large tumours, the antibody titre increased up to the time of appearance of palpable tumours, and in the third, which had an unusual small slow growing tumour in the foot, the titre has continued to increase after the appearance of the tumour until it has so far reached 1 : 20,480. In the mice with the large tumours the same antibody titre was reached where the tumour appeared at 10 weeks and where it appeared at 23 weeks. Haemagglutination inhibition antibodies were therefore present in the mice during the entire period of growth after virus infection. Further data on this point are given in Table X, and these also show that the presence of palpable tumours is always associated with a high titre of inhibition antibodies.

In hamsters, although inhibition antibodies are also present after virus inoculation, the titres are lower in animals with palpable tumours than in the case in mice (Table X). Palpable tumours in hamsters develop earlier than in mice (Eddy *et al.*, 1958c). The earliest palpable polyoma induced tumours in our hamsters developed about 4 weeks after inoculation in contrast to about 9 weeks before the earliest palpable tumours are found in mice (Sachs *et al.*, 1959b).

TABLE IX.—*Haemagglutination Inhibition Tests for Inhibitors in Extracts from Normal and Tumour Bearing Mice*

		Extract or serum dilution 1 :													Saline control
		10	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960	
Extracts from normal mice	Liver, lung, kidney, and heart (pooled)	Unheated	-	-	-	+	+	+	+	+	+	+	+	+	+
		Heated at 56° C. for 30 min.	+	+	+	+	+	+	+	+	+	+	+	+	+
	Spleen, lymph-nodes, and thymus (pooled)	Unheated	H	+	+	+	+	+	+	+	+	+	+	+	+
		Heated at 56° C. for 30 min.	+	+	+	+	+	+	+	+	+	+	+	+	+
Extracts from tumour-bearing mice	Liver, lung, kidney and heart (pooled)	Unheated	-	-	-	-	-	-	-	-	-	-	-	-	+
		Heated at 56° C. for 30 min.	+	+	+	+	+	+	-	-	-	-	-	-	+
	Spleen, lymph-nodes and thymus (pooled)	Unheated	-	-	-	-	-	-	-	-	-	-	-	-	+
		Heated at 56° C. for 30 min.	+	+	+	+	+	+	-	+	+	+	+	+	+
Extracts from parotid tumours		Unheated	-	-	-	-	-	-	-	-	-	-	-	-	+
		Heated at 56° C. for 30 min.	+	+	+	+	+	+	-	+	+	+	+	+	+
Serum from normal mice		Unheated	-	-	+	+	+	+	+	+	+	+	+	+	+
		Heated at 56° C. for 30 min.	-	-	+	+	+	+	+	+	+	+	+	+	+
Serum from tumour-bearing mice		Unheated	-	-	-	-	-	-	-	-	-	-	-	-	+
		Heated at 56° C. for 30 min.	-	-	-	-	-	-	-	-	-	-	-	-	+

H = Haemolysis.

TABLE X.—*Haemagglutination Inhibition Titres at Various Intervals After Virus Inoculation into Mice and Hamsters*

Time after inoculation (weeks)	Mice			Hamsters		
	Titre 1 :	Palpable tumours	Age at time of inoculation (days)	Titre 1 :	Palpable tumours	Age at time of inoculation (days)
2	640*	—	2	2560	—	7
3	2560**	—	N.B.	2560	—	8
5	2560	—	N.B.	2560	+	2
5	5120	—	N.B.	5120	+	2
5	5120	—	N.B.	2560	+	2
7	5120	—	N.B.	2560	+	2
7	10240	—	N.B.	2560	+	2
9	10240	—	N.B.	2560	+	2
11	20480	—	N.B.	1280	+	5
11	40960	+	N.B.	2560	+	8
11	10240	+	N.B.	2560	+	3
12	10240	—	2	N.T.	N.T.	N.T.
12	40960	+	2	1280	+	3
13	> 20480	+	N.B.	640	+	5
13	40960	+	N.B.	640	+	5
14	20480	+	N.B.	640	+	3
15	20480	—	N.B.	2560	+	3
16	N.T.	N.T.	N.T.	640	+	3
20	> 5120	+	N.B.	N.T.	N.T.	N.T.
22	40960	+	N.B.	N.T.	N.T.	N.T.
22	5120	—	N.B.	N.T.	N.T.	N.T.

* Litter of 4 mice pooled. ** Litter of 7 mice pooled. N.B. = Newborn. N.T. = Not tested.
+ = Present. — = Absent. Each test represents the result from a different animal.

TABLE XI.—*Haemagglutination Inhibition Antibodies in AKR Mice*

Number of mice	Age in weeks at 1st test	Antibody titre 1 :		
		1st test	2nd test 4 weeks later	3rd test 8 weeks later
5	13	20	20	20
3	15	20	20	20
1	15	10	20	20
1	17	20	40	40
1	17	20	20	20
1	23	20	40	20
1	23	20	40	20
1	32	320	5120	N.T.
1	32	640	5120	2560
1	32	1280	2560	2560
1	32	1280	5120	2560
1	32	1280	5120	2560
1	32	1280	5120	2560
1	34	20	20	20
1	34	1280	N.T.	N.T.
1	35	20	10	20
1	35	20	N.T.	N.T.
1	36	10	N.T.	N.T.
1	36	2560	N.T.	N.T.

N.T. = Not tested.

Hamsters also have a different distribution of tumour types (Eddy *et al.*, 1958c), an observation that we have confirmed. The difference in inhibition antibodies between mice and hamsters does not seem to be due to the existence of an initially weak immune response in the hamster, since animals inoculated at 7 or 8 days of age (Table X) have antibody titres of 1 : 2560 after 2 or 3 weeks. The difference may be due to differences in cell virus relationships and the production of haemagglutinating particles. In addition hamsters may not produce titres higher than 1 : 5120, and there may be more antibody adsorption *in vivo* in hamsters than in mice. These possibilities are now being examined.

FIG. 2.—Haemagglutination inhibition antibody titres in relation to the appearance of palpable tumours in three mice inoculated as newborn with polyoma virus.

Haemagglutination inhibition antibodies in AKR mice

The existence of haemagglutination inhibition antibodies against polyoma virus in some AKR mice 32 weeks of age or older has already been reported (Sachs *et al.*, 1959b). These animals have now been further studied in order to obtain additional information on the existence of these antibodies in AKR, a strain of mice with a high incidence of spontaneous leukaemia. The results of two sets of tests, 4 and 8 weeks after the original titrations, are given in Table XI. These show that the animals with antibodies were also positive at the later tests.

All the mice have been kept as breeding pairs, and the 6 mice, 32 weeks old when first tested and found to have antibodies, are all litter mates belonging to the same family. In addition however, the 5 negative mice 13 weeks old at the first test, are litter mates derived from one of the pairs positive at 32 weeks. If the parents had been positive from the beginning, it may be assumed that their offspring should also be positive (Sachs *et al.*, 1959b, and unpublished), so that it appears that the mice positive at 32 weeks were negative at an earlier age. Four week old litters (7 animals tested) born from the negative mice 13 weeks old at the first test were also negative. Furthermore, both of the other positive mice shown in Table XI (one 34 and one 36 weeks old at the first test) have negative litter mates. These litter mates have been kept since birth in the same animal cage.

If the presence of inhibition antibodies in AKR were due merely to polyoma contamination in our laboratory, it would be expected that both members of the litter should have inhibition antibodies, since the presence of animals in the same cage seems to be the best means of ensuring contamination (Sachs *et al.*, 1959b, and unpublished). All AKR mice have been kept by us since their introduction in a different room from animals inoculated with polyoma virus. The existence of inhibition antibodies against polyoma in some AKR mice has also been observed by Rowe *et al.* (1958), who seem to interpret their results as due to contamination of the animals with polyoma.

DISCUSSION

The present studies have further shown the value of combining the *in vivo* with an *in vitro* approach in defining the properties of a mammalian tumour virus. The data on the haemagglutinating spectrum, and the rates of adsorption and elution, may serve as markers for the finding of mutants of the polyoma. Regarding the epidemiology of this virus, its high temperature stability would be a potent factor in determining its ability for survival and re-infection. In common with non-tumour forming viruses, infectivity is more sensitive than haemagglutination to ultraviolet inactivation, and although polyoma has a high resistance to ultraviolet inactivation, this could be a suitable method for virus inactivation and the maintenance of antigenicity. However for this latter purpose formalin has been found very satisfactory (Sachs and Fogel, unpublished).

The finding of haemagglutination inhibition antibodies in both mice and hamsters throughout their entire period of growth after virus infection, and the demonstration that tumour cells can continue to produce haemagglutinins (Sachs *et al.*, 1959b), makes it possible to determine the presence of this virus in tumours by testing the serum of animals for inhibition antibodies. An equivalent method has also been of value in studies on tumours induced in rabbits with papilloma virus (Rous, 1935).

It appears from the results obtained with AKR mice that some animals of this high leukaemic strain may develop inhibition antibodies against polyoma. Although it has been experimentally established that animal contamination with polyoma can take place (Sachs *et al.*, 1959b), the data presented, which include the observation that antibodies are found in older but not in young mice, make it unlikely that all the results with AKR are merely due to laboratory contamination. The possibility exists that virus activation may occur in AKR mice. Activation of a provirus may thus actually be demonstrable with a tumour virus, and further experiments in this direction are under way. In contrast to activation towards lytic action, i.e. a state that causes only cell destruction, as is found in lysogenic strains of bacteria, this would be activation to a stable cell virus relationship that under the appropriate conditions causes cell proliferation, i.e. the development of tumours.

SUMMARY

A study has been made of the haemagglutinating system of the polyoma virus with particular reference to the properties of the haemagglutinating particles, the formation of haemagglutination inhibition antibodies in relation to tumour formation, and the existence of such antibodies in AKR mice.

The polyoma can haemagglutinate in the cold (4° C.), the virus has a specific haemagglutination spectrum when tested with red blood cells from various species, and haemagglutination can take place at pH from 5.4–8.4.

Experiments on adsorption and elution have shown that the virus does not seem to possess a receptor destroying enzyme, that there is a rapid adsorption at 4° C., and a rapid elution at 37° C.

Sedimentation studies at 105,000 *g* have shown that haemagglutinins can sediment after 1 hour of centrifugation, but that even after 3 hours the supernatant still has some virus particles.

Ultraviolet irradiation experiments have demonstrated that in common with non-tumour forming viruses, infectivity is more sensitive than haemagglutination to inactivation with ultraviolet.

Temperature inactivation studies have shown a high stability of both infectivity and haemagglutination after 30 minutes at 56° C., and that there may still be some infective virus after 30 minutes at 70° C.

Virus isolation from polyoma induced parotid tumours, mammary adenomas and an adenocarcinoma in mice, and a kidney sarcoma in rats, gave cross haemagglutination inhibition, so that haemagglutinins from these different tumours have common antigens.

It was found that extracts of normal mouse organs possess haemagglutination inhibitors to a titre of 1 : 80, and that these inhibitors are destroyed after 30 minutes at 56° C. Extracts from organs of tumour bearing mice and from the tumours themselves contain inhibition antibodies in addition to these heat labile inhibitors.

Haemagglutination inhibition antibodies were shown to be present in animals throughout the entire period of growth after virus infection. In mice the presence of palpable tumours was always associated with a very high titre of inhibition antibodies, whereas hamsters with palpable tumours do not have such high titres.

Evidence has been presented to show that AKR mice may naturally develop haemagglutination inhibition antibodies against polyoma virus.

We are indebted to the Winfield Baird Foundation for a grant in support of this work.

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