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Hemagglutination Studies of the Viral Antigen in a Murine Leukemia (Rauscher).* (31199)

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Hemagglutination (HA) and hemagglutination-inhibition (HAI) reactions with tannic acid, protein treated sheep red blood cells (SRBC) have been employed for the study of a wide variety of immunological problems (1). Although these methods are potentially suited for detection of viruses and virus antibodies, relatively few viruses have been studied(2). The recent availability of highly purified tissue culture virus has made it possible to adapt the HA method to measure antibodies to a murine leukemia virus (Rauscher)(3). The present report has two aims: to determine the sensitivity of this method for detection and estimation of small amounts of virus and of antibody, and to study the primary and secondary antibody responses of Rhesus monkeys immunized with Rauscher virus.

Materials and methods. Viruses. For sensitization, the supernatant fluids from embryonic Balb/c spleen/thymus cultures inoculated *in vitro* with Rauscher virus (JLS-V5) were used(4). A similar preparation (JLS-V6) from the same culture, but not inoculated with Rauscher virus, served as a control(4). These antigens were enzyme treated and concentrated by zonal ultracentrifugation as previously described(5). The virus preparation had a nitrogen concentration of 30 μg/ml, an RNA concentration of 47 μg/ml, and a relative virus concentration of approximately 500 particles/square on a standard electron microscope grid. All of these materials and assays were obtained from Mr. Irving Toplin, John L. Smith Memorial Laboratory, Charles Pfizer & Co., under contract to the National Cancer Institute.

Rauscher virus preparations and their controls tested in HAI studies included the following: 10% homogenate of spleen from infected and from normal Balb/c mice(6); and 10-fold concentrate of viremic and of normal Balb/c mouse plasma prepared by differential centrifugation(3); and the 1.16 density isolates obtained on centrifugation of these 10-fold concentrates of viremic and of normal plasmas on sucrose gradients(7).

Sera. Monkey anti-Rauscher serum was prepared by the inoculation of 10 times concentrated plasma from Balb/c mice as previously described(8). The course for immunization consisted of a primary intraperitoneal inoculation of 1.0 ml or 2.0 ml of virus emulsified with an equal quantity of complete Freund's adjuvant. This was followed 28 days later by a subcutaneous inoculation of 0.25 ml of virus without adjuvant. Sera were obtained 7-10 days after the booster inoculation and stored individually at −20°C.‡ Rabbit anti-mouse plasma serum was prepared by a similar procedure using a 1:2 dilution of mouse plasma as the antigen.

All of the serums used in these experiments were inactivated for 30 minutes at 56°C and absorbed with washed SRBC until no reactions were observable by HA. Monkey anti-Rauscher virus serums were then absorbed to completion with normal Balb/c erythrocytes (MoRBC). This was followed by absorption with normal Balb/c plasma (NMoP) until an excess of Balb/c plasma could be

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‡ All monkey sera were prepared at Bionetic Research Laboratories, Inc., Falls Church, Va.

demonstrated by micro-double diffusion tests and no reactions occurred with normal Balb/c plasma(8).

Tanned cell HA tests. The methods used were essentially those of Stavitsky(1). SRBC were washed 3 times and resuspended to 2.5% in pH 7.2 phosphate buffered saline (PBS). Equal volumes of cell suspensions and tannic acid dilutions in pH 7.2 PBS were incubated for 10 minutes at 37°C. The cells were washed once in pH 7.2 PBS and resuspended to the original volume in pH 6.4 PBS. Tanned SRBC, viral antigen, and pH 6.4 PBS were then mixed in the following proportions: v/v 1:1:4. After incubation of the cells for 10 minutes at room temperature, they were washed twice and resuspended in 1% normal rabbit serum (NRS) in pH 6.4 PBS. Serial 2-fold dilutions of antiserum were made in 10 × 75 mm acid-cleaned tubes in 0.5 ml volumes. To each dilution in a series was added 0.05 ml of 2.5% sensitized SRBC. HA patterns were read after 3 hours of incubation at room temperature, and the titers were expressed as the highest dilution of antiserum showing complete HA. Diluent and cell controls, as well as titrations of known positive and negative serums, accompanied each test. Specificity of all reactions was also tested by simple HAI (blocking) with antigen.

HAI tests. The unit of antibody or the highest dilution of serum necessary to produce complete HA was determined on each date of testing. For viral assay tests, materials to be examined for the presence of antigen were diluted serially. One-tenth ml of serum, diluted to contain 2, 4, or 8 hemagglutinating units, was then added to each dilution in a series. The mixture was incubated for 15 minutes at 37°C before the addition of sensitized SRBC. HA patterns were read after 3 hours of incubation at room temperature and expressed as the highest dilution of material showing definite inhibition.

Complement fixation tests (CF). Standard CF reactions(9) in barbital buffer containing Mg and Ca salts were carried out on various dilutions of inactivated, unabsorbed monkey serums for the purpose of titrating the antibody reaction to normal mouse plasma. Using a 1 ml system, the following conditions were maintained: Two units of guinea pig complement contained in a 0.2 ml volume were added to 0.2 ml of Balb/c mouse plasma antigen (1:320 dilution) and 0.2 ml of monkey antiserum. After overnight fixation at 4°C, a mixture of 0.2 ml of 3% SRBC and 0.2 ml of hemolysin containing four 100% hemolytic units/ml was added to each tube in a series. The mixture was incubated for 30 minutes at 37°C and read for hemolysis with 2+ considered as the endpoint. In addition to the standard controls, anticomplementary control tubes of each dilution of antigen or antiserum were always included.

Experimental procedures and results. Conditions of the test. Optimal conditions for testing were determined for the purpose of utilizing the smallest amount of the highly purified viral antigen. The concentration of tannic acid, tested from 1:10,000 through 1:80,000 with a constant dilution of JLS-V5 virus, had no effect on the titer of pooled monkey antiserum. A 1:20,000 dilution of tannic acid was routinely employed. The effect of concentration of the antigen on the ability to sensitize SRBC was then tested, and the results are given in Fig. 1. Maximal titers were obtained between 25 and 200 μg protein/ml; at a concentration of 12 μg/ml or lower there was a sharp decline in sensitivity. The optimal concentration of viral antigen was 25 μg protein/ml or a 1:8 dilution. Five different batches of virus containing similar concentrations of protein, RNA,

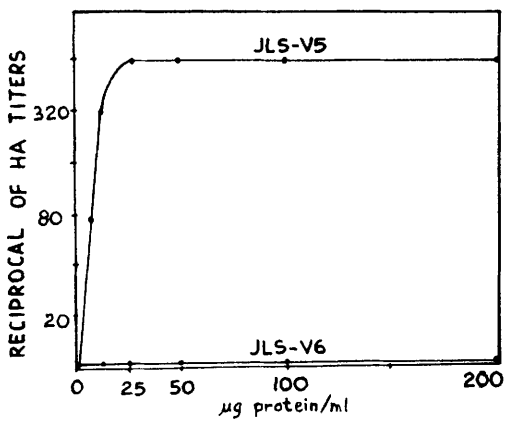

FIG. 1. Effect of concentration on HA titers of pooled monkey anti-Rauscher serum.

TABLE I. Specificity of Hemagglutination Reaction of JLS-V5 Sensitized SRBC and Pooled Monkey Anti-Rauscher Serum.

Serum treatment	Pooled monkey sera	Reciprocal of hemagglutination titers SRBC	MoRBC	JLS-V5 sensitized SRBC	JLS-V6 sensitized SRBC	Presence of antibodies to NMoP*
Inactivation at 56°C for 30 min	Normal	112	1,792	NT†	NT	+
	Immune	224	28,672	NT	NT	+
Complete absorption with SRBC	Normal	0	112	0	0	+
	Immune	0	14,336	1,280	0	+
Complete absorption with NMoP	Normal	0	112	0	0	0
	Immune	0	7,168	1,280	0	0
Complete absorption with MoRBC	Normal	0	0	0	0	0
	Immune	0	0	640	0	0

* Micro-double diffusion test(8). † NT = Not tested.

and virus particles gave identical results, but no reactions occurred when JLS-V6 served as the antigen.

Various other factors such as the time, temperature and pH of sensitization and the pH of the diluting fluids were tested, but no marked differences in the sensitivity of the JLS-V5 sensitized SRBC were found. Although they were used within a short time after preparation, the stability of the cells was evaluated at various periods up to 48 hours. When stored at 4°C, no changes in the endpoints of replicate titrations occurred throughout this period. Leaching of antigen was not detectable until 24 hours and washing of the cells with fresh 1% NRS in pH 6.4 PBS just prior to testing eliminated this problem.

Specificity and sensitivity of the reaction. As recorded in Table I, pooled normal and immunized monkey serums were tested for antibodies to JLS-V5 and JLS-V6 as well as for antibody to SRBC, MoRBC, and NMoP before and after absorption with these antigens. Before absorption, both normal and immune monkey serums possessed agglutinins to SRBC and MoRBC, and reacted with NMoP in micro-double diffusion tests. HA titers against JLS-V5 were not significantly affected when antibodies to SRBC, MoRBC, and NMoP were completely removed by stepwise absorption. The serum from both immune and normal monkeys failed to react to JLS-V6 sensitized SRBC. The results suggested that if JLS-V5 virus contained mouse protein contaminants, they were adsorbed in extremely minute amounts, if at all, on the red cell surfaces. This was later supported by the observation that high-titered rabbit anti-mouse plasma serum failed to react with JLS-V5 or JLS-V6 treated cells in an HA test.

Experiments were undertaken to determine whether an HAI test might be used for detection and assay of virus. The effects of various inhibiting materials on the reaction, given in Table II, are representative of the findings. Virus or virus-containing materials were inhibitory at relatively high dilutions depending on the degree of sensitivity of the tests. Corresponding control materials showed minimal inhibitory activity only when the most sensitive tests were performed. A direct relationship exists between the concentration of antiserum (units of antibody) and the dilution of virus or virus-containing material permitting a rough estimation of the sensitivity of the tests. For example, when standard JLS-

TABLE II. Effect of Various Inhibiting Materials on Reaction of JLS-V5 Sensitized SRBC and Pooled Monkey Anti-Rauscher Serum.

Inhibiting material	No. of HA units of Ab added 2	4	8
JLS-V6	20*	0	0
JLS-V5	1,280	640	160
10% normal Balb/c spleen extract	40	0	0
10% infected Balb/c spleen extract	2,560	640	160
10× normal Balb/c plasma	20	0	0
10× infected Balb/c plasma	10,240	2,560	640

* Reciprocal of HAI titers.

TABLE III. Response of 14 Rhesus Monkeys Given Rauscher Virus Vaccine.*

Animal No.	CF titers on unabsorbed sera against NMoP		HA titers of absorbed sera against virus			
			Pre-booster†		Post-booster	
	Pre-booster†	Post-booster	JLS-V5	JLS-V6	JLS-V5	JLS-V6
49A	0‡	160	0	0	640	0
58A	80	160	0	0	2,560	0
64A	20	80	0	0	1,280	0
66A	40	160	0	0	40	0
43A	80	320	10	0	2,560	0
44A	AC§	160	0	0	1,280	0
52A	160	320	20	0	5,120	0
57A	0	160	10	0	160	0
60A	0	10	0	0	20	0
63A	10	80	0	0	80	0
65A	AC	160	0	0	80	0
74A	40	320	0	0	640	0
811	40	640	10	0	2,560	0
815	AC	AC	0	0	0	0
GMT‖	15	160	2	0	320	0

* Animals were given a 1 ml (49A, 58A, 64A, 66A) or 2 ml (remaining numbers) intraperitoneal injection of Rauscher virus vaccine in Freund's adjuvant followed 28 days later by 0.25 ml of virus vaccine without adjuvant. Final bleedings were made 7-10 days after the final injection.

† Expressed as reciprocal of the titer.

‡ 0 = Negative at lowest dilution tested, 1:10.

§ AC = Anticomplementary at all dilutions tested.

‖ GMT = Geometric mean titers.

V5 virus was tested against 2 units of antibody (0.1 ml of a 1:128 monkey anti-Rauscher), definite inhibition at a dilution of 1:1,280 was demonstrable (Table II). This corresponds to a virus protein concentration of less than 0.2 μg/ml.

In a separate experiment, HAI by sucrose gradient purified virus obtained from concentrated mouse plasma was examined. The single, highly opalescent 1.16 density band isolated from the gradient showed the same degree of inhibitory activity as the standard JLS-V5 preparation. Density gradients of the normal Balb/c plasma concentrates showed only slight opalescence at the 1.16 density; these failed to inhibit the reaction.

Monkey anti-viral responses. The antibody responses of 14 Rhesus monkeys given Rauscher virus vaccine are given in Table III. Four of 14 animals demonstrated minimal anti-JLS-V5 titers 28 days after a single intraperitoneal injection of antigen and adjuvant. In contrast, 10 days after a second subcutaneous injection of virus alone, 13 of 14 animals responded to JLS-V5 virus with markedly higher titers ranging from 1:20 to 1:5,120, with a geometric mean of 1:320. These titrations have been performed many times with the same and different batches of antigen and are typical of the results. HA tests for antibodies to SRBC and MoRBC as well as CF tests for antibodies reactive with mouse plasma proteins were performed on each monkey serum. In general, the ability of individual animals to respond to viral antigen corresponded to the response to mouse plasma antigens as measured by CF. The geometric mean titers of pre-booster and post-booster groups were 14 and 160 respectively (Table III).

Discussion. Murine leukemia viruses have not been shown to cause direct agglutination of erythrocytes. The work presented herein indicates that a murine leukemia virus (Rauscher) can be attached to the surface of tannic acid-treated SRBC which are then rendered agglutinable by immune monkey serum. Specific inhibition of this reaction by minute amounts of virus or virus-containing materials also occurs and makes possible the assay of this murine leukemia virus.

Since Rhesus monkeys in these experiments not only reacted to viral antigen(s) but to normal mouse proteins as well (Table III), the specificity of this reaction has been rigidly controlled. Evidence for the specificity of the

anti-viral response is as follows: (a) the reaction was completely inhibited by high dilutions of JLS-V5 and by various preparations of virus including the density gradient purified virion; (b) the reaction was minimally, if at all, inhibited by fluids of the control cell line JLS-V6, which had never been inoculated with virus, or plasma or spleen of uninfected mice; (c) the reaction was negative when the antigen was prepared from fluids of the control cell line, JLS-V6; and (d) the reaction against JLS-V5 was negative when tested against sera from uninoculated, control monkeys. In addition, other observations tend to support the interpretation that these reactions are virus-specific. The amount of highly purified JLS-V5 virus used in the sensitization process had a direct effect upon the HA titers of the serums tested (Fig. 1). The virus preparation itself did not sensitize cells to mouse proteins since high-titered rabbit anti-mouse plasma failed to react with JLS-V5 treated SRBC. In addition, absorbed and unabsorbed monkey antiserums demonstrated essentially identical titers to JLS-V5. Either the virus used in these experiments was relatively free of murine antigens or the low concentration of virus necessary to sensitize the cells tended to minimize or eliminate the reactions to these foreign proteins. Finally, it should be emphasized that the monkey serums were completely absorbed in order to remove any extraneous or nonspecific reactions to non-viral antigens that might be present.

The very slight degree of inhibition produced by normal tissue culture fluids or spleen extracts or plasma from uninoculated mice was only demonstrable when the most sensitive inhibition tests were performed. Thus, the inhibition produced by these materials might be nonspecific, resulting simply from the high degree of sensitivity of the test; or specific, resulting from the occurrence of viral material already present in normal mice which cross reacts in this system. The possibility that Balb/c mice might harbor an agent antigenically similar to the Rauscher virus was proposed by Fink and Rauscher in an earlier study(6). More information is necessary to clarify this point. Whatever the reason, however, very slight reduction of the sensitivity of the test, using 4 units instead of 2 units of antibody, eliminates this problem for practical testing (Table III).

In this report the HA procedure was adapted for measuring the antibody responses to viral antigen(s) of the Rauscher agent in the Rhesus monkey. The second injection of virus antigen 28 days after the primary stimulus engendered the synthesis of a considerable amount of anti-viral antibody and seems essential for production of high-titered antiserums. HA tests with SRBC and MoRBC and CF tests to NMoP were carried out concomitantly with anti-viral titrations to determine whether a correlation between these responses might exist. The reaction to the erythrocytes did not appear to be useful for predicting anti-viral response, but the CF reaction to the normal mouse proteins tended to parallel the anti-viral response in most animals.

Several techniques have been applied to the problem of detection of murine leukemia viruses using the Rauscher virus as a model. Infectivity assays(10), electron microscopy (11) and fluorescence microscopy(12) studies, and micro-immunodiffusion tests(8) are useful as viral detection methods and for elucidating the antigenic structure of murine leukemia viruses. The HA and HAI tests, however, offer the first *in vitro* semi-quantitative methods for estimation of virus antibodies and of virus in the murine leukemia system. This test may also prove similarly useful in leukemia studies in man.

Summary. The tanned cell HA and HAI methods were adapted for study of a murine leukemia virus (Rauscher). The tests using antibody prepared in Rhesus monkeys were shown to be sensitive and specific for detection and estimation of virus and of anti-viral antibody. Antisera produced in 14 individual monkeys had titers ranging from 1:20 to 1:5,120 with a geometric mean of 1:320 when tested against JLS-V5 sensitized SRBC. As little as 0.2 μg/ml of viral protein was detected by HAI.

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Relative Oöporphyrin Content and Porphyrin Forming Capacity of Wild-Type and White-Egg Japanese Quail Uterine Tissue. (31200)

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A previous study of eggshell pigmentation in wild-type and mutant white-egg strains of Japanese quail (*C. coturnix japonica*) showed that oöporphyrin is the principal pigment found on the eggshells and in the uterus of both quail types(1). It also was found that the oöporphyrin content of the wild-type uterus is reduced immediately following deposition of superficial eggshell pigment, *i.e.,* 2-3 hours before oviposition. These findings led to two questions relating to functional differences between the 2 quail phenotypes. 1) Does oöporphyrin occur in the mutant uterus in amounts equal to those previously reported in the wild-type uterus? 2) If not, might there be a difference between the 2 quail types in their capacity to produce oöporphyrin in the uterus?

Homogenates of chicken uterine tissue are able to synthesize oöporphyrin *in vitro* when incubated with the porphyrin precursor, delta-aminolevulinic acid (ALA). Furthermore, homogenates of white-egg layers synthesize as much oöporphyrin as do homogenates of brown-egg layers(2). By using similar methods I hoped to gain an insight into the relative porphyrin synthesizing capacities of wild-type and mutant quail uteri.

Materials and methods. The environmental regime and the method for recording the time of ovipositions in our quail colony have been described(1). The procedure for preparation and analysis of quail uterine pigment extracts—an adaptation of earlier work by Polin(2)—is described in the same publication(1). These were 3N HCl extracts; the amount of pigment present was expressed in optical density units at wavelength 410 mμ, determined with a Beckman DU spectrophotometer.

The method for estimating porphyrin synthesis in quail uteri was also a modification of Polin's techniques(2). All manipulations, up to incubation of homogenates, were carried out in an ice bath. Quail hens were decapitated and allowed to bleed freely. From each, 1 g of uterine tissue (almost the entire uterus) was homogenized in a Ten-Broeck tissue grinder with 20 ml of cold phosphate buffered saline adjusted to pH 7.2 with NaOH. This homogenate, plus a 5 ml aliquot of buffered saline used to rinse out the grinder, was poured into a 30 ml tube, mixed, and allowed to stand for 15 minutes. The upper 10 ml of homogenate was pipetted into another tube and thoroughly mixed. From here 1.6 ml aliquots of homogenate were placed in each of 4 screw cap tubes containing 0.08, 0.175, 0.35, and 0.7 ml of 0.008 M ALA* in buffered

* δ-aminolevulinic acid • HCl, Calbiochem, Los Angeles.