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Non-Specific Hemadsorption by Rhesus Monkey Kidney Cells. (30735)

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The value of primary rhesus monkey kidney tissue culture for the isolation of myxoviruses of human origin has been well established. The hemadsorption and the hemadsorption-inhibition tests, with their many modifications, have been accepted as indispensable tools for myxovirus detection and identification(1,2). However, the problems associated with adventitious simian agents, and the suspected frequency of occurrence of these agents, have caused the usefulness of both the tissue system and the hemadsorption technique to be seriously questioned.

Many laboratories have reported that a lot of monkey kidney tissue completely free of at least some hemadsorption was rare. This has also been our finding. Of particular concern is the SV_5 virus of Hull(3) which has all the characteristics of a myxovirus: it hemadsorbs, hemagglutinates, elutes from red blood cells (RBC's) by enzymatic action, and demonstrates minimal cytopathic effect in early passage. Prior experience with SV_5 contamination, and the assumption by many laboratory workers that SV_5 may behave similarly to SV_{40}(4) or SV_{13}(3,5), have resulted in the general impression that SV_5 contamination is almost universal. Any adsorption of RBC's to uninoculated tissue has been thought to indicate the presence of SV_5 or some other myxovirus that either multiplies very slowly or is only prevented from exploding into a full blown infection by the use of specific antiserum in the tissue culture medium.

It has been our contention that hemadsorption resulting from the presence of adventitious myxoviruses may be easily recognized and identified. However, we have been unable to find in the literature any detailed explanation of the nature of hemadsorption unassociated with virus infection. This report, based on the use of rhesus monkey kidney tissue for a controlled period of over 2½ years, attempts to define the phenomenon more clearly.

Materials and methods. Monkey kidney cultures. Immature rhesus monkeys were obtained directly from India through a commercial import agency. Monkeys were received at the Communicable Disease Center approximately every 3 months in lots of 100 and held for a quarantine period of 3 to 4 weeks. No attempts were made to keep individual monkeys isolated.

Kidneys for tissue culture were obtained from a single monkey and blood was collected at the time of sacrifice for later serologic studies. Kidney cells were prepared by the Tissue Culture Unit at the Communicable Disease Center and grown in screw-capped tubes in Melnick's medium of 5% calf serum and 95% Hanks' solution containing 0.5% lactalbumin hydrolysate. Penicillin and streptomycin were added to this and all following

media to a concentration of 200 units and 100 μg respectively. On the sixth day after seeding, tubes were washed once with Hanks' solution and changed to maintenance medium consisting of Eagle's MEM without serum.

Red blood cells (RBC's). RBC's were obtained from healthy donors and collected at the rate of 1 part whole blood to 4 parts Alsever's or citrate solutions. RBC's were washed 3 times in phosphate buffered saline (PBS), pH 7.2, and diluted to a final concentration of 0.4%.

Hemagglutination (Ha) and hemagglutination-inhibition (HI) tests. Ha and HI tests were performed as described previously(6).

Hemadsorption (HAd) and hemadsorption-inhibition (HAdI) tests. Tissue cells were tested for HAd with 0.4 ml of a 0.4% suspension of guinea pig RBC's. Tubes were read after 10 and 45 minutes at room temperature for virus isolation. For all later tests for non-specific HAd, tubes were read after 30 minutes at room temperature. HAdI tests were performed by washing the cell sheets three times with Hanks' solution and adding 1 ml of Hanks' containing the appropriately diluted serum. Tubes were allowed to incubate at room temperature for 30 minutes and were read for HAd in the usual manner.

Results. Virus isolations. Over the past 2½ years, a single rhesus monkey was sacrificed each week to meet the nomal requirements of the Respirovirus Unit diagnostic laboratory. The bulk of the tissue was employed for routine virus isolations and tubes were inoculated with throat swab extracts or throat washings. At least ⅓ of the inoculated tubes were tested for HAd 7 to 10 days after inoculation (13 to 16 days after seeding tissue). Uninoculated tubes were treated similarly. Tubes negative for virus isolation underwent one additional blind passage and all tubes were tested for Ha and/or HAd 7 to 10 days later.

The myxoviruses isolated from diagnostic specimens during this period included parainfluenza types 1, 2, 3, and 4, influenza types A and B, and mumps. The origin of these isolates for the most part was confirmed by a diagnostic antibody rise in paired sera of patients from whom the viruses were recovered. At no time were these viruses associated with uninoculated tissue.

From 144 monkeys sacrificed for tissue culture, SV_5 virus was isolated from kidney tissue on 3 occasions (2%): in May and September, 1964, and in March, 1965. In all instances some cytopathic evidence of SV_5 was observed at the time of testing for HAd. By the 10th to 13th day after seeding, tissue sheets demonstrated 3 to 4+ HAd with Ha titers of 1:40 to 1:80. SV_5 contamination observed in the 1965 lot involved approximately 10% of the tubes while nearly 90% of the tubes were involved on the two previous occasions.

Antibody patterns of sacrificed monkeys. HI tests performed with sera collected from over 100 monkeys at time of sacrifice showed 69% had antibody titers of 1:20 or greater against SV_5 and 82% had similar titers against parainfluenza type 3. However, SV_5 was recovered from monkey kidney on only 3 occasions and isolates similar to parainfluenza type 3 could not be associated with the host tissue at any time. Over ½ of these sera were tested by HI against parainfluenza type 1 (HA-2), type 2 (CA), and mumps, and by neutralization against SV_{41}(7). Antibody titers of all sera were less than 1:20 indicating there was little or no involvement of these viruses in natural infections of the monkeys sacrificed for tissue culture.

Non-specific HAd. Condition of RBC's. Non-specific HAd, which continued to occur in the absence of virus isolation, appeared in some way to be related to the age or condition of the RBC's. Fresh (used on day of collection) RBC's yielded no non-specific HAd regardless of the solutions used for collecting, washing, or suspending (Fig. 1). However, HAd patterns of maximum density could be demonstrated with sterile RBC suspensions in either PBS, unbuffered saline, or Hanks' solutions after storage for periods as short as 24 hours at 37°C, 48 hours at room temperature, or 7 to 10 days at 4°C (Fig. 2). In general, RBC's stored at 4°C in PBS demonstrated detectable HAd after 72 hours and gradually increased with continued storage. RBC's stored in unbuffered saline or Hanks' solution under the same conditions demon-

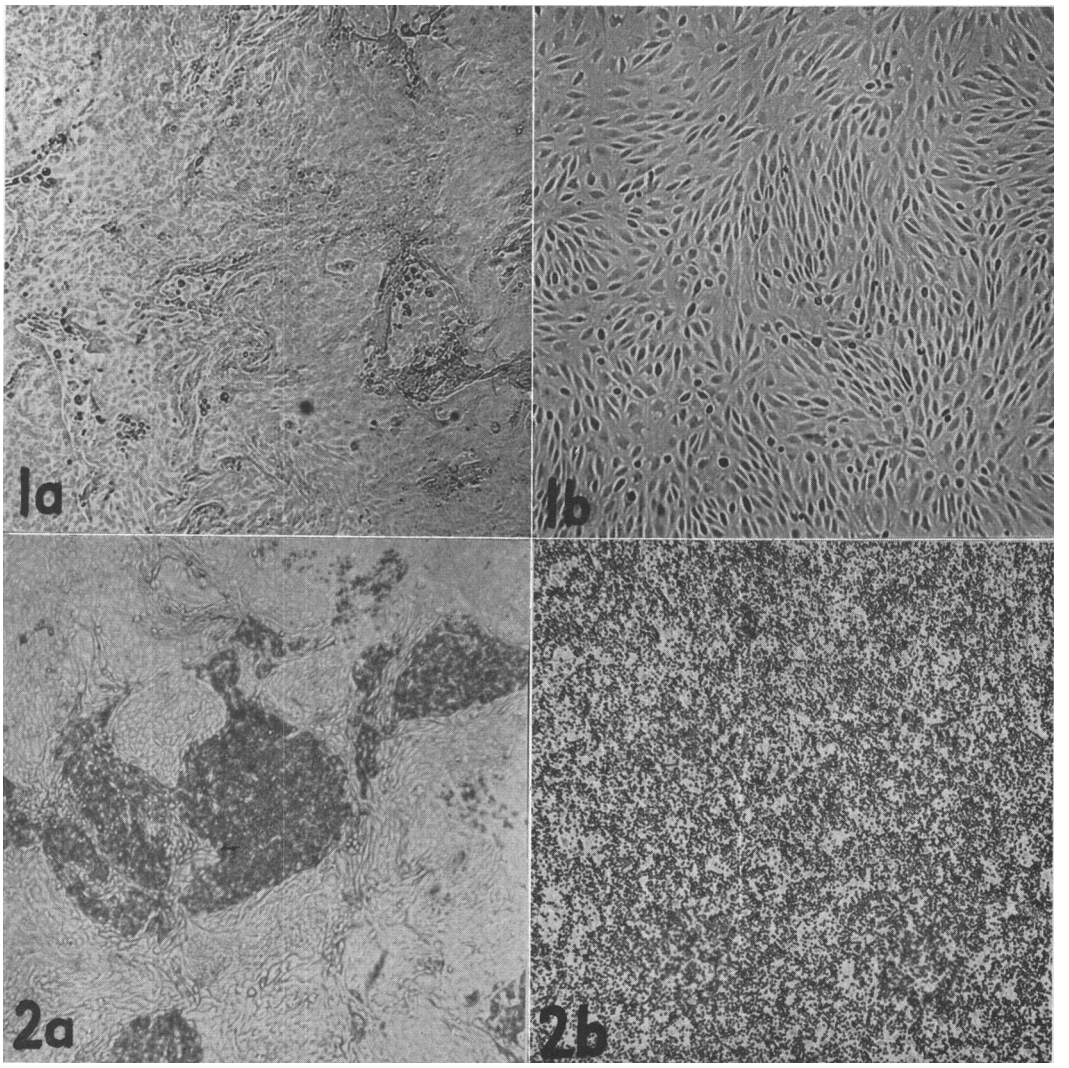

FIG. 1. Negative hemadsorption. Fresh guinea pig RBC's. 1a) Normal primary rhesus monkey kidney tissue culture. 1b) Normal rhesus kidney cell strain Rh#6, 26th passage.

FIG. 2. Non-specific hemadsorption (THAd). Aged guinea pig RBC's. 2a) Normal primary rhesus monkey kidney tissue culture. 2b) Normal rhesus kidney cell strain Rh#6, 26th passage.

strated somewhat less HAd. RBC's stored in the original Alsevers' or citrate collecting solutions showed only slight adsorption after 1 to 2 weeks at 4°C, but near-maximum non-specific HAd titers were obtained after 3 to 4 weeks (Table I).

Fresh guinea pig RBC's could be caused to adsorb non-specifically by prior treatment with trypsin, potassium periodate, or the receptor destroying enzyme (RDE) of *Vibrio cholerae*. Incubation of 0.4% PBS suspensions of RBC's with equal volumes of 10^{-3} M potassium periodate for 30 minutes yielded maximum non-specific HAd patterns on monkey kidney tissue. Treatment of RBC suspensions for 1 hour with commercial 1:300 trypsin at a dilution of 1:10, or high titered RDE at the same dilution yielded some hemadsorption, but of a much lower order than that seen with potassium periodate. Higher dilutions of trypsin or RDE were less effective.

TABLE I. Comparison of Specific (SV_5) and Non-Specific HAd (THAd) of Primary Monkey Kidney Tissue Cultures.

Test conditions*	HAd†	
	Specific (SV_5)	Non-specific (THAd)
"Fresh" RBC's	+	—
"Aged" RBC's		
Test temp 4°C	+	+
" " 25°C	+	+
" " 37°C	—‡	+
pH range	+ (<5.5 to >8.5)	+ (<5.5 to 7.5)
HAdI (SV_5 antiserum)	—	+
RDE treated RBC's	—	+
SV_5 " "	—	+
Water treated tissue	+	—
Merthiolate treated tissue	+	—
Heat " "	+	—

* Room temperature unless otherwise indicated.
† Tubes read 30 min after adding RBC's.
‡ Elution or desorption of RBC's.

Guinea pig RBC's held for 2 days at room temperature in sterile PBS (hereafter referred to as "aged") were most commonly employed in this study but extensive non-specific HAd also could be readily demonstrated with aged RBC's from man, sheep, chickens, rhesus monkeys, vervet monkeys, and rats.

Temperature requirement. Aged guinea pig RBC's were added to normal primary monkey kidney tubes and incubated at various temperatures. Tubes held at room temperature or 37°C for 15 minutes demonstrated approximately 50-75% complete HAd. Tubes held at 4°C showed somewhat less. After 30 minutes maximum patterns were obtained at all 3 temperatures. No elution occurred after prolonged incubation. SV_5 controls containing fresh or aged RBC's characteristically showed nearly 50% elution (desorption) after 30 minutes at room temperature and complete elution at 37°C during the same period of time. Tubes held at 4°C remained strongly positive.

pH range. Uninoculated monkey kidney tissue tubes and tubes inoculated with parainfluenza type 1 and SV_5 were washed 3 times with Hanks' solution adjusted to specific pH levels with 0.2 M phosphate buffer. Aged guinea pig cells were added after 30 minutes. Maximum non-specific HAd patterns occurred at pH levels ranging from 5.5 to 7.5. Some reduction was noted at pH 8.0 and tubes held at pH 8.5 were essentially negative. In contrast, specific viral HAd appeared to be constant over the entire pH range with no obvious reduction up to pH 8.5.

HAdI with specific antiserum. Uninoculated monkey kidney tubes were treated in the usual manner with specific virus antisera at final dilutions of 1:20. HAdI test controls consisted of specific inhibition of homologous viral HAd. Non-specific HAd with aged RBC's could not be inhibited by antisera to parainfluenza type 1 (HA-2), type 2 (CA), type 3 (HA-1), type 4 (M-25), SV_5, or mumps, or pools of normal monkey sera.

SV_5 treatment of RBC's. PBS suspensions of 1% human "O" RBC's from outdated blood bank stock were added to equal quantities of fresh SV_5 tissue culture harvests having HA titers of 1:40 or 1:80. Virus suspensions were allowed to adsorb to RBC's at room temperature for 30 minutes and eluted by incubating at 37°C for the same time period. This adsorption and elution cycle was repeated 2 to 4 times. RBC suspensions thus treated were washed 3 times in PBS, diluted to 0.4%, and added to uninoculated monkey kidney and control SV_5 tubes. Treated RBC's did not adsorb to SV_5 infected tissue. The same treatment had no effect on non-specific HAd patterns.

Receptor destroying enzyme (RDE) treatment of RBC's. PBS suspensions of 1% human "O" RBC's from outdated blood bank stock were treated with equal quantities of varying dilutions of RDE. The mixtures were

TABLE II. Titration of SV_{40} Virus by Inhibition of Tissue Hemadsorption on Rhesus Monkey Kidney Cell Strain Rh#6.

	Days after incubation*							
	1		5		9		13	
Virus dilution	THAd†	CPE‡	THAd	CPE	THAd	CPE	THAd	CPE
10^0	++++	—	—	±	—	+	—	+
10^{-1}	++++	—	±	—	±	±	—	±
10^{-2}	++++	—	++++	—	+	—	—	±
10^{-3}	++++	—	++++	—	++++	—	+	—
10^{-4}	++++	—	++++	—	++++	—	+++	—
10^{-5}	++++	—	++++	—	++++	—	++++	—
Tissue control	++++	—	++++	—	++++	—	++++	—

* Tubes in stationary racks at 33°C.
† Tissue hemadsorption, average of 5 tubes, maximum = ++++.
‡ Cytopathic effect, average of 5 tubes, maximum = ++++.

incubated at 37°C for 1 hour. Treated cells were washed twice in PBS, diluted to 0.4% and added to normal monkey kidney tissue and SV_5 infected controls. RBC receptors for SV_5 were destroyed by all dilutions of RDE tested (through 1:80). Non-specific HAd was unaffected.

Inhibition of non-specific HAd. Adsorption of RBC's appeared to be an active process of specialized kidney cells. Tubes exposed to alkaline conditions (pH 7.5-8.0) for several days either demonstrated very poor non-specific HAd or none at all. Non-specific HAd could be completely inhibited by heating normal tissue in Eagle's medium at 56°C for 10 minutes, by treatment with distilled water for as little as 30 seconds, or by a 20-minute exposure to 1:10,000 dilution of merthiolate. Specific HAd with SV_5 was not affected under these same conditions.

It was observed that tissues infected with high multiplicities of SV_{40} virus also failed to show non-specific hemadsorption. The resulting inhibition permitted direct titration of SV_{40} virus in primary and continuous rhesus monkey kidney tissue in the absence of a clearly recognizable cytopathic effect (Table II). Pretreatment of the virus inoculum with varying dilutions of specific SV_{40} rabbit antiserum or rhesus monkey serum pools reversed the inhibition of HAd. Specific serum neutralizing titers of 1:640 or greater were obtained with virus dosages of $100TCD_{50}$.

Spectrum of non-specific HAd. Adsorption of aged RBC's was not limited to rhesus kidney tissue. Significant non-specific HAd was found to occur also with tissue culture derived from kidneys of African green monkeys, rabbits, cats, and dogs. Of the few fetal human and monkey kidney tissues available for testing, either non-specific HAd did not occur or was limited to an occasional cluster of kidney cells. Non-specific HAd could not be demonstrated with strains of human diploid lung fibroblasts or continuous cell lines of non-kidney origin such as HEp-2, KB, Hu-Am (FL), Chang's liver, and Detroit 6.

All of the primary kidney tissue cultures examined have shown non-specific HAd. It may be seen in Fig. 2a, however, that all kidney cells are not involved. Adsorption occurred on individual tissue cells or islands of cells which varied widely in size and number. Thinly seeded tubes requiring 7 to 8 days to develop confluent tissue sheets appeared to yield the most advanced HAd patterns. With continued passage of primary tissue the percentage of kidney cells capable of hemadsorbing increased considerably. $LLC\text{-}MK_2$, a rhesus continuous cell line developed by Hull (8), and Rh#6, a rhesus kidney cell strain developed in this laboratory, continued to demonstrate HAd after 40 and 30 passages respectively (Fig. 2b). A green monkey kidney line developed in Dr. John Enders' laboratory at Harvard showed strong HAd after 101 passages. The ability of kidney tissue to hemadsorb appears to be a permanent characteristic.

Discussion. It has been shown that hemadsorption in monkey kidney tissue culture may occur by two different mechanisms, one involving the classic specific reaction of RBC's with viral receptors and the other in-

volving a completely unrelated, non-viral, non-specific reaction by certain kidney cells with partially denatured or altered RBC's. This phenomenon of non-specific hemadsorption, or tissue hemadsorption (THAd), seems to be a characteristic of kidney cells and was not found to occur in cell cultures of non-kidney origin.

While this manuscript was in preparation, Neuman and Tytell reported similar findings and showed that the degree of non-specific hemadsorption could be related to the concentration of calcium ions(9). We concur with their finding that calcium is essential for adsorption. However, evidence presented here suggests that calcium ions are not the initiating factor. The usual concentration of calcium in Eagle's medium is 1.8 mM. Under these conditions we have found no evidence of non-specific adsorption of fresh RBC's. Yet RBC's which have been preconditioned or aged (in the absence of calcium) were found to yield maximum HAd patterns when tested in the same medium.

Non-specific adsorption may be avoided under the usual conditions for specific viral HAd tests simply by using fresh RBC's. However, the observed inhibition of THAd by SV_{40} infection of rhesus monkey kidney and preliminary studies with rubella in LLC-MK_2 suggest that THAd inhibition (THAdI) may offer some advantage as a practical research tool.

Summary. Hemadsorption in rhesus monkey kidney tissue culture has been shown to occur by two different mechanisms, a specific reaction of red blood cells with viral receptors and a non-specific, non-viral, reaction of partially denatured or altered red blood cells with specialized kidney cells. Non-specific tissue hemadsorption (THAd) was found not to be limited to rhesus kidney tissue culture but also occurred in kidney tissue strains and lines derived from other animal species. The finding that some viruses inhibit THAd suggests the possible use of this phenomenon as a tool in diagnostic virology.

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Effect of Thyroxine on Spontaneous Nephrosis in the Rat.* (30736)

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The occurrence of spontaneous nephrosis with proteinuria, hyperglobulinemia and hypercholesterolemia in the rat was described previously(1). Proteinuria was observed at weaning and changes in the glomerular capillary basement membrane were noted at 60 days of age. Incidence and severity of renal lesions increased with age and proteinuria became correspondingly greater. Earlier studies(2,3,4) showed that onset of nephrosis as

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