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# Hemagglutination by BK Virus, a Tentative New Member of the Papovavirus Group

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Some characteristics of hemagglutination (HA) by the BK virus, a new candidate for the papovavirus group, have been studied. Hemagglutinin prepared from cell cultures was found to be partially masked by inhibitors which could be dissociated from the virus by incubation at 37 C or by fluorocarbon extraction. Optimal conditions for HA are outlined. In routine tests, 0.5% human erythrocytes were used. The reaction was carried out at *p*H 7.0 on ice-water slurry. BK hemagglutinin receptors on human erythrocytes were found to be more resistant to neuraminidase than polyoma receptors. By gradient centrifugation analysis, two types of particles were found to be responsible for HA: (i) full, deoxyribonucleic acid-containing particles with a density of 1.325 g/cm$^3$ and (ii) empty capsids with a density 1.29 g/cm$^3$. Based on particle counting, one HA unit was calculated to correspond to $3 \times 10^6$ virus particles.

An apparently new virus was recently isolated from urine of a patient suffering of complications of a renal transplantation (5). The virus, tentatively named BK virus, morphologically resembles papovaviruses. Reciprocal serological tests suggested a possible antigenic relationship between BK and simian virus 40 (SV40), but there was no cross-reactivity between the BK virus and polyoma or human wart virus. Like polyoma virus, the BK virus was found to hemagglutinate human type O erythrocytes. In the present experiments, the hemagglutination (HA) reaction of the BK virus was studied, and some properties of the hemagglutinin were characterized.

## MATERIALS AND METHODS

**Viruses.** The BK virus seed was kindly provided by Sylvia Gardner (Central Public Health Laboratory, London, England). Influenza virus strain A/England/334/68 (H3N2) was obtained from Kari Cantell (Central Public Health Laboratory, Helsinki, Finland). The latter was grown in chicken embryos and used at the fourth passage for neuraminidase treatment of erythrocytes. Polyoma virus strain was sent by T. Wesslen, Department of Virology, University of Uppsala, Sweden.

**Cell cultures.** Vero cells (Biocult Laboratories, Glasgow, Scotland), a continuous line of African green monkey kidney cells, were used to propagate BK virus. Cells were dispersed with 0.25% trypsin and grown in BME diploid (Gibco, Grand Island, N.Y.) supplemented with 10% calf serum and 5% Tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). Primary mouse embryo cell cultures were prepared by trypsinization from decapitated BALB/c mouse embryos. Growth medium was the same as for Vero cells.

**Preparation of hemagglutinins.** Vero cells infected with BK virus were maintained in BME medium with 2% calf serum. The medium was changed once a week, and virus was harvested after 3 weeks of incubation. At that time cell cultures showed the typical cytopathic effect (5). For radioactive labeling, $^3$H-thymidine was added to the medium (3 $\mu$Ci/ml; thymidine [methyl-T], 5 Ci/nmole, Radiochemical Centre, Amersham, England) for the period from the 7th to the 14th day of the incubation, after which the virus was harvested. Some hemagglutinating virus was found in the medium, but 70 to 90% of the activity remained cell bound. Infected cells were scraped off the glass and harvested by centrifugation or they were detached by freezing and thawing in 0.05 M phosphate buffer, *p*H 7.0, with 0.15 M NaCl (PBS). Infected cells in PBS (about $5 \times 10^6$ cells/ml) were lysed by adding Nonidet P-40 (Shell Chemical Co.) to 0.1%. Cells were then broken in an ultrasonic bath (Bransonic 12, Branson Europa N.V. Soest, The Netherlands), and the cell debris was removed with low-speed centrifugation. The supernatant fluid was extracted twice with fluorocarbon (Genetron 113, Allied Chemical, New York) on a mixer (Whirlimixer, Fisions Scientific Apparatus, Loughborough, England).

Polyoma virus hemagglutinin was prepared identically from infected mouse embryo cells.

**Serological tests.** A microtitration method was used (12). Erythrocytes from various species were tested as indicated in Results. The effect of *p*H on HA was studied in a *p*H range of 5.6 to 8.6 using 0.15 M NaCl in 0.05 M phosphate or barbiturate buffer. These tests were carried out at four temperatures, 0 C (ice-

water slurry), 4 C, room temperature and 37 C. In routine tests, human type O erythrocytes were used at a 0.5% concentration. Fresh cells were obtained weekly, and the cell concentration was determined for each lot against a cyanmethemoglobin standard (14). PBS (*p*H 7.0) was used as diluent throughout. During the settling of the erythrocytes, the plates were kept on ice-water slurry.

**Tests for virus receptors on erythrocytes.** Human type O erythrocytes were treated with influenza A2 virus neuraminidase, as was described by Hirst (9). Sensitivity of receptors to receptor-destroying enzyme (RDE) was also studied. Based on preliminary tests, a 1:2 dilution of RDE (cholera filtrate, N.V. Philips-Duphar, Amsterdam, Holland) in PBS was used. A 1% suspension of erythrocytes in RDE was incubated at 37 C for various times as indicated in Results. Control cells were incubated similarly in PBS. After incubation, cells were centrifuged, washed, and suspended to a 0.5% concentration in PBS.

**Gradient centrifugation.** Virus samples for gradient centrifugation were prepared in the same way as for HA. In some experiments, deoxyribonuclease treatment was an additional preparation procedure. Virus in PBS with 5 mM $MgCl_2$ was incubated with 50 $\mu$g of deoxyribonuclease (Miles-Seravac, Maidenhead, England) per ml at 37 C for 30 min. This treatment had no effect on the distribution of hemagglutinin in the gradient.

For centrifugation a 0.5-ml sample of virus (cold or labeled with $^3$H-thymidine) was mixed with CsCl in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.0) with 1 mM ethylenediaminetetraacetic acid (EDTA) to give final density of 1.295 g/cm$^3$. Gradients were centrifuged in an SW50.1 rotor in a Beckman L3-50 centrifuge at 103,000 $\times$ *g* for 40 hr. Fractions were collected through the bottom of tubes. Densities were obtained by measuring refractive indexes and by comparing these with a standard curve made by weighing a series of CsCl solutions in 100-$\mu$liter capillary tubes. Samples of radioactive fractions were plotted on filter papers, treated with 5% trichloroacetic acid and ethanol, dried, and counted in a toluene-based scintillation liquid.

**Electron microscopy and particle counting.** A drop of the sample was placed on a Formvar-covered grid and stained with 2% phosphotungstic acid, *p*H 7.0, for 30 sec. Grids were examined in a Siemens Elmiskop 1A electron microscope at an instrumental magnification of 40,000 at 60 kV.

Virus for particle counting was prepared from extracellular fluid by concentration and partial purification with differential centrifugation (9,300 $\times$ *g* for 15 min, and 103,000 $\times$ *g* for 2 hr) and fluorocarbon extraction. Dilutions of 1:10 and 1:100 of virus were made with 0.5-ml volumes, and titrated for hemagglutinin. Two virus preparations were tested, and the final HA titers used for calculations were an average of 6 or 12 separate titrations for the two preparations, respectively. Particle counting was done by using a negative staining-latex particle method (10). Uniform polystyrene latex particles (Dow Chemical Co., Indianapolis, Ind.) with a diameter of 126 nm were used as reference. Grids were examined at a magnification of 20,000. Micrographs were taken of 10 representative areas over the whole grid, and viral and latex particles were counted.

## RESULTS

**Unmasking of the BK hemagglutinin.** It was found in early experiments that both the maintenance medium and the extract from infected cells obtained with ultrasonic treatment contained HA inhibitors. Nonidet P-40, which was later added in the preparation procedure to enhance the release of hemagglutinin from cell extracts, had no effect on the inhibitors. Inhibition appeared in the form of partial, granular agglutination at the end point of the HA titer. Inhibitory activity in the medium seemed to be caused by calf serum. Five lots of calf serum tested were found to cause similar partial inhibition of HA in variable titers up to 1:160. This inhibitory activity was almost completely eliminated by RDE treatment of sera according to the procedure used to remove nonspecific influenza HA inhibitors (11). The inhibitors could be eluted from virus at 37 C, as indicated by 4- to 16-fold increase in HA titer after incubating virus at 37 C for 30 min. The inhibitors could also be removed both from extracellular virus preparations and from cell extracts with fluorocarbon treatment. Fluorocarbon extraction was therefore included in the standard hemagglutinin preparation procedure.

**Variables of the HA reaction.** No HA was obtained with erythrocytes from African green monkey or mice (strains CBA, BALB/c, and C57 were tested). A positive reaction was given by human, guinea pig, and young chicken erythrocytes. At 0 C (ice-water bath), human and guinea pig erythrocytes showed similar HA pattern (Table 1): both reacted within a wide *p*H range

TABLE 1. *Effect of pH and incubation temperature on hemagglutination by BK virus*

| *p*H | Hemagglutinin titer with human type O erythrocytes at | | | | Hemagglutinin titer with guinea pig erythrocytes at | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 C[a] | 4 C | RT[b] | 37 C | 0 C[a] | 4 C | RT[b] | 37 C |
| 5.6 | 4 | 8 | 4 | 4 | 4 | 4 | 8 | 2 |
| 6.1 | 8 | 16 | 8 | 8 | 8 | 4 | 2 | <1 |
| 6.5 | 16 | 16 | 8 | 8 | 16 | 8 | 1 | <1 |
| 7.0 | 16 | 16 | 8 | 2 | 4 | 4 | <1 | <1 |
| 7.4 | 8 | 16 | 8 | <1 | 4 | 4 | <1 | <1 |
| 8.0 | 8 | 8 | 1 | <1 | 2 | 1 | <1 | <1 |
| 8.6 | <1 | <1 | <1 | <1 | <1 | <1 | <1 | <1 |

[a] Incubated on ice water slurry.
[b] Room temperature.

with an optimum at *p*H 6.5 to 7.0. The reaction was sensitive to an increase of the incubation temperature and of the *p*H, especially when guinea pig erythrocytes were used. With young chicken erythrocytes, HA was obtained at 0 C at *p*H 5.6 to 7.0, but the titers were lower than with human or guinea pig erythrocytes; at room temperature and at 37 C, a barely noticeable HA was observed at *p*H 5.6.

In view of the results described above, a study was undertaken to determine the possibility of adsorbing and eluting the virus from erythrocytes by changing temperature and *p*H. Virus was incubated for 1 hr with human erythrocytes on ice-water slurry at *p*H 7.0. Cells were centrifuged, washed once with cold PBS (*p*H 7.0), and resuspended in the original volume of PBS, *p*H 8.0. After 1 hr of incubation at 37 C, the supernatant fluid was brought back to *p*H 7.0 and titrated for hemagglutinin. One-third of the adsorbed virus was eluted under these conditions.

**BK virus receptors on erythrocytes.** The effect of neuraminidase on BK virus receptors on human erythrocytes was studied by using influenza A2 virus. Erythrocytes were incubated at 37 C as a 1% suspension in undiluted allantoic fluid which had an influenza HA titer of 1:256. In 5 hr, the cells became "stabilized" (i.e., no more agglutination occurred when the cells were left to settle). After four washings the cells were suspended to a 0.5% concentration in PBS. Control cells incubated in PBS were prepared identically. No agglutination of influenza A2-treated erythrocytes was obtained with the influenza virus which, with control cells, showed a titer of 1:128. The result with polyoma virus was the same: a virus preparation having a titer of 1:512 with control cells was completely negative with A2-erythrocytes. In contrast to this, A2-erythrocytes were agglutinated by BK virus, although the titers were only ⅛ of those obtained with control cells.

In another series of experiments, human erythrocytes treated with RDE as described in Materials and Methods were used to titrate BK, polyoma, and influenza A2 virus (Table 2). Again, the receptors for polyoma virus were found to be more sensitive to RDE than the receptors of the BK virus. In terms of titers, influenza A2 receptors seem to be more resistant than BK virus receptors, but the agglutination of RDE-treated cells by influenza virus was very fragile. Tilting the plate for a while made the cells flow down in the same way as in negative wells.

**Characteristics of the hemagglutinin.** When virus preparations were centrifuged at 103,000 × *g* for 2 hr, all the HA activity was found in the pellet; no soluble hemagglutinin could be identified. Density gradient centrifugation was used to characterize the particles responsible for HA. Figure 1 shows the results of a representative run. Two peaks of hemagglutinin were found at densities 1.325 and 1.29 g/cm³, respectively. In some similar experiments, especially when the virus was not treated with fluorocarbon, HA reactivity was also found at the top of the gradient. This light hemagglutinin showed, in contrast to the hemagglutinin in the two denser peaks, a 4- to 64-fold increase in titer after incubation at 37 C for 30 min. Therefore, it could also represent

TABLE 2. *Effect of receptor-destroying enzyme treatment on virus receptors on erythrocytes*

| Incubation time (min)[a] | Hemagglutinin titer of | | |
|---|---|---|---|
| | BK | Polyoma | Influenza A2 |
| 15 | 16 | <2 | 32 |
| 30 | 8 | <2 | 32 |
| 60 | 4 | <2 | 16 |
| 120 | <2 | <2 | <2 |
| 240 | <2 | <2 | <2 |
| Control | 128 | 512 | 64 |

[a] Human erythrocytes were incubated in cholera filtrate receptor-destroying enzyme for the given times. After incubation, cells were washed, suspended to a 0.5% solution, and used to titrate the three viruses. Control cells were incubated in phosphate-buffered saline, *p*H 7.0, for 240 min.

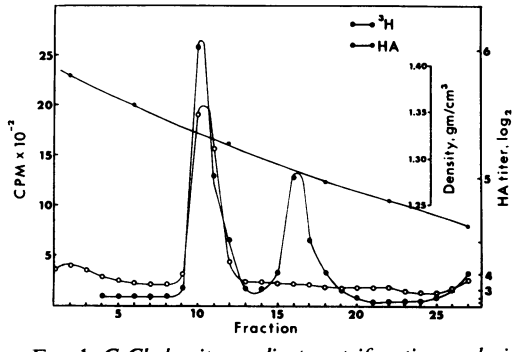


FIG. 1. *CsCl density gradient centrifugation analysis of the BK virus hemagglutinin. BK virus labeled with ³H-thymidine was extracted from infected cells by lysing the cells with Nonidet P-40 (0.1%) followed by ultrasonic treatment. After fluorocarbon treatment, 0.5 ml of the extract was treated with deoxyribonuclease (50 μg/ml; 37 C for 30 min) and mixed with CsCl solution in 0.05 M Tris-hydrochloride buffer (pH 7.0) with 1 mM EDTA to yield a density of 1.295 g/cm³. Gradients were centrifuged in a Spinco SW50.1 rotor at 103,000 × g for 40 hr. Fractions were collected and tested for hemagglutinin. Fractions were plotted on filter papers, treated with 5% trichloroacetic acid, and counted in toluene-based scintillation liquid. Symbols: ○, radioactivity; ●, hemagglutinin.*

virus particles attached to light inhibitor-containing cellular structures. In the curve of acid-precipitable radioactivity, only one peak was found coinciding with the 1.325 g/cm³ HA peak. Figure 2 shows an electron microscope analysis of the two HA fractions. According to these results, the two hemagglutinins separable in density gradient centrifugation seemed to be (i) complete virus particles containing deoxyribonucleic acid (DNA), and (ii) empty virus capsids. Both types of hemagglutinin were found in somewhat variable relative amounts both in extracellular fluids and in extracts from infected cells.

Two virus preparations were examined for determination of the number of virus particles needed for one HA unit, using latex spheres as reference. The ratios of the number of virus particles to the number of latex particles, counted from the electron micrographs, were found to be 724/88 and 664/76, respectively. The corresponding HA titers were 1:6,190 and 1:6,400. Calculated from these figures one HA unit would correspond to $3.0 \times 10^6$ or $3.1 \times 10^6$ virus particles.

## DISCUSSION

The BK virus has been tentatively proposed as a member of the papovavirus group (5). It shows some cross-reactivity with SV40 antisera in immune electron microscopy, but a high-titered polyoma antiserum had no inhibitory effect on BK virus HA (5). Similarly, we did not get any HA inhibition reaction against polyoma virus with a rabbit anti-BK serum, which had a homologous titer of 1:12,000 (*unpublished results*). On the other hand, the HA capability of the BK

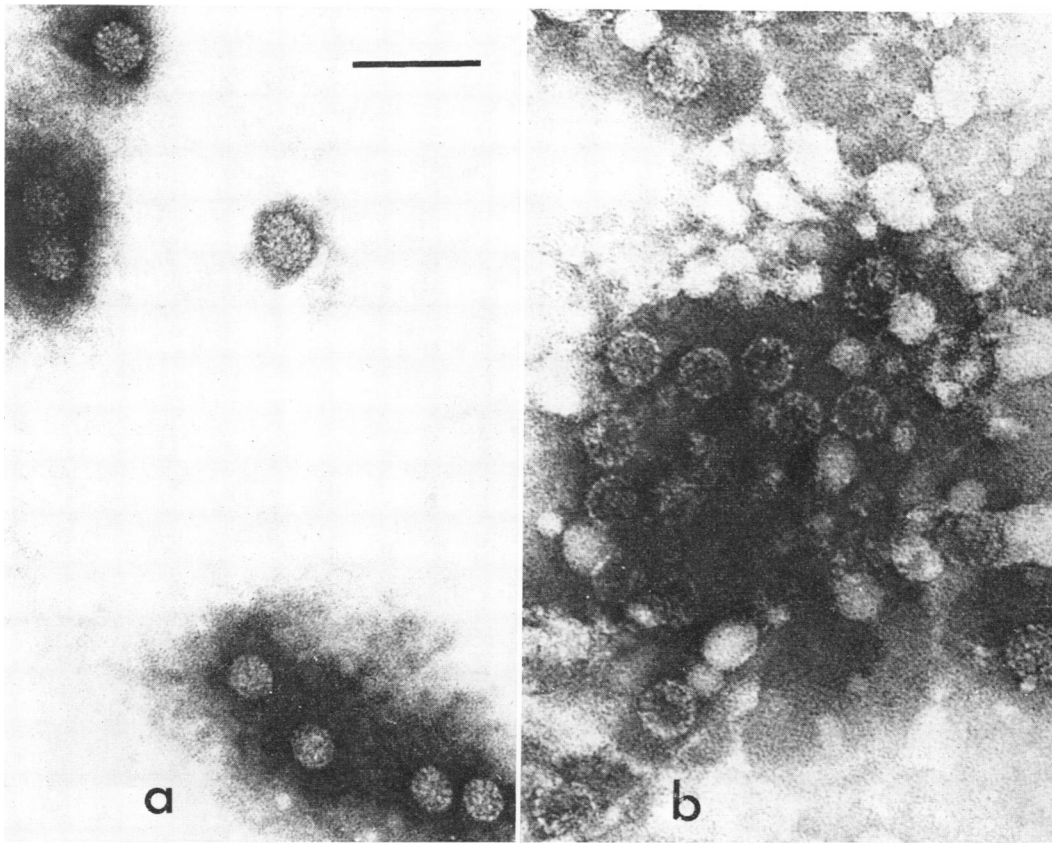


FIG. 2. *Electron micrograph of the two BK virus hemagglutinins. Hemagglutinin was extracted from infected cells lysed with Nonidet P-40 (0.1%) followed by ultrasonic fluorocarbon treatment. The two hemagglutinin peaks in a CsCl density gradient were collected separately. They were diluted with PBS and centrifuged in a Spinco SW-50.1 rotor at 103,000 × g for 2 hr. Pellets were suspended in 0.05 ml of distilled water and stained on Formvar-covered grids with 2% phosphotungstic acid, pH 7.0. a, Particles from the 1.325 g/cm³ fraction; b, particles from the 1.29 g/cm³ fraction. The bar represents 100 nm.*

virus is a property shared within papovavirus group only by polyoma virus and K virus of mice.

In the present experiments, some characteristics of HA by BK virus were outlined. Most of the hemagglutinin in a cell culture was found to be associated with cells, an observation compatible with the nuclear appearance of BK virus (5). The masking of a part of the hemagglutinin by inhibitors is a phenomenon also observed with polyoma virus, as is the release of the inhibitors from hemagglutinin at elevated temperatures (7) and with fluorocarbon extraction (6).

As has been reported earlier, the BK virus agglutinated human erythrocytes, but equal HA titers were also obtained with guinea pig erythrocytes under proper conditions. The adsorption and elution of hemagglutinin from erythrocytes by means of *p*H and temperature changes could probably be used as one step in virus purification procedure.

Erythrocytes treated with RDE or influenza virus neuraminidase become unsusceptible to agglutination by polyoma virus (4, 8). In the same way, myxovirus receptors are RDE sensitive to a degree characteristic for a given myxovirus; this is the basis of the so-called receptor gradient of myxoviruses (2). In the present experiments, polyoma virus receptors on human erythrocytes were found to be more sensitive than BK virus receptors to both cholera filtrate RDE and influenza A2 virus neuraminidase. This difference suggests that a relationship similar to the receptor gradient of myxoviruses also exists between polyoma and BK virus.

Density gradient centrifugation experiments seem to indicate that HA by BK virus is connected with viral capsid, in the form of either full virus particles or empty shells. This again is identical with polyoma virus (1). Also the densities of full and empty BK particles are similar to those of polyoma virus (3). Preliminary results using zonal centrifugation with more shallow S-shaped CsCl gradient suggest that virus population may be more heterogenous than is revealed by density gradient analysis (*unpublished observations*). The appearance of $^3$H-thymidine label in the denser virus peak of the gradient suggests that the nucleic acid of BK virus is DNA. Characteristics of the DNA, especially the conformation, are under investigation.

Virus particle-HA unit ratio of BK virus is $3 \times 10^6$ according to our results which are based on a "semiquantitative" (10) particle-counting method. This is of the same order as reported for polyoma virus (13). Virus preparations used for particle counting still contained a little contaminating material. This was morphologically heterogenous, but theoretically it is possible that some of the clumps observed contained virus-derived material capable of causing HA.

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