Duvenhage Virus: Morphological, Biochemical, Histopathological and Antigenic Relationships to the Rabies Serogroup

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SUMMARY

Duvenhage virus was originally isolated in South Africa from the brain of a man who had been bitten by a bat and died after a rabies-like illness. Previous immunofluorescence tests indicated that the virus was distinct from rabies virus. In the present study an antigenic relationship of this virus to rabies is defined, and pathological, morphological and further serological characterization is presented. Duvenhage virus-infected mice developed a central nervous system disease characterized by a short incubation period, a moderate degree of inflammatory infiltration of brain parenchyma and by small intraneuronal inclusion bodies. By electron microscopy typical rhabdovirus particles were found budding upon endoplasmic reticulum and plasma membranes of brain neurons. In these characteristics Duvenhage virus resembled laboratory or 'fixed' strains of rabies virus. The structural polypeptide composition of Duvenhage virus was very similar to that of rabies virus. Duvenhage virus could be distinguished from rabies by in vivo neutralization and cross-challenge tests in mice, and to a lesser extent by complement-fixation and fluorescent antibody tests. Antibody to purified ribonucleoproteins used in indirect immunofluorescence tests did not distinguish between rabies and Duvenhage virus. In vitro neutralization tests using antisera against whole virus, and against purified virus glycoprotein, confirmed the distinction; consequently Duvenhage virus should be considered a new member of the rabies serogroup (i.e. Lyssavirus genus, Rhabdoviridae family).

INTRODUCTION

The rabies serogroup (which will constitute the Lyssavirus genus in the Rhabdoviridae family) consists of rabies and four other viruses: Lagos bat, Mokola, Obodhiang, and kotonkan (Schmidt et al. 1965; Shope et al. 1970; Kemp et al. 1973). A more recently isolated rabies serogroup virus, Duvenhage, has also been reported to differ in antigenic properties from rabies virus (Meredith, Rossouw & van Praag Koch, 1971).

Duvenhage virus was isolated from the brain of a man bitten on the lip by a bat in South Africa. The man subsequently died of a rabies-like illness. Antigen in the brain of the
deceased failed to react with a rabies immunofluorescent conjugate but reacted with a homologous conjugate. To confirm this apparent difference from rabies, the present morphological, biochemical, histopathological and antigenic study of Duvenhage virus was undertaken.

METHODS

Viruses. Duvenhage virus was used at the 6th passage at Yale University and was sent to the Center for Disease Control (CDC) where it was used at the 9th passage level. Duvenhage virus was also received at CDC directly from South Africa and used at the 5th passage level. Other rabies serogroup viruses were: Lagos bat, passage 17; Mokola, strain 1bAn 27377, passage 7; street rabies from a dog in Louisiana, passage 1; rabies, strain CVS; rabies, Flury HEP; rabies from a monkey in Trinidad, TR 5843 (Tignor & Shope, 1972), passage 11; rabies from a bat in Maryland (supplied by C. Wisseman and O. Eylor, University of Maryland), passage 17; rabies from a rodent, strain 1820B (Sodja, Lim & Matouch, 1971), passage 2 at Yale University; rabies, Pasteur, passage > 200; and rabies strain ERA. All viruses were passaged intracerebrally (i.c.) in infant mice to prepare working stocks except rabies strain ERA which was propagated in BHK-21 cells.

Adaptation to cell culture and virus cloning. Duvenhage virus, 6th baby mouse brain passage, was inoculated into BHK-21 cell cultures which were incubated at 35 °C in Eagle’s minimal essential medium containing 10% foetal calf serum. Cell cultures were sub-passaged with a 1:4 splitting ratio at bi-weekly intervals. Virus from the 10th BHK-21 cell passage was thrice clone-purified and used to prepare BHK-21 cell origin virus stocks for antigenic and biochemical characterization.

Preparation of immune reagents. Antibody to whole virus propagated in mouse brain was produced in female mice with multiple intraperitoneal (i.p.) inoculation of virus and Freund’s complete adjuvant (FCA; March & Hetrick, 1967) and with fresh Sarcoma-180 cells or FCA to stimulate the formation of ascitic fluids. In some cases, β-propiolactone was used to inactivate the initial immunogens.

Virus for immunization of rabbits was propagated and purified by methods previously described (Sokol et al. 1968; Sokol & Clark, 1973). Glycoprotein prepared from purified rabies (strain ERA) and Duvenhage viruses by the method of Bishop et al. (1975), was kindly supplied by Dr Pat Repik, University of Alabama Medical Center, Birmingham, Alabama. Rabbits immunized against purified virions were given an initial intravenous inoculation of 2 to 5 µg of virions in NTE buffer (0.13 M-NaCl, 0.05 M-tris-HCl, 0.001 M-EDTA, pH 7.8; Sokol et al. 1968) followed by one to three additional inoculations of a similar dose of virions emulsified in FCA and inoculated at several sites i.m. at bi-weekly intervals. Rabbits were bled 2 weeks after the final inoculation. Rabbits immunized against glycoprotein received three inoculations at bi-weekly intervals of 10 to 20 µg/ml in FCA administered i.m., and were bled 2 weeks after the final inoculation.

Histopathology. Three-week-old ICR mice and LVG hamsters (Charles River Farm) were inoculated i.c. with Duvenhage virus and sacrificed 6 days after infection when moribund. Infant mice were sacrificed 4 days after i.c. inoculation. Sections were stained by haematoxylin and eosin and by inclusion body stains (VO, unpublished stain developed by A. Van Orden, CDC; cited by Murphy et al. 1973).

Electron microscopy. Infant mice were inoculated i.c. with Duvenhage virus and sacrificed 5 days later. Although 3-week-old mice inoculated i.c. developed encephalitis, no virus
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Particles were detected in brains by electron microscopy. Brain specimens from moribund mice were processed as described previously (Murphy et al. 1973).

Immunofluorescent conjugates. The conjugates used were: Duvenhage (guinea pig immunized with infected mouse brain) prepared in South Africa; CVS-rabies, Mokola and Lagos bat conjugates (all mouse ascitic fluids), prepared from antisera to whole viruses. These conjugates were made by isolating IgG on DEAE-Sephadex columns (0.02 M, pH 8.0 phosphate buffer). The IgG was conjugated with fluorescein isothiocyanate (FITC) at a ratio of 8 μg of FITC/mg of protein (Hebert et al. 1972). Immunofluorescence tests were conducted according to the CDC protocol for routine laboratory and reference work including the extinction test for specificity (Murphy et al. 1973).

Complement-fixation and immunization challenge tests in mice. Techniques described by Tignor & Shope (1972) were used. Mice were of the ICR strain.

Neutralization tests. Neutralization tests in mice were performed as described by Shope et al. (1970). Plaque reduction neutralization tests were done in Vero and BHK-21 cells (Wiktor & Clark, 1973; Clark & Wiktor, 1974; Buckley, 1975).

Biochemical techniques. Cultures of BHK-21 cells were infected with 5 p.f.u./cell of Duvenhage or rabies (ERA strain) virus as described previously (Sokol & Clark, 1973). To the infected cells was added 100 ml of Eagle’s minimum essential medium with 0.2% bovine serum albumin (BSA). To label the virus, a 3H-amino acid mixture (15 amino acids, New England Nuclear Corp., Boston, Massachusetts, NTE-250) at 1 μCi/ml was added to the Duvenhage infected cells, while a 14C-amino acid mixture (15 amino acids, New England Nuclear, NEG-445) at 0.5 μCi/ml was added to the rabies infected cells. The cells were incubated at 33 °C and harvested when c.p.e. was evident, about 120 h after inoculation. The supernatant fluid was clarified by centrifugation at 800 g for 15 min, then centrifuged in a type 19 rotor at 19,000 rev/min for 120 min at 4 °C to pellet the virus. The pellet was resuspended in a small volume of NTE, layered on a pre-formed 10 to 60% (w/v) sucrose gradient in NTE, and centrifuged in an SW27 rotor at 22,000 rev/min for 90 min at 4 °C. The virus band was removed from the gradient and dialysed extensively against NTE at 4 °C in a Collodion bag (No. 100; Schleicher and Schuell, Inc., Keene, New Hampshire). Polyacrylamide electrophoresis of the labelled virions was as described by Sokol, Stancek & Koprowski (1971).

RESULTS

Pathogenicity for laboratory animals

Intracerebral inoculation of 10,000 infant mouse LD₅₀ of Duvenhage virus killed suckling and adult mice with average survival times of 4 and 6 days, respectively. Suckling mice receiving 10 LD₅₀ intracerebrally survived 7 days. Suckling hamsters died following intramuscular inoculation of 10,000 LD₅₀ with average survival of 5 days. These survival times most closely resemble those of laboratory strains of rabies virus.

Histopathology in mice

In 3-week-old mice, histopathological lesions were seen only in the brain. Mononuclear inflammatory infiltration was observed as perivascular cuffs in several areas of the brainstem and midbrain. No neuronophagia or neuronal damage was seen. Very few small, indistinct inclusions were seen in the cytoplasm of neurons. The amount of inflammation was far in excess of that expected in mice of this age with street rabies viruses which have been studied, and resembled that seen with fixed rabies virus strains (Fig. 1). In infant mice,
Fig. 1. Perivascular mononuclear inflammatory infiltration in the midbrain of a 3-week-old mouse 6 days after intracerebral inoculation of Duvenhage virus. Cuffing was much more extensive than expected with typical street rabies virus in the same host. Magnification × 520.

Fig. 2. Direct immunofluorescence using a guinea pig anti-Duvenhage virus FITC conjugate. Substrate is an impression of the brain of a suckling mouse which was moribund 4 days after intracerebral inoculation of virus. Magnification × 520.
Fig. 3. Neuron from suckling mouse brain with inclusion body (IB, top) in cytoplasm, and Duvenhage virus particles associated with neuronal processes near its plasma membrane (arrowhead). Magnification × 28000.

Fig. 4. Dense virus inclusion (IB) in the cytoplasm of a neuron undergoing cytopathic changes – vacuolization, condensation. Magnification × 23000.
Fig. 5. Higher magnification of virus inclusion (IB) illustrating the finely filamentous structure (randomly massed nucleocapsid strands) and nearby maturation of virus particles (arrowhead) upon endoplasmic reticulum membranes of the infected neuron. Magnification × 53,000.

Fig. 6. Anomalous branched virus in neuronal cytoplasm. Magnification × 68,000.

Fig. 7. Virus particles budding upon the plasma membrane of neuronal processes, and a free virus particle in extracellular space (arrow). Magnification × 65,000.

Fig. 8. Two virus particles budding into extracellular space from neuronal processes. Such virion maturation upon plasma membranes is rare in street rabies. Magnification × 67,000.
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Fig. 9. Duvenhage, second virus passage, partially separated from BHK-21 cell debris by differential centrifugation. Negative contrast sodium silico-tungstate, pH 6.8. Left: Four virus particles, bullet-shaped, amid endoplasmic reticulum of host cells. Magnification $\times 111,000$. Right: Three virus particles illustrating increasing stain penetration from top to bottom; top: virus envelope with projections; middle: virus envelope and axial channel; bottom: interior of virus particle. Magnification $\times 178,000$. 
Table 1. Rabies serogroup complement-fixation reactions*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Rabies CVS</th>
<th>Rabies TR5843</th>
<th>Duvenhage</th>
<th>Lagos bat</th>
<th>Mokola</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies CVS</td>
<td>512</td>
<td>256</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Rabies TR5843</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Duvenhage</td>
<td>64</td>
<td>16</td>
<td>128</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Lagos bat</td>
<td>32</td>
<td>8</td>
<td>32</td>
<td>1024</td>
<td>128</td>
</tr>
<tr>
<td>Mokola</td>
<td>256</td>
<td>128</td>
<td>32</td>
<td>256</td>
<td>1024</td>
</tr>
</tbody>
</table>

* Results expressed as reciprocal of the ascitic fluid titre. Test conditions included 2 units of C, twofold serum dilution, fourfold antigen dilution, hyperimmune ascitic fluids (four injections of virus).

virus inclusions were also observed by immunofluorescence (Fig. 2), but not by light microscopy.

Ultrastructural pathology in mice

In infant mice, ultrastructural evidence of virus infection was widely distributed. Virus inclusions were usually smaller than those in brain specimens of mice inoculated with street rabies virus (Fig. 3). There was a range in density of virus inclusions from rather dispersed (Fig. 3) to compact and granular (Fig. 4). At high magnification, the convoluted filamentous structure of inclusions was evident (Fig. 5); ultrastructural details were indistinguishable from those in standard rabies strains. Some inclusions were spatially associated with the sites of virion maturation (Fig. 5), some were relatively near (Fig. 3), and in many cases there were no virions near inclusions (Fig. 4). The latter observation most closely resembles that with fixed rabies strains. Virus particles were indistinguishable from standard rabies strains; the particles were bullet-shaped, 180 x 70 nm in dimension, with cross-striations and an envelope with projections (Fig. 6, 7, 8, 9). Most particles matured within neurons upon endoplasmic reticulum membranes. Anomalous particles were also present (Fig. 6). Additionally a modest number of virus particles were found budding from plasma membranes of neuronal cell bodies or neuronal processes (Fig. 7, 8). Budding from plasma membranes of neurons has been considered a characteristic of fixed virus strains (Murphy, 1975). Anomalous virus particles were common as in fixed rabies infections (Fig. 6). Some cytopathology was associated with infection; this was characterized by neuronal cytoplasmic vacuolization, increase in cytoplasmic density, minimal changes in organelle structure and an influx of inflammatory cells into the immediate area of infected neurons. By electron microscopy, there were more inflammatory cells evident than seen by light microscopy.

Infectivity of cell culture

Mouse brain passaged Duvenhage virus produced c.p.e. and plaques in Vero cells within 6 to 8 days after inoculation; plaques were 3 mm in diam. Plaque-cloned virus was found to be indistinguishable by neutralization tests from its parent stock. Duvenhage virus, propagated in BHK-21 cells, induced plaques on agarose-suspended BHK/13S cells (Sedwick & Wiktor, 1967), following only two passages. Thus, adaptation of Duvenhage virus to plaque formation in cell culture was much more rapid than that commonly experienced with street rabies virus strains. The virus stock was tested by complement-fixation tests and was found to be free of LCM, herpes simplex, vaccinia, and over 300 other mouse pathogenic reference viruses in the collection of the Yale Arbovirus Research Unit. This testing was
Table 2. Immunization and challenge of mice with Duvenhage and strains of rabies virus

<table>
<thead>
<tr>
<th>Group*</th>
<th>Vaccination virus</th>
<th>Challenge virus</th>
<th>Log LD₅₀ of challenge virus</th>
<th>Log protection index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Rabies (HEP)</td>
<td>Duvenhage</td>
<td>3.4</td>
<td>1.5</td>
</tr>
<tr>
<td>None</td>
<td>Duvenhage</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Rabies (HEP)</td>
<td>Rabies (Pasteur)</td>
<td>≤ 0.5</td>
<td>≥ 5.9</td>
</tr>
<tr>
<td>None</td>
<td>Rabies (Pasteur)</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Rabies (HEP)</td>
<td>Maryland bat</td>
<td>≤ 0.5</td>
<td>≥ 6.0</td>
</tr>
<tr>
<td>None</td>
<td>Maryland bat</td>
<td>6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Rabies (HEP)</td>
<td>Trinidad monkey TR₅₈₄₃</td>
<td>≤ 0.5</td>
<td>≥ 5.2</td>
</tr>
<tr>
<td>None</td>
<td>Trinidad monkey TR₅₈₄₃</td>
<td>5.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Duvenhage</td>
<td>Rabies (CVS)</td>
<td>4.7</td>
<td>0.2</td>
</tr>
<tr>
<td>None</td>
<td>Rabies (CVS)</td>
<td>4.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Duvenhage</td>
<td>Duvenhage</td>
<td>≤ 0.5</td>
<td>≥ 4.4</td>
</tr>
</tbody>
</table>

* Groups A to D, six-week-old mice immunized i.c. (0.03 ml) and challenged 30 days later i.c. with serial tenfold dilutions of challenge virus, 6 mice per dilution. Groups E and F, Six-week-old mice immunized i.p. (Habel Test) and challenged i.c.

done to rule out contaminating virus in the Duvenhage stock as the explanation for the antigenic differences between Duvenhage and rabies viruses.

Complement-fixation tests

In complement-fixation tests using hyperimmune ascitic fluids, significant differences were shown between Duvenhage virus and other viruses of the rabies serogroup (Table 1). The magnitude of the cross-reactions between Duvenhage virus and rabies was equal to or greater than the cross-reactions between Duvenhage virus and Mokola or Lagos bat virus. There were no cross-reactions between Duvenhage virus and kotonkan or Obodhiang viruses (data not shown).

Immunization-challenge tests

Immunization with rabies virus (HEP) i.c. gave minimal protection against Duvenhage challenge, and protection was complete when rabies immunized mice were challenged with diverse isolates of rabies virus (Table 2). In tests employing Duvenhage immunization i.p. followed by rabies virus challenge, either i.c. or i.p., there was no significant cross-protection, but once again, homologous protection was effective.

In other experiments, sera of mice immunized with Duvenhage virus were found to have a log neutralization index of 4.0 when tested in a baby mouse neutralization test with rabies virus; the homologous log neutralization index was 5.1. These same Duvenhage virus immune mice did not resist i.c. challenge with rabies virus (0.2 log protective index).

Structural polypeptides

Analysis of structural polypeptides of the ERA strain of rabies virus and Duvenhage virus, labelled respectively with ^14C- and ^3H-amino acid mixtures, showed similar patterns. Using the known mol. wt. of the G, N, M₁, and M₂ proteins of rabies virus (ERA strain) as markers (Sokol et al. 1971), we estimated the mol. wt. for the G, N, M₁, and M₂ proteins of Duvenhage virus by the procedure of Shapiro, Viñuela & Maizel (1967). The mol. wt. for
the G, N, M₁, and M₂ proteins are 80,000, 62,000, 41,000 and 28,000, respectively. The L protein of rabies virus has been shown to co-migrate with the L protein of vesicular stomatitis virus (H. P. Madore & J. N. England, unpublished observations) which has been estimated to have a mol. wt. of 190,000 (Wagner et al. 1972). The L protein of Duvenhage co-migrates with that of rabies virus and, therefore, has an apparent mol. wt. of 190,000.

**Immunofluorescence tests**

In initial immunofluorescence tests, infected brain impressions were stained with a rabies conjugate diluted to twofold less than the last dilutions giving 4+ specific homologous fluorescence. All Duvenhage specimens were negative; all CVS-rabies control specimens were positive (4+). In a subsequent test, Duvenhage virus was passaged in suckling mouse brain and impressions were stained with concentrated rabies conjugate, just as used in routine and reference diagnostic testing. Positive (4+) reactions were obtained with Duvenhage virus infected mice as well as with CVS-rabies infected mice. Controls were negative; these included tests with uninfected mouse tissues and tests in which homologous virus suspension was used to pre-adsorb conjugate reactivity. A Duvenhage virus conjugate, received from South Africa gave positive (4+) brightness with homologous and CVS-rabies suckling mouse brain impressions when an arbitrary dilution of 1:4 was used. Therefore, in a diagnostic setting, where conjugate dilutions are arbitrary, identification of Duvenhage virus might be inconsistent. Furthermore, these results confirmed the reality of disparities noted in the initial tests on Duvenhage virus carried out in several laboratories in the U.S.A. In order to explain further the relationship between rabies and Duvenhage virus as detected by immunofluorescence, quantitative testing was done.

As shown in Table 3, suckling mouse brain impressions were stained with varying dilu-
Table 5. *Mouse neutralization test reactions of rabies and Duvenhage viruses using undiluted antibody*  

<table>
<thead>
<tr>
<th>Viruses (suckling mouse brain stock)</th>
<th>Rabies</th>
<th>Duvenhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse immune ascitic fluid</td>
<td>CVS</td>
<td>Street</td>
</tr>
<tr>
<td>Rabies (CVS)</td>
<td>$2.0 \ (5.3)^\ddagger$</td>
<td>$\leq 0.5 \ (\geq 2.8)$</td>
</tr>
<tr>
<td>Duvenhage A</td>
<td>$2.8 \ (4.5)$</td>
<td>$\leq 0.5 \ (\geq 2.8)$</td>
</tr>
<tr>
<td>Duvenhage B</td>
<td>$2.0 \ (5.3)$</td>
<td>$\leq 0.5 \ (\geq 2.8)$</td>
</tr>
<tr>
<td>Titre in normal mouse ascitic fluid</td>
<td>$7.3$</td>
<td>$3.3$</td>
</tr>
<tr>
<td>Titre in diluent prior to incubation</td>
<td>$8.5$</td>
<td>$3.6$</td>
</tr>
</tbody>
</table>

* In suckling mice/0.03 ml i.c.; virus dilution–undiluted antibody.  
† A and B represent different passage levels in replicate titrations.  
‡ ( ), log neutralization index (Kärber, 1930).  
§ Diluent was 0.75 % bovine albumin in phosphate-buffered saline.

Table 6. *Mouse neutralization test reactions of rabies and Duvenhage viruses by antibody dilution technique*  

<table>
<thead>
<tr>
<th>Viruses (suckling mouse brain stock)</th>
<th>Rabies</th>
<th>Duvenhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse immune ascitic fluid</td>
<td>CVS</td>
<td>Street</td>
</tr>
<tr>
<td>Rabies (CVS)</td>
<td>$3125^\ddagger$</td>
<td>$1325$</td>
</tr>
<tr>
<td>Duvenhage A</td>
<td>$1420$</td>
<td>$5500$</td>
</tr>
<tr>
<td>Duvenhage B</td>
<td>$342$</td>
<td>$940$</td>
</tr>
<tr>
<td>Inoculum LD$_{50}$</td>
<td>$113$</td>
<td>$75$</td>
</tr>
</tbody>
</table>

* In suckling mice/0.03 ml i.c. using the virus constant–antibody dilution technique; dilutions were fivefold; reagents were prepared at CDC.  
† A and B represent different passage levels in replicate titrations.  
‡ Reciprocal of 50 % ascitic fluid dilution end-point (Kärber, 1931).  

Further cross-reactions by direct fluorescent antibody tests are shown in Table 4; in these tests suckling mouse brain impressions were used as substrates and conjugates were diluted twofold less than the terminal dilution. The conjugates used were: Duvenhage (guinea pig, prepared in South Africa), CVS-rabies, Mokola and Lagos bat. The results indicated that at these single dilutions the South African Duvenhage conjugate was rather specific, but all other conjugates showed a degree of cross-reactivity which has been typical in most comparisons of the rabies-related viruses.

Indirect immunofluorescence testing was also used to compare the cross-reactivities of immune reagents. In two-way box comparisons, homologous Duvenhage and rabies titres (fluorescent brightness end-points) were $1:320$ and $1:640$, respectively; both heterologous...
Table 7. Cross-plaque neutralization tests in Vero cells with cloned rabies and Duvenhage viruses

<table>
<thead>
<tr>
<th>Cloned virus</th>
<th>Hyperimmune mouse serum</th>
<th>Rabies CVS (135 p.f.u.)</th>
<th>Duvenhage (124 p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies (CVS)</td>
<td>65536*</td>
<td>4096</td>
<td></td>
</tr>
<tr>
<td>Duvenhage</td>
<td>2048</td>
<td>8192</td>
<td></td>
</tr>
</tbody>
</table>

* Titres expressed as reciprocal of serum dilution that gave a 50% reduction of plaques; antisera were prepared against cloned virus.

Table 8. Comparison of Duvenhage and rabies virus anti-glycoprotein and anti-virion sera by plaque reduction neutralization test in BHK/13S cells

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Rabies (LNI)*</th>
<th>Duvenhage (LNI)</th>
<th>Rabies (SN)†</th>
<th>Duvenhage (SN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies (ERA) glycoprotein</td>
<td>&gt;4.7</td>
<td>&gt;6.0</td>
<td>15000</td>
<td>1480</td>
</tr>
<tr>
<td>Duvenhage glycoprotein</td>
<td>2.4</td>
<td>&gt;6.0</td>
<td>240</td>
<td>1440</td>
</tr>
<tr>
<td>Rabies (ERA) virion</td>
<td>6.6</td>
<td>4.0</td>
<td>10000</td>
<td>380</td>
</tr>
<tr>
<td>Duvenhage virion</td>
<td>0.5</td>
<td>4.5</td>
<td>38</td>
<td>1280</td>
</tr>
</tbody>
</table>

* LNI = log neutralization index: ERA $10^{6.8}$ p.f.u./0.1 ml; DUV $10^{6.3}$ p.f.u./0.1 ml.
† SN = reciprocal of serum dilution neutralizing 50% plaques; 100 p.f.u./0.1 ml.

titres were 1:160. Thus, smaller differences between the two viruses were observed than with any other serological approach including complement-fixation testing.

Neutralization tests

The results of virus dilution and serum dilution neutralization tests in mice are given in Tables 5 and 6, respectively. In all the tests homologous reactions are greater than heterologous; the amount of cross-reaction seems to depend upon the test. There was a suggestion of an asymmetric neutralization pattern in the virus dilution test; this 'one-way' cross-reaction was extreme in the serum dilution test. In each instance, rabies antiserum did not completely neutralize Duvenhage virus. In the serum dilution test it may be seen that undiluted rabies immune ascitic fluid protected against Duvenhage virus, but 50% protection end-points were reached at 1:7 and 1:9 dilutions; whether these extremely low titres are indicative of real antibody effects is not known. Differences of this order of magnitude have not been found when strains of street rabies virus have been compared by serum dilution mouse neutralization tests (Crandell, 1966), but have been consistent in comparisons of Mokola and Lagos bat viruses with rabies virus (Shope et al. 1970).

Plaque-cloned Duvenhage and CVS-rabies viruses were compared by plaque reduction neutralization tests in Vero cells; the rabies ascitic fluid titre was 16-fold higher with the homologous than the heterologous virus while less difference was seen with the Duvenhage ascitic fluid (Table 7). The differences between Duvenhage and rabies (CVS) by this test were only slightly greater than those found by Wiktor & Clark (1973) among four fixed rabies virus strains.
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Fig. 10. Comparison of Duvenhage and rabies virus structural polypeptides. Duvenhage (■—■) and rabies (-——-) viruses were labelled with \(^3\)H- and \(^{14}\)C-amino acids respectively, purified, and co-electrophoresed on 7.0% polyacrylamide gels as described in Methods.

Plaque reduction neutralization tests in BHK/13S cells using rabbit anti-glycoprotein and anti-purified virion sera also showed differences between Duvenhage and rabies viruses (Table 8). However, the asymmetric cross-neutralization pattern, which was evident in the in vivo neutralization tests, was reversed in these plaque reduction tests. The rabies antisera (both anti-purified virion and anti-glycoprotein) were much more reactive with homologous viruses than heterologous. In the case of rabies (ERA) antibody in the virus dilution test, no conclusion can be drawn because the end-points were not reached.

DISCUSSION

Even from the original observations made in South Africa by Meredith et al. (1971), Duvenhage virus was considered to be distinct from rabies virus. In the present study, the distinctions between the two viruses were shown to extend to several biological, biochemical and immunological characteristics.

Taken together, Duvenhage virus characteristics did not follow the pattern of any particular rabies virus strain, but the differences between Duvenhage and rabies viruses were of a magnitude similar to those previously shown between rabies and Mokola and Lagos bat viruses (Shope et al. 1970).

The character of Duvenhage virus morphogenesis in mouse brain and the prominence of the inflammatory infiltration evoked by the infection were unexpected in view of the relatively low passage level of the virus stock. The fact that Duvenhage virus was of bat origin may provide a partial explanation; Hurst & Pawan (1931, 1932) found that bat strains of rabies produced lesions in man and in rabbits which were indistinguishable from those caused by fixed virus, i.e. the bat strains caused much more inflammation than other street viruses.
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The structural polypeptide composition of Duvenhage virus was remarkably similar to that of rabies virus. This is in contrast to the marked differences previously found between the polypeptides of Lagos bat and Mokola viruses and that of rabies virus (Sokol & Koprowski, 1975). This evidence suggests that Duvenhage virus is more closely related to rabies virus than are either Mokola or Lagos bat viruses.

Despite these biological, morphogenetic and physicochemical comparisons which indicated that Duvenhage virus is very similar to rabies virus, the precise distinction of the virus comes from serological testing—immunization challenge, immunofluorescence, complement-fixation and neutralization. Immunization challenge tests in mice indicated significant differences between Duvenhage and rabies viruses. Mice immunized with rabies virus intracerebrally, a route of inoculation which produces high serum antibody titres, were more than 10000 times more susceptible to Duvenhage virus challenge than to homologous challenge. Duvenhage virus immunization was ineffective in protecting against rabies virus challenge. These cross-reactions were similar in magnitude to those previously observed in immunization challenge tests between rabies and other rabies-related viruses (Tignor & Shope, 1972), but were much greater than differences found by Wright & Habel (1948) in their studies of various strains of the Pasteur fixed rabies virus. In other studies, no differences were found among bat isolates of rabies by cross-protection and other tests (Johnson, 1948; Schroeder et al. 1952).

Several neutralization testing techniques were included in this study to represent the variety being used in different laboratories for strain comparisons. Variations included mouse and cell culture substrates, sera and/or immune ascitic fluids produced in rabbits or mice against crude, purified or sub-unit virus immunogens. In all neutralization tests Duvenhage virus was distinguishable from rabies virus. A one-way cross-reaction occurred in the \textit{in vivo} tests; Duvenhage antibody neutralized rabies virus much more than did the converse pairing. This asymmetric neutralization pattern was reversed in \textit{in vitro} tests using antisera against purified virions or their glycoproteins, and when cloned viruses were used in \textit{in vitro} tests there was no asymmetry and more pronounced cross-reaction. As in all neutralization testing, particular methods may be chosen to emphasize similarities or differences, but direct comparisons of the results of varying methods are not straightforward. The work of Clark & Wiktor (1974) on the plasticity of phenotypic characteristics of Lagos bat and Mokola viruses bears on this point. It was shown that neutralization of individual clones of these two rabies-related viruses varied; virus derived from one clone of Lagos bat virus was consistently neutralized to a greater degree by rabies antiserum than was uncloned Lagos bat virus or two other clones. Thus, the qualities of the viruses themselves, as well as the qualities of immune sera, influence comparative results. There is at present conflicting evidence as to whether rabies virus has one or two glycoproteins (Gyorgy, Sheehan & Sokol, 1971; Neurath et al. 1972; Sokol & Koprowski, 1975); if antigenic determinants are expressed in multiple, complex structural entities on the surface of virus particles, the variability of neutralization may reflect a varying dominance of different determinants in different tests.

The degree of specificity of diagnostic fluorescent antibody tests in distinguishing Duvenhage virus from rabies depended upon the reagents employed and their dilution. When immunofluorescent conjugates were used in low dilutions on rabies and Duvenhage virus substrates, cross-reactivity was complete. However, at terminal dilutions, conjugates only reacted with their homologous antigens. It was clear that in South Africa, in a diagnostic setting, the use of an arbitrary single anti-rabies conjugate dilution, as is customary throughout the world, resulted in the human disease remaining undiagnosed (terminal homologous dilution of conjugate) but could have resulted in an identification of Duvenhage virus as a
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conventional rabies strain (excess conjugate). In this case, the exceptional pattern obtained with the human virus isolate was recognized by the South African workers (Meredith et al. 1971). If, in a diagnostic setting, viruses like Duvenhage are to be distinguished then there must be more consideration to the breadth of specificity and relative potency of immunofluorescence reagents. Perhaps two dilutions of the conventional rabies conjugate should be used; rabies isolates would be positive with low and terminal dilutions, but viruses like Duvenhage only with low dilutions. Alternatively, conjugates with broad specificity (anti-ribonucleoprotein) could be used together with narrowly specific reagents (anti-glycoprotein).

When an exceptional virus like Duvenhage emerges from the diagnostic setting, further understanding of the varying contribution of each virus structural component in each serologic test system is needed. It is well known that neutralization specificity depends upon the antigenicity of glycoproteins exposed at the surface of virus particles, and that complement-fixation specificity is dominated by virus ribonucleoprotein which is present within virus particles and which accumulates in large amounts within infected cells (the Negri body). Results of neutralization and complement-fixation tests taken together with tests employing antisera to purified virus glycoproteins, clearly showed that the major differences between rabies and Duvenhage viruses are expressed at the virus surface. In further complementary testing, antisera were prepared against purified ribonucleoprotein of rabies and Duvenhage viruses; these rabbit antisera were tested by indirect immunofluorescence, by a solid phase indirect radioimmunoassay and by neutralization. These sera, as expected, did not have neutralizing activity, but in both the immunofluorescence and radioimmunoassays Duvenhage virus was not distinguishable from rabies virus (Lee, 1975). Similar comparisons have been made previously with other rabies-related viruses (Schneider & Schoop, 1974).

For the compelling reason that Duvenhage virus represents a potential public health problem in South Africa, it deserves classification as a distinct virus—related to, but different from rabies virus. Criteria for establishing subdivision of the rabies serogroup were originally suggested by Schneider et al. (1973); more recently the International Committee on Taxonomy of Viruses has proposed a division of the Rhabdoviridae family to include a genus, Lyssavirus, for the rabies-related viruses (Fenner, 1976). On the basis of the evidence presented here, Duvenhage virus should be accorded the status of a specific virus in this genus, along with rabies, Mokola and Lagos bat viruses. At this time, the taxonomic status of kotonkan and Obodhiang viruses is uncertain.

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