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RADIAL HAEMOLYSIS IN GEL USED IN SEROLOGICAL STUDIES ON VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

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Summary. — Radial haemolysis in gel was successfully used for the detection of antibody in convalescents after laboratory infection with Venezuelan equine encephalomyelitis (VEE) virus at remote intervals after infection (up to 23 years). The antibodies reacted only with VEE antigen but not with Sindbis and Chikungunya alphavirus antigens. Radial haemolysis in gel was sensitive as the haemagglutination inhibition test but the antibody could be titrated by the diameter of the haemolysis zone omitting the serum dilutions. Slight deviations in the virus dose used for sensitization of erythrocytes or in pH values were not critical. For the test, the sera were heated at 56 °C for 30 min. Both sera stored for long periods and those collected on paper disks could be used.

Key words: radial haemolysis; *Alphavirus*; *laboratory diagnosis*

Introduction

Recently, we have modified the radial haemolysis in gel (RH) test for arboviruses (Gaidamovich and Melnikova, 1979). In the present study we explored the possibility of using RH for serological investigations of human arbovirus infections. The model was Venezuelan equine encephalomyelitis (VEE) virus and the blood sera were collected from convalescents after a laboratory infection which had occurred more than 20 years before. At that time, the diagnosis of the disease was verified by virus isolation or seroconversion (Shubladze *et al.*, 1959; Slepushkin, 1959). We compared the sensitivity of RH with that of the haemagglutination inhibition (HI) test for antibody detection, and investigated the use of RH for measuring humoral immunity levels at remote intervals post infection, optimal conditions for RH performance including the use of sera collected on filter paper disks.

Materials and Methods

Antigens. A sucrose-acetone (SA) antigen from the brains of VEE virus-infected suckling mice (Trinidad strain) inactivated with beta-propiolactone, and a tissue culture antigen from the attenuated VEE-230 strain were used. The latter was the culture fluid from virus-infected chick

Table 1. Examinations of sera from VEE convalescents and healthy persons in RH and HI tests with SA antigens

Serum No.	Years after infection	Haemolysis zone diameter (mm)			HI titre		
		VEE	Sindbis	Chik	VEE	Sindbis	Chik
1	9	10	0	0	160	0	0
2	9	11	0	0	160	0	0
3	9	6	0	0	40	0	0
4	9	5	0	0	20	0	0
5	23	9	0	0	40	0	0
6	12	10	0	0	80	0	0
7	12	8	0	0	40	0	0
8	9	6	0	0	80	0	0
9	17	7	0	0	40	0	0
10	11	11	0	0	160	0	0
11	11	7	0	0	40	0	0
12	4	5	0	0	20	0	0
13	9	11	0	0	160	0	0
14	9	7	0	0	40	0	0
15	9	9	0	0	80	0	0
Controls (15 sera)		0	0	0	0	0	0
IAF VEE		15	0	0	640	nt	0
IAF Sindbis		0	12	0	nt	320	0
IAF Chik		0	0	17	nt	nt	1280

nt = not tested.
0 means no reaction.

fibroblast cultures grown in medium 199 without serum, and was concentrated 6-8-fold with polyethylene glycol (Loginova *et al.*, 1973). For specificity control, the tests were performed with SA brain antigens of Sindbis and Chikungunya (Chik) viruses belonging, like VEE virus, to the genus Alphavirus. The haemagglutinin titres of the antigens were from 1280 to 2560.

Sera. Fifteen sera from convalescents and an equal number of sera from laboratory workers who had had no contact with VEE virus were tested. Serum specimens collected at various

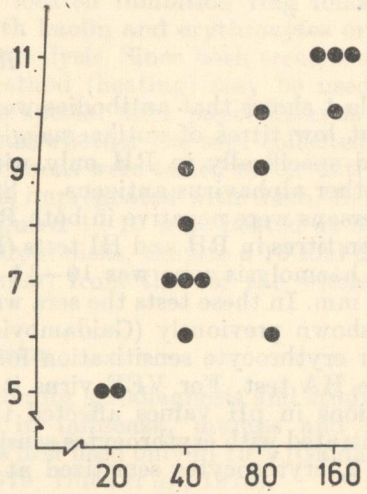

Fig. 1.

Comparison of antibody titres to VEE virus as determined by RH and HI tests in convalescent sera
Abscissa: HI titre (dilution reciprocals); ordinate: diameter (mm) of haemolysis zone

Table 2. Titre of IAF to VEE virus in RH test with sheep erythrocytes sensitized at pH 6.0, 6.2, and 6.4

IAF dilutions	Haemolysis zone diameter (mm) with erythrocytes sensitized at pH 6.0	6.2	6.4
Undiluted	15	15	13
1 : 2	15	14	12
1 : 4	13	13	11
1 : 8	12	12	10
1 : 16	10	10	9
1 : 32	10	9	6
1 : 64	9	8	5
1 : 128	7	6	4

periods of convalescence (from 4 to 23 years) were stored in the laboratory in lyophilized form. The sera were reconstituted to the original volume with distilled water. Depending on the aim of the experiment, they were treated with kaolin and erythrocytes (Clarke and Casals, 1958), or heated at 56 °C for 30 min or used native. To test the possibility of using sera collected on filter paper disks in RH 0.1 ml of serum was placed on each disk (Schleicher and Schuell No. 740-E). A few days later the serum was extracted in 1 ml of phosphate buffer, pH 7.2, for 18 hr at 4 °C. In addition to convalescent sera, mouse immune ascitic fluids (IAF) to VEE, Sindbis and Chik viruses were used.

Haemagglutination (HA) and haemagglutination inhibition (HI) tests (Clarke and Casals, 1958) were performed by the micromethod. Sera for the tests were treated with goose erythrocytes and kaolin. The HI test was run with 8 agglutinating units at pH 6.0 (VEE, Sindbis) and pH 6.4 (Chik).

Radial haemolysis (RH). Sheep erythrocytes were sensitized with VEE and Sindbis virus antigens at pH 6.0 and Chik antigen at pH 6.4, and then together with complement added to melted (42 °C) 1.5% agarose A-37 (IBF, France) as described in detail by Gaidamovich and Melnikova (1979; 1980). The mixture was pojured on a 6 × 8 cm plate and after the agarose had solidified, wells of 2 mm diameter were cut in it; 5-10 μl of the test sera were added to the wells. After 18 hr of exposure at 37 °C in a humid chamber the results were read by the diameter of the haemolysis zone expressed in mm. The results was considered to be positive at a haemolysis diameter ≧ 4 mm.

Results

Table 1 shows that antibodies were found by RH in all convalescent sera even at low titres of antihaemagglutinins (20—40). The convalescent sera reacted specifically in RH only with the homolous VEE antigen but not with other alphavirus antigens — Sindbis and Chik. The sera from the control persons were negative in both RH and HI tests. There was a correlation between titres in RH and HI tests (Fig. 1). At a HI titre of 160 the diameter of the haemolysis zone was 10—11 mm, and at a low HI titre of 20 it was only 5 mm. In these tests the sera were heated at 56 °C for 30 min.

As shown previously (Gaidamovich and Melnikova, 1979), the optimum pH for erythrocyte sensitization for RH corresponded to the optimum pH for the HA test. For VEE virus it was 6.0. To find out how much slight deviations in pH values affected the results of tests, IAF to VEE virus were titrated with erythrocytes sensitized at pH 6.0, 6.2, and 6.4. As shown in Table 2, erythrocytes sensitized at pH 6.2 yielded the same results as at

Table 3. RH with sera from VEE convalescents

Serum No.	Haemolysis zone diameter (mm) with	
	Whole sera	Sera extracted from filter paper disks diluted 1 : 10
1	10	9
2	11	9
3	6	5
5	9	8
6	10	9
7	8	6
8	6	5

the optimal pH 6.0 but erythrocytes sensitized at pH 6.4 revealed somewhat lower antibody titres.

Nor was the dose of antigen used for sensitization critical; it could be varied slightly. Similar antibody titres were obtained after sensitization of erythrocytes with both undiluted SA antigen (2560 haemagglutinating units — HAU) and diluted 4-, 8-, and 16-fold (640, 320, and 160 HAU, respectively). Both culture antigens and SA brain antigens could be used for erythrocyte sensitization. Selective examinations of sera Nos. 3, 6, 7, 10, 11, and 14 showed that the degree of haemolysis was similar in both cases.

It is known that sera to be examined by HI tests have to be pretreated to remove inhibitors and heteroagglutinins. To find out whether these factors affect the results of RH, both native sera and sera treated with kaolin and erythrocytes or heated at 56 °C for 30 min were used in RH. Selected results of this experiment are shown in Fig. 2 (Plate V). Untreated sera produced incomplete, opaque haemolysis with indefinite borders. Two zones were formed around the wells: a centrally located inhibition ring followed by a haemolysis ring. The sera treated with kaolin and erythrocytes or heated at 56 °C produced clear transparent haemolysis. Since both treatments gave similarly good results, the simplest method (heating) may be used alone.

Currently, blood serum collection on special filter paper disks is finding wider and wider application. To find out whether the sera collected in this way were suitable for RH, 7 serum specimens were tested in the native form and after extraction of filter paper disks impregnated with them. Before the test, both native and extracted sera diluted 1 : 10 were heated at 56 °C for 30 min. The titre of the sera extracted from disks, despite a 10-fold dilution, did not differ significantly (by 1—2 mm) from that of the whole serum Table 3).

Discussion

RH has already found wide application for serodiagnosis and study of the immune structure of the population in influenza, mumps and rubella. With arboviruses, however, this test was first used only in 1979 (Gaidamovich and Melnikova, 1979, 1980; Odelola, 1979; Duca *et al.*, 1979).

In the present study RH was first used for serological studies on antibodies to VEE virus. Close agreement of the results of RH and HI tests suggests that RH is as sensitive as the routine HI test. Odelola (1979) and Duca *et al.* (1979) gave the same evaluation of RH in studies on antibodies to West Nile and yellow fever viruses.

The simplicity of the technique and the possibility of determinig antibody titres by the haemolysis diameter without using serum dilutions allow examination of large numbers of sera within a short time. The advantages of RH also include the simple treatment of sera consisting only in heating at 56 °C for 30 min. Slight deviations from optimal pH values and antigen doses for sensitization of erythrocyte do not affect significantly the results of the test. Sera collected on filter paper disks may be used.

As shown by our study, antibodies to VEE virus circulate in the hosts for a long time (the observation period — 23 years). This permits using RH for serodiagnosis and for the detection of virus circulation in natural foci at remote intervals.

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