THE USE OF RIVANOL FOR THE REMOVAL OF NON-SPECIFIC INHIBITORS OF RUBELLA VIRUS HAEMAGGLUTININ

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HOŘEJŠÍ AND SMETANA (1956) have shown that rivanol, when added in increasing quantities to human or animal serum, will precipitate successively albumin, alpha-globulins and beta-globulins. The resulting filtrate contains 97–98 per cent. of the total gamma-globulin. Geft and Polyak (1963) successfully employed this simple and rapid technique to remove non-specific inhibitors of influenza virus from horse serum. It has also been used to remove non-specific inhibitors of arbovirus haemagglutinins (HA) from human and various animal serum (Duca and Buiuc, 1970; Grešíková and Sekeyová, 1970).

The non-specific serum inhibitor of rubella virus HA has been identified as a beta-lipoprotein (Sedwick, Furukawa and Plotkin, 1968), and Stastný and Hořejší (1961) have reported that beta-lipoproteins can be precipitated from human serum by rivanol at low ionic strength. We have evaluated this technique and compared it with the dextran sulphate-CaCl₂ extraction method (Liebhaber, 1970*b*) for the preparation of sera for the rubella haemagglutination-inhibiting (HI) antibody test.

MATERIALS AND METHODS

Diluent

This was HEPES-serum albumin-gelatin-buffered saline (HSAG) composed of 0.025M-HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), 0.14M-NaCl, 0.0005M-CaCl₂, 1 per cent. bovine serum albumin (BSA) and 0.0001 per cent. gelatin (Toms and Mostratos, unpublished; Liebhaber, 1970*a*).

Erythrocytes

These were obtained from adult pigeons, unfed day-old chicks and adult fowls. Whole blood was collected into approximately four volumes of Alsever's solution (Bukantz, Rein and Kent, 1946). The red blood cells (RBC) were washed three times in PBS(A) (Dulbecco's phosphate-buffered saline, solution A, Oxoid Ltd, London), resuspended to 10 per cent. (v/v) in Alsever's solution and stored at 4°C. Pigeon cells were generally discarded after 4 days' storage, chick and fowl cells after 7 days'. For use, the cells were washed a further three times in PBS(A) and resuspended to the initial volume in the same diluent. HA and HI tests were performed with a 1 in 40 dilution of this suspension in HSAG, i.e., with a 0.25 per cent. suspension of red cells.

Haemagglutinins

The following batches of rubella virus HA were used in the various experiments. (a) Rubella HA Antigen batch C961268, purchased from Flow Laboratories (Rockville, Maryland,

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USA) (Flow HA 1). (b) Rubella HA Antigen batch C961273, purchased from Flow Laboratories (Flow HA 2). (c) Rubella HA, kindly supplied by Dr C. M. P. Bradstreet (Public Health Laboratory Service Standards Laboratory for Serological Reagents, Colindale Avenue, London), prepared by the method of Halonen, Ryan and Stewart (1967) (Colindale HA). (d) Rubella HA, kindly supplied by Dr M. Longson of the Pasteur Institute (Paris, France). This antigen consisted of the supernatant of rubella virus-infected baby hamster kidney cells (BHK21), treated with 1 in 4000 formaldehyde to remove infectivity (Pasteur HA). (e) Rubella HA prepared in this laboratory by the following method. Monolayer cultures of BHK21 cells were infected with rubella virus "West Point", kindly supplied by Professor A. Dudgeon (Children's Hospital, Great Ormond Street, London), at a multiplicity of 0.03 plaque-forming units per cell, and these were maintained on Eagle's minimal essential medium containing 2 per cent. kaolin-extracted foetal calf serum (Stewart *et al.*, 1967). Four days later the supernatant was collected, centrifuged at low speed to remove cell debris and stored, as antigen, at -20°C (WP22164 HA). (f) Rubella HA prepared in this laboratory as above but treated with Tween 80 and ether (Norrby, 1962) before storage (WP12264T/E HA).

Haemagglutination tests

Except where stated otherwise, titrations of HA were carried out by the method of Stewart *et al.*

Collection and storage of sera

Human test sera were obtained from female medical students and laboratory personnel. The blood was allowed to clot and the serum was separated. Residual RBC were removed by low-speed centrifugation and the serum specimens were then divided into 0.2-ml amounts and stored at -20°C .

Dextran sulphate- CaCl_2 method of serum extraction

Serum in 0.2-ml amounts was extracted with dextran sulphate and CaCl_2 by the method of Liebhaber (1970b). If day-old chick RBC were used in the HI test, the sera were absorbed with adult fowl cells to remove any red-cell agglutinins.

Rivanol method of serum extraction

Unless stated otherwise, sera were extracted by the following modification of the method of Hořejší and Smetana. A 1 per cent. solution of rivanol (2-ethoxy 6, 9-diaminoacridine lactate, Winthrop Chemical Corporation, Newcastle upon Tyne) was prepared in cold distilled water. This was diluted 2 in 5 in borate saline buffer, pH 9.0 (Clarke and Casals, 1958), to give a 0.4 per cent. solution of rivanol at pH 9.0. The final, clear bright yellow solution was stored at 4°C .

Serum (0.2 ml) was first absorbed with an equal volume of a 50 per cent. suspension of adult fowl or pigeon erythrocytes in diluent. The mixture was shaken thoroughly and held at 4°C for 30 min. The RBC were then removed by centrifugation at 2000 r.p.m. for 15 min. in the cold. Rivanol—0.7 ml of 0.4 per cent. solution—was added slowly to the absorbed serum with constant agitation. This resulted in the formation of a bright yellow precipitate. The serum was kept for 10 min. at 4°C and the precipitate was then sedimented by centrifugation at 2000 r.p.m. for 10 min. The yellow-tinted supernatant was decanted into a Kahn tube containing 0.03 g of activated charcoal to remove excess rivanol. The tube was agitated violently for 15 s on a Rotamixer, by which time the yellow tinge had usually disappeared. The contents of the tube were immediately decanted into a 2 ml syringe and expelled through a 13-mm Swinney membrane filter. The resulting clear, colourless filtrate was used in the HI test as a 1-in-4 dilution of the original serum specimen.

The haemagglutination inhibition (HI) test

HI test were performed by the method of Liebhaber (1970b). The WP12264T/E HA antigen was used in all tests.

RESULTS

Extraction of serum with rivanol dissolved in distilled water or in buffer at pH 9.0

Six serum specimens, known to be without neutralising activity for rubella virus, were first extracted by the original method of Hořejší and Smetana, i.e., with a 0.4 per cent. solution of rivanol in distilled water. This method proved to be unreliable for removing non-specific inhibitors of rubella virus HA. Results were variable and the serum, extracted on different days, showed HI activity ranging from <1 in 4 to 1 in 32. The same six sera, when extracted by the dextran sulphate-CaCl₂ method always showed complete absence of non-specific activity.

Several workers have reported that precipitation of serum proteins is dependent upon pH (Boettcher, Kistler and Nitschmann, 1958; Saifer and Lipkin, 1959), and Duca and Buiuc have successfully removed inhibitors of arbovirus HA from animal and human sera with rivanol dissolved in dilute borate saline buffer, pH 9.0. When the six sera were extracted with a 0.4 per cent. solution of rivanol in borate saline buffer, at pH 9.0, there was complete and reproducible loss of non-specific inhibitor activity.

Schmidt and Lennette (1970) have reported that human sera have higher levels of natural agglutinins for pigeon erythrocytes than for chick erythrocytes, and that these are often difficult to absorb from test sera. Early work in this laboratory with the dextran sulphate-CaCl₂ method of serum extraction confirmed these findings. It was found, however, that when the rivanol method of serum extraction was employed, the natural agglutinins for pigeon cells could then be easily removed by a single 30-min. absorption with 50 per cent. pigeon erythrocytes. Comparable HI antibody titres were obtained in duplicate tests with pigeon erythrocytes and chick erythrocytes.

Comparison of the buffered rivanol and dextran sulphate-CaCl₂ extraction techniques in the HI antibody test

The buffered rivanol and dextran sulphate-CaCl₂ extraction methods were used on 25 serum samples collected from unselected undergraduates and laboratory personnel. Duplicate samples of each serum were extracted with dextran sulphate-CaCl₂ or rivanol and were then titrated for rubella HI antibodies, at the same time but on separate plates. The plates were assigned coded identification numbers and the results were read blindly by an uninvolved colleague (table I). With 24 of the 25 sera, the two methods of extraction gave comparable results. The occasional two-fold differences in antibody titres are within the limits of experimental error and are not significant.

One serum sample, RR, had an HI antibody titre of 8 after rivanol extraction, but no detectable antibody after dextran sulphate-CaCl₂ treatment. Further testing of this serum showed this difference to be reproducible; the false positive reactions previously obtained with rivanol in distilled water were characterised by their lack of reproducibility. Because the serum possessed

rubella virus neutralising antibody to a dilution of 1 in 7, the findings suggested that the rivanol method was more reliable than the dextran sulphate- CaCl_2

TABLE I

Comparison of the rubella haemagglutination-inhibiting (HI) antibody titres of 25 human sera after extraction with rivanol or dextran sulphate- CaCl_2

Serum	HI antibody titre after extraction with		Serum	HI antibody titre after extraction with	
	dextran sulphate- CaCl_2	rivanol		dextran sulphate- CaCl_2	rivanol
SP	<4	<4	FD	128	256
MP	<4	<4	BK	256	128
AS	<4	<4	PB	256	128
SM	<4	<4	MK	256	128
JH	<4	<4	SP	256	128
GT	<4	<4	JG	256	256
RR	<4	8	AM	256	256
EC	32	64	NM	256	256
TP	128	128	JO	512	128
EM	128	128	HB	512	256
HJ	128	128	E	512	256
JK	128	128	CL	512	512
HJI	128	256			

TABLE II

The effect of calcium ions on the activity of rubella virus haemagglutinin (HA)

Antigen	HA titre* in the presence of the following molar concentrations of CaCl_2							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	0
Flow HA 1	16	16	16	16	16	16	16	8
WP22164 HA	32	32	32	32	32	32	32	16
Flow HA 2	512	128	64	32	32	32	32	32
Colindale HA	64	32	32	16	16	16	16	16
Pasteur HA	256	256	128	128	64	64	64	64
WP12264T/E HA	512	256	128	128	64	64	64	32

* Reciprocal of the highest dilution of antigen producing a complete shield of agglutinated pigeon or chick RBC.

method for detecting low titres of HI antibody. A possible explanation for this is the high concentration of calcium present in the initial titration dilutions of the dextran sulphate- CaCl_2 extracted serum.

The effect of divalent cations on rubella HA

The necessity for divalent cations in haemagglutination by rubella virus is well documented (Auletta *et al.*, 1968; Liebhaver, 1970a), although no quantitative analysis of this phenomenon has been attempted. Table II shows

the results of an experiment to determine the effect of calcium-ion concentration on the activity of rubella virus HA. Antigens from six different sources were studied. HA and RBC were diluted in buffered saline, pH 7.0–7.2, consisting of 0.005M-sodium barbital, 0.147M-NaCl, 1.0 per cent. BSA, 0.0001 per cent. gelatin and CaCl_2 in concentrations ranging from 0 to 10^{-1}M . Two distinct patterns of reactivity were found. With two antigens (Flow HA 1 and WP22164 HA) HA activity was unaffected by the changes in CaCl_2 concentration, but in the absence of CaCl_2 there was a two-fold fall in titre. With the other four antigens, there was a four- to 16-fold drop in the HA titre as the CaCl_2 concentration was reduced from 10^{-1}M to 10^{-5}M , but little or no further drop in the absence of CaCl_2 . In a similar experiment with MgSO_4 instead of CaCl_2 and antigens Flow HA 2, Pasteur HA and Colindale HA, only the

TABLE III

Effect of charcoal absorption on the rubella haemagglutination-inhibiting (HI) antibody titre of seven rivanol-extracted human sera

Amount of charcoal used (g per ml of serum)	HI antibody titre of the following seven serum specimens after absorption						
	JK	NM	TP	EC	NL	AM	SP
0	64	256	64	32	32	64	<16
0.03	64	256	64	64	32	128	<4
0.06	64	256	64	64	32	128	<4
0.10	64	128	64	64	32	32	<4

highest salt concentration, 10^{-1}M , had any effect, producing a small, two-fold, increase in the HA titres, compared with those obtained in diluent without MgSO_4 .

These results indicate that calcium ions, in concentrations present in low dilutions of serum that has been extracted with dextran sulphate- CaCl_2 , have a considerable enhancing effect upon the haemagglutinating activity of some rubella virus antigens. This might produce in the HI test an apparent absence of antibody in serum specimens containing low titres of antibody.

The effect of charcoal absorption on the antibody titre of rivanol-extracted serum

Rivanol is capable of lysing RBC. Shaking extracted serum for 15 s with the standard 0.03 g of charcoal sometimes fails to remove residual rivanol completely. However, charcoal may also absorb specific antibody. Tests were therefore carried out to determine the maximum quantity of charcoal that could be added to serum to remove rivanol without lowering the titre of specific antibody. Portions of extracted serum were shaken for 15 s without charcoal or with 0.03, 0.06 or 1.0 g of charcoal and were then filtered and titrated for HI antibodies. The results of tests with seven sera are presented in table III. In one serum only, AM, was there any reduction in antibody titre after absorption

with 0.1 g of charcoal. In none of the sera was there any significant reduction in titre after absorption with 0.03 or 0.06 g of charcoal. With 0.06 g of charcoal, residual rivanol was completely removed from all the sera tested. On the other hand, in HI tests with sera which had been extracted with rivanol but not absorbed with charcoal, the RBC were lysed only by serum dilutions up to 1 in 16. Since rubella HI antibody is usually present in serum in higher concentrations than this, the charcoal absorption step could be omitted from the extraction procedure without serious disadvantage.

DISCUSSION

The rivanol extraction technique offers a cheap and rapid method of removing non-specific inhibitors of rubella virus haemagglutination from human serum. The value of this technique must ultimately depend on its ability to remove inhibitor completely from any serum sample without affecting the level of specific antibody.

Hořejší and Smetana (1956) and subsequent workers (Boettcher *et al.*, 1958; Saifer and Lipkin, 1959) found that the purity of gamma-globulin prepared by rivanol treatment depends both on pH and on the batch of rivanol. Human serum treated with the batch of rivanol used throughout the present study, dissolved in distilled water, had previously been shown to retain 96 per cent. of its gamma-globulin, as well as significant amounts of alpha-globulin and traces of beta-globulin (Mostratos and Beswick, 1969). The presence of the latter impurities may explain our failure to remove all non-specific inhibitor from serum specimens with rivanol dissolved in distilled water. By the use of rivanol dissolved in dilute borate saline at pH 9.0, inhibitor was completely eliminated.

Isliker (1957) predicted that extraction of serum at high pH might lead to precipitation of gamma-globulin and, subsequently, Stastný and Hořejší (1961) reported that IgG may be precipitated out of human serum at pH 10; at lower pH no significant loss occurred. We, however, were unable to detect any significant lowering of rubella HI antibody titres in serum specimens extracted with rivanol at pH 9.0, in comparison with those extracted with dextran sulphate-CaCl₂. On the other hand, Duca and Buiuc (1970) reported significant losses of arbovirus HI antibody from mouse, rabbit and human sera after treatment with 0.4 per cent. rivanol in dilute borate saline buffer, pH 9.0. In the human and rabbit sera, significant, four-fold drops in antibody titre occurred only in sera that also showed a fall in titre after treatment with 2-mercaptoethanol. These losses of antibody, therefore, are probably due to precipitation of IgM antibody. Such precipitation of IgM by rivanol has been reported by Stastný and Hořejší. Although we carried out no confirmatory tests, it is likely that the bulk of the antibody in the sera we examined was IgG. Further work is required on the effect of rivanol on IgM in serum as this may provide a convenient and rapid method for differentiating between IgM and IgG antibodies.

No antibody was removed when the extracted serum was absorbed with

charcoal, 0.06 g per ml of serum, to remove residual rivanol. However, this step in the procedure could well be omitted, since amounts of rivanol that might interfere with the HI test are no longer present when the test serum is diluted beyond 1 in 16. Alternatively, excess rivanol could be removed by precipitation with sodium chloride or potassium bromide (Matthaeus and Matheka, 1963) without loss of gamma-globulin.

Direct comparison of the rivanol and dextran sulphate- CaCl_2 methods of extraction indicates that the latter might produce falsely low HI antibody titres because of the effect on the activity of the rubella HA antigen of the high concentrations of calcium remaining in the serum. Thus, the HA activity of four out of six antigens, from different sources, could be raised four- to 16-fold by increasing the calcium-ion concentration. In the HI test proper there would, therefore, be an inadvertent boosting of the challenge dose of HA antigen beyond the standard 4 HA units, with a consequent fall in the serum antibody titre as read in the test. The significance of these results for the accuracy of the HI test, particularly in sera with small amounts of antibody, has yet to be determined.

The major advantages of rivanol extraction for routine diagnostic work are its speed and simplicity, especially if the charcoal absorption step is omitted. A further bonus is the fortuitous removal of natural erythrocyte agglutinins from sera. This makes it easier to use pigeon RBC in the HI test, instead of the less sensitive chick RBC, since repeated or prolonged absorptions of the test sera with pigeon RBC are no longer required.

SUMMARY

Extraction with 0.4 per cent. rivanol, at pH 9.0, provides a rapid and economical method of removing non-specific inhibitors of rubella virus HA from human serum without loss of specific antibody; natural agglutinins for pigeon erythrocytes are largely removed. Serum HI antibody titres are comparable to those obtained with the dextran sulphate- CaCl_2 extraction method.

Possible sources of error in the HI test due to the method of serum extraction are discussed.

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