Comparative Studies on the Agglutination of Fowl Red Blood Cells by the Haemagglutinins of Vaccinia and Coxsackie A7 Viruses

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SUMMARY

Red blood cells from a group of fowls were sensitive to the haemagglutinins of vaccinia and Coxsackie A7 viruses but the red cells from certain fowls exhibited differences in their relative sensitivities. Differences between the reaction of these haemagglutinins with susceptible red cells of the same sensitivity were shown by treatment with chemical and physical agents. There were differences in the effect of pH value on the haemagglutination titres and, unlike Coxsackie A7 haemagglutinin, the adsorption of vaccinia haemagglutinin to susceptible red cells was inhibited by divalent cations. Red cell receptors for both haemagglutinins were insensitive to RDE (receptor destroying enzyme) but were inactivated by treatment with potassium periodate, papain or α -chymotrypsin. There were quantitative differences in the degrees or rates of receptor destruction by these reagents. Haemagglutination by vaccinia and Coxsackie A7 haemagglutinins was inhibited only by homologous antiserum. These qualitative and quantitative differences indicate separate red cell receptors for the two haemagglutinins.

INTRODUCTION

Red blood cells from certain fowls are highly susceptible to agglutination by the haemagglutinin specifically associated with vaccinia-infected tissue. Other fowls and most other animals possess red cells which are either agglutinated to a much decreased titre or are completely insensitive (Clark & Nagler, 1948; Burnet & Boake, 1946). Fowl red cells which are agglutinable by the vaccinia haemagglutinin show a parallel sensitivity to agglutination by normal tissue lipids (Burnet & Stone, 1946). Physicochemical studies have shown that vaccinia haemagglutinin is particulate and distinct from the infective particle (Chu, 1948; Neff, Ackermann & Preston, 1965). The activity of this haemagglutinin is associated with lipid and it is probably a lipoprotein (Stone, 1946*a*; Chu, 1948).

The restriction of the range of sensitive red cells to 'lipid-sensitive' fowl red cells appeared to be a phenomenon peculiar to vaccinia and other poxvirus haemagglutinins (Burnet & Boake, 1946; McCarthy & Helbert, 1960). However, Grist (1960) described a haemagglutinin specifically associated with tissue infected with an enterovirus, the Coxsackie A7 virus, which will also agglutinate only vaccinia-agglutinable

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fowl red cells. This Cocksackie A7 haemagglutinin was shown to be particulate and separable from the infective particle. Unlike vaccinia haemagglutinin, the activity of the Cocksackie A7 haemagglutinin appears not to be associated with lipid but with protein (Williamson & Grist, 1965).

Although both haemagglutinins will agglutinate only 'lipid-sensitive' fowl red cells, differences in the chemical nature of the reactive sites of vaccinia and Cocksackie A7 haemagglutinins suggested that they might attach to susceptible red cells by different mechanisms. This hypothesis has been investigated by comparative studies of the reactions of these haemagglutinins with susceptible fowl red cells.

METHODS

Viruses. The viruses used were the Evans strain of vaccinia virus and the 1034 strain of Cocksackie A7 (Grist, 1960); sometimes the Lee strain of influenza type B virus was also used.

Preparation of haemagglutinins. Extracts of suckling mouse tissue infected with Cocksackie A7 virus were prepared as described previously (Williamson & Grist, 1965) and used without further treatment as Cocksackie A7 haemagglutinin.

Stock suspensions of vaccinia virus passaged in chick embryos were used to inoculate the chorioallantoic membranes of 12-day chick embryos to give confluent lesions. Membranes were removed 48 or 72 hr later and extracted with physiological saline by disruption of the infected membranes in a mechanical homogenizer. The extracts were clarified by low-speed centrifugation and the supernatant fluid used as vaccinia haemagglutinin.

The Lee strain of influenza type B virus was passaged in the allantoic cavities of 10-day chick embryos. The influenza virus haemagglutinin was obtained by harvesting infected allantoic fluid 48 hr after inoculation.

Haemagglutination and haemagglutination inhibition tests. The preparation of vaccinia-agglutinable fowl red blood cells (r.b.c.) and the performance of these tests in Perspex depression trays were described by Williamson & Grist (1965). Haemagglutination inhibition tests were used routinely to test the specificity of vaccinia haemagglutinin preparations. The haemagglutinin specifically associated with vaccinia-infected tissue is inhibited by vaccinia antiserum but not by normal serum (Stone, 1946*a*).

Preparation of antisera. Cockerels were immunized by four intramuscular injections at weekly intervals of 1.0 ml. of an extract of suckling mouse tissue infected with Cocksackie A7 virus, emulsified with an equal volume of incomplete Freund's adjuvant. The fowls were bled 7 days after the final injection.

Vaccinia antisera were prepared similarly in fowls by injection with adjuvant of an extract of vaccinia-infected chick embryo chorioallantoic membrane.

Treatment of red cells. It was established in the early stages of these investigations that vaccinia-agglutinable fowl r.b.c. were rapidly rendered auto-agglutinable by even limited exposure to RDE, potassium periodate or proteolytic enzymes. This prohibited the quantitative assessment of the effect of treatment by direct use of these cells in haemagglutination tests. In order to overcome this problem, the following indirect procedure was adopted.

Washed vaccinia-agglutinable fowl r.b.c. were resuspended to 1% (v/v) in dupli-

cate serial dilutions of 5.0 ml. amounts of 0.0025 M-potassium periodate in physiological saline. Similar duplicate 1% (v/v) r.b.c. suspensions were made in 0.01% trypsin, 0.1% α -chymotrypsin, 1% papain and *Vibrio cholerae* filtrate (Burroughs Wellcome Ltd.). The proteolytic enzymes were dissolved in phosphate buffer saline, 0.14 M-sodium chloride + 0.01 M-phosphate buffer (pH 7.2). Papain was activated by addition of 3% (w/v) cysteine hydrochloride. *Vibrio cholerae* filtrate was reconstituted as directed by the suppliers. Treatment with KIO_4 was at 18° for 1 hr; with proteolytic enzymes at 37° for up to 6 hr; with *V. cholerae* filtrate at 37° overnight. Controls included in each series consisted of r.b.c. at a similar concentration incubated at the appropriate temperature in the absence of the particular reagent. After treatment, the r.b.c. were deposited by centrifugation at 500 g for 5 min. and washed three times with 0.85% NaCl. Periodate-treated r.b.c. were washed with 0.85% NaCl containing 1% (w/v) glucose.

One of the duplicate sets of treated r.b.c. was then resuspended in 1.0 ml. of a solution containing four haemagglutinating units of Cocksackie A7 haemagglutinin. The other set was resuspended in 1.0 ml. of a solution containing vaccinia haemagglutinin at a similar concentration. Mixtures, together with controls, were kept at 18° for 2 hr with resuspension of the r.b.c. at frequent intervals. After exposure to the haemagglutinins, the r.b.c. were deposited by low-speed centrifugation and the titre of haemagglutinating activity remaining in the supernatant fluids determined.

RESULTS

Range of susceptible fowl red cells

The sensitivity of washed r.b.c. from 172 Rhode Island Red fowls to vaccinia and Cocksackie A7 haemagglutinins was determined. Suspensions of r.b.c. were adjusted to 0.5% (v/v) by using a haematocrit. Serial twofold dilutions of each haemagglutinin were made from stock preparations containing 16 haemagglutinating units when titrated with the same susceptible r.b.c. All dilutions were made in bulk before dispensing on to Perspex plates in order to make each dilution series strictly comparable. Results were read 2 hr after the addition of 0.5% (v/v) fowl r.b.c. suspensions and again after standing at 4° overnight.

Of the 172 samples of fowl r.b.c. tested, 60% gave identical titres with both haemagglutinins, 17% gave a twofold lower titre and 17% gave a twofold or slightly higher titre with Cocksackie A7 haemagglutinin than with vaccinia haemagglutinin. There was an eightfold difference between the maximal and minimal titres obtained with both haemagglutinins. The remaining 6% of the fowl r.b.c. tested were not agglutinated even by higher concentrations of either haemagglutinin. Insensitive r.b.c. were also obtained from 12 chick embryos which were tested.

The fowl r.b.c. used in the experiments reported here were equally sensitive to both vaccinia and Cocksackie A7 haemagglutinins.

Effect of physical agents on haemagglutination

Temperature. Haemagglutination titres obtained with vaccinia and Cocksackie A7 haemagglutinins were similarly affected by the temperature at which the tests were made. Parallel tests showed a twofold increase at 37° and a twofold decrease at 4° as compared with titres obtained at room temperature (15–18°). The patterns of

agglutinated r.b.c. at each temperature differed, however, in that vaccinia haemagglutinin gave a uniformly distributed shield pattern, whereas Cocksackie A7 haemagglutinin gave a more densely packed pattern of coarsely agglutinated r.b.c.

Electrolytes. Vaccinia and Cocksackie A7 haemagglutinins were dialysed overnight against isotonic solutions of glucose, NaCl, MgCl₂, CaCl₂ or Na₂SO₄. Haemagglutination titres were then determined with the use of isotonic solutions for the preparation of 0.5 % (v/v) fowl r.b.c. suspensions. With both vaccinia and Cocksackie A7 haemagglutinins, haemagglutination was not detectable in the absence of electrolytes. Cocksackie A7 haemagglutinin gave identical haemagglutination titres in each of the isotonic salt solutions. Identical haemagglutination titres were obtained with vaccinia haemagglutinin in isotonic solutions of NaCl and Na₂SO₄. Haemagglutination with the vaccinia haemagglutinin, however, was decreased 16-fold in isotonic MgCl₂ and was completely inhibited in isotonic CaCl₂. Unlike Cocksackie A7 haemagglutinin, agglutination of susceptible fowl r.b.c. by vaccinia haemagglutinin was inhibited by the divalent cations tested.

In other experiments, vaccinia haemagglutinin of a known haemagglutination titre in 0.85 % NaCl solution was dialysed overnight against isotonic CaCl₂. Susceptible fowl r.b.c. equilibrated to isotonic CaCl₂ were added to the dialysed preparation and the mixture kept at room temperature for 2 hr with resuspension of the r.b.c. at frequent intervals. The r.b.c. were then deposited by low-speed centrifugation and the supernatant fluid dialysed against isotonic NaCl. Haemagglutination titres determined after dialysis indicated complete recovery of vaccinia haemagglutinin in the supernatant. In control experiments with isotonic NaCl as the suspending medium, haemagglutinin was not detectable in the supernatant fluid following exposure to susceptible r.b.c. Ca²⁺ ions therefore prevented the adsorption of vaccinia haemagglutinin to susceptible fowl r.b.c. Further experiments established that Ca²⁺ ions similarly inhibited agglutination of susceptible r.b.c. by a 0.4 % (w/v) suspension of lecithin prepared as described by Stone (1946*b*).

Effect of pH values. Haemagglutination titres were determined at room temperatures in 0.85 % NaCl buffered with citrate buffers (pH 5.0–6.0), phosphate buffers (pH 7.0–8.0) and glycine buffers (pH 9.0–10.0). Preliminary experiments had shown that both haemagglutinins and fowl r.b.c. were stable over these ranges. There were marked differences in the effect of the pH value of the diluent on the titres obtained with vaccinia and Cocksackie A7 haemagglutinins (Fig. 1). With Cocksackie A7 haemagglutinin, maximal titres were obtained between pH 5.0 and 8.0 but there was a marked decrease in titre as the diluent became progressively more alkaline. Conversely, titres were minimal in acid diluents with the vaccinia haemagglutinin, maximal titres being obtained in the alkaline range. Titres obtained with vaccinia haemagglutinin were closely paralleled by those obtained with the lecithin preparation under the same conditions.

Effect of treatment of fowl red blood cells

Vibrio cholerae filtrate. A receptor destroying enzyme (RDE) present in *V. cholerae* filtrate destroys the red cell receptors for influenza virus (Burnet & Stone, 1947). Treatment of lipid-sensitive fowl r.b.c. with an RDE preparation under the conditions described destroyed their ability to absorb the type B influenza virus strain

Lee, but was without effect on the receptors for the vaccinia and Cocksackie A7 haemagglutinins.

Potassium periodate. Although r.b.c. receptors for vaccinia and Cocksackie A7 haemagglutinins were destroyed by the highest concentration of periodate used (0.0025 M), there were quantitative differences in the degree of receptor destruction at lower concentrations (Fig. 2). Thus, susceptible fowl r.b.c. treated with 0.001 M-potassium periodate completely absorbed the Cocksackie A7 haemagglutinin, whereas only 50 % of the vaccinia haemagglutinin was absorbed. Untreated r.b.c. completely absorbed both haemagglutinins under the same conditions. These results

Fig. 1

Fig. 1. Effect of pH value on the agglutination of lipid-sensitive fowl red blood cells by vaccinia haemagglutinin, Cocksackie A7 haemagglutinin and a 0.4 % (w/v) lecithin suspension. ●—●, Vaccinia haemagglutinin; ■—■, Cocksackie A7 haemagglutinin; ▲—▲, lecithin suspension.

Fig. 2

Fig. 2. Absorption of vaccinia and Cocksackie A7 haemagglutinins by potassium periodate-treated fowl red blood cells. ●—●, Vaccinia haemagglutinin; ■—■, Cocksackie A7 haemagglutinin.

suggest that the r.b.c. receptors for vaccinia haemagglutinin are more sensitive to the action of periodate than those for Cocksackie A7 haemagglutinin.

Proteolytic enzymes. Papain and α -chymotrypsin destroyed receptors for both vaccinia and Cocksackie A7 haemagglutinins but quantitative differences were again established. Under the conditions described, treatment with 1 % papain for 2 hr completely destroyed the capacity of susceptible r.b.c. to absorb vaccinia haemagglutinin. Identically treated r.b.c., however, absorbed 75 % of the Cocksackie A7 haemagglutinin to which they were exposed. Similarly, r.b.c. receptors for vaccinia haemagglutinin were completely destroyed by 0.1 % α -chymotrypsin after 2 hr, but such r.b.c. retained the capacity to absorb 50 % of the Cocksackie A7 haemagglutinin. Extended treatment with papain or with α -chymotrypsin rendered r.b.c. completely insensitive to Cocksackie A7 haemagglutinin.

Treatment of r.b.c. with 0.01 % trypsin up to 6 hr did not diminish their capacity

to absorb both haemagglutinins. Modification of the conditions of treatment by increasing the incubation period or the concentration of enzyme rendered the r.b.c. unsuitable for absorption experiments.

Exclusion experiments

Attempts were made to saturate susceptible r.b.c. with one of the haemagglutinins and then to determine whether such r.b.c. were capable of absorbing the second haemagglutinin. Saturation of intact r.b.c. with Cocksackie A7 haemagglutinin was prevented by lysis of the r.b.c. by the haemolysin present in extracts of suckling mouse tissue infected with Cocksackie A7 virus (Williamson & Grist, 1965). To overcome this difficulty experiments were made with stromata prepared by lysis of susceptible r.b.c. in distilled water. Such stromata were repeatedly resuspended in preparations of either the vaccinia or Cocksackie A7 haemagglutinin. After thorough washing in 0.85 % NaCl solution at 4°, the stromata were resuspended in dilutions of the homologous haemagglutinin containing 4 haemagglutinating units. Failure to decrease the haemagglutination titre indicated complete saturation. After further washing, the stromata were resuspended in similar dilutions of the heterologous haemagglutinin. Neither stromata saturated with vaccinia haemagglutinin nor stromata saturated with Cocksackie A7 haemagglutinin showed any detectable absorption of heterologous haemagglutinin.

Stromata saturated with vaccinia or Cocksackie A7 haemagglutinin were also resuspended in dilutions of the influenza virus preparation containing 4 haemagglutinating units. Although control preparations completely absorbed the influenza virus haemagglutinin, there was no detectable adsorption to stromata saturated with vaccinia or Cocksackie A7 haemagglutinin.

Table 1. *Haemagglutination inhibition tests with vaccinia and Cocksackie A7 haemagglutinins using homologous and heterologous immune sera*

Haemagglutinin	Serum titres			
	Vaccinia		Cocksackie A7	
	Normal	Immune	Normal	Immune
Vaccinia	32	1024	32	32
Cocksackie A7	< 32	< 32	< 32	512

Haemagglutination inhibition tests

Differences in the serological specificity of vaccinia and Cocksackie A7 haemagglutinins were established by haemagglutination inhibition tests (Table 1). Homologous immune sera inhibited both haemagglutinins to high titre but there was no specific inhibition of either haemagglutinin by heterologous immune sera.

DISCUSSION

Quantitative differences revealed in the relative sensitivity of certain fowl r.b.c. to the two haemagglutinins suggest that although there is a close relationship between sensitivity to vaccinia and Cocksackie A7 haemagglutinins, there are quantitative differences in the capacity of some fowl r.b.c. to absorb these haemag-

glutinins. Failure of Cocksackie A7 haemagglutinin to agglutinate chick embryo r.b.c. indicates that red cell receptors for this haemagglutinin appear only as the fowls mature as previously described for receptors for vaccinia haemagglutinin (McCarthy & Helbert, 1960).

The qualitative and quantitative differences in the effect of physical and chemical agents on the reaction of vaccinia and Cocksackie A7 haemagglutinins with susceptible fowl r.b.c. reflect differences in the physicochemical properties of both the haemagglutinins and the red cell receptors. Inhibition by divalent cations of the agglutination of r.b.c. by lipid-associated haemagglutinins has been previously reported with vaccinia haemagglutinin (Briody, 1951) and with the psittacosis haemagglutinin (Gogolak & Ross, 1955). In the present study, only vaccinia and lecithin haemagglutinins were inhibited by either Ca^{2+} or Mg^{2+} ions. Physical and chemical studies have indicated that the activity of the Cocksackie A7 haemagglutinin is not associated with lipid but with protein (Williamson & Grist, 1965).

The results of treatment of r.b.c. with RDE, periodate and proteolytic enzymes suggest that the red cell receptors are probably mucoproteins but distinct from myxovirus receptors (Andrewes, Bang & Burnet, 1955). Quantitative experiments indicate that, if the receptors are chemically similar, there are more receptors for the Cocksackie A7 haemagglutinin than for the vaccinia haemagglutinin. Conversely, if there are the same number of receptors for each haemagglutinin, it may be argued that the receptors are chemically different. Attempts to resolve the relationship between the receptors by saturation experiments gave equivocal results and suggest only that there may be a close spatial proximity between the receptors for the two haemagglutinins. However, either interpretation of the quantitative experiments points to a distinction between the receptors for vaccinia and Cocksackie A7 haemagglutinins.

Previous studies have shown that there are differences in the chemical nature of the red cell receptors for the haemagglutinins in the main groups of viruses (Klenk & Lempfrid, 1957; Buckland & Tyrell, 1963; Philipson *et al.* 1964). In addition to chemical differences (Williamson & Grist, 1965), vaccinia and Cocksackie A7 haemagglutinins have been shown to be serologically distinct. Both qualitative and quantitative differences have been established in the reaction of these haemagglutinins with susceptible fowl r.b.c. It is concluded that these results indicate that, although closely linked, there are separate red cell receptors for vaccinia and Cocksackie A7 haemagglutinins.

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