Studies on the Purification of Chlamydial Agents Grown in Yolk Sacs of Embryonated Eggs Using Disulphide-linked Immunosorbents and Enzymes

By I. S. SIM* AND J. STEPHEN

Department of Microbiology, University of Birmingham, Birmingham B15 2TT

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SUMMARY

Immunosorbents were derived from avid and non-avid sera raised in rabbits to multiple or single injections of chlamydiae passaged once or three times in HeLa cells after routine passage in eggs. Egg-derived suspensions of chlamydiae required pretreatment before application to immunosorbent columns; this was most conveniently done by fractionation on Sepharose 4B. Immunosorbents derived from avid serum had greater capacity than those from non-avid sera. However, organisms were desorbed in low yield from an avid immunosorbent, but in higher yields from non-avid immunosorbents. Even under the best conditions, the immunosorbents yielded suspensions of organisms still contaminated with egg antigens.

Partially purified suspensions of chlamydiae were also treated with phospholipase C to yield suspensions of high purity.

INTRODUCTION

Several procedures have been used for purifying chlamydiae grown in the chorioallantoic or yolk sac membranes of embryonated eggs or in tissue culture cells. These include: centrifugation (Colón & Moulder, 1958; Jenkin, 1960); centrifugation with prior treatment with 1 M-KCl (Taverne, Blyth & Reeve, 1964; Sowa *et al.* 1969) or trypsin and celite (Weiss *et al.* 1964); density gradient centrifugation as a main or terminal step (Tamura & Higashi, 1963; Vedros, 1967; Detels, Wang & Grayston, 1968; Sarov & Becker, 1968); DEAE-cellulose chromatography (Ormsbee & Weiss, 1963; Katzenelson & Bernkopf, 1965; Provost & Vickers, 1972); liquid-liquid partition using fluorocarbons (e.g. Wang, Kenny & Grayston, 1967); fractional precipitation with ammonium sulphate (Wang *et al.* 1967); and, as a terminal step, use of antiserum to normal yolk sac antigens (Moulder & Weiss, 1951). The techniques are often lengthy and may inactivate the agent, although for some purposes this is not important. It is difficult to extract exact information on the yields, infectivity and purity of 'purified' preparations from many of these papers.

We describe attempts to purify trachoma and inclusion conjunctivitis (TRIC) agent grown in yolk sac membrane, using disulphide-linked antibody immunosorbents (Stephen, Gallop & Smith, 1966; Crook *et al.* 1972*a*). With other antigens this approach has shown high specificity (Stephen *et al.* 1966; Wood, Stephen & Smith, 1968; Chidlow, Stephen & Smith, 1970; Wood, Gould & Smith, 1970) and has been used successfully to purify proteins (Crook, Stephen & Smith, 1972*b*; Nicklin & Stephen, 1974), vaccinia virus antigens (Birkbeck

* Present address: Department of Virology, Medical School, University of Birmingham, Birmingham B15 2TJ.

& Stephen, 1970, 1971), bacterial toxins (Bird, Low & Stephen, 1974), Semliki Forest virus (Wood *et al.* 1970) and influenza virus (Sweet, Stephen & Smith, 1974). Yolk sac-grown agent is recognized as more difficult to purify than agent from allantoic fluid (Weiss *et al.* 1970), but we attempted to purify it because there are no suitable tissue culture systems for some TRIC strains, and eggs still offer the cheapest source of the large quantities of organisms as would be required for vaccine production (Collier, 1972).

METHODS

Chlamydiae. The agents used, followed by the abbreviations used in the text in parentheses, were as follows: TRIC/ /GB/MRC-4/ON (Jones, 1961) (MRC-4), and the fast-killing variant (MRC-4f) (Taverne *et al.* 1964); the fast-killing variant of TRIC/ /USA-Cal/Cal-1/OT (Hanna *et al.* 1959) (Bour f); guinea-pig inclusion conjunctivitis agent (Murray, 1964) (gp-ic).

Growth of chlamydiae in eggs. Stocks of each agent were prepared by infecting six- to eight-day-old chick embryos (Tang *et al.* 1957) with a thousand 50% egg lethal doses (ELD₅₀)/egg. After approximately 50 % of eggs had died the remaining live eggs were taken and 20 % (w/v) crude yolk sac homogenates made in phosphate buffered saline (Dulbecco & Vogt, 1954) containing 0.25 M-sucrose.

Growth of MRC-4f in cells. HeLa cells (Wellcome Reagents Ltd) were grown in Eagles medium (Wellcome Reagents Ltd) with 10 % (v/v) calf serum (Virology Department, University of Birmingham) and streptomycin (500 µg/ml). Confluent monolayers were infected with KCl-fractionated MRC-4f (see below). The agent was allowed to adsorb on to cell monolayers for 4 h at 37 °C in maintenance medium (Eagles medium with 2 %, v/v, heat-inactivated calf serum) after which fresh maintenance medium was added. The cells were harvested after 44 h and the agent released by ultrasonication (MSE ultrasonicator, 60 W, 3 min at 0 °C). The resultant suspension was centrifuged (500 g for 10 min at 4 °C) and the supernatant collected. The TRIC agent (designated once-passaged HeLa cell-grown MRC-4f) was used for immunizing rabbits directly or after two further passages through HeLa cells.

Measurement of infectivity. Suspensions of MRC-4f were assayed in eggs or HeLa cells (Reeve & Taverne, 1967).

Measurement of chlamydial particle concentration. This was determined by dark-field microscopy (Reeve & Taverne, 1962). The coefficient of variation of particle counts was estimated for a number of suspensions and was never greater than 15 %.

Antisera. Avid (and non-avid, as defined by Nicklin & Stephen, 1973) anti-TRIC antisera were raised in rabbits by three or four subcutaneous injections of live MRC-4f which had been once passaged in HeLa cells (approx. 10⁷ ELD₅₀ per injection). The agent was given in complete Freund's adjuvant at not less than weekly intervals followed by an intravenous boost of live agent (10⁷ ELD₅₀) without adjuvant at least 40 days after the final subcutaneous injections. Rabbits were bled 10 days thereafter.

Non-avid anti-TRIC antisera were obtained from rabbits after a single injection of live MRC-4f as above; the latter had been passaged once or three times through HeLa cells. Rabbits were bled 10 days after injection.

Rabbits were also immunized with antigen-antibody complexes following the schedule of Birkbeck & Stephen (1971). Immune aggregates were prepared in immunodiffusion gels (Watson & Wildy, 1969) using soluble antigens derived from egg-grown MRC-4f and avid anti-TRIC antisera which contained no detectable anti-egg antibodies either at the outset or after adsorption with normal yolk sac antigens.

Anti-egg antisera were raised in rabbits by multiple subcutaneous injections of 5 mg amounts of normal yolk sac antigens in complete Freund's adjuvant on days 0, 2, 4 and 9 and intravenous injections of 100, 200 and 200 μ g of normal yolk sac antigens without adjuvant on days 47, 49 and 51, respectively. Rabbits were bled on days 61 and 62.

γ -Globulin concentrates of immune sera were prepared by Na_2SO_4 precipitation (Smith *et al.* 1962) and used throughout this work, but are referred to as antisera, for brevity.

Immunodiffusion. The method of Crowle (1958) was used. Soluble antigens from *chlamydiae* were obtained by ultrasonication (MSE ultrasonicator, 60 W, 10 min at 0 °C) of suspensions of KCl-fractionated organisms (10^{10} particles/ml). Normal yolk sac antigens were prepared from uninfected 14-day-old yolk sacs subjected to a single cycle of differential centrifugation and ultrasonication.

Infectivity of MRC-4f after exposure to various pH buffers. KCl-fractionated MRC-4f was diluted 1:100 in the appropriate buffer, incubated for 1 h at 5 °C and immediately titrated in eggs. The buffers (I 0.26) used were citrate buffers pH 3.0, 4.0 and 5.3, phosphate buffers pH 6.0 and 8.0, borate buffers pH 9.0 and 10.0 (compositions as given by Nicklin & Stephen, 1973), and glycine-NaOH-NaCl, pH 10.5 and 11.0 (compositions as given by Miller & Golder, 1950). A similar method was applied for determining the effect of pH on particle integrity.

Preparation and use of disulphide-linked immunosorbents. Immunosorbent polymers were prepared by thiolating γ -globulin concentrates, precipitating the thiolated protein with Na_2SO_4 (as above) and cross-linking the pellet of thiolated protein with potassium ferri-cyanide (modification I procedure, Crook *et al.* 1972*a*). Columns (Mark III-type, Crook *et al.* 1972*b*) were prepared by placing a mixture of -S-S-linked immunosorbent and Sephadex G-15 on top of more Sephadex G-15; the column was equilibrated in phosphate-buffered saline (PBS), containing (g/l): Na_2HPO_4 , 8.09; KH_2PO_4 , 2.44; NaCl, 15.9. This arrangement allows the use of extreme acidic (or alkaline) pH-buffers in desorbing labile antigens from homologous immunosorbent and recovering them in neutral buffer after only brief exposure to the potentially denaturing desorbent (Crook *et al.* 1972*b*). Buffers used in this work were PBS for adsorbing agent, and either 0.2 M-glycine adjusted to pH 10.5 with NaOH or 0.2 M-arginine adjusted to pH 11.3 with HCl for desorbing TRIC agent.

For some experiments a batch procedure (Sweet *et al.* 1974) was used. Immunosorbent polymer was stirred with TRIC agent suspension for 3 h at 4 °C, centrifuged (500 g for 10 min at 4 °C) and the supernatant withdrawn. The immunosorbent was washed with at least five 5 ml volumes of PBS and desorbed with at least three 5 ml volumes of the appropriate buffer.

Immunosorbents, either in column or batch procedure, could be recycled several times. After desorption, immunosorbent was re-equilibrated in PBS and the adsorption-desorption procedure repeated.

Preliminary purification of MRC-4f. It was usually necessary to effect some preliminary purification of *chlamydiae* obtained as homogenates of infected yolk sac membranes. Four methods were used: (i) KCl fractionation. An equal volume of 2 M-KCl at 0 °C was added slowly to egg-derived TRIC agent suspension (Taverne *et al.* 1964) and centrifuged (12 000 g for 20 min at 4 °C). The pellet was resuspended in PBS containing sucrose and centrifuged (500 g for 10 min at 4 °C). The supernatant was collected and the two centrifuging procedures repeated. The final supernatant was designated KCl-fractionated MRC-4f. (ii) Modified KCl fractionation. Homogenates of MRC-4f were subjected to the first two centrifugal steps described above. The pellet thus obtained was suspended in borate-buffered saline pH 7.3 (72.3 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 5.84 g NaCl/l; pH adjusted to 7.3 with conc. HCl) containing

CaCl_2 (final concn 0.05 %, w/v). The organisms were centrifuged and finally resuspended in borate-buffered saline. The product was designated modified KCl-fractionated MRC-4f. (iii) Trypsin-celite fractionation. Crude yolk sac homogenates were adjusted to pH 7.5 and treated with trypsin (0.2 %, w/v, for 15 min at 37 °C) (Wang & Grayston, 1967). An equal volume of 2 M-KCl was added and the suspension centrifuged (12 000 g for 20 min at 4 °C). The pellet was resuspended in PBS containing sucrose, and celite added (0.1 g celite/g original yolk sacs). After about 5 min the celite was removed with the flocculated protein by centrifugation (500 g for 10 min at 4 °C). The TRIC agent was pelleted again and resuspended in PBS containing sucrose to the desired volume. The product was designated trypsin-celite-fractionated MRC-4f. (iv) Sepharose 4B fractionation. Crude yolk sac homogenates were fractionated on columns of Sepharose 4B (Pharmacia) equilibrated in PBS. Up to 50 % of the applied TRIC agent was recovered in the breakthrough peak. This peak also contained approx. 50 % of the applied protein. Several other fractions (containing no TRIC particles) were obtained from the column and some material was strongly adsorbed to the Sepharose.

Purification of MRC-4f using phospholipase C. The source of crude enzyme was a *Clostridium welchii* type A freeze-dried culture filtrate, lot AD1043D2, a gift from Dr R. O. Thomson, Wellcome Research Laboratories; it contained 19 LV units of α -toxin/mg, where 1 LV unit is that amount of toxin which in the presence of 1 i.u. antitoxin will cause a turbid reaction in egg yolk. Excess substrate present in crude yolk sac homogenates of MRC-4f was removed by modified KCl fractionation of MRC-4f. Suspensions were incubated at 37 °C with phospholipase (particle to enzyme ratios were varied) for the times indicated and immediately chilled to 0 °C. During overnight storage, visible gel-like aggregates of material formed which were separated from the TRIC agent suspension by rapid filtration through approx. 1 ml Sephadex G-15. The MRC-4f suspension was centrifuged (12 000 g for 30 min at 4 °C), resuspended in borate-buffered saline, and analysed for total particles, nitrogen, lipid phosphorus and dry weight.

Estimation of purity of MRC-4f. The purity of MRC-4f preparations was assessed by total particle counts, total nitrogen analysis (by micro-Kjeldahl digestion followed by direct Nesslerization), by lipid phosphorus analysis [lipid extraction (Bligh & Dyer, 1959) followed by phosphate determination (Bartlett, 1959)] and dry weight determination. Guinea-pig hypersensitivity tests (Hughes *et al.* 1974) were used to detect normal yolk sac antigens and normal rabbit serum proteins in MRC-4f preparations.

RESULTS

Production of antisera

The rationale for raising the antisera was as follows: To isolate, by immunosorbents, MRC-4f from egg yolk sacs required antisera containing antibodies to MRC-4f antigens but not to egg antigens. This involved the use of a second host (HeLa cells) for growing the organism. Thus, rabbits immunized with MRC-4f grown in HeLa cells should contain antibodies to MRC-4f and to HeLa cell antigens but not to egg antigens. Alternatively, the requirement for the two-host system could be overcome by using antigen-antibody complexes isolated from immunodiffusion gel media and injecting these into rabbits. Here the specificity of the resultant antisera should depend only on the degree to which the isolated immune-complex was contaminated with other antigens in the immunodiffusion-gel.

Production of antiserum against MRC-4f grown in HeLa cells. Avid antisera raised in rabbits by multiple injections of MRC-4f passaged once in HeLa cells were analysed by

eT, soluble antigens derived from yolk sac-grown MRC-4f; NYS, normal yolk sac antigens.

Fig. 1 (a) and (b). Immunodiffusion analyses of avid anti-Hela cell-grown MRC-4f antisera from individual rabbits (3, 4, 5 and 6).

Fig. 2. Immunodiffusion analysis of non-avid sera (aT) raised in rabbits in response to thrice-passaged HeLa cell-grown MRC-4f.

Fig. 3. Immunodiffusion analysis of an antiserum (aT) raised in a rabbit by injection of antigen-antibody complexes.

micro-immunodiffusion. Individual sera showed up to seven precipitins (Fig. 1a) with no evidence of precipitating anti-egg antibodies (Fig. 1b). Non-avid antisera showed fewer precipitins, and in one instance detectable anti-egg antibodies; the presence of the latter was attributed to egg antigens in some preparations of MRC-4f which had been passed once in HeLa cells before being used to immunize the rabbits. In further experiments, to ensure complete removal of contaminating egg antigens, MRC-4f was passed three times through HeLa cells and used to raise non-avid sera in a separate group of rabbits. These resultant antisera showed no precipitating anti-egg antibodies but four TRIC-specific precipitins were detected (Fig. 2); no egg antigens were detected in the preparations of MRC-4f passed three times in HeLa cells.

Production of antiserum against immune complexes. An attempt was made to avoid multiple passage of the agent through a second host by immunizing rabbits with MRC-4f-specific immune complexes. Rabbits were immunized with antigen-antibody complexes prepared from egg-derived MRC-4f antigens and an avid serum raised to MRC-4f grown in HeLa cells and which contained no detectable precipitating anti-egg antibodies. However, the

Table 1. *Recovery of MRC-4f from disulphide-linked polymers derived from unrelated γ -globulin*

KCl-fractionated MRC-4f was added to three batches of polymer (total protein of each, 60 mg) and the amounts of TRIC agent recovered unadsorbed were determined by total particle counts. Batch 1 was washed with 0.2 M-glycine-NaOH buffer, pH 10.5, after cycle 1 and re-equilibrated in PBS before cycle 2.

Batch	Cycle	MRC-4f		
		Total particles		Percentage of applied recovered
		Applied	Recovered unadsorbed	
1	1	7.4×10^9	6.3×10^9	85
	2	1.0×10^{10}	8.2×10^9	82
2	1	8.3×10^9	6.6×10^9	80
3	1	8.3×10^8	9.5×10^8	116

resultant antisera contained both TRIC-specific and egg-specific precipitins (Fig. 3); pre-immune sera showed no precipitating activity in immunodiffusion tests against normal yolk sac antigens.

Infectivity of MRC-4f after exposure to various pH buffers

Over a period of 1 h at 5 °C the agent was stable in buffers of pH values 6.0 to 10.0. Above pH 10.0 and below pH 6.0 significant losses of infective agent were recorded; the losses were greater on the acid than on the alkaline side. Initially a 0.2 M-glycine-NaOH buffer, pH 10.5, was selected for desorption, since this was the limiting pH at which the agent remained infective and the number of particles recovered was high (98 %, after 1 h). Desorption was later done with a 0.2 M-arginine-HCl buffer, pH 11.3, although this caused apparent disruption of the particles in the pH stability tests; only 11 % of particles were recovered after 5 min and none was detectable by total particle assay after 1 h at 5 °C.

Purification of MRC-4f using immunosorbents

Specificity of disulphide-linked polymers. At high doses [from 7×10^9 to 1×10^{10} particles (batch 1, cycles 1 and 2; batch 2, cycle 1)] the amount of TRIC agent recovered unadsorbed at pH 7.0 from polymers derived from unrelated γ -globulin was not significantly different from the amount applied, i.e. there appeared to be little or no non-specific adsorption of the agent (Table 1), although, because of the limitations of the total particle count assay it was possible that up to 3×10^9 particles were retained in the polymer. However, when the third batch of polymer was loaded with approximately one-tenth the amount of MRC-4f used previously, again a high yield of particles was recovered (batch 3, cycle 1; Table 1).

The recovery of unadsorbed egg antigens was not measurable. However, if egg antigens were adsorbed at pH 7.0, they were not desorbed (as judged by immunodiffusion tests) when the polymers were washed with 0.2 M-arginine-HCl buffer, pH 11.3.

Purification of MRC-4f using anti-TRIC immunosorbents derived from avid antisera. Two samples of disulphide-linked immunosorbent polymer were prepared from avid anti-MRC-4f sera pooled from several rabbits and were used in batch and column procedures. Each polymer was loaded with KCl-fractionated MRC-4f, washed with PBS, and desorbed with 0.2 M-glycine-NaOH buffer, pH 10.5; in all, 5 cycles were carried out. The polymer

Fig. 4

Fig. 5

Fig. 4. Immunisorbent fractionation of trypsin-celite fractionated MRC-4f. The immunosorbent was derived from a non-avid serum raised to once passaged HeLa cell-grown MRC-4f and contained 30 mg polymer. The column was loaded with 2.1×10^9 particles. Fraction 1 (Fr. 1) is the unadsorbed material (54 % of the applied TRIC agent); the neutral fraction (Fr. 2) contains the desorbed MRC-4f (33 % of the adsorbed agent); no particles were observed in the alkaline fraction (Fr. 3). Later experiments (see text) suggested that the material in Fr. 3 was of egg origin and was bound by the Sephadex G-15. —, E_{280} ; ----, pH; ▨, neutral desorbed fraction.

Fig. 5. Effect of incubation of partially purified suspensions of MRC-4f with phospholipase C on the ratios particles:dryweight, particles:lipid phosphorus and particles:nitrogen. ○, Total particles/g dry wt; ●, total particles/g lipid phosphorus; □, total particles/g nitrogen.

adsorbed approximately 3×10^8 particles of MRC-4f/mg polymer protein, but the amount of MRC-4f desorbed was only about 4 % of that adsorbed.

Purification of MRC-4f using anti-TRIC immunosorbents derived from non-avid antisera raised to MRC-4f passaged once through HeLa cells. In an attempt to desorb the agent in higher yield, non-avid immunosorbents were investigated. Two polymers were prepared from different non-avid antisera and packed in columns. These were loaded with trypsin-celite-fractionated MRC-4f and washed with PBS. Desorption with 0.2 M-glycine-NaOH buffer, pH 10.5, gave better but variable yields of particles than had been obtained from avid immunosorbents. More consistent yields were obtained when columns of similar capacities were desorbed with 0.2 M-arginine-HCl buffer, pH 11.3. Thirty mg of polymer adsorbed 5.5×10^8 particles (mean of 8 runs). Desorption with 0.2 M-arginine-HCl buffer, pH 11.3, yielded 35 % of adsorbed particles in the neutral pH fraction (see Fig. 4 for a typical elution profile with this type of starting material). Columns were recycled eight times without apparent loss of capacity or decrease in the yield of agent desorbed.

Particle to ELD₅₀ ratios of 119:1 and 160:1 were obtained before and after immunopurification, respectively; in a separate experiment the respective ratios were 110:1 and 80:1. This indicates little or no inactivation of MRC-4f during the procedure.

Suspensions of immunopurified MRC-4f dialysed against PBS to remove traces of arginine buffer contained from 2.4×10^{12} to 1.0×10^{13} particles/g nitrogen, whereas suspensions of trypsin-celite-purified MRC-4f contained 1×10^{13} to 3×10^{13} particles/g

nitrogen. Thus, in terms of nitrogen, immunopurified MRC-4f was less pure than other TRIC isolates for which there are data (Weiss *et al.* 1964; Wang *et al.* 1967; Detels *et al.* 1968). Nevertheless, considerable purification with respect to E_{280} adsorbing (and presumably nitrogen poor) materials was achieved (Fig. 4).

The most probable sources of residual nitrogenous contamination were: egg antigens which had been specifically or non-specifically adsorbed and subsequently desorbed, or immunosorbent protein dissolved by the pH 11.3 buffer. The nature of the contaminants in the immunopurified MRC-4f suspensions was investigated by guinea-pig hypersensitivity tests. Egg antigen was detected in five out of six samples (tested in guinea pigs sensitive to a minimum of 500 ng of normal yolk sac antigens) whilst normal rabbit serum antigens (i.e. immunosorbent proteins) were detected in only one of the six samples tested (< 500 fg/ml). To confirm the presence of egg antigens, all desorbed samples were pooled, concentrated and used to immunize two rabbits following the schedule described in Methods for the production of anti-egg serum. Anti-egg antibodies were detected by immunodiffusion in the sera of both rabbits.

The egg antigens in the desorbed pools of MRC-4f might have come from non-specific adsorption of egg by the immunosorbent column or specific adsorption by the immunosorbent itself. The latter might have occurred if the serum from which the polymer was derived contained non-precipitating anti-egg antibodies undetected by immunodiffusion tests. [In this context, Nicklin & Stephen (1974) demonstrated that non-precipitating anti-lysozyme antibody when polymerized in an immunosorbent was capable of adsorbing its homologous antigen and that the antigen could be subsequently desorbed.]

Purification of MRC-4f, and other chlamydiae, using anti-TRIC immunosorbents derived from non-avid antisera raised to MRC-4f passaged three times through HeLa cells. Experiments were conducted with immunosorbent derived from a non-avid serum raised in rabbits by a single injection of thrice-passaged, HeLa cell-grown MRC-4f. Preliminary fractionation of crude homogenates of egg-grown MRC-4f on Sepharose 4B was introduced to simplify the purification procedure. Three columns were prepared, each having a similar capacity for MRC-4f (5×10^6 to 12.5×10^6 particles/mg immunosorbent protein). The yield of MRC-4f desorbed was similar for all columns and was approximately 33 %. The elution profile was similar to that in Fig. 4, the main difference being that the unadsorbed peak was more heterogenous, reflecting the nature of the starting material. Columns were re-cycled up to 11 times without loss of capacity.

The adsorption and desorption of several chlamydiae using a single anti-MRC-4f immunosorbent column was investigated. Each of three strains of chlamydiae (MRC-4, gp-ic and Bour f) were grown in the yolk sac. The capacity of the immunosorbent for all three heterologous agents (6×10^6 to 11×10^6 particles/mg immunosorbent protein) was similar to that for the homologous agent (MRC-4f), and yields of desorbed gp-ic and Bour f were similar to those of desorbed MRC-4f. The recovery of strain MRC-4 on desorption was approximately 50 % less than the yields for the others.

A suspension of immunopurified MRC-4f from one experiment contained 1.1×10^{12} particles/g nitrogen but suspensions from others contained only 1×10^{11} to 3×10^{11} particles/g nitrogen. The samples were still contaminated with egg antigens whose presence could hardly be attributed to anti-egg antibodies in the serum from which the immunosorbent was derived. A considerable purification was again achieved with respect to E_{280} absorbing materials but not in terms of nitrogen.

Adsorption of egg antigens by Sephadex G-15 in immunosorbent columns. The previous experiment, together with the first experiment in this section, suggested that the egg antigens

in the desorbed fractions of MRC-4f arose by non-specific adsorption by the Sephadex component of the immunosorbent column. This was tested by applying Sepharose-fractionated MRC-4f to a column of Sephadex G-15, washing with PBS and eluting with 0.2 M-arginine-HCl buffer, pH 11.3. Egg antigens were detected in both the unadsorbed and in the desorbed fractions. A similar result was obtained with a fresh column of Sephadex G-15. All the MRC-4f applied was recovered unadsorbed at neutral pH.

Further attempts to purify immunosorbent-fractionated MRC-4f. Since contamination of immunopurified MRC-4f with soluble egg antigens was attributable to Sephadex G-15 (a necessary component in the construction of immunosorbent columns; Crook *et al.* 1972b), attempts were made to exploit Sephadex to purify MRC-4f further. A sample of immunopurified MRC-4f was passed through a column of Sephadex G-15 (equivalent to the volume of Sephadex in the immunosorbent column) equilibrated in PBS. Some of the egg antigens were adsorbed by the Sephadex but the remainder were recovered in the eluate, together with approximately 50 % of the applied TRIC agent. The unadsorbed material was re-run on the same column after elution with 0.2 M-arginine-HCl buffer, pH 11.3, and re-equilibration in PBS; it resolved again into an unadsorbed fraction (smaller than in the first cycle) containing egg antigens and no detectable TRIC, and a fraction which adsorbed to the Sephadex G-15. This approach was therefore abandoned.

Attempts were then made to remove egg antigens specifically using an anti-egg immunosorbent column derived from an avid anti-egg serum. Three adsorption-desorption cycles were performed with immunopurified MRC-4f. The unadsorbed PBS eluate never contained any MRC-4f and had only slight 280 nm absorption. No MRC-4f was detected in the neutral desorbed fraction when the anti-egg immunosorbent was eluted with 0.2 M-arginine-HCl buffer, pH 11.3. Two adsorption-desorption cycles were then performed using two separately prepared batches of trypsin-celite-fractionated MRC-4f. With each, a high percentage of the applied MRC-4f was adsorbed by the anti-egg immunosorbent but a small quantity (10 and 21 %, respectively) of MRC-4f was recovered unadsorbed in the PBS eluate. These results suggested that some egg antigens were closely associated with TRIC particles and were responsible for binding particles to the anti-egg immunosorbent. Recovery of some particles - derived by partial purification of egg-grown TRIC by the trypsin-celite procedure - unadsorbed by anti-egg immunosorbent suggested that the particle-associated egg antigen might be removed by treatment of the organisms with an appropriate enzyme.

Purification of MRC-4f using phospholipase C

Since treatment of MRC-4f with trypsin might have removed particle-associated egg antigens, further (but only partially successful) studies with this enzyme were made. The effect of phospholipase was also examined because of the probable nature of the contaminants. Phospholipid contents and dry weights were used as additional criteria of purity.

Purification of MRC-4f with crude phospholipase C. Modified KCl-fractionated MRC-4f (4.5×10^9 particles/ml) was treated with crude *Clostridium welchii* culture filtrate (1.6 mg/ml, 25 LV units phospholipase C/ml). Samples were taken after 5, 15, 60 and 120 min incubation, stored, filtered, centrifuged, resuspended and analysed. The most rapid change in particle to nitrogen, lipid phosphorus or dry weight ratios occurred within 10 min of incubation (Fig. 5); the experiment was stopped at 2 h. Whilst enzyme treatment gave only a 2.2-fold increase in purity as measured by total nitrogen, 3.6-fold and 6.6-fold purifications were achieved in terms of lipid phosphorus and dry weight, respectively. From the nitrogen measurements, the suspension of MRC-4f was approximately tenfold more pure than preparations of

MRC-4f purified by the trypsin-celite procedure. Guinea-pig hypersensitivity tests did not detect egg antigens in the suspension of MRC-4f treated with enzyme for 2 h. The recovery of TRIC particles at the modified KCl-fractionation stage was 46 % of the original crude homogenate and the overall recovery after all subsequent treatments was 45 %, i.e. little loss was sustained from the modified KCl-fractionation stage to the final product.

In four experiments in which separate suspensions of MRC-4f were treated with crude *Cl. welchii* culture filtrate (25 LV units phospholipase C/ml, 2 h at 37 °C), the increases in purity achieved were approximately twofold by total nitrogen measurements (mean 6.6×10^{13} particles/g nitrogen) and 3.8-fold by total lipid phosphorus (mean 1.6×10^{15} particles/g lipid phosphorus).

Purification of MRC-4f with pure Cl. welchii phospholipase C. To confirm that, of the large number of enzymes known to be present in the crude *Cl. welchii* culture filtrate, the phospholipase C was the enzyme responsible for the observed purification of MRC-4f, a sample of phospholipase was purified from the culture filtrate by immunosorbent fractionation (Bird *et al.* 1974). A batch of modified KCl-fractionated MRC-4f (3×10^9 particles/ml) was treated with purified enzyme (13 units phospholipase C/ml) using the method described above, except that the time of incubation was restricted to 60 min because of the known thermal lability of the purified enzyme. The purified suspension of MRC-4f contained 5.8×10^{13} particles/g nitrogen and 8.7×10^{14} particles/g lipid phosphorus. The degree of purification achieved was similar to that recorded above.

Attempts to detect contaminating egg antigens in phospholipase C-purified MRC-4f using anti-egg immunosorbents. Anti-egg immunosorbent was used to determine whether treatment of suspensions of yolk sac-grown MRC-4f with phospholipase C had removed egg antigen complexes from the TRIC particles. This technique was employed since it was predicted that 'egg-free particles' should not be adsorbed. Moreover, a batch process was used to overcome the adsorptive effects of Sephadex G-15. Phospholipase C-treated MRC-4f was compared with KCl-fractionated MRC-4f. Each suspension was divided into three parts and added to 5 mg quantities of three polymers: an avid anti-egg immunosorbent, a non-avid anti-MRC-4f immunosorbent (from serum raised to thrice passaged-HeLa cell-grown MRC-4f) and a polymer derived from unrelated γ -globulin; the latter had previously been shown not to react with normal yolk sac antigens in immunodiffusion tests. KCl-fractionated MRC-4f behaved as predicted: there was no retention of the TRIC particles by the unrelated γ -globulin polymer; the anti-MRC-4f immunosorbent exhibited its expected capacity for the homologous agent (8×10^6 particles/mg polymer protein); MRC-4f particles were adsorbed to the anti-egg immunosorbent.

The suspension of MRC-4f purified by phospholipase C did not behave as predicted for there was non-specific adsorption of the enzyme-purified MRC-4f to the unrelated γ -globulin polymer; this had not previously been detected. The anti-MRC-4f immunosorbent adsorbed the predicted amount of agent; some TRIC particles were also adsorbed by the anti-egg immunosorbent. In a second experiment, phospholipase C-purified MRC-4f was pretreated with egg antigens; the latter were derived from the supernatant from the first high-speed centrifugation in the preparation of modified KCl-fractionated MRC-4f. The rationale here was to convert the phospholipase C-treated particles to the form in which they were not adsorbed. Such pretreatment caused partial inhibition of the non-specific adsorption of the TRIC particles by unrelated polymers: only 45 % of the applied load was adsorbed by 5 mg of polymer compared with 85 % of phospholipase C-purified MRC-4f adsorbed when not pretreated with egg antigens. The kinetics of adsorption of phospholipase C-treated MRC-4f by its homologous anti-MRC-4f immunosorbent and by the polymer

derived from unrelated γ -globulin did not distinguish between the rates of specific and non-specific adsorption, although the former might be predicted to be more rapid.

Effect of phospholipase C treatment on the viability of MRC-4f. In two experiments, phospholipase C treatment gave a 100-fold drop in infectivity (measured in HeLa cells) of MRC-4f below that of the modified KCl-fractionation stage. This loss occurred in two stages: a tenfold loss during incubation at 37 °C for 2 h, and a further tenfold loss after over-night standing, filtration and centrifugation. In an attempt to reduce the loss of infectivity, the conditions of phospholipase treatment were altered. The ionic strength (approximately 0.5) of the borate buffer saline was reduced by half (but without changing the concentration of calcium chloride – 0.05 %, w/v) and the tonicity of the buffer was increased by the addition of 0.25-M-sucrose. The concentration of enzyme was increased fourfold and the time of incubation at 37 °C reduced to 30 min. Under these conditions, a quantity of modified KCl-fractionated MRC-4f was treated with crude phospholipase C and immediately filtered, centrifuged and assayed. There was no significant inactivation of the particles arising from this purification procedure. The final product contained 5.7×10^{13} particles/g nitrogen and 1.5×10^{15} particles/g lipid phosphorus.

DISCUSSION

The main objective of this work was to apply immunosorbent technology to purify yolk sac-grown MRC-4f. A column procedure was developed, resulting in considerable – but not complete – purification of the organisms with full retention of infectivity. The enzymic and serological studies, together with the adsorption properties of the purified particles, indicated that the principal contaminants in preparations of immunopurified MRC-4f were soluble and particle-associated phospholipid-containing antigens.

The experiments also provided information on the mechanism of antigen desorption and the interactions of phospholipids in these disulphide-linked immunosorbent systems. Detailed arguments have been presented elsewhere (Crook *et al.* 1972*b*) for the view that successful desorption of homologous antigen with buffers of extreme pH depends on a rapid rate of protonation (or alternatively, hydroxylation) of the antigen and its rapid return to neutral conditions. In practice this is achieved when the desorbing buffer front elutes as a sharp pH-gradient and the immunosorbent column is appropriately constructed with suitable molecular exclusion material such as Sephadex G-15. This work provides direct evidence that the period of contact between bound antigen and desorbing buffer must be short: infective particles were recovered using as desorbent a buffer which destroyed the particles in suspension.

Consideration of the interactions of phospholipids will be restricted to those present in semi-purified preparations. Specific adsorption of TRIC particles by homologous immunosorbent is in part dependent on an egg-derived phospholipid fraction associated with the particles. It cannot cover the whole surface, otherwise no TRIC-specific adsorption would have been possible. Removal of this material with phospholipase C removes the specificity of particle uptake by –S–S–linked immunosorbents. It is not clear whether the phospholipid prevents non-specific adsorption by altering the properties of the particles *per se* or by excess of such material saturating non-specific binding sites on the polymer.

The interaction of soluble phospholipids with Sephadex G-15 also proved important and caused contamination of immunopurified MRC-4f particles. These substances were adsorbed by the Sephadex G-15 component of immunosorbent columns and desorbed by the alkaline buffer used to desorb TRIC particles from –S–S–linked antibody. The presence of phospho-

lipid prevented the adsorption of TRIC particles by Sephadex G-15 on its own, whereas the absence of phospholipid allowed this. It appears, therefore, that to exploit fully a two-step, immunospecific fractionation system for isolating infective TRIC (or other chlamydiae) from the complex lipid-rich mixture that comprises yolk sac homogenates, a substitute for Sephadex G-15 must be found. This should possess the molecular exclusion properties of Sephadex G-15, and should not adsorb phospholipid materials or 'clean' TRIC particle at neutral pH. However, it is unlikely that such improvements would succeed in removing particle-associated host contaminants; the latter may best be removed by chemical or, as here, by enzymic methods. The most highly purified preparations of chlamydiae were either obtained from sources other than yolk sac (Jenkin, 1960; Garrett, Harrison & Manire, 1974), or purified by methods which include chemical (Wang *et al.* 1967) or enzymic (Weiss *et al.* 1964) treatments.

The experiments with phospholipase C suggest a new approach to obtaining good yields of egg-grown chlamydiae which retain infectivity. However, relatively less pure preparations of modified KCl-fractionated MRC-4f yielded relatively less pure preparations of MRC-4f (by the criteria of nitrogen and lipid phosphorus) after treatment with phospholipase C. The most probable explanation for the variations observed in the purity of the final product is that the standard conditions of enzyme treatment – concentration of enzyme in relation to the available substrate, present both as free and particle-associated phospholipid, and the time of incubation – were too varied to yield reproducible results. A second explanation is that the method for separating the purified particles from the gel-like aggregates was not good enough. A third possibility is that the differences in the purity of the suspensions of modified KCl-fractionated MRC-4f are due to differing degrees of contamination with substances other than phospholipid.

Viable TRIC particles were obtained with the highest measured particle:g nitrogen ratio ever reported for yolk sac-grown agent. Purities were measured as particle:phospholipid (lipid phosphorus) ratios. Reproducible retention of infectivity is clearly a function of optimizing conditions for enzyme treatment and removal of the gel-like aggregates which form as a result. An additional point arising from these studies is that the enzyme may remove a particle-specific component without affecting infectivity. Gogolak & Ross (1955) and Ross & Gogolak (1957) reported a phospholipid (lecithin) as part of the chlamydial group-specific complement-fixing antigen. Analysing highly purified suspensions of allantoic-grown meningopneumonitis agent, Jenkin (1960) also detected phospholipid in the chlamydial particle. Thus the effect of enzyme treatment on the group antigen must be investigated, especially because there is no information about the role of the group-specific complement-fixing antigen in infectivity. It would be interesting to examine organisms treated with phospholipase C, with their apparently altered surface properties, in the biological system of Woodland, Griffiths & Pearce (1973) who are investigating adherence phenomena as the possible basis of specificity in chlamydial infections.

Finally, in immunodiffusion tests, seven TRIC-specific precipitins were detected in avid sera. This is similar to the results of Katzenelson & Bernkopf (1967) and Kuo, Kenny & Wang (1971). Immunodiffusion analysis indicated three common antigens in MRC-4f (*Chlamydia trachomatis*) and gp-ic (*Chl. psittaci*). Kuo *et al.* (1971) suggested three antigens are common to members of the genus. There was no apparent difference between the two f variants tested, MRC-4f and Bour f, but MRC-4f could be distinguished from its parent MRC-4. Because of these antigenic similarities, all four agents tested could be 'purified' on a single (anti-MRC-4f) immunosorbent column. Non-avid anti-MRC-4f antisera usually showed fewer precipitins in immunodiffusion tests.

After this manuscript had been submitted, our attention was drawn to the work of Howard, Orenstein & King (1974), who purified *Chl. trachomatis* from egg yolk sacs by centrifugation on Renografin density gradients. The purity of their final preparations was 5.7×10^{12} to 7.0×10^{12} particles/g dry weight and 7.8×10^{12} to 9.7×10^{12} particles/g protein. These are not so good as the best data in this paper but we think that this technique could be valuable as a first-stage purification step.

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