

## Neutralization of TRIC organisms by antibody: enhancement by antisera prepared against immunoglobulins

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### SUMMARY

The neutralizing activities of fowl, rabbit and mouse antisera prepared against TRIC agents were enhanced, in some cases over a 100-fold, by the addition of antiserum against the appropriate IgG. Significant non-specific inactivation of the organisms was observed in the absence of antiserum against TRIC agents, in reaction mixtures containing normal serum and antiserum against IgG. Since the extent of this inactivation showed a prozone, control tests with dilutions of normal serum equivalent to the full range of dilutions of the antiserum tested are necessary to show that enhancement of neutralization is specific.

### INTRODUCTION

Antibodies that neutralize *Chlamydia* can be found in serum from naturally infected hosts and from immunized animals, but usually their titres are so low as to make the neutralization test of little use either in laboratory work or in diagnosis. Williams & Hahon (1970) reported, however, that the titre of neutralizing antibody in serum from roosters immunized against psittacosis was enhanced significantly in the presence of antiserum against rooster immunoglobulins. They also obtained higher titres of neutralizing antibodies in the sera of patients suffering from subclinical or overt psittacosis when antiserum against human immunoglobulins was included in the neutralization test.

We report here that although the neutralizing activity of antisera against TRIC agents can be significantly enhanced by adding antiserum against IgG, commensurate inactivation was sometimes obtained when normal serum was substituted for antiserum against the TRIC agent.

### MATERIALS AND METHODS

#### *TRIC agents*

The following strains were used: PK-2*f* (T'ang, Chang, Huang & Wang, 1957), MRC-4 (Jones, 1961) and MRC-4*f*. The suffix *f* indicates a fast-killing variant (Taverne, Blyth & Reeve, 1964).

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### *Preparation of pools*

Pools were made from infected chick embryo yolk sacs; they were treated with m-KCl and stored in 0.25 M sucrose in buffer (Taverne & Blyth, 1971).

### *BHK-21 cells*

Methods and media for culture of these cells, and for infectivity titrations of TRIC agents in them, have been described (Blyth & Taverne, 1974). Cultures were inoculated with MRC-4 by centrifugation at 2000 g for 30 min at 35° C.; they were fixed with methyl alcohol 3 days later. For inoculation with PK-2f and MRC-4f, cultures were centrifuged at 600 g for 30 min. at 35° C. and fixed 2 days later. Iodine stained inclusions were counted in 30 microscope fields chosen at random from each of three replicate cultures and the mean number per field was calculated.

### *Antisera*

*Rabbits* were injected at monthly intervals with strain PK-2f grown in BHK-21 cells. They were given six intravenous injections of 1 ml. each, containing about  $5 \times 10^9$  infective organisms, and were bled 1 week after the last injection.

*Mice* received equal parts of a yolk sac suspension of PK-2f containing  $7 \times 10^9$  infective organisms/ml. and Freund's complete adjuvant. They were injected intramuscularly with 0.2 ml. into each hind leg; a month later they received by the same route a second dose of suspension without adjuvant. They were bled a fortnight later and the serum was pooled.

*Fowl.* A cockerel was given six intraperitoneal injections of a suspension of MRC-4f, each equivalent to one infected yolk sac, at weekly intervals. It was bled 8 days after the last injection.

*Baboons.* The conjunctivae of six baboons were inoculated with a yolk sac suspension of MRC-4. All animals developed an infection and the mean score (Collier & Blyth, 1966) for the group was 42.6; they were bled 6 weeks after inoculation and the serum was pooled.

### *Antisera against immunoglobulins*

'Species precipitating sera' against hen, mouse and rabbit serum globulins were obtained from Wellcome Reagents Ltd. Antisera prepared in rabbits against hen IgG and in goats against rabbit IgG (Cappel Laboratories, Downington, Pa., U.S.A.) were also tested. Goat antiserum against human IgG was kindly given to us by Dr T. Phillips, MRC Blood Group Reference Laboratory, London. Although the activity of the antisera differed quantitatively, precipitation and non-specific inactivation were observed with those prepared against purified IgG as well as with the 'species precipitating sera'.

### *Neutralization tests*

Dilutions of antisera, none of which contained complement, were made in maintenance medium containing 50% calf serum. Reaction mixtures containing 0.5 ml. of an appropriate concentration of TRIC organisms and 0.5 ml. of a

Table 1. *Enhancement of neutralization by antisera against IgG*

TRIC agent	Source of antiserum v. TRIC agent	Dilution of antiserum v. TRIC agent*	Dilution of antiserum v. IgG*					
			2	4	8	16	32	128
MRC-4f	Fowl							
	Batch 1	10	.	.	> 93†	.	> 93	4.2
	Batch 2	16	.	> 300	.	50	.	.
PK-2f	Rabbit	8	10	.	3	.	1.5	1.1
PK-2f	Mouse	4	.	.	203	.	65	8.5

\* Reciprocal.

† Enhancement of neutralization:  $\frac{\text{no. of inclusions/field with antiserum v TRIC alone}}{\text{no. of inclusions/field with antiserum v TRIC and antiserum v IgG}}$ .

Table 2. *Neutralization titres of antisera against TRIC agents in the presence and absence of antisera against IgG*

TRIC agent	Source of antiserum	Dilution of antiserum v. IgG*	Titre of antiserum v. TRIC*	
			Alone	With antiserum v. IgG
MRC-4f	Fowl	15	12	1280
PK-2f	Rabbit	2	32	2560

\* Reciprocal.

dilution of homologous antiserum were incubated for 30 min. at 35° C. Each mixture then received 0.5 ml. of a dilution of antiserum against the IgG of the species in which the antiserum against the TRIC agent had been prepared (or 0.5 ml. of medium with 50% calf serum), and was incubated for a further 15 min. Final dilutions were therefore 3-fold higher than those quoted. From each reaction mixture, 0.4 ml. volumes were added to each of three cultures of BHK-21 cells which were then centrifuged at 35° C. The end-point of neutralization was calculated as the reciprocal of that dilution of test serum which decreased the number of inclusions by 50% compared with the controls containing calf serum.

## RESULTS

*Titration of antisera prepared against IgG*

The enhancing effect of antiserum against IgG was tested on organisms sensitized by incubation with specific antisera prepared in various animals against TRIC agents. For each experiment a dilution of sensitizing antiserum was chosen which decreased the number of infective organisms by about half during the initial incubation. Dilutions of antiserum against the appropriate IgG were then added to the mixtures, which were incubated for a further 15 min. A control containing one dilution of antiserum against IgG was included in each test with a dilution of normal serum from the same animal species substituted for the sensitizing serum. There were no significant differences between the numbers of inclusions in these controls and in others containing 50% calf serum.

Table 3. *Non-specific inactivation of TRIC organisms in the presence of normal serum and antiserum against IgG*

Source of normal serum	TRIC agent	Dilution of normal serum*	Dilution of antiserum against IgG*	Inactivation (%)†	Precipitation
Fowl	MRC-4f	8	.	5	—
		.	4	27	—
		8	4	17	+
		16	4	46	++
		32	4	32	+++
Baboon	MRC-4	8	.	0	—
		.	2‡	0	—
		8	8	0	+
		16	8	17	++
		32	8	94	+++
		64	8	64	+++
Rabbit	MRC-4f	10	.	0	—
		.	8	0	—
		8	8	43	—

\* Reciprocal.

† Compared with control containing 50 % calf serum.

‡ Antiserum against human IgG.

Antisera against fowl, rabbit and mouse IgG all enhanced neutralization to some extent (Table 1); that against rabbit IgG was weak and another batch showed no activity. On the basis of these results, a dilution of each antiserum against IgG was chosen for use in subsequent experiments.

*Titration in the presence of antiserum against IgG of specific antisera prepared against TRIC agents*

The neutralizing activities of two antisera against TRIC agents were enhanced about a 100-fold in the presence of the selected dilution of antiserum against the appropriate IgG (Table 2). By itself, a 1/4 dilution of pooled serum from a group of baboons infected conjunctivally with strain MRC-4 did not neutralize, but even higher dilutions did so when a 1/8 dilution of antiserum against human IgG was added: after a prozone, neutralization rose to a maximum of 64 % in the reaction mixture containing a 1/64 dilution of baboon serum. In this titration, however, a precipitate which also showed a prozone was observed in some reaction mixtures. This finding suggested that the decrease in infectivity might not have been caused by specific antibody but be related to the precipitate, since the two effects occurred in parallel. TRIC organisms were therefore incubated with a range of dilutions of normal serum from different animal species and the appropriate antiserum against IgG (Table 3). Some inactivation occurred in all these mixtures which with baboon serum reached 94 %. A precipitate formed in some reaction mixtures of the series containing fowl or baboon serum and prozones occurred in both precipitation and inactivation.

Table 4. Neutralization of strain MRC-4f by homologous antiserum prepared in a cockerel; enhancement by antiserum against fowl IgG

Antiserum v. IgG	Normal serum			Antiserum v TRIC			Titre†
	Dilution*	Inactivation (%)	Precipita- tion	Dilution*	Neutraliza- tion (%)	Precipita- tion	
None	8	5	—	8	85	—	12
				10	76	—	
				16	21	—	
				40	18	—	
				160	20	—	
1/4	8	17	+	8	> 99	+	2560
	16	46	++	16	> 99	++	
	32	32	+++	32	> 99	+++	
	160	13	++	160	99	++	
	640	13	+	640	97	+	
	2560	12	—	2560	47	—	

\* Reciprocal.

† Reciprocal of dilution giving 50% neutralization.

In the light of these findings the fowl antiserum was titrated again, using as controls one dilution of antiserum against fowl IgG and the complete range of dilutions of normal fowl serum (Table 4). Normal serum alone at a dilution of 1/8 did not diminish infectivity significantly but with antiserum against fowl IgG some inactivation occurred; it reached a maximum of 46% with a 1/16 dilution of normal serum. By itself the antiserum against TRIC agent had a titre of 1/12, which was increased to 1/2560 in the presence of antiserum against IgG, an enhancement of about 200-fold. It is apparent that this enhancement is specific since mixtures containing dilutions of normal serum above 1/32 did not inactivate the organisms significantly. Precipitation was greatest at a dilution of 1/32 both with normal serum and with antiserum against TRIC agent.

## DISCUSSION

Our demonstration that specific neutralization of TRIC agents can be enhanced by antiserum against IgG confirms with *Chlamydia* group A the findings of Williams & Hahon (1970) with *Chlamydia* group B. Specific neutralization was significantly greater than the extensive non-specific inactivation produced by normal serum in the presence of antiserum against IgG. With viruses, non-specific inactivation of this kind does not appear to have been a major problem, although Brown, Elsner, Zebovitz & Allen (1969), who studied Group A arboviruses, reported 'a rare case of non-specific inhibition in mouse fluids up to 1:5 dilution'. It is not possible to determine from most reports whether sufficient controls were included to cover the range of dilutions of normal serum equivalent to that of antiserum. Although it is likely that gross precipitation would have been noticed, we emphasize that non-specific inactivation sometimes occurs in the absence of visible precipitation.

It is well known that not all specific antibodies that combine with virus particles

neutralize infectivity, and that particles may sometimes become sensitized so that subsequent reaction with antibody against immunoglobulin renders them un-infective. Results obtained with chlamydiae may, however, be only superficially similar; nothing is known about the mechanism of their neutralization and there is no reason to believe that it is the same as for viruses. Neutralization of chlamydiae may not involve a specific alteration of the organism, or the blocking of a particular site on its surface, but rather an altered reaction of the host cell to an organism coated with antibody.

Infective elementary bodies are phagocytosed selectively by the host cell and the vacuole in which they multiply does not fuse with lysosomes (Lawn, Blyth & Taverne, 1973). By contrast, in the macrophage – a cell in which the organism fails to multiply – elementary bodies enter lysosomes and are inactivated. An organism coated with antibody is perhaps not recognized by the host cell and so fails to enter, or on entry is immediately transferred to a lysosome; in either case no chlamydial inclusion would develop and the organism would apparently be ‘neutralized’. Furthermore, if the host cell fails to recognize elementary bodies coated non-specifically with globulin combined with antibody against globulin, inactivation in the absence of specific antibody would be explained. If this hypothesis is correct, then measurements of neutralizing antibodies against chlamydiae and enhancement of their titre are of no special value, and merely provide an insensitive way of assaying antibodies that are more easily measured by complement fixation or immunofluorescence.

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