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DETERMINATION OF ANTIBACTERIAL ANTIBODIES IN SERUM BY IMMUNORADIOMETRIC ASSAYS

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THE early detection of serum antibody is one possible means of making a rapid diagnosis of an infectious disease. For this purpose, immunoradiometric assays appear to offer the advantages of high sensitivity and precision. Of the several different principles that can be used as a basis for immunoradiometric assay, two were selected for rigorous assessment. These were, first, competition between antibody in the test sample and added isotopically-labelled homologous antibody for attachment to antigen and, second, attachment of antibody in the test sample to solid-phase antigen, followed by reaction of the resulting complex with isotopically-labelled homologous anti-globulin (Rosenthal, Hayashi and Notkins, 1972; Nielsen, Parratt and White, 1973; Restall, Thomas and Pennant, 1973; Smith, Gehle and McCracken, 1974; Nassau, Parsons and Johnson, 1975). When assay procedures based on the two principles were applied to samples containing known concentrations of immunopurified anti-bacterial globulins in saline-phosphate buffer, sensitive detection and reproducible results were obtained. However, normal human and rabbit sera interfered in both assay systems, markedly decreasing their sensitivity and precision. Attempts were made to eliminate this interference.

We report details of the immunoradiometric assays and their performances, means of decreasing interference by serum, and a comparison of the sensitivities of these assays with the sensitivity of a conventional agglutination test.

MATERIALS AND METHODS

Micro-organisms. *Francisella tularensis* (Live Vaccine strain, "LVS"; Eigelsbach and Downs, 1961; Tigertt, 1962; Scharer, Klein and Lincoln, 1968) was grown in batch culture in the medium of Scharer *et al.* (1968). Washed live and formaldehyde-killed suspensions

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were prepared as described by Hambleton *et al.* (1974). Washed live and killed suspensions of *Serratia marcescens* strain 8UK were prepared as described by Strange and Martin (1972).

Trichloroacetic acid-soluble bacterial antigens. A pellet of washed live bacteria (about 4×10^{12}) was suspended in five times its volume of water, an equal volume of cold 10% (w/v) trichloroacetic acid added, and the suspension held at 4°C for 24 h. The suspension was centrifuged (10 000 g, 15 min.) and the pellet discarded. The pH of the extract was adjusted to 6.5, 2 volumes of cold (−10°C) ethanol were added and the mixture was held at −10°C for 16 h. Precipitated material was sedimented (20 000 g, 20 min.), dispersed in water and dialysed against water (24 h). The dialysate was centrifuged (10 000 g, 10 min.) and the supernate ("antigen preparation") volume adjusted to 5 ml with water.

Purification of antibodies. Rabbit antibacterial globulins and sheep anti-rabbit (SAR) globulins were immunopurified as described previously (Strange, Powell and Pearce, 1971; Strange and Hambleton, 1976).

Radioactive labelling of antibodies. Immunopurified globulins were iodinated with ^{125}I by the method of Hunter and Greenwood (1962) as described previously (Strange *et al.*, 1971; Strange and Hambleton, 1976).

Animals. New Zealand White rabbits were supplied by Allington Farm, Porton Down, Salisbury SP4 0JG.

Vaccination of rabbits. Rabbits (2–3 kg) received a single intraperitoneal injection of a washed suspension of live *F. tularensis* strain LVS (1 ml, 5×10^8 bacteria). Blood samples, taken from an ear vein before and at intervals after vaccination, were centrifuged and the separated sera filtered on Millipore membranes (pore size 0.22 μm).

Pre-treatment of sera

DEAE-cellulose chromatography. Serum samples (1 ml) were loaded on to columns (4×1 cm) of DEAE-cellulose (DE52, Whatman Laboratory Sales Ltd, Maidstone, Kent, pre-equilibrated with 10mM sodium-phosphate buffer, pH 8.0). Columns were eluted with about 14 ml of half-strength saline-phosphate buffer (0.055M NaCl, 0.01M sodium phosphate, pH 7.7) and the total eluate in each case was concentrated to 1 ml by pressure dialysis and filtered on a Millipore membrane (0.22 μm). Cellulose acetate electrophoresis of eluates showed that all the γ globulin together with other protein components in the original sera were eluted.

Heat treatment. Sera were heated at 56°C for 30 min.

Ammonium sulphate fractionation. Unheated and heated sera were mixed with an equal volume of saturated (at 20°C) $(\text{NH}_4)_2\text{SO}_4$ solution and the suspensions held at 4°C for 1 h. Precipitates were separated by centrifugation, dissolved in saline-phosphate buffer (0.11M NaCl, 0.02M sodium phosphate, pH 7.7), reprecipitated and finally dialysed against the same buffer until free of detectable ammonium ion. The volume of the solution was adjusted to that of the original serum sample and the solution filtered on a Millipore membrane (0.22 μm).

Other treatments. Sera were exhaustively dialysed against saline-phosphate buffer, pH 7.7; for some tests, NaCl and EDTA (disodium, adjusted to pH 7.6 with NaOH) were added to dialysed sera to final concentrations of 0.5M and 10mM, respectively.

Direct competitive immunoradiometric assay

All solutions were filtered through Millipore membranes (0.22 μm) before use. Samples (0.1 ml) of normal serum or saline-phosphate buffer with and without added antibody, or immune serum, were mixed with ^{125}I -labelled homologous antibody (20 μl ; 440 ng of antibody protein; 3.5–13 μCi per mg) in plastic tubes (7.5×1 cm) and the reaction started by adding a suspension of washed killed bacteria (10 μl , 2×10^8 bacteria per ml). Reaction mixtures were incubated at 25°C for 3 h and the resulting immune complexes were separated and washed on Millipore membranes (diameter 2 cm; 0.45 μm pore size) as described by Strange *et al.* (1971). The filtration areas (diameter 7 mm) of the filters were punched out and their radioactivities measured with an automatic γ counter (Model 8311; Nuclear Enterprises Ltd, Sighthill, Edinburgh).

Indirect immunoradiometric assay

Portions of killed bacterial suspensions ($10\ \mu\text{l}$, 5×10^{10} bacteria per ml) or trichloroacetic-acid-soluble antigens ($5\text{--}10\ \mu\text{l}$) were measured into the wells of plastic Microtitre plates (MRC-96, Linbro Chemical Co., New Haven, New York, USA), spread with a fine glass rod and dried at 60°C . Methanol ($50\ \mu\text{l}$) was added to each well and evaporated to dryness at 20°C . Samples ($20\text{--}50\ \mu\text{l}$) of sera or saline-phosphate buffer, with and without added rabbit antibacterial antibodies were placed in the wells and allowed to react for $30\text{--}120\ \text{min.}$ at 20°C . The wells were then washed several times with saline-phosphate buffer containing 1% (w/v) Brij 35 (polyoxyethylene lauryl ether; BDH Chemicals Ltd, Poole, Dorset) followed with saline buffer alone and allowed to dry. ^{125}I -labelled SAR globulin reagent ($20\ \mu\text{l}$; $440\ \text{ng}$ of antibody protein; $1.5\ \mu\text{Ci}$ per μg) was added to each well and after 2 h at 25°C , the wells were washed as before. Individual wells were cut out, placed in vials and counted as above. In some cases, immune complexes in the wells were extracted with 0.1N NaOH ($0.1\ \text{ml}$, 3 min.) and the solutions with washings transferred to vials and counted.

In attempts to decrease adsorption of non-immune globulins from rabbit sera (see *Results*), the wells were treated with normal sheep, horse, bovine or human serum, or globulin fractions from them, before or after fixing antigen, or both, or after reaction of fixed antigen with samples of rabbit sera, or the former and last treatments combined.

Agglutination test

Serial doubling dilutions of sera or solutions of antibacterial globulins in saline-phosphate buffer ($\text{pH } 7.4$) in Microtitre-plate wells were mixed with an equal volume ($50\ \mu\text{l}$) of a suspension of killed homologous bacteria (5×10^9 per ml) and incubated at 37°C for 2 h. The plates were held for 48 h at 4°C before reading.

RESULTS

Measurement based on the response of immunopurified antibodies

Immunoradiometric assays and agglutination tests were calibrated by measuring the responses of known concentrations of immunopurified antibodies. The antibody proteins migrated as γ globulins on electrophoresis (cellulose-acetate strips) and had high specific precipitating activity against homologous bacteria (Strange and Martin, 1972) but their actual purity is not known. For calibration purposes, however, antibody preparations were assumed to be pure and hence the sensitivities of all the assay systems tested are probably underestimated.

Direct competitive immunoradiometric assay

In a given reaction period, or when equilibrium is reached, the amount of antibody combining with bacteria depends on the antibody concentration (fig. 1). Therefore, to compare calculated values and experimental results for the inhibition of uptake of labelled antibody by unlabelled antibody in a sample, uptake over the range of total antibody (labelled+unlabelled) concentrations tested must be known. These data were obtained by measuring uptake in the presence of the appropriate concentrations of labelled antibody alone. Tests with varying concentrations of labelled antibody and bacteria established the optimum conditions for determining $25\text{--}2000\ \text{ng}$ of antibody in saline-phosphate

buffer (fig. 2). If a sample contains x ng of unlabelled antibody (Ab) and y ng of labelled antibody (Ab*), the calculated inhibition value is $100 - \left(\frac{x}{x+y} \times 100 \right) \%$ and the experimental inhibition value,

$$100 - \frac{(\text{test signal} - \text{blank}) \text{ with } x \text{ Ab} + y \text{ Ab}^*}{(\text{signal} - \text{blank}) \text{ with } (x+y) \text{ Ab}^*} \times 100 \%$$

calculated and experimental inhibition values were in fair agreement (fig. 2) indicating that iodination of antibody with ^{125}I did not affect its ability to combine with antigen significantly.

When sera from apparently normal human beings and rabbits were tested with the competitive assay, uptake of labelled antibody by homologous bacteria was inhibited to a variable degree and almost completely by certain sera. It seemed unlikely that the sera tested contained antibodies against *F. tularensis* and *S. marcescens* in sufficient concentration to account for the inhibitory effects, and attempts were therefore made to eliminate this interference. Various pre-treatments of sera (see *Materials and methods*) including heating (56°C ; 0.5 h), separation of a crude globulin fraction by ammonium-sulphate fractionation, chromatography on DEAE-cellulose and exhaustive dialysis against saline-phosphate buffer (pH 7.7) all substantially decreased but usually did not

FIG. 1

FIG. 2

FIG. 1.—Effect of antibody concentration on antibody uptake by homologous bacteria. Reaction mixtures containing *Francisella tularensis* (2×10^6 cells), ^{125}I -labelled anti-*F. tularensis* globulin (0.175–2.64 μg ; 13 μCi per μg) in saline-phosphate buffer (pH 7.7) were incubated for 3 h at 25°C . Immune complexes were separated and washed on Millipore membranes (0.45 μm) and their radioactivities (signal minus blank) measured.

FIG. 2.—Inhibition of uptake by bacteria of ^{125}I -labelled antibody by unlabelled antibody in the competitive immunoradiometric assay. Reaction mixtures containing *F. tularensis* (2×10^6 cells), ^{125}I -labelled anti-*F. tularensis* globulin (0.44 μg ; 6.5 μCi per μg) with and without unlabelled anti-*F. tularensis* globulin (0–2.2 μg) in saline-phosphate buffer (pH 7.7; 40 μl) were incubated for 3 h at 25°C . Immune complexes were separated and washed on Millipore membranes (0.45 μm) and their radioactivities measured. Experimental inhibition (O); calculated inhibition (●).

entirely eliminate interference (tables I and II). None of these treatments significantly decreased the activity of antibody added to serum; for example, the effect of DEAE-cellulose chromatography on serum with and without added anti-*F. tularensis* globulin is shown in table II. Residual interference was further decreased by adding 0.5M NaCl and 10mM EDTA to serum before assay; with dialysed rabbit serum, inhibition values with increasing concentrations of added antibody were then close to those with similar concentrations in saline-phosphate buffer (fig. 3). Similar pre-treatment and reaction conditions also decreased interference by normal human serum but to a lesser extent; generally, inhibition was decreased from about 95% to about 25%. If the concentration of antibody added to serum was sufficiently high to allow dilution (at least 1 : 20) with saline-phosphate buffer before assay, pre-treatment of serum was unnecessary.

The minimum quantity of added antibody detectable in 50 μ l of dialysed rabbit serum was 25 ng (fig. 3), i.e., 500 ng of antibody per ml; the minimum concentration of the same antibody detectable by visible agglutination of homologous bacteria was 5–10 μ g per ml.

FIG. 3

FIG. 4

FIG. 3.—Competitive immunoradiometric assay on samples of treated rabbit serum (see text) and saline-phosphate buffer (pH 7.7) with and without added anti-*F. tularensis* globulin (0.1–1.0 μ g). Samples (0.1 ml) incubated with *F. tularensis* (2×10^6 cells) and 125 I-labelled anti-*F. tularensis* globulin (20 μ l; 0.44 μ g; 6.5 μ Ci per μ g) for 3 h at 25°C were processed as described in *Materials and methods*. Treated rabbit serum (●), saline-phosphate buffer (○).

FIG. 4.—Indirect immunoradiometric assay of antibody against *F. tularensis* in sera of rabbits before and after vaccination. Portions (50 μ l) of dilutions of rabbit serum in sheep serum (NSS) containing EDTA (10mM) were placed in wells of Microtitre plates containing fixed extracted antigens of *F. tularensis* pre-treated with NSS. After 1.5 h at 25°C, the wells were washed and 125 I-labelled sheep anti-rabbit globulin added (20 μ l; 0.44 μ g; 1.5 μ Ci per μ g). After 1.5 h at 25°C the wells were washed, cut out and their radioactivities (count minus blank) measured. Serum before vaccination (●), and after vaccination (○).

TABLE I
Effect of pre-treatment of normal sera on the competitive assay*

Sample	Treatment*	Mean test radioactivity (c.p.s.)†	Percentage of signal in saline buffer
Saline-buffer	Nil	886	100
Normal rabbit serum (NRS)	Nil	69	7.8
	1. Heat (56°C, 0.5 h)	264	30
	2. (NH ₄) ₂ SO ₄ precipitation of globulins	558	63
	1+2	568	64
Saline-buffer	Nil	589	100
NRS (i)	Nil	405	69
	Dialysis	589	100
NRS (ii)	Nil	249	42
	Dialysis	495	84
NRS (iii)	Nil	226	38
	Dialysis	545	93
Saline-buffer	Nil	552	100
NRS	Nil	36	6.5
	Dialysis	331	60

Reaction mixtures, in triplicate, contained sample, killed *Francisella tularensis* and ¹²⁵I-labelled anti-*F. tularensis* globulin.

* See *Materials and methods*

† Minus mean blank (no bacteria).

TABLE II
Effect of chromatography on a DEAE-cellulose column on interference by normal rabbit serum in the competitive assay*

Sample	Mean test radioactivity (c.p.s.)†	Percentage of signal in saline buffer
Saline-buffer	428	100.0
Normal rabbit serum (NRS)	195	45.6
NRS fraction from DEAE-cellulose column‡	409	95.6
Saline buffer + 1.3 µg of antibody	143	33.4
(NRS + antibody) fraction from DEAE-cellulose column (1.3 µg of antibody before treatment)	166	40.0

* Reaction mixtures as in table I.

† Minus mean blank (no bacteria).

‡ See *Materials and methods*.

TABLE III

Indirect immunoradiometric assay* of immunopurified anti-*S. marcescens* globulin in saline buffer and normal rabbit serum

Diluent	Antibody added (ng)	Mean response (c.p.s.)	Response—blank (c.p.s.)
Saline-phosphate buffer (pH 7.7)	0	38 ± 2.9†	...
	2	46 ± 3.1	8
	10	63 ± 2.7	25
	20	83 ± 10.8	45
	50	113 ± 3.9	75
	100	220 ± 10.5	182
	200	352 ± 15.8	315
Pooled normal rabbit serum	0	370 ± 25	...
	2	372 ± 18	...
	10	346 ± 29	...
	20	367 ± 20	...
	50	406 ± 25	36
	100	464 ± 18	94
	200	577 ± 33	207

* Triplicate samples (50 μ l) were assayed with the solid-phase indirect assay with trichloroacetic-acid-extracted antigens of *Serratia marcescens* (see *Materials and methods*).

† Mean deviation.

TABLE IV

Comparison of the competitive assay* and agglutination† tests for determining antibody in serum samples from a rabbit vaccinated with *F. tularensis* (strain LVS)

Sample	Competitive assay			Agglutination titre†
	Test radioactivity (c.p.s.)‡	Percentage inhibition	Antibody (µg per ml)	
Pre-vaccination	16 937	0	0	4
Hours post-vaccination	72	11 489	32	6.0
	96	10 826	36	7.2
	103	13 114	23	3.6
	120	6 128	64	21.6
	127	6 782	60	18.8
	144	3 202	81	38.8
	168	1 022	94	> 120.0

* Reaction mixtures containing samples (50 μ l) of dialysed serum with added NaCl (0.5M) and EDTA (10mM), *F. tularensis* (2×10^6 ; 10 μ l) and 125 I-labelled anti-*F. tularensis* globulin reagent (20 μ l; 440 ng of antibody; about 3.5 μ Ci per μ g) were incubated for 3 h at 25°C. The assay response was calibrated by testing samples of dialysed pre-vaccination serum containing known amounts of added anti-*F. tularensis* globulin.

† See *Materials and methods*.

‡ Minus blank (no bacteria).

Indirect two-antibody immunoradiometric assay

With killed bacteria or trichloroacetic acid-soluble bacterial antigens fixed to wells of plastic plates, 2 ng of immunopurified antibody in 50 μ l of saline-phosphate buffer was reproducibly detected with the indirect assay. However, when samples of normal rabbit sera without added antibody were assayed, radioactive counts were 10–20 times those obtained with saline-phosphate buffer and the agreement between results for replicate tests was poor (table III). Apparently, globulins in normal rabbit serum became attached to the plastic wells or fixed antigen, or both, and subsequently combined with ^{125}I -labelled anti-rabbit globulin. For this reason, pre-treatment of serum that decreased interference in the competitive assay (see above) had little effect on the indirect assay. Trials with other support media (glass, various plastics, Teflon-coated plastic and various membrane-filter materials) failed to resolve the interference problem. Adjustment of the pH of serum to 5.0 and addition of M NaCl or 10mM EDTA, or both, before assay decreased interference by up to 50%, but it was still relatively high and variable. Surprisingly, treatment of the plastic wells with various sera or globulin fractions (sheep, horse, bovine or human) before or after antigen was fixed or after incubation of fixed antigen with rabbit serum, or both, did not improve the results. Thus, because of non-specific interference and the variability of replicate results, less than 50–100 ng of antibody in 50 μ l of undiluted rabbit serum could not be detected with the indirect assay.

If the antibody concentration in serum was sufficiently high to allow the sample to be diluted 1 in 50 with saline-phosphate buffer before assay by the indirect method, interference was eliminated (fig. 4) and precision improved.

Assay of sera from vaccinated rabbits

When samples of serum taken from rabbits before and after vaccination with *F. tularensis* (strain LVS) were assayed with the immunoradiometric assays and agglutination tests, newly synthesised antibody was usually detected earliest by the direct competitive assay (table IV; results for only one rabbit are given but those for several other rabbits were similar). The indirect assay unequivocally did not detect antibody sooner than agglutination tests.

Restall *et al.* (1973) determined antibody titres of antibacterial sera by assaying serially diluted samples of pre-vaccination and immune sera with the indirect assay. Provided that the antibody concentration was sufficiently high, this method gave satisfactory results with rabbit anti-*F. tularensis* serum (fig. 4).

DISCUSSION

One of the parameters selected for measurement during a study of tularaemia in rabbits was the circulating antibody level. It was expected that immunoradiometric techniques would be suitable for this purpose, but application of two assays based on different principles apparently showed that all the

samples of normal sera tested contained small concentrations of antibodies against *F. tularensis* and other bacteria. One explanation was that all the sera contained "natural antibodies" against these bacteria (Jerne, 1955, 1960; Boyden, 1966; Haimovich *et al.*, 1970; Inman, 1974) and another that non-immune components in normal sera interfered in the assay systems. In the case of a direct competitive assay, the latter explanation largely accounted for the results, since interference was markedly decreased by dialysis of serum and further decreased by the inclusion of M NaCl and 10mM EDTA in the reaction mixture. The competitive immunoradiometric assay gave reproducible results and usually detected the first appearance of anti-*F. tularensis* globulins in sera from vaccinated rabbits before agglutination tests.

Indirect immunoradiometric assays based on solid-phase antigens fixed in plastic wells have been used to detect antiviral antibodies (Rosenthal *et al.*, 1972) and antibacterial antibodies (Restall *et al.*, 1973; Nassau *et al.*, 1975). As found by Nassau *et al.* (1975) and confirmed here, a serious difficulty with the indirect technique is adsorption of non-antibody globulin by antigen or supporting medium, or both, and this subsequently combines with labelled anti-globulin. In our assay system, the response of adsorbed globulin was equivalent to 5–10 μ g of antibody per ml serum. High blank values and the relatively poor agreement between the results for replicate samples decreased the sensitivity of the indirect assay, and in practice it was no better than the agglutination test for detecting antibody.

SUMMARY

When used to determine immunopurified antibacterial globulins in saline-phosphate buffer, the detection sensitivities of direct competitive and indirect immunoradiometric assays were respectively 25 and 2 ng. Normal human and rabbit sera interfered in both types of assay, markedly decreasing sensitivity and precision. Various pre-treatments of sera and modifications of reaction conditions substantially decreased interference in the competitive assay without affecting the activity of added antibody, but they had only marginal effects in the indirect assay. When serum samples taken from rabbits after vaccination with the Live Vaccine strain of *Francisella tularensis* were tested with the two assays and agglutination tests, newly synthesised circulating antibody was usually detected earlier by the competitive assay than by the other methods.

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