Staphylococcal protein A; its preparation and an application to rubella serology

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SYNOPSIS Good yields of staphylococcal protein A are obtained by growing the staphylococcus Cowan type 1 on cellophane agar. The activity of these preparations in removing immunoglobulin G (IgG) from human serum can be readily measured by the Mancini radial-diffusion technique and the correct in-use dilution determined. Treatment with protein A of sera from women with a history of rubella may help in the identification of those having specific antibody in the IgM and IgA fractions. This relatively simple procedure may have worthwhile application in the diagnosis of rubella.

In the serological diagnosis of recent rubella infection, problems arise when the antibody titre by haemagglutination-inhibition (HAI) or complement-fixation (CF) tests has already reached a high level which does not show any significant change when paired sera are tested. In such cases the diagnosis of recent infection depends on the demonstration of rubella antibody in the IgM or IgA classes.

Ankerst *et al* (1974) showed that 95% of IgG could be removed from serum by absorption with staphylococcal protein A without significantly affecting the IgA, IgD or IgM content. The technical simplicity of the method may make it more suitable for immunoglobulin analysis than current methods such as sucrose density, gel-filtration or fluorescence techniques for the serological diagnosis of rubella.

We describe here a comparison of two methods for the production of protein A and its effect on the immunoglobulin content of sera from women with serological evidence of recent rubella infection.

Material and methods

SERA

The sera investigated were 33 samples from women in whom HAI and CF tests had indicated rubella infection within the previous 12 days to 20 weeks. A human reference serum for immunoglobulin determinations was obtained from a healthy adult male and stored in aliquots at -70°C .

PROTEIN A

Preparation

Two methods were tested for the cultivation of *Staphylococcus aureus* Cowan 1 (NCTC 8530):

(a) in liquid medium, in 200 ml volumes of casein casein yeast (CCY) broth (Arvidson *et al*, 1971) in 80 oz Winchester bottles loosely plugged with cotton wool which, after inoculation with 10 ml volumes of six-hour, log-phase, CCY broth cultures, were incubated at 37°C for 18 hours with rolling at 3 rev/min to promote aeration;

(b) on solid medium, on CCY agar (2% Difco agar in CCY broth) in 150 ml volumes in 23 cm square plastic dishes (NUNC-BIO assay plate no. 1015). This was overlaid with dialysis membrane (AutoAnalyzer type C, DHAAI Laboratory, Pharmaceutical and Industrial Services, Shipley, Yorkshire). The cellophane-covered agar was flooded with six-hour, log-phase broth culture and incubated at 37°C for 18 hours after removal of excess fluid. The cellophane was then removed in sections, which were transferred to 20 ml aliquots of Dulbecco phosphate-buffered saline, pH 7.3 (PBS), and shaken to remove the staphylococci.

The staphylococcal cultures from method (a) and suspensions from method (b) were centrifuged at 2500 rev/min for 45 minutes in an MSE Major centrifuge. Then using an MSE Mistral centrifuge (4000 rev/min for 10 minutes) the deposits were washed three times in PBS. The washed bacteria were treated for 3 hours at room temperature with 100 ml of 0.5% formaldehyde in PBS, washed twice more with PBS, then, following the method of Kronvall (1973), heated briefly at 80°C and resuspended at a concentration of 10% (vol/vol) in PBS containing

0.1% sodium azide. The stock 10% suspensions were stored at 4°C until used.

Measurement of activity

The affinity of each 10% staphylococcal suspension for IgA, IgM, and IgG was assayed by mixing 0.5 ml of a reference serum diluted in PBS with the centrifuged deposits from 2.0 ml volumes of serial two-fold dilutions of the suspensions. The organisms were removed by centrifugation after standing for 30 minutes at room temperature. Tri-partigen and LC-partigen immunodiffusion plates (Hoechst) were used to determine the immunoglobulin levels of the original reference serum dilutions and the residual IgA, IgM, and IgG after their absorption. An initial 10-fold dilution of the reference serum was suitable for IgG estimations while a twofold dilution was used in the measurement of the IgA and IgM.

TESTS FOR RUBELLA ANTIBODY

Preliminary treatment of sera

Before carrying out chick cell haemagglutination-inhibition tests it was necessary to remove from test sera non-specific agglutinins of chick erythrocytes and non-specific inhibitors of haemagglutination by rubella virus. This was achieved by adding 0.1 ml of 1 M MnCl₂ and 0.1 ml of heparin solution (5000 units/ml) to 0.2 volumes of unheated serum samples diluted in 0.4 ml dextrose gelatin veronal buffer (DGV).

After 30 minutes at 4°C 0.05 ml of a 50% suspension of chick erythrocytes was added; after a further one hour at 4°C the mixture was centrifuged at 2000 rev/min for 20 minutes to remove precipitated inhibitors and chick erythrocytes. The supernatant fluid was diluted with DGV to give a final 10-fold dilution of the original serum.

Absorption of sera by protein A

Two-millilitre volumes of 10% staphylococcal suspensions were centrifuged in an angle head centrifuge in bijou bottles and the supernatant was removed. The sedimented staphylococci were resuspended in 0.5 ml volumes of the 10-fold dilutions of treated test sera. After standing at room temperature for 30 minutes the mixtures were centrifuged to remove the staphylococci.

Haemagglutination-inhibition tests

0.1 ml of serial doubling dilutions of the pretreated serum were allowed to react with 0.1 ml of rubella HA antigen (Burroughs Wellcome & Co) diluted in DGV to give four haemagglutinating doses either at room temperature for one hour (short incubation) or at 4°C for 18 hours (long incubation) before addition of 0.5% erythrocytes from one-day-old chicks.

Results

PRODUCTION OF PROTEIN A

Comparison of the two methods, in liquid or solid media, for the cultivation of *Staphylococcus aureus* showed that the 10% staphylococcal suspensions obtained were of about equal protein A activity (figure). However, cultivation on cellophane agar

Figure Effect on immunoglobulin levels of neat (0.2 ml) and decreasing volumes of staphylococcal protein A from agar (—) and broth (---) cultures.

was the more convenient method because it involved less centrifugation and was more productive. Typical yields of the 10% staphylococcal suspensions used in these experiments were 150 ml from 240 ml of staphylococcal suspension harvested from 12 cellophane agar plates but only 25 ml from 1260 ml of broth culture in six Winchester bottles.

ABSORPTION OF IMMUNOGLOBULINS BY PROTEIN A

The absorption by protein A suspensions of IgG, IgM, and IgA from a normal human reference serum (containing IgG 12.0 g/l, IgM 0.9 g/l, and IgA 1.5 g/l) was determined by radial-diffusion tests before and after treatment of the serum. The figure shows the proportion of IgG removed by various dilutions

of stock suspensions obtained from either liquid or solid media. There is clearly little difference in this respect between the suspensions produced by the two methods. At dilutions of 1/32 or 1/16 they reduced the IgG level by about 50%, at 1/8 by more than 85%, and at 1/4 by more than 90%. The one preparation that showed much less activity than this had been inadvertently held in PBS at 4°C for two days before treatment with formaldehyde and heat. The effect on IgM and IgA levels of treatment of the serum with lower dilutions of suspensions prepared on solid media is also shown in the figure. Although there was a reduction in these levels it is clearly much less than that of IgG, and the loss of IgM and IgA tends to be reduced by decreasing the staphylococcal dose to which sera are exposed.

RUBELLA ANTIBODY LEVELS AFTER PROTEIN A ABSORPTION

Table I shows the rubella HAI titres (long incubation method) of 33 sera collected between 12 days and 20 weeks after the onset of rash; titres were determined before and after absorption with protein A. The

Serum no.	Days after appearance of rash	Rubella haemagglutination inhibition titre	
		Serum not absorbed	Serum absorbed with protein A
1	12	2560	120
2	13	1280	20
3	16	3840	160
4	16	> 5120	320
5	17	640	20
6	17	640	30
7	17	640	40
8	17	1920	120
9	18	> 5120	40
10	18	2560	120
11	19	2560	160
12	21	1280	40
13	21	1280	40
14	23	2560	40
15	24	2560	80
16	24	640	20
17	27	1280	20
18	27	1280	< 10
19	30	1280	10
20	32	2560	40
21	35	640	< 10
22	42	2560	40
23	42	1920	10
24	42	> 5120	10
25	47	1280	10
26	49	640	< 10
27	49	1280	10
28	49	320	< 10
29	52	1920	< 10
30	70	2560	< 10
31	77	480	< 10
32	84	1920	10
33	140	960	< 10

Table I Effect of staphylococcal protein A absorption on HAI titres of sera collected 12 to 140 days after rubella rash

earliest serum sample in which there was no detectable HAI activity after absorption with protein A was collected on day 27. Sera collected earlier than that had post-absorption titres in the range 20 to 320. Among sera collected before day 27 none had a post-absorption titre of less than 20, and among those collected after day 42 none had a post-absorption titre of more than 10.

FACTORS AFFECTING THE TEST FOR RUBELLA ANTIBODY

Sensitivity of the HAI test

With the 'long incubation' test 12 of 14 sera gave titres that were more than twofold greater than when the 'short incubation' method was used. This difference in sensitivity was important in the case of one serum collected 17 days after onset of rash which had a post-absorption titre of less than 10 by the 'short incubation' method but a titre of 20 after 'long incubation'.

Dose of protein A

The adequacy of the selected dose of protein A was investigated by measuring the residual HAI titre of serum no. 4 (table I) after two consecutive absorption treatments. This was the serum with the highest post-absorption titre (320) in our series but it remained unchanged after the second treatment.

IgG content of sera

To investigate the effect of individual variation in the level of total serum IgG on the residual HAI titre after protein A absorption, nine sera with high HAI titres were examined (table II). It is noteworthy that three of the four sera with the highest total IgG levels had residual HAI titres of less than 10.

Serum no.	Total serum IgG ¹ (g/l)	Post-absorption HAI titre
18	20.6	< 10
29	15.8	< 10
33	12.8	< 10
27	12.8	10
24	12.0	10
17	10.6	20
32	10.4	10
30	9.5	< 10
25	8.6	10

Table II Comparison of total serum IgG levels with rubella HAI titres after staphylococcal protein A absorption

¹Normal range 6-16 g/l

Discussion

Staphylococcus aureus Cowan 1 grown on dialysis membrane spread on the surface of casein casein

yeast agar gave a high yield of protein A with IgG-absorbing activity at least equal to that of staphylococci grown in liquid cultures. Growth of staphylococci on a solid medium in this way provides diagnostic laboratories with a readily available source of protein A. The activity of these protein A preparations in absorbing IgG and the correct dose for use in serological tests can be assessed by determining IgA, IgG, and IgM by radial-diffusion techniques on sera before and after treatment. The reported day-to-day variations in protein A production by Cowan 1 and other strains of staphylococci (Kronvall *et al*, 1971) indicates the need for routine monitoring of the potency of protein A batches. Being technically simple and also commercially available, the Mancini technique offers advantages over the radioisotope method described by Ankerst *et al* (1974).

In relation to rubella serology, the value of the protein A absorption technique lies in the demonstration of residual IgM and IgA antibody indicative of recent infection after removal of most of the IgG. In this investigation we have demonstrated the importance of using the 'long incubation' haemagglutination-inhibition test which is more sensitive than the 'short incubation' method and more likely therefore to detect low titres of IgM antibody after absorption. There is some reduction in IgM and IgA titres after absorption with protein A, greater in our hands (IgM 29%, IgA 19%) than that reported by Ankerst *et al* (1974) (IgM 2.5%, IgA 4%). The reason for this difference is not clear but might be related to the use of myeloma protein by Ankerst *et al* or to the presence in our reference serum of anti-staphylococcal IgM or IgA, although we have not investigated this. We have shown, however (figure), that loss of IgM and IgA may be reduced by careful adjustment of the dose of protein A.

Our results suggest that protein A absorption is effective in reducing rubella IgG antibody to a level less than the initial dilution, 1 in 10, used in the HAI test. In our series (table I), eight sera with initial titres in the range 320 to 2560 were all reduced to less than 10 after absorption. Although protein A combines with about 95% of serum IgG, it has no effect on subclass 3, which represents about 5% of the total. The role of subclass 3 could be significant

in relation to the effect of protein A in any serum with an initial titre of 200 or more in which a 95% reduction might leave antibody detectable at the initial (1 in 10) dilution of the HAI test. Our findings evidently support the suggestion of Ankerst *et al* (1974) that IgG of subclass 3 does not affect antibody titres determined by the rubella HAI test.

Using the protein A absorption technique, Ankerst *et al* (1974) were able to demonstrate residual post-absorption HAI activity in sera collected between 7 and 14 days after the onset of rubella. We have extended the survey period to between 12 days and 20 weeks after onset. Our findings (table I) show that it may be possible, by demonstrating residual post-absorption HAI activity, to diagnose recent rubella even when serum is first obtained 14 days or more after the onset of the rash, by which time the HAI and CF titres have already risen. Current investigations on a direct comparison with the sucrose density gradient technique may help to define the diagnostic precision of the protein A absorption method. However, the results presented here already indicate that the test is of value since it enables us to diagnose recent rubella in a number of patients without resorting to the more complex immunological methods.

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