DETERMINATION OF ULTRAMICRODOSES OF ANTIGENS BY MODIFIED VIROIMMUNOASSAY

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Highly sensitive immunologic methods, such as radioimmunoassay and ELISA, are extensively used in various branches of molecular biology and medicine. Even greater sensitivity of determination might be expected by the use of viruses (bacteriophages) as the label. However, methods based on phage neutralization, and also on the mixed immunoadsorption test, are no more sensitive than the methdos mentioned above [4,7]. To increase sensitivity of determination of antibodies to bacteriophage the writer has suggested extracting complexes containing phage and antigen with an immunosorbent, followed by determination of the quantity of extracted phage (viroimmunoassay) [11]. This same principle has been used for determining antibodies to hapten [9]. Later a variant of viroimmunoassay was developed for determination of antigens based on competitive inhibition by antigen of binding of phage–antibody conjugates with an immunosorbent [1].

In this paper a direct and more sensitive method of determination of antigens by their specific extraction from solution by directionally immobilized antibodies (Fab'-fragments of antibodies) [2] is suggested. The dose of antigen was judged from the number of conjugates (phage–Fab'-fragments of the same antibodies), added to molecules of the antigen extracted by the immunosorbent.

The basis for the immunosorbent was coarse Sephadex G-25 (Pharmacia, Sweden), which was modified by the method described in [8], with some variations. To 3 g of swollen gel in 0.3 N NaOH 0.3 ml of epichlorhydrin was added and the mixture was heated on a water bath at 60°C for 1 h with mixing in an extraction cupboard. The gel was washed on a glass filter with 500 ml of distilled water and 100 ml of 0.1 M carbonate-bicarbonate buffer, pH 9.2, 20 mg of egg albumin in 2 ml of the same buffer was added, and the mixture was left overnight at room temperature. Unreacted oxirane groups were blocked by 1 M glycine in the same buffer for 24 h. Sephadex was washed off the unbound protein consecutively with 0.1 M acetate buffer, pH 5.0, and 0.1 M carbonate-bicarbonate buffer, pH 10.6. Free thio groups were then introduced into the protein by addition of 20 mg N-acetyl-homocysteinethiolactone in 0.1 M carbonate-bicarbonate buffer, pH 10.6, and leaving the mixture to stand for 40 min at 4°C. The thiol derivative of Sephadex was washed in 0.1 M Tris-HCl buffer, pH 8.0, with 0.001 M Trilon B, and 5 ml of 0.001 M Ellman's reagent [5,5-dithio-bis-(2-nitrobenzoic acid)] was then added. A mixed disulfide was formed by the thiol-disulfide exchange reaction, and it reacted readily with the free thio group of the Fab'-fragments of the antibodies. In that case 0.5 mg of Fab'-fragment of rabbit antibodies against rat IgG was added to 1 g of modified Sephadex. The antibodies and their fragments were obtained by the method described previously [3].

Bacteriophage ØX174 was obtained and purified by the method in [10]. Addition of the Fab'-

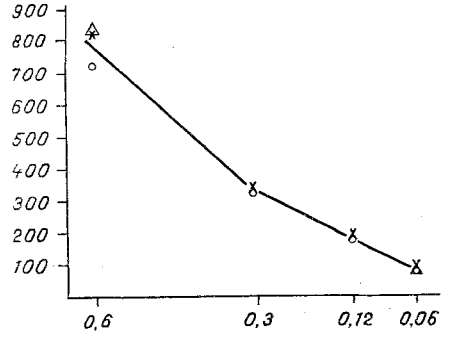

Fig. 1. Number of PFU determined as a function of quantity of antigen (rat IgG) added per sample. Abscissa, quantity of rat IgG (in pg per sample); ordinate, number of PFU determined above background. Symbols indicate number of PFU for different quantities of rat IgG in different experiments.

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TABLE 1. Reproducibility of Parallel Determinations in Separate Experiments on Addition of 0.06 pg of Rat IgG per Sample

Expt. No.	Conjugate No. 1		
	experiment	control	difference between number of PFU in experimental samples and mean number in control
1 (conjugate No. 1)	240	156	90
	254	139	104
	220	150	70
	224	159	74
Mean	235±8	150±5	85±8
2 (conjugate No. 2)	132	89	29
	142	110	39
	128	98	25
	130	105	27
	120	112	17
Mean	130±3	103±4	27±4
3 (conjugate No. 3)	130	87	43
	112	83	25
	135	94	48
	132	82	45
Mean	127±5	87±3	40±5

TABLE 2. Reproducibility of Results of Determination of Rat IgG (0.06 pg per sample) in Different Experiments

Expt. No.	Number of PFU determined (conjugate No. 1)		
	experiment	control	difference between number of PFU in experimental and control samples
1	235	150	85
2	239	143	96
3	320	235	85
4	205	130	75
Mean	250±25	164±24	85±4

fragments of the antibodies to phage was carried out in the same way as the sorbent, by a reaction of thiol-disulfide exchange. To 0.5 mg of phage ($2 \cdot 10^{11}$ PFU/ml) was added 0.1 ml of 0.2 M N-acetyl-homocysteinethiolactone in 0.1 M carbonate-bicarbonate buffer, pH 10.6 and the mixture was allowed to stand for 1 h at 4°C. The phage was freed from excess of lactone on a column with P-4 (Bio-Rad, USA), equilibrated with 0.1 M Tris-HCl buffer, pH 8,0 with 0.001 M Trilon B, after which 50 µl of 0,001 M Ellman's reagent was immediately added, and the mixture was allowed to stand for 30 min at 4°C. This chemical modification of the phage did not reduce its biological activity. The phage was freed from excess of Ellman's reagent on a column with P-4, equilibrated with the same buffer, 65-100 µg of Fab'-fragments of rabbit antibodies against rat IgG in a volume of 0.1 ml was added, and the mixture allowed to stand overnight at 4°C. The conjugate of phage with Fab'-fragments thus formed was freed from unbound fragments on a column with Sepharose 6B or 4B (Pharmacia), equilibrated with 0,1 M Tris-HCl buffer, with 0.001 M Trilon B and 0.01% of sodium azide. The phage in the conjugate prepared in this manner lost 70-80% of its biological activity. The biological activity of the phage could be completely restored by treatment of the conjugates with 5-10 mM dithiothreitol (Koch-Light, Eng-

land) as the result of reduction of the disulfide bonds between phage and the antibody fragments.

To each of several conical test tubes was added 50 µl of packed immunosorbent gel in buffered physiological saline (BPS) (0.14 M NaCl, pH 7.0) with 0.05% Tween-20 or Triton X-100, and 0.01% of sodium azide. The solution above the precipitated sorbent was drawn off and equal quantities of antigen (rat IgG) in a volume of 10-20 µl were added. BPS was added to the control samples. After incubation for 3 h at 37°C the sorbent was washed off 5 times with BPS and transferred into clean conical test tubes and washed once with BPS with 0.05% Tween-20 and 0.01% sodium azide; the solution above the sorbent was drawn off and 10^5 conjugates in a volume of 10-20 µl was added. The mixture was incubated for 4 h at 37°C. After incubation the sorbent was washed 5 times with BPS, transferred to round-bottomed 10-ml test tubes, and the solution above the sorbent was drawn off. The added conjugate was removed from the sorbent during standing for 30 min at room temperature by the addition of 0.1 ml of 5-10 mM dithiothreitol. Next, 0.2 ml of a fresh culture of *E. coli* C and 5 ml of 0.8% agar with 1% peptone in BPS, melted and cooled to 47°C, was then added to the test tubes and seeding carried out on Petri dishes. The dishes were incubated for 5-6 h or overnight at 37°C and the number of plaques formed was counted.

During assay by the method described above considerably more PFU were found in samples containing the antigen than in the control samples. If 0.3-0.6 pg antigen was present in the samples, 300-700 more PFU were found in them than in the control. This excess was smaller, but still significant ($P < 0.05$) in cases when the samples contained a smaller amount of antigen. The results of three such experiments, in which the samples contained 0.06 pg of antigen, are given in Table 1. Good reproducibility was observed when the results of different experiments were compared (Table 2). In every case the standard error was less than 15%. Using different quantities of antigen, we plotted a calibration curve (Fig. 1), from which the quantity of antigen in test samples could be determined starting from 0.06 pg per sample.

The suggested method is more sensitive than the better versions of ELISA by 1-2 orders of magnitude for the analogous antigen [5, 6]. The effectiveness of the suggested method is due to the new way of obtaining the immunosorbent [2] and conjugates, based on oriented addition of Fab'-fragments of the antibodies to the matrix of the sorbent and to the phage particles. This method not only is more sensitive, but has other advantages also. It does not require expensive equipment and it is simple to carry out. Another distinguishing feature of viroimmunoassay is that its results can be obtained in absolute values.

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