Acta vet. scand. 1978, *19*, 153—155.

Brief Communication

RADIOIMMUNOASSAY FOR ANTIBODIES AGAINST MYCOBACTERIUM PARATUBERCULOSIS USING ^{125}I-LABELLED PPD*

A prerequisite for control and eradication of paratuberculosis is access to diagnostic procedures with high diagnostic sensitivity and diagnostic specificity making it feasible via serological mass screening to identify infected animals at a preclinical stage.

The present investigation was carried out to evaluate the diagnostic features of a radioimmunological procedure employing ^{125}I-labelled purified protein derivative (PPD) from Mycobacterium paratuberculosis. The antibody concentration in serum was measured by determining the primary binding capacity. Contrary to inconclusive results with M. tuberculosis (*Minden & Farr* 1969) the present observations were promising.

PPD purification and iodination

Outgrown cultures of M. paratuberculosis were heated at 102°C for 20 min. and cleared of particulate material including cells. The extract was dialyzed 5 times against deionized water followed by dialysis against a solution of disodium phosphate, 0.1 M, to render PPD-1.

Nucleic acids were removed by precipitating 100 volumes of PPD-1 with 1 volume of a 10 % solution of dihydrostreptomycin sulfate (*Long & Pardee* 1967). Subsequently the proteins were precipitated by adding 210 mg of ammonium sulfate per ml solution. The protein precipitate was reconstituted in phosphate buffer, 0.1 M, pH 7.1, followed by dialysis against this buffer. The solution, PPD-2, was subjected to gel filtration on a 2.5 × 40 cm column of Sephadex G-200 using phosphate buffer, 0.1 M, pH 7.1 as the eluant. A high molecular weight protein making up 55 % of the total amount of eluted protein appeared in the V_e/V_t interval 0.41 to 0.51. V_e and V_t is the elution volume and the volume of the gel bed, respectively. Fractions representing the V_e/V_t interval 0.44 to 0.48 were pooled to form PPD-3.

Phosphate buffer, 0.04 M, pH 7.4 was the solvent of all reagents used for iodination.

To 20 µl of PPD solution with an absorbance of 4.0 at 280 nm and 10 mm light path the following reagents were added in rapid succession: 5 µl carrier-free ^{125}I corresponding to 0.5 mCi and 10 µl of a freshly prepared solution of chloramine-T, 1 mg/

* This work was supported by the Danish Agricultural and Veterinary Research Council.

ml. The iodination was terminated after 60 sec. by adding 10 µl of a solution of sodium metabisulfite, 3 mg/ml. Iodinated PPD (^{125}I-PPD) was separated from non-protein bound ^{125}I by gel filtration on a 0.9 × 10 cm column of Sephadex G-25, coarse grade. Elution was performed with phosphate buffer, 0.04 M, pH 7.4, containing human serum albumin (HSA), 5 mg/ml.

Radioimmunoassay (RIA)

One hundred µl of serum diluted 10^{-4} with Tris buffer, pH 7.4 (0.01 M Tris, 0.14 M sodium chloride, 0.1 % sodium azide, 0.1 % gelatine and hydrochloric acid for adjustment to pH 7.4) was subjected to the test. The above Tris buffer was used as diluent for all reagents employed in the test. In all test series blanks containing diluent buffer but no serum were included. To each test tube were added 100 µl of Tris buffer containing 2 % HSA to prevent binding of ^{125}I-PPD to the polystyrene test tubes. Subsequently 100 µl of ^{125}I-PPD solution (4×10^4 counts per min.) was added. The reaction mixture was incubated for 22 hrs. at 4°C. Precipitation of complexes of ^{125}I-PPD and bovine IgG was achieved by addition of 100 µl of a solution of rabbit IgG (0.67 mg/ml) containing anti bovine IgG antibodies. This reagent had been rendered non-reactive to bovine serum albumin (BSA) via absorption with this compound. Immediately after addition of rabbit anti bovine IgG, 100 µl of a solution containing bovine IgG for coprecipitation was added to each test tube. This reagent was a 10^{-3} dilution of bovine serum with no complement fixing (CF) activity against PPD. Complete precipitation was achieved during incubation for 2 hrs. at room temperature. Following centrifugation at 3000 × g at 4°C for 30 min. the supernatant was aspirated and the radioactivity in the precipitate measured in a Selektronik well type gamma counter. Prior to the centrifugation the precipitate had been washed with 1 ml of a solution of sodium chloride, 0.15 M, containing 0.5 % Tween-20.

Perfection of RIA

The binding affinity of iodinated PPD-1, PPD-2 and PPD-3 was compared using bovine sera grouped according to their CF-titers (Table 1). It may be seen that the approximate binding affinity of PPD-2 and PPD-3 was 20 and 40 times greater, respectively, than that of PPD-1. Furthermore, using PPD-3 the difference in binding capacity of CF-negative and CF-positive sera could only be revealed after the sera had been diluted 30,000 times. PPD-3 stored in buffer solution at —75°C was readily soluble. At —18°C, ^{125}I-PPD-3 was stable for about 1 month.

Intraassay variation was determined on 215 serum samples. For 75 samples with binding below 5 % the coefficient of vari-

ation (SD_{rel}) was 7.6 %, while the comparable figure for 140 samples with binding above 5 % was 5.5 %. Interassay variation was determined on 15 sera each subjected to analysis repeated on 5 different days. SD_{rel} was 8.7 % and 5.3 % for 4 and 11 sera with binding below and above 5 %, respectively.

Chi-square analysis revealed highly significant ($P < 0.001$) association between CF-titers and binding capacity in the RIA of bovine sera. Using the CF-grouping of the sera as the category criterium the nosographic specificity and nosographic sensitivity was found to be 97 % and 74 %, respectively.

The RIA described here has proved to be convenient and rendering results in approx. 48 hrs. One person may readily analyse 50 sera per day.

Table 1. Binding of ^{125}I-PPD from different purification steps in tests employing sera covering a variety of CF-titers.

CF-test		% ^{125}I-PPD bound in RIA			
Dilution of sera		Sera diluted 1/3,000			Sera diluted 1/30,000
		PPD-1*	PPD-2*	PPD-3*	PPD-3
negative	< 1:8	0.76	17	35	8.6
	< 1:8	0.80	16	31	
doubtful	(1:10)	0.60	13	34	4.8
	(1:10)	0.53	14	25	5.0
positive	1:40—80	1.04	21	41	31
	1:80	0.69	20	40	
	1:80—160	0.82	21	41	
	1:80—160	0.92	18	35	
	1:160	1.16	20	38	22

* For explanation see text.

ACKNOWLEDGEMENTS

Dr. J. Berg Jørgensen, the Danish State Veterinary Serum Laboratory, kindly provided material for preparation of PPD and performed the complement fixation analyses on the sera.

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(Received January 18, 1978).

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