

The Use of Ferromagnetic Dacron as Solid-phase in Enzyme Immunoassays

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Ferromagnetic dacron is proposed as an alternative solid-phase for magnetic enzyme immunoassays. Human serum albumin (HSA) was covalently immobilized onto ferromagnetic dacron and an enzyme immunoassay was developed using anti-HSA rabbit sera. Peroxidase, o-phenylenediamine (OPD) and hydrogen peroxide were used as the enzymatic label and substrates, respectively. Best results were observed when particles of 63-100 µm (diameter) and 10 µg of immobilized antigen were used. Positive reactions were detected until dilutions of 1:51200 of immune sera. Its reproducibility was similar to standard ELISA. Disruption of the immunocomplexes formed and recuperation of the immobilized antigen in other immunoassays also proved to be reliable.

Key words: magnetic ELISA - ferromagnetic dacron - human serum albumin

Immobilized biosystems have been widely employed as described in extensive and recent reviews (Coughlan et al. 1988, Kennedy et al. 1989, Powell 1990). Heterogeneous immunoassays require the use of antigen or antibody immobilized on a solid phase. The most common solid phases used in immunology are different kinds of plastics, such as PVC, nylon, glass and polystyrene (Guesdon & Avrameas 1981a, Kemeny & Challacombe 1988). Magnetic supports were firstly used in immunology by Hersh and Yaverbaum (1975) employing silanized magnetite as solid phase in a radioimmunoassay for digoxin. Lately other magnetic supports were proposed like polyacrylamide-agarose (Guesdon & Avrameas 1977, 1981b, Camargo et al. 1984, 1986), Sepharose (Mosbach & Anderson 1977), polystyrene (Robinson et al. 1985) and cellulose (Paus & Nustad 1989).

Among a lot of supports described in literature, Dacron has been successfully used in enzyme immobilization (Weetall 1970, Goldstein et al. 1977). It is a polyester widely employed as a plastic in several industrial applications. Lately, an easier method was proposed to immobilize enzymes on dacron consisting in three steps: (1) *Hydrazinolysis*, (2) *Conversion of Hydrazide*

Groups to Azide and (3) *Immobilization of Protein* (Carvalho Jr et al. 1986, 1987, Oliveira et al. 1989). Its application has been extended to other proteins such as antigen from *Yersinia pestis* (Montenegro et al. 1991, 1993).

Recently, ferromagnetic Dacron® was proposed as a matrix to immobilize proteins covalently (Carneiro Leão et al. 1991). It presents good mechanical properties, is resistant to solvents and to microbial attack, and is easily recovered from the medium using a magnetic field, replacing centrifugation or filtration.

In this work, antigen (human serum albumin) has been immobilized on ferromagnetic dacron and magnetic ELISA has been carried out successfully.

MATERIALS AND METHODS

Proteins and reagents - Dacron® was produced by Rhodia do Brasil SA. Human serum albumin (HSA) fraction V and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. FeCl₃.6H₂O and FeCl₂.4H₂O were acquired from Reagen SA. NUNC Immunoplates 96 wells were purchased from Intermed, Denmark. All other reagents were analytical grade obtained from Merck SA.

Sera and immunization procedure - Normal and anti-HSA sera were obtained in our laboratory. Two New Zealand white three months rabbits were inoculated with two injections of HSA proximal to the lymphonode (10 mg with complete Freund adjuvant/each) on days 1st and 21st. On days 40th, 47th and 54th the animals were inoculated with alumen precipitated HSA (5

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mg/ml) i.m. On day 59th rabbits were bled. The pool of sera contained 2.47 mg/ml of specific antibodies as determined by immunoprecipitation (Hudson & Hay 1989). Pig anti-rabbit IgG conjugated to peroxidase was bought to Dako (Denmark).

Activation and magnetization of dacron - This stage was proceeded according to Carneiro Leão et al. (1991). Hydrazide-dacron was freeze-dried and the particles were separated into 63-100 μm (small particles) and 100-250 μm (large particles) sizes through appropriate sieves. The resulting ferromagnetic-hydrazide-dacron was converted to ferromagnetic-azide-dacron as described previously.

Immobilization of HSA ferromagnetic dacron - HSA (5 ml of a 5.0 mg/ml solution prepared in 0.15 M NaCl) was incubated with the ferromagnetic-azide-dacron (0.5 g) for 3 hr at 4°C under mild stirring. Then, the ferromagnetic powder of HSA-dacron was successively washed with 0.15 M NaCl (100 ml), 1.0 M NaCl (100 ml) and 0.15 M NaCl (100 ml), and stored at -20°C in 0.15 M NaCl until use.

Antibody assay - A suspension of the ferromagnetic HSA-dacron particles was adjusted to a protein content of 250 $\mu\text{g/ml}$ in 0.15 M NaCl and aliquots of 50 μl were withdrawn to the assay tubes. Skinned milk (1% w/v) prepared in 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) was used as a blocking buffer during 1 hr at 25°C. Incubation with anti-HSA serum and with swine anti-rabbit IgG conjugated to peroxidase (diluted 1:5,000) were carried out at 37°C during 1 hr. Washing between incubations were proceeded using PBS Tween 0.05% v/v (PBS/Tween) by precipitation of the ferromagnetic HSA-dacron particles under a magnetic field (6,000 Oe) and discarding the supernatant. The development of the reaction was carried out using 0.026% v/v H_2O_2 and 0.17 M o-phenylenediamine (OPD) prepared in 0.3 M Tris/citrate buffer, pH 6.0 as substrates during 15 min and read at 492 nm. Protein contents were determined according to Lowry et al. (1951), using a BSA standard curve.

Classical ELISA - It was done following the general procedure described by Voller et al. (1977). In brief: 5 $\mu\text{g/ml}$ (0.25 $\mu\text{g/well}$) of HSA were adsorbed to a NUNC immunoplate using carbonate buffer pH 9.0 overnight. First antibody was incubated during 1 hr at 37°C. The rest of the reaction followed some general procedure described previously to ferromagnetic-dacron. Anti-rabbit IgG conjugated to peroxidase was diluted at 1:5000 after checkerboard titration.

Recuperation of immunocomplexes - The final immunocomplexes obtained from an antibody assay using ferromagnetic-dacron were washed in 500 μl of PBS/Tween for five times. Magnetic

field was used to recover the magnetic modified immobilized antigen preparation. Subsequently, this derivative was vigorously stirred in 1,000 μl of 0.05 M glycine/HCl buffer, pH 2.3, by using a vortex mixer, and kept resting for 60 min at 28°C, with similar stirring each 15 min intervals. Then, Tris/HCl buffer (1,500 μl of 0.05 M solution, pH 8.6) was added to the magnetic preparation, with stirring. Finally, the material was washed with PBS/Tween for five times. The recovered material in the last washing was used for another antibody assay as described above.

RESULTS AND DISCUSSION

Ferromagnetic-dacron using amyloglucosidase as a model has been previously tested as a suitable matrix for protein immobilization (Carneiro Leão et al. 1991). The ferromagnetic dacron derivative was completely recovered by using a magnetic field and no enzymatic activity was detected in the supernatant. Else, the presence of magnetite did not interfere with the hydrazide/azide conversion step and with the catalytic property of the immobilized enzyme.

In order to obtain larger particles (powder), making recovery by magnetic force more efficient, incubation time of the dacron with hydrazine hydrate was reduced from 72 to 48 hr (Carvalho Jr et al. 1986, 1987).

The relationship between offered HSA for the immobilization onto ferromagnetic-azide-dacron and fixed protein was determined. A linear correlation was observed until concentration of 5.0 mg/ml (10 μg of protein are fixed onto 1 mg of support per each mg of offered protein). A maximum value of about 120 μg of fixed protein per g of ferromagnetic-dacron was calculated if protein concentration of about 200 mg/ml would be used during immobilization. Here, protein concentration of 5.0 mg/ml (50 μg of fixed protein per mg of support) was proposed since it should be advisable to avoid antigen overloading that can yield steric hindrance for the antigen-antibody complex formation. This preparation was used to calculate the optimum amount of protein per assay.

Figure 1 presents the relationship between the amount of antigen (μg of HSA-ferromagnetic-dacron derivative) used in the proposed assay and the absorbance at 492 nm by using ELISA procedure. It was compared with the standard procedure employing polystyrene plates. The amount of 12.5 μg of protein per assay used throughout this work were selected from these results. Optimization of this technique would improve its performance so that lower amount of protein is required.

The critical influence of the ferromagnetic-dacron particle size is demonstrated in Fig. 2. More scattered values are observed when ferromagnetic-dacron with uncontrolled particle size

was used compared with those from controlled particle size and classical ELISA. These data can be better analyzed by the average and standard deviation of the absorbances at 492 nm obtained by using these materials for the magnetic ELISA: uncontrolled particles size - 0.441 ± 0.216 ; 100-250 μm size - 0.128 ± 0.031 ; 63-100 μm - 0.158 ± 0.012 . The mean OD value obtained from classical ELISA was 0.391 ± 0.013 . Considering these results, small particles (63-100 μm) were selected to be used throughout this work.

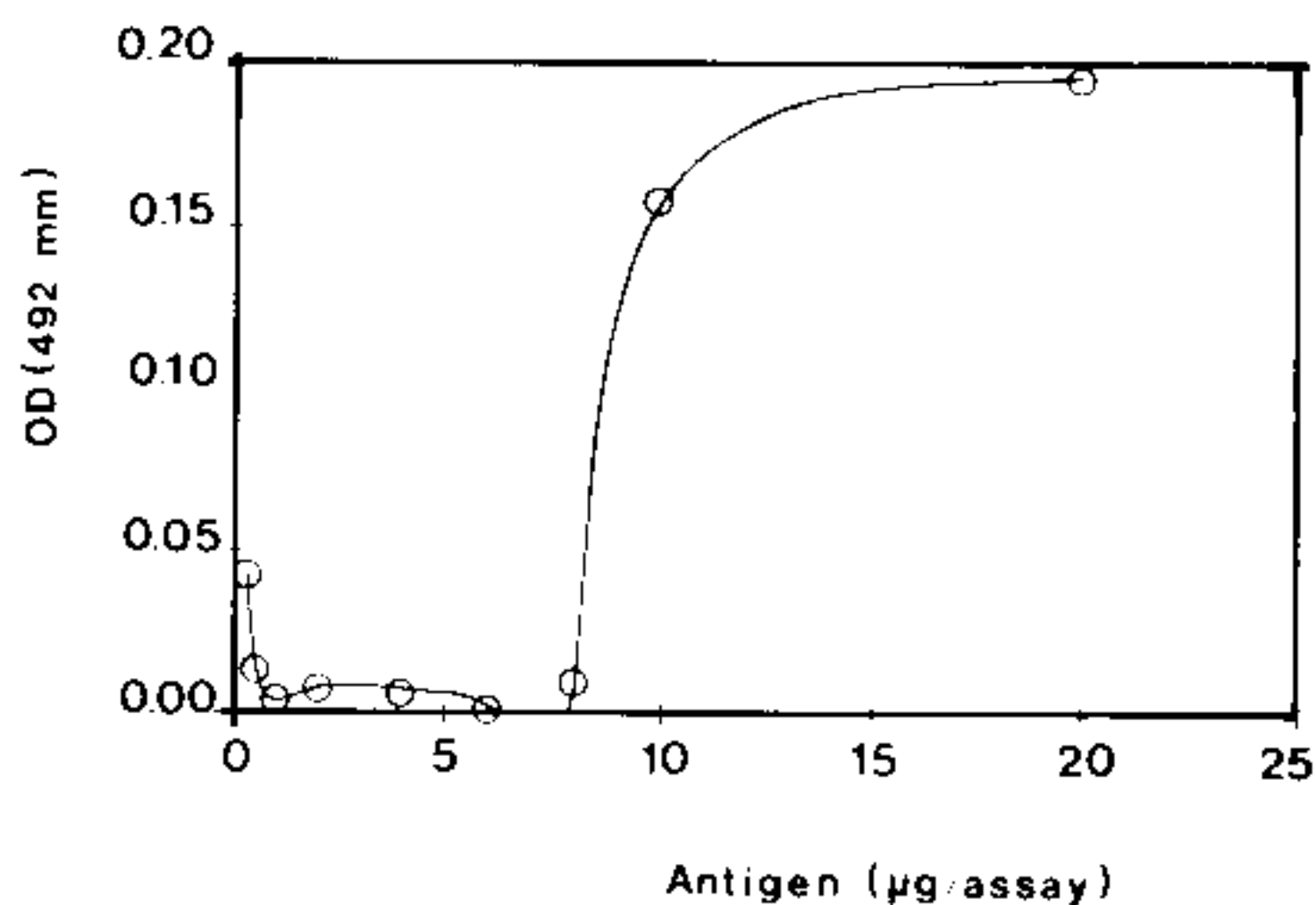


Fig. 1: titration of antigen (HSA) using magnetic enzyme-linked immunosorbent assay employing ferromagnetic dactron. A suspension of the ferromagnetic HSA-dactron particles (0.05 - 20 μg of protein) was introduced into the assay tubes and ELISA proceeded as described. Washing between incubations was carried out using PBS/Tween by precipitation of the ferromagnetic HSA-dactron particles under a magnetic field and discarding the supernatant.

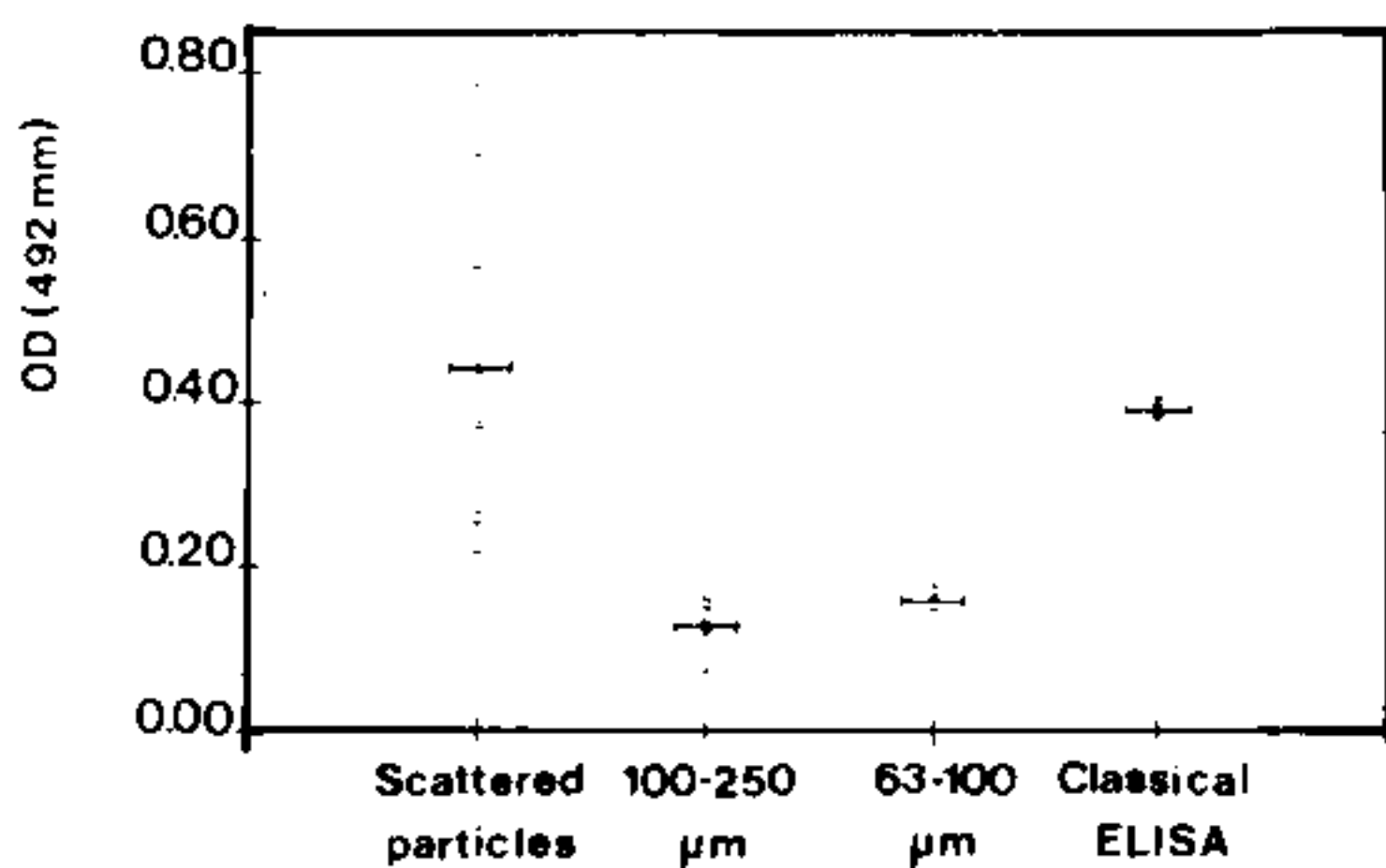


Fig. 2: magnetic enzyme-linked immunosorbent assay employing uncontrolled and controlled ferromagnetic dactron particle sizes compared with classical ELISA. Ferromagnetic HSA-dactron particles (12.5 μg /assay) were used and ELISA carried out as described in magnetic ELISA. Rabbit anti-HSA sera were diluted at a ratio of 1:100. Column 1 displays the absorbances at 492 nm using uncontrolled size of HSA-ferromagnetic-dactron particles, whereas Columns 2 and 3 present the values using 100-250 μm and 63-100 μm particle sizes, respectively. Column 4 depicts the results obtained using standard ELISA.

Figure 3 shows a typical antibody titration by using ferromagnetic dactron (63 - 100 μm) as solid phase in ELISA. A ratio of 2.5 was still estimated between absorbances at 492 nm of the immunized rabbit sera at the dilution of 1/51,200 (4,82 μg /100 ml of sample) and of the normal rabbit serum diluted at 1/100.

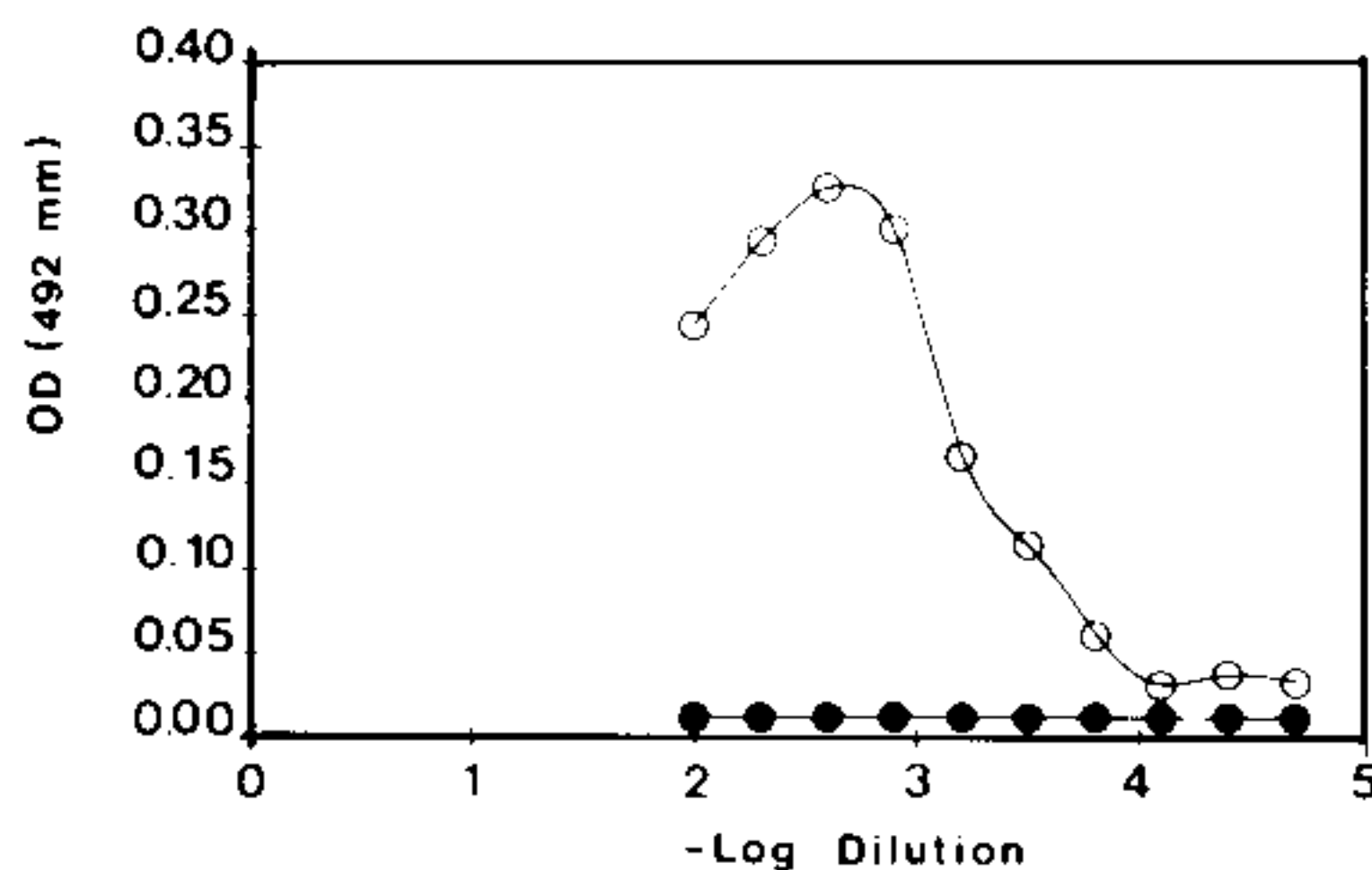


Fig. 3: titration of antibodies using ferromagnetic dactron as solid phase in ELISA. Ferromagnetic HSA-dactron particles (12.5 μg of protein/assay) were used and ELISA carried out as described. Rabbit anti-HSA serum (O) was used in dilution from 1/100 to 1/51,200. As control was employed rabbit normal sera (●). Washings between incubations were proceeded using PBS/Tween by precipitation of the ferromagnetic HSA-dactron particles under a magnetic field and discarding the supernatant.

Table presents the titration of rabbit anti-HSA serum using magnetic enzyme-linked immunosorbent assay employing ferromagnetic dactron and the recuperation of this derivative. Firstly, the method showed to be effective using 12.5 μg of HSA per assay comparing with those values obtained for the normal rabbit sera. Furthermore, the same water insoluble HSA-ferromagnetic-dactron derivative used for a second time, after a previous treatment to remove the complex with the first antibody (rabbit anti-HSA serum) and the second antibody (pig anti-rabbit IgG conjugated to peroxidase), presented reliable values to evaluate the titration of rabbit anti-HSA serum. The data are relatively higher than those described for the first assay because of some remnant antibody not removed by the treatment with glycine/HCl buffer although the correlation factor (F) between absorbances of immunized and normal sera remained similar. Finally, a third use of the same derivative also showed significant values to estimate the titration of rabbit anti-HSA serum.

The summary of these findings indicates that antigen-ferromagnetic-dactron derivative can be used for enzyme-linked immunosorbent assay with some advantages: magnetic field replaces

TABLE

Titration of rabbit anti-HSA serum using magnetic enzyme-linked immunosorbent assay and the recuperation of this derivative

Assay	Sera				
	Normal ^a		Imunized ^a		
	1:100 (O.D.)	1:100 (O.D.)	[F]	1:1.600 (O.D.)	[F]
First assay	0.059	0.463	7.85	0.309	5.24
After recuperation	0.118	0.835	7.08	0.687	5.82
Second recuperation	0.162	0.956	5.90	0.684	4.22

^a: these values represent the means of duplicates of OD at 492 nm.

[F]: correlation factor

Ferromagnetic HSA-dacron particles (63-100 µm; 12.5 µm of immobilized protein) were introduced into the assay tubes and antibody assay proceeded as described. The used HSA-ferromagnetic-dacron derivative was washed in PBS/Tween and kept overnight. Subsequently, it was treated with glycine/HCl buffer and Tris/HCl buffer, successively. Finally, the material was washed with PBS/Tween and used for another assay as described above. A second reuse was proceeded. The ferromagnetic HSA-dacron particles were recovered by using magnetic field.

centrifugation or filtration and the antigenic complex can be used more than once. However it is important to observe that relatively higher amounts of antigen were required in this technique with some loss of sensitivity if compared with polystyrene matrix, probably due to steric hindrance of antigenic sites. Probably the big amount of protein required for immobilization modify the presentation of epitopes (Kricka et al. 1980, Venkatesan & Wakelin 1993). This phenomenon was also observed when complex antigenic extract (parasite crude extract) were used (not shown). Optimization of this method will certainly offer best results. So, it is possible to conclude that this method should be economic relevant for immunological purposes, specially considering its recuperation. In our laboratory, the diagnosis of some tropical diseases by using this method is under investigation.

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