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Enzymoimmunoassay of the Main Core Protein (p28) of Mouse Mammary Tumour Virus (MMTV)*

MARC DUBUCQ,† JEAN-CLAUDE HENDRICK,‡ PAUL M. OSTERRIETH,§ CAMILLE FRANÇOIS|| and PAUL FRANCHIMONT‡

†*Institut National des Radioéléments, Service Analyse Médicale, B-6220 Fleurus, Belgium,*
‡*Laboratoire de Radio-Immunologie, Institut de Médecine, Université de Liège, Belgium,*
§*Laboratoire de Microbiologie Générale et Médicale, Université de Liège, Belgium, and*
||*Laboratoire de Biochimie, Université de Liège, Belgium*

Abstract—*An enzymoimmunoassay was developed to detect p28, the main core protein of mouse mammary tumour virus. It is an ELISA test (enzyme-linked immunosorbent assay) using polystyrene tube as solid phase.*

The method was assessed according to two techniques: the one-step technique in which tubes coated with anti-p28 antibodies are incubated only once after simultaneous addition of both antigen (sample or standard) and enzymatic tracer (anti-p28 antibodies conjugated to glucose oxidase); and the two-step technique in which coated tubes are incubated first with antigen, then, after washing, submitted to a second incubation with the enzymatic tracer. In both techniques, after the last incubation, tubes are washed and submitted to enzymic detection.

The dose–response curves of the reaction are investigated and appear as different according to the technique used. As regards sensitivity, precision, accuracy and reproducibility, these methods were equal to competition radioimmunoassay and the results obtained by enzymoimmunoassay and radioimmunoassay for a series of tissue extracts are closely related. The advantages of the enzymoimmunoassay over radioimmunoassay are simplicity, speed, absence of radioactive material and stability of the tracer.

INTRODUCTION

A PREVIOUS publication [1] described a radioimmunoassay (RIA) for p28, the main core protein of mouse mammary tumour virus (MMTV). As ^{125}I-labelled p28 is fairly unstable, a fact already noted by Parks *et al.* [2], we carried out an enzyme immunoassay for the detection of this protein.

The present paper deals with two variants of this enzymoimmunoassay (EIA) for p28, according to the technique of enzyme-linked immunosorbent assay (ELISA) using polystyrene tubes as solid phase [3]. In this method, the tube wall is coated with antibodies. Secondly the antigen is allowed to react with the coated antibodies, forming complexes able to bind, in the last step, glucose oxidase-conjugated antibodies. The amount of enzymatic tracer bound depends on the amount of antigen present in the sample tested and is revealed by measurement of its glucose oxidasic activity.

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MATERIALS AND METHODS

Antigen

Polypeptide p28 was prepared according to Hendrick *et al.* [1], by filtration chromatography on Ultrogel ACA 54 (L.K.B., Bromma, Sweden) followed by chromatography on hydroxyapatite. The p28 obtained is 99% pure as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Antibodies

Rabbit anti-p28 antiserum was prepared as described in a previous paper [1] by intradermal injection of p28 emulsified in Freund's complete adjuvant (Difco Laboratories, Inc., Detroit, Michigan). This antiserum diluted 1:64,000 precipitates 25% of the ^{125}I-labelled p28.

The immunoglobulins precipitated by sodium sulfate precipitation (18% w/v), were resolubilized with a sufficient amount of 0.05 M $NaHCO_3$ to restore the initial volume. The protein concentration of this immunoglobulin solution, as determined by absorbance at 280 mm, is 12 mg/ml. This immunoglobulin solution will be referred to as anti-p28 antibodies or p28 Ab.

Washing buffer

When washing is needed at the different steps of the method, phosphate buffer (0.05 M, pH 7.5) containing 5 ml/l Tween 20 and 4 g/l ovalbumin was used. It was filtered on Selecta filter No. 595 1/2 extemporaneously.

Coating of polystyrene tubes with anti-p28 antibodies

Polystyrene tubes (L.K.B. 2174-086) were activated with glutaraldehyde by a modification of the technique of Boenisch [4]. One ml glutaraldehyde diluted 1:1000 v/v in sodium carbonate–HCl buffer 0.1 M pH 9.0 was added to the tubes and left 1 hr at room temperature. After the activation, the tubes were washed twice with demineralized water, filled with 1 ml of p28 Ab solution (diluted 1:1000 in 0.05 M $NaHCO_3$) and immediately incubated at 4°C for 24 hr. Tubes containing the antibody solution were stored in the cold until used. The p28 concentration was determined, after preliminary experiments, as the smallest quantity required for maximal fixation of antigen.

Conjugation of rabbit immunoglobulin to glucose oxidase

Conjugation of 10 mg of glucose oxidase (Boehringer Mannheim Grad I) or G.O.D. to 2.4 mg of p28 Ab (0.2 ml of p28 Ab solution) was carried out according to Nakane and Kawaio [5]. The product of the reaction was submitted to gel filtration in 0.05 M phosphate buffer pH 7.5, on a 60 × 1 cm column of Ultrogel ACA 34 (L.K.B., Bromma, Sweden) previously calibrated with a solution of pure G.O.D. Absorbance of the eluate was estimated at 280 nm in 1 cm light-path cells. The conjugated molecules (G.O.D.–p28 Ab) may be defined by:

1. Having a mol. wt around 310,000 since they must be constituted of one immunoglobulin (IgG) molecule (mol. wt: 150,000) bound to one G.O.D. molecule (mol. wt: 160,000).
2. Displaying glucose oxidase activity.
3. Maintaining the immunoreactivity of the original antibody.
4. Not being fixed on the antibody-coated tubes in absence of antigen.

The eluted fractions which contained material displaying these four characteristics were pooled.

The enzymoimmunoassay

Just before use, the antibody solution was poured out from the coated tubes and 2 ml of washing buffer were added to each tube. This buffer was left in the tube for 10 min in order to saturate with albumin the possibly unoccupied activated sites.

The immunoreaction

Two kinds of procedure were investigated.

A two-step procedure. The washing buffer was discarded and the coated tubes were filled with 0.8 ml of washing buffer, 0.1 ml of the sample to be assayed or of a solution containing p28 in concentration ranging from 0.1 to 30,000 ng/ml, 0.1 ml of normal human serum and 10 µl of a mixture of protease inhibitors (3 mg/ml phenylmethylsulfonyl fluoride, 5000 U/ml Trasylol in phosphate buffer pH 7.5, 0.05 M). The tubes were then incubated 6 hr at 20°C. Thereafter they were washed three times with 2 ml of washing buffer. Finally they were filled with 1 ml of G.O.D.–p28 Ab (1:3000 in washing buffer) and incubated overnight at room temperature (20°C). The dilution 1:3000 was found optimal for the tests as determined by a dilution curve (see Results).

A one step procedure. The tubes were filled at the same time with antigen and G.O.D.–p28 Ab. The filling was as above except for the replacement of 0.1 ml washing buffer by 0.1 ml of a 1:3000 dilution of G.O.D.–p28 Ab. The tubes were then incubated for 18 hr at room temperature.

Detection of G.O.D. activity

In both procedures, after incubation with conjugate, the tubes were washed three times with washing buffer to eliminate unbound conjugate as well as the various substances present in serum or in organ extracts. Some of these substances could interfere with the measurements of the G.O.D. activity. In order to detect the enzymic activity fixed, the tubes were then filled with 1 ml of a freshly prepared solution of 50 mg/ml β-D-glucose, 30 µg/ml horseradish peroxidase (Grad II—100 U/mg, Boehringer Mannheim) and 750 µg/ml 2.2′-azino-di(3-ethyl-benzthiazoline sulfonate [6]) crystallized diammonium

salt (Boehringer Mannheim) in citrate phosphate buffer (0.1 M citric acid, 0.1 M $Na_2H PO_4$, pH 5.6) for 45 min at room temperature.

Enzymic activity was estimated from absorbance at 410 nm, measured in the Automatic 2074 L.K.B. printing absorptiometer which allows for lecture of absorbance in the coated tube itself. All experiments were performed in duplicate.

Radioimmunoassays

They were performed as described by Hendrick *et al.* [1].

Mouse tissue extracts

Mammary tumours of MMTV-infected Swiss albino mice were prepared as described by Zangerle *et al.* [6].

RESULTS

Preparation of the G.O.D.–conjugate antibodies

After the Nakane's conjugation procedure, the unpurified conjugate is submitted to a gel filtration. As shown in Fig. 1, the proteins are eluted in three peaks from the ACA 34 chromatography when monitored by absorbance at 280 nm. In order to find which fractions contains molecules presenting the required properties of the conjugate, p28 antibody-coated tubes are incubated for 18 hr at room temperature with 1 ml of a 1:500 dilution of each fraction to which is added 0.1 ml of washing buffer containing or not p28 (0.1 μg/ml). Thereafter the tubes are treated to detect G.O.D. activity bound to the walls as described in Materials and Methods. The tubes incubated with the fractions of peak I and II displayed G.O.D. activity.

Peak I (fractions 14–16) corresponds to the void volume of the column and contain substances of a mol. wt greater than 400,000 according to the characteristics of the gel. These molecules are efficiently bound to the coated tube in presence of antigen p28 (1.9 absorbance at 410 nm for fraction 15) but binding remains significant in absence of antigen (0.34 absorbance for the same fraction). This is probably due to non-specific binding of these molecules to coated tubes.

Peak II (fractions 17–23) presents a maximum of absorption at 280 nm for fraction 22. In these fractions, the material has a mol. wt between 400,000 and 160,000. Proteins of all fractions of this peak are efficiently bound to the coated tube in presence of antigen p28 (2.6 absorbance at 410 nm for fraction 20) but not in its absence (0.03 absorbance at 410 nm for the same fraction).

Peak III (fractions 24–29) corresponds to substances of a mol. wt around 160,000. The proteins eluted here do not present G.O.D. activity coupled to antibody. Thus anti-p28 antibodies conjugated to G.O.D. (GOD–p28 Ab) are eluted in fractions 17–23. These fractions were pooled and the titer of the pool determined (Fig. 2) in a one-step test using a

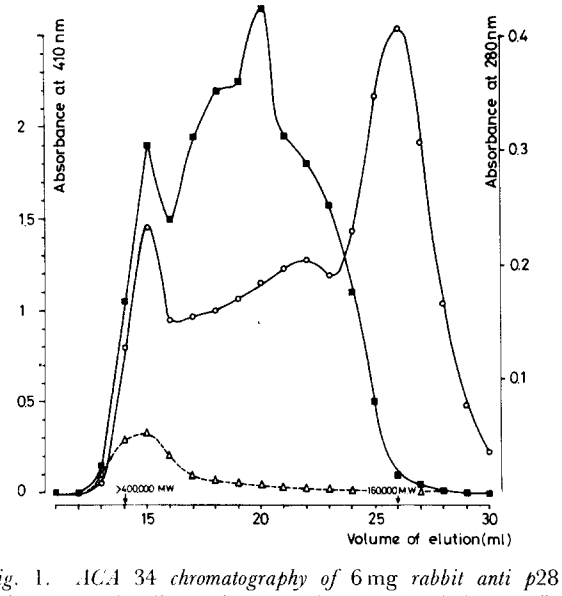

Fig. 1. ACA 34 chromatography of 6 mg *rabbit anti p28 IgG immunoglobulin conjugates with* 10 mg *G.O.D. The chromatography was carried out on a* 60 × 1 cm *column, flow rate* 11 ml/hr. (○—○) *Absorbance at* 280 nm, (■—■) *absorbance at* 410 nm *after ELISA one-step test of the fractions with* 10 ng *p28 per tube, and* (△---△) *control (without p28).*

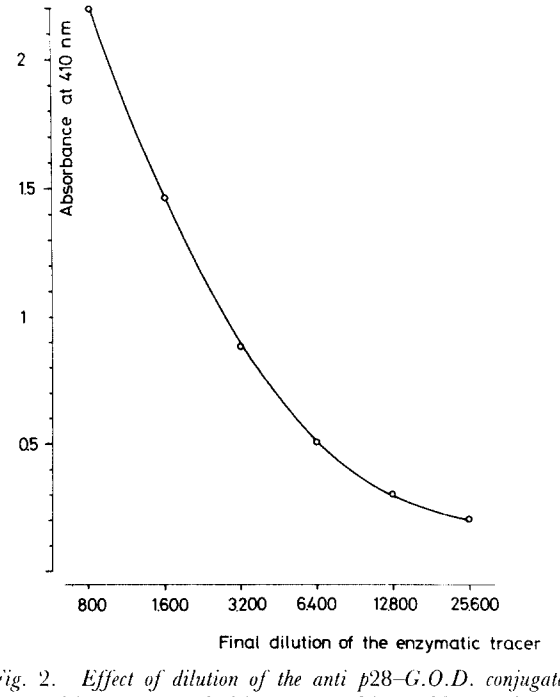

Fig. 2. Effect of dilution of the anti p28–G.O.D. conjugate tested in one-step method in presence of 6 ng *p28 per tube.*

constant amount of p28 (6 ng in 0.1 ml) and various dilutions of the G.O.D.–p28 Ab solution. A 1:3000 final dilution was found adequate since this dilution allows for a good level of absorbance (1.1) using 6 ng p28 per tube and for enzymatic tracer economy.

Comparison between one-step and two-step enzymoimmunoassays

In both techniques, the respective amounts of p28-Ab bound to the tube-walls, of purified p28, and of G.O.D.–p28 Ab were the same. Both techniques measure the quantity of antigen p28 fixed to the p28 Ab-coated tubes as revealed by subsequent fixation of a constant dilution 1:3000 of G.O.D.–p28 Ab used as tracer.

The antigen is thus sandwiched between coating antibody and conjugated antibody. Results are expressed as a dose–response curve in Fig. 3.

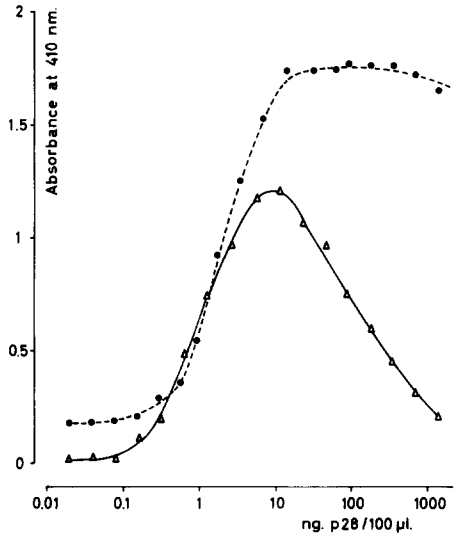

Fig. 3. Assay of antigen p28 by the one-step and the two-step procedures. (△—△) *One-step method,* (●---●) *two-step method.*

Two-step technique (*dotted line*). In absence of antigen the absorbance is 0.2. The reaction becomes positive when the amount of added antigen reaches 0.5 ng. A plateau (1.8 of absorbance) is obtained when this amount attains 16 ng and more. A slight decrease is observed with more than 1000 ng antigen per tube.

One-step technique (*continuous line*). A bell shaped dose–response curve is obtained. The reaction becomes positive with the same amount of antigen as in the two-step assay. A maximum of absorbance (1.2) is reached at an amount of antigen corresponding to the onset of the plateau described in the two-step method: 15 ng p28 per tube. Further increase of the amount of antigen leads to a decrease of the absorbance which, for 3000 ng of antigen, is down almost to the base line, (absorbance 0.01 in absence of antigen). The slope of the ascending part of the curve is somewhat less steep than that of the two-step technique.

For large amounts of antigen, the two-step method gives higher absorbance values than those obtained with the one-step method. To investigate the causes of these discrepancies (Table 1), three large amounts of antigen p28 (500, 750 and 3000 ng) were tested comparatively by the two-step and the one-step techniques, and a series of tubes, which were assayed with these same amounts of p28 by the one-step technique up to the end of incubation, were washed and incubated for a further 6 hr after addition of 1 ml of G.O.D.–p28 Ab (1:3000 dilution in washing buffer). After another washing, these tubes were submitted to detection of G.O.D. activity as usual. Table 1 shows that if the one-step technique leads to lower absorbances than the two-step technique, the effect of the second incubation with addition of G.O.D.–p28 Ab is to increase the final absorbance to the values precisely obtained by the two-step method.

Table 1. Absorbances obtained by one-step procedure with and without a second incubation and two-step procedure in presence of the same amounts of antigen p28

Amount of p28 (ng per tube)	One-step procedure: Unmodified	One-step procedure: Submitted to a second incubation with tracer after washing	Two-step procedure
3000	0.14	1.56	1.48
750	0.34	1.72	1.71
500	0.41	1.75	1.73

Characteristics of the enzymoimmunoassay (*Table 2*)

Table 2 gives the comparative values for one-step and two-step enzymoimmunoassays and for the competition radioimmunoassay of antigen p28.

The sensitivity calculated on the basis of two S.D.'s of the absorbance in absence of antigen is 0.5 ng per tube in all tests.

Precision seems a little better for E.I.A. as estimated by the precision index of Gaddum

Table 2. Comparison of the characteristics of RIA and ELISA of p28

	ELISA (Two-step)	ELISA (One-step)	RIA
Sensitivity	500 pg	500 pg	500 pg
Precision (λ of Gaddum)	0.005	0.007	0.015
Intra-assay C.V.	<12%	<12%	<10%
Interassay C.V.	<15%	<15%	<15%
Accuracy	3.87%	4.1%	4.2%

[7] calculated on the linear ascending part of the curves.

The reproducibility is estimated with the intra-assay coefficient and the interassay variation coefficient: they are identical with the three techniques.

The accuracy was determined by two ways: first by testing a series of dilution of a p28-containing mouse mammary tumour extract and secondly by recovery of added material in a serum. The curve obtained with serial dilutions of the extract is parallel to the standard curve (Fig. 4). The mean values calculated from these different dilutions was 193.2 ng p28 per 0.1 ml ± 7.48 (S.D.) of the undiluted sample containing 2.5 mg protein/ml and 772 ng p28/mg protein. Calculated accuracy is 3.87 for the two-step method and 4.1 for the one-step assay. Similar values were obtained with the competition RIA technique. The recovery of 1 ng of p28 diluted in 0.1 ml normal human serum was 0.89 ± 0.03 ng by the one-step procedure.

The specificity of the sandwich method depends upon the monospecificity of the antibody molecules coupled with the enzyme and coating the tubes. In order to test this specificity we used gp17, another MMTV antigen. This antigen does not bind p28 Ab. Moreover the addition of 0.1 ml of normal human serum in the tube does not induce any non-specific fixation of G.O.D.–p28 Ab.

Open circles of Fig. 4 shows a dilution curve of a suspension of MMTV virus pretreated with desoxycholate [1] tested in a one-step enzymoimmunoassay. This curve is identical to that obtained with the standard curve (open triangles). The undiluted virus preparation contained 186 ng p28/ml and 1 mg protein/ml.

Comparison between results obtained with EIA and RIA of p28 in tissue extracts

To ascertain the validity of the absolute values obtained with the enzymoimmunoassay for the determination of the p28 content of organ extracts, 13 extracts of mammary tumours of MMTV-infected Swiss albino mice were tested both by RIA and one step EIA. Both tests were carried out in presence of protease inhibitors. The results (Table 3) obtained by one technique shows excellent correlation with these obtained by the other one.

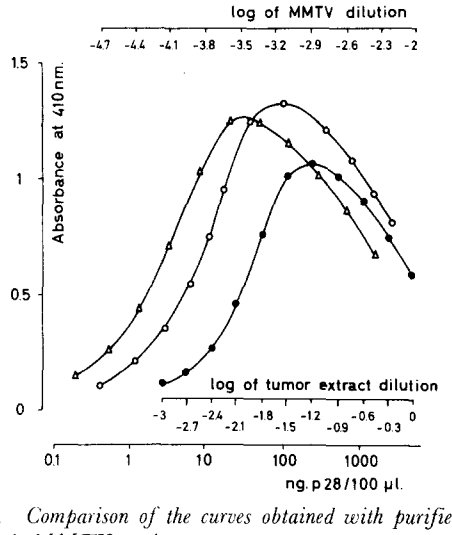

Fig. 4. Comparison of the curves obtained with purified p28, disrupted MMTV and mammary tumour extract. (△—△) *Purified p28* (ng/100 µl), (○—○) *disrupted MMTV* (*log of dilution*), *the undiluted preparation contained* 186 ng *p28*/mg *protein and* 1 mg *protein*/ml, (●—●) *Swiss mouse mammary tumour extract* (1930 ng *p28*/ml *and* 2.5 mg *protein*/ml = 772 ng *p28*/mg *protein*). *The maximum of the curves corresponds in all three cases to values between* 10 *and* 12 ng *p28 per tube.*

Table 3. Detection of p28 in Swiss albino mice mammary tumour extracts: comparison of RIA and one-step ELISA results

Tumour	ELISA	RIA
1	173	44.2
2	616	705.5
3	22.2	307.2
4	5.6	24.1
5	25	40
6	224.1	275.5
7	141.6	130.2
8	173.9	159.5
9	20	28.8
10	2777	1368
11	588.2	1038
12	390.6	920.7
13	6944.4	1962

These results are expressed in ng p28/mg protein. The coefficient of correlation is 0.85 with $P<0.005$ (regression line: $y=2.7x-529$).

The results of one-step enzymoimmunoassays were always calculated on the ascending part of the curve by means of several dilutions.

Organ extracts of Swiss mice at the 15th day of lactation were similarly tested comparatively by both methods. Extracts of brain liver and lung were found devoid of antigen p28 by both techniques; conversely p28 was detected in mammary gland extracts (EIA: 27.9 ng p28/mg protein, RIA: 30.6 ng p28/mg protein), spleen extracts (EIA: 0.98 ng p28/mg protein, RIA: 1.46 ng p28/mg protein) and colon extracts (EIA: 0.5 ng p28/mg protein, RIA: 1.16 ng p28/mg protein).

DISCUSSION

In the preparation of glucose-oxidase-conjugated anti-p28 antibodies we selected the material present in the second peak of elution because it presented the required characteristics, i.e., mol. wt around 300,000, antibody and G.O.D. activities. The material present in peak I, because of its high mol. wt, probably corresponds to polymers of immunoglobulins and glucose oxidase.

Since it had also some non-specific binding properties it was discarded. Peak III, according to its position in the elution pattern, corresponds to unconjugated material: glucose oxidase and/or IgG both of $\pm$ 160,000 mol. wt.

The final reagent brings a large excess of substrate (50 mg/ml of β-D-glucose) to insure that H_2O_2 will be the only limiting factor in the measurement of enzyme activity. Indeed β-D-glucose, oxidised by glucose oxidase, is transformed into gluconic acid and hydrogen peroxide which is the substrate of horse radish peroxidase. The color change of A.B.T.S. monitors this latter oxidation. As the antigen–antibody reaction was performed, the fixation of G.O.D.–p28 Ab is only limited by the amount of antigen p28 as long as it remains lower than 10 ng per tube. When antigen amount becomes greater than 10 ng per tube, discrepancies between the one- and two-step methods appear which may be explained as follows: During the first part of the two-step procedure, the antigen reacts solely with the coating antibodies. If the amount of antigen rises, more antigen will bind to these coating antibodies up to the point when all active sites are occupied. The plateau of the two-step procedure is reached. Further amount of antigen will not be fixed. The second part of the assay will only reveal how much antigen reacted with the coating antibodies. The slight decrease in presence of very large amounts of antigen may result from some steric hindrance phenomenon. The one-step procedure differs significantly from the latter firstly by the fact that for the same amount of antigen, maximal absorbance obtained with the two-step procedure is greater than the corresponding one obtained with the one-step test and secondly by its bell-shaped dose–response curve. we think that these discrepancies may be related to the fact that during the same incubation the antigen can react both with coating antibodies and with G.O.D.–p28 Ab, up to equilibrium.

In these conditions, for high amounts of Ag, the difference of absorbance with the two steps method can be explained by the formation of 'p28 . G.O.D.–p28 Ab' complexes which will not fix themselves to the coating antibodies already saturated with the antigen. These complexes will be eliminated by the last washing step. Consequently, the amount of 'coating Ab . p28 . G.O.D.–p28 Ab' sandwich decreases and so does absorbance.

This interpretation is enforced by the results given in Table 1 which shows that, in the presence of large amounts of antigen, after the one-step procedure, some 'coating antibody–p28' complexes remain free of G.O.D.–p28 Ab. Indeed further addition of G.O.D.–p28 Ab reveals its presence as shown by the absorbance values increasing to those obtained by the two-step assay.

Between the competition RIA and the here described EIA there are some basic differences:

(a) In the RIA the specificity of the assay depends upon the degree of purity of the labelled antigen. The specificity of the sandwich EIA depends on the specificity of the antibodies used for tube coating and enzymic tracer. Nevertheless in both kinds of tests cross-reactions remain possible.

(b) Our experience shows that EIA requires more antiserum than RIA. This is presumably a limitation for EIA.

(c) In RIA, as the one used for the detection of p28, proteolysis occurring during incubation [8] may result in false positive values, since digested labelled antigen will not bind antibodies anymore. This leads to an overestimation of unlabelled antigen. In the sandwich EIA, proteolysis, destroying the antigen during incubation, will decrease the fixation of G.O.D.–p28 Ab and result in lower absorbance values. This leads to

an underestimation of the amount of antigen present. Comparison of results obtained by both kinds of assays will indicate proteolysis if the values obtained by RIA are significantly larger than those obtained by EIA.

(d) The one-step EIA presents a bell-shaped dose-response curve. Testing two dilutions of the sample will show on which arm of the curve the values are to be read. The curves of the two-step EIA and of the RIA are sigmoidal.

(e) EIA presents well-known advantages over RIA: a very stable tracer (we worked for 1 yr with the same batch of G.O.D.–p28 Ab), no radioactive material to handle, simplicity and speed of the whole system (photometric reading, no centrifugations). Furthermore, the one-step method allows for the coverage of a larger range of antigen amounts. Both EIA tests are easily performed in small series which is specially useful for the detection of p28 since this antigen becomes very labile when ^{125}I-labelled for RIA (J. C. Hendrick, personnal communication). Comparison of results obtained with RIA and EIA shows that they are both as sensitive, precise, accurate and reproducible for the detection of p28 in biological extracts.

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