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## ANTIGEN REQUIREMENTS AND SPECIFICITY OF A MICROPLATE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR MAREK'S DISEASE VIRAL ANTIBODIES IN CHICKEN, MOUSE AND RABBIT SERA

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### Résumé

ÉTUDE D'UN TEST ELISA POUR LA MISE EN ÉVIDENCE DES ANTICORPS VIRAUX DE LA MALADIE DE MAREK DANS DES SÉRUMS DE POULET, DE LAPIN OU DE SOURIS. — Ce travail étudie les différents paramètres qui interviennent au cours du test ELISA appliqué au virus de la maladie de Marek. Les anticorps ont été produits chez trois espèces différentes: le poulet, la souris et le lapin. Le maximum de sensibilité a été obtenu en utilisant une concentration de 10 µg/ml de protéines virales partiellement purifiées. Une heure d'incubation à 37 °C en présence de l'antisérum nous a donné les meilleures réponses. Différents essais, afin d'éliminer les réponses non spécifiques sont décrits. L'incorporation de NP40 à 0,1 % a amélioré sensiblement nos résultats. La polylysine a également réduit les réactions non spécifiques de l'antisérum produit chez la souris.

The enzyme linked immunosorbent assay (Elisa), initially developed by Engvall and Perlmann (1971) has since been extensively used in studies involving antigen-antibody reactions. This test is an efficient diagnostic tool for viral diseases since it can detect specific antibodies against a large number of viruses in both human and animal sera.

In avian virology, for example, this technique has been employed to detect serum antibodies against infectious bronchitis virus (Mockett and Darbyshire, 1980; Soula and Moreau, 1981), reovirus (Slaught *et al.*, 1978) and infectious bursal disease virus (Marquardt *et al.*, 1980)...

The present paper describes the application of Elisa to detect antibodies against Marek's disease

viral antigens, using chicken, mouse and rabbit sera.

The optimal antigen requirements and incubation time as well as the different parameters employed to eliminate non-specific reactions with chicken and mouse sera are described.

### Materials and Methods

#### 1. Virus purification

The HPRS-16 (Purchase and Biggs, 1967) and JM-19 (Witter, 1973) strains of Marek's disease herpes virus were used. The HPRS-16 strain was purified from feather follicle epithelium and JM-19 strain from primary kidney cell cultures.

The method of purification used for HPRS-16 was similar to that reported by Cauchy (1974). Virus was extracted in phosphate buffered saline (PBS) from the skin of feather follicle tips. After eliminating the gross

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contamination by low speed centrifugation at 16 000 rev/minute for 1 hour, the virus was further purified by zonal centrifugation on a 20% to 50% (w/w) linear cushion sucrose gradient in PBS (pH 7.4) for 2 hours at 26 000 rev/minute at 4 °C. The fractions of the gradient were scanned at 254 nm. The fractions absorbing at 254 nm were pooled and checked for the presence of virus by electron microscopy using a negative staining technique. The fractions between 47 and 49% sucrose revealed the presence of virus. These fractions were pooled, diluted with PBS and sedimented at 36 000 rev/minute for 1 hour. The virus pellet was resuspended in PBS and stored at -70 °C.

For JM-19 strain of Marek's disease virus, kidney cells from 3-4 week old chickens which had been inoculated with virus at one day old were cultured *in vitro*. The primary kidney culture was performed in MEM containing 7% inactivated calf serum.

After 24 hours, the primary culture medium was replaced by one containing 1% serum and 96 hours later, the heavily infected cell cultures were harvested. These cells were swelled in RSB (reticulocyte standard buffer) for 10 minutes at 4 °C and then disrupted with 4-10 strokes in a Dounce homogenizer as described by Lee *et al.* (1973). After low speed centrifugation to remove the nuclei, the virus was sedimented at 16 000 rev/minute for 1 hour. It was then subjected to zonal centrifugation and the virus pellet obtained as described above.

For both virus strains, the protein content of the viral antigen was evaluated by Lowry's method.

### 1.1. Sera

#### Chicken.

Antisera were obtained from specific pathogen-free birds inoculated at one day old with Marek's disease virus strain of MDV. Sera were collected after 5 weeks, inactivated at 56 °C for 30 minutes and stored at -70 °C. Negative serum was obtained from a pool of SPF chickens.

#### Mouse.

Antisera were prepared in Balb/c strain of mice. A preliminary intraperitoneal injection of 80 µg of purified feather follicle Marek's disease virus (HPRS-16) with Freund's complete adjuvant was followed 7 days later by 3 or 4 weeks intravenous injections of 50 µg purified virus in the tail region. Sera were collected one week after the last injection, inactivated at 56 °C for 30 minutes and stored at -70 °C. Negative serum was obtained from a pool of uninoculated mice.

#### Rabbit.

Antisera were prepared by subcutaneous injections of 5 mg of partially purified feather follicle virus (HPRS-16) with Freund's complete adjuvant at multiple sites on the back of several rabbits. After one week the same procedure was repeated. It was followed by three intravenous injections of 3 mg partially purified antigen in the vein of the ear at weekly intervals. Serum was collected one week after the last injection, inactivated at 56 °C for 30 minutes and stored at -70 °C. Negative serum was obtained from an uninoculated rabbit. The specificity of the rabbit serum was increased by absorbing it with normal chick tissue powder consisting of liver and kidney cells prepared by passing concentrated cell suspensions through acetone. The dried tissue powder was mixed with rabbit serum at 1:2 v/v and gently stirred overnight at 4 °C, after which the tissue powder was removed from the serum by centrifugation.

#### Mercaptoethanol treatment.

One volume of serum was treated with 1/20 volume of 2M 2-mercaptoethanol in PBS for 2 hours at room temperature.

#### Kaolin treatment.

The method used was similar to that described by Shillitoe (1982). To 0.2 ml of chicken serum was added 0.8 ml of PBS (pH 7.4) and 1 ml of 25% w/v of kaolin (hydrated aluminium silicate, particle size 0.1, Sigma Chemical Company). This mixture was stirred for 20 minutes at room temperature and then centrifuged for 10 minutes to remove kaolin at 1000 × g.

### 1.2. ELISA technique

The technique was adapted from that described by Engvall and Perlman (1972). Four different methods of fixing to microplate wells were tested.

#### 1.2.1. Fixation with poly-L-lysine.

The technique of Kelose and Weller (1978) was adapted. Polystyrene microplates (Nunc Laboratories) were used. Wells were coated with 0.1 ml poly-L-lysine solution (100 µg/ml in PBS). After incubation for one hour at room temperature, the plates were washed twice with PBS followed by the addition of 0.1 ml of antigen preparation diluted in PBS. The plates were then incubated for 30 minutes at room temperature. The antigen was fixed with 0.1 ml of 1% glutaraldehyde in PBS, for 10 minutes at room temperature. The wells were washed three times with PBS. To each well 0.2 ml of 0.2 mg/ml gelatin solution containing 0.2% sodium azide was added. The plates were kept at 4 °C until use.

#### 1.2.2. Fixing on serum coated plates.

The technique used was similar to that described by Slaughter *et al.* (1979). The plates were initially coated

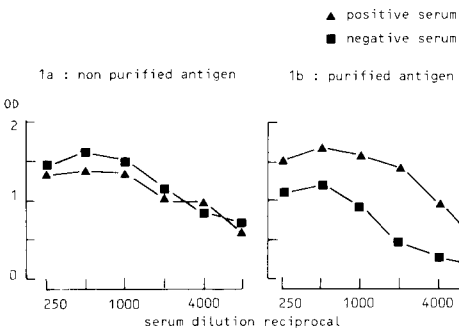
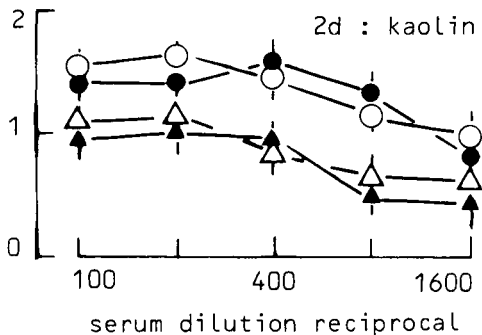
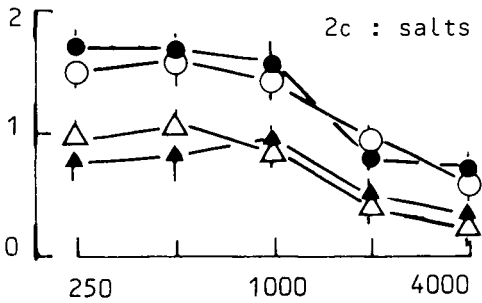
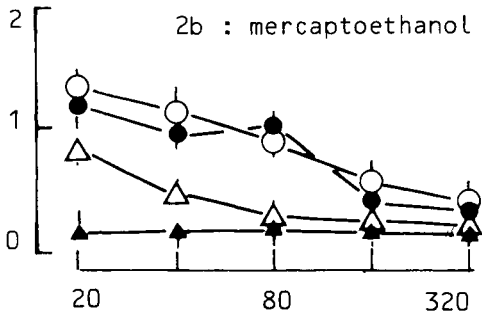
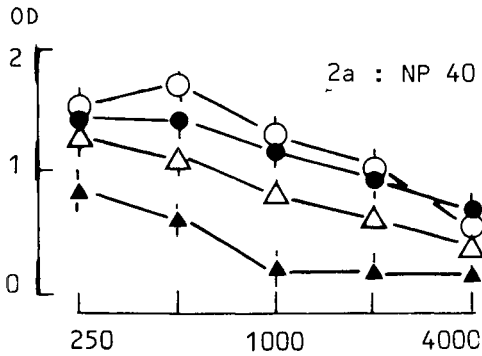


Fig. 1. — Influence of virus purification on the behavior of normal serum and positive anti Marek's chicken serum Elisa. Antigen concentration used was 10 µg/ml.



with 0.1 ml of 1.25 % heat-inactivated foetal calf serum in distilled water and dried after which 0.1 ml of 1 % glutaraldehyde in PBS was added and allowed to react for 30 minutes. The plates were then washed and dried and 0.1 ml of antigen diluted in distilled water was added to each well and allowed to dry at room temperature. The plates were washed before use in Elisa test.

1.2.3. Fixing with coating buffer.

Bicarbonate buffer pH 9.6, 0.05 M, was used as coating buffer to sensitise the plates. The plates were sensitised at 37 °C for 3 hours or overnight at room temperature.

1.2.4. Fixing the antigen by drying.

The antigen was diluted in distilled water and air-dried in a dust-free atmosphere or kept at 37 °C overnight.

Incubation time of antibodies with the antigen.

Three different time periods (3 hours, 1 hour and 1/2 hour) were tested at 37 °C.

Dilution of the antisera and reaction with conjugate.

Mouse and rabbit sera were diluted in PBS (pH 7.2) containing 0.05 % Tween 20 (PBST). Chicken serum was diluted in PBS (pH 7.2) containing 0.01 % NP-40 (PBSN). For chicken sera, NP-40 has given better results in the suppression of the non-specific response than the Tween 20. Before adding the sera, plates were washed 3 times with the respective solutions used for dilution. The same washing procedure was carried out after every step.

After washing, rabbit anti-chicken serum (H + L) (Nordic) was used at 1/1000 dilution in PBSN. Rabbit anti-mouse serum was prepared by us and was used at 1/500 in PBSN or PBST. 0.1 ml of the diluted serum was added to each well and incubated for 1 hour at 37 °C.

After washing, 0.1 ml of alkaline phosphatase-conjugated goat anti-rabbit serum (IgG whole molecule) (Sigma) diluted at 1/2000 in PBSN or in PBST was added to each well and incubated for 1 hour 30 minutes. The wells were washed, and 0.1 ml of substrate solution was added. The substrate was prepared immediately

Fig. 2. — Effect of various treatments for eliminating non-specific reactions.

- a) The non-specific fixation of normal serum is reduced by using NP-40.
  - b) No background binding appeared when the serum was treated with 2-mercaptoethanol.
  - c) No effect of salt concentrations on the background binding.
  - d) Proportionate increase in optical density is observed in normal and positive serum when treated with kaolin.
- The results refer to the means of 20 different samples of sera.

	treatment	
serum	+	-
+	●	○
-	▲	△

before use. The solution consisted of 1mg/ml p-nitrophenyl phosphate (Sigma) in 10% diethanolamine buffer (pH 9.8) containing  $10^{-3}$  M magnesium chloride. The enzyme reaction was allowed to proceed for 1 hour at 37 °C and a yellow colour indicated a positive reaction.

The enzyme reaction was stopped by adding 0.05 ml of 4N sodium hydroxide. The intensity of the colour was measured using a Titertek Multiskan plate reader at 405 nM.

## Results

### Adsorption of Marek's disease antigen to microplates

Between the four assays tested to detect the best technique for fixing viral antigen (coated plate with serum, with tampon, with polyLysine or fixed by drying, the best was found to be that of air drying the antigen solution at 37 °C overnight. The final coloration obtained at the end of the test was the more specific in relation to the control.

### Antiserum incubation time and temperature

Determination of an optimal incubation time for each serum was assayed at room temperature and at 37 °C. A 60 minute incubation period at 37 °C was found to be satisfactory. Increasing the time resulted in an increase of non-specific reactions while 30 minutes incubation was insufficient.

### Determination of optimal antigen concentration

Microplates were coated with different antigen concentrations, namely 40 µg/ml; 20 µg/ml, 10 µg/ml; 5 µg/ml and 2.5 µg/ml. A chequer board titration was performed with different dilutions of

Marek's disease-specific chicken antiserum. Equivalent dilutions of Marek's disease negative chicken serum were used as controls. Very little difference in optical density was observed when 40, 20 or 10 µg/ml concentrations of antigen were used. As the binding of negative serum was minimum and the mean optical density obtained was maximum when 10 µg/ml antigen was used, it was decided to employ this concentration throughout our study.

### Elimination of non specific reactions

In the initial trials positive reactions with both normal chicken serum and the positive anti-Marek's disease serum were obtained. When non-purified Marek's disease antigen was used, it was found that the normal chicken serum reacted even more strongly than the positive anti-Marek's disease serum (Text fig. 1a). However with purified virus, the positive anti-Marek's disease chicken serum reacted more strongly than the negative one (Text. fig. 1b). Partially purified Marek's disease virus was therefore chosen as the antigen for routine use in Elisa, although the background binding produced by normal chicken serum was still significant. In order to reduce this, the non-ionic detergent, NP-40 was added at a concentration of 0.01% (Text. fig. 2a). By using this method we have tested 20 different sera and the results obtained were quite satisfactory.

The background colour produced by normal chicken serum was eliminated by treating the serum with mercaptoethanol, while the intensity of the specific reaction was not affected (Text. fig. 2b). However, to obtain such a result, it was necessary to concentrate the sera and use the

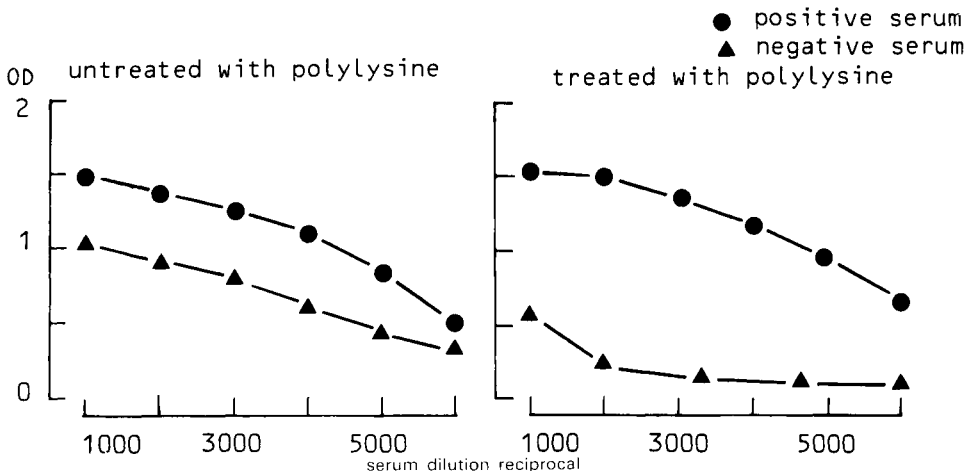


Fig. 3. — Effect of poly-L-lysine treated plates in eliminating the non-specific reaction. Antigen concentration used was 10 µg/ml. (Results are the means of 5 different mice sera).

preparation immediately, because after 2 days sera had lost complete activity.

There was no effect of high salt concentrations (8% NaCl + 0.15 M NaI) on the degree of the non specific reactions and the background colour caused by normal serum was unchanged (Text. fig. 2c). An attempt was made to reduce the non-specific reactions by treating the serum with kaolin. Treatment with kaolin enhanced the activity of the serum (fig. 2d).

When virus purified from primary kidney culture was used in Elisa, an additional step of adsorbing the serum with normal chicken tissue powder was required. To avoid this, we have always used viral antigens purified from feather follicles.

In order to find out which sera would be the best for our experimentation, we decided to raise hyperimmune sera against Marek's disease viral antigens in mice and rabbits.

#### 1. Mouse sera

The fixation of antigen in the presence of poly-L-lysine was found to reduce the non specific binding of normal mouse serum in the Elisa. Text. fig. 3 indicates the elimination of non specific reactions by poly-L-lysine and the activity obtained after hyperimmunisation.

#### 2. Rabbit sera

Rabbit sera were repeatedly absorbed with normal chicken tissue powder in order to eliminate antibodies against chicken antigens. The activity of rabbit sera in the Elisa is indicated in the text, fig. 4.

### Discussion

This study has established the optimal conditions for Elisa when it is used to detect antibodies to Marek's disease virus in chicken, mouse and rabbit sera. The results demonstrate the need for purified virus for adsorption onto microplates. A similar requirement has been shown for other viruses such as avian infectious bronchitis virus (Dawson *et al.*, 1980; Soula and Moreau, 1981).

Satisfactory adsorption of purified virus was achieved by drying overnight at 37 °C. This procedure was superior to other methods attempted. The optimal concentration of Marek's disease viral antigen coated to microplates was found to be 10 µg/ml; this is the same as the concentration used with infectious laryngotracheitis virus (Meulemans and Halen, 1982). Best results were obtained when the antiserum and antigen were incubated for one hour at 37 °C. NP-40 at a concentration of 0.01% was found to reduce substantially the background binding. NP-40 at this concentration did not denature the viral antigens nor did it inhibit the activity of the enzyme alkaline phosphatase.

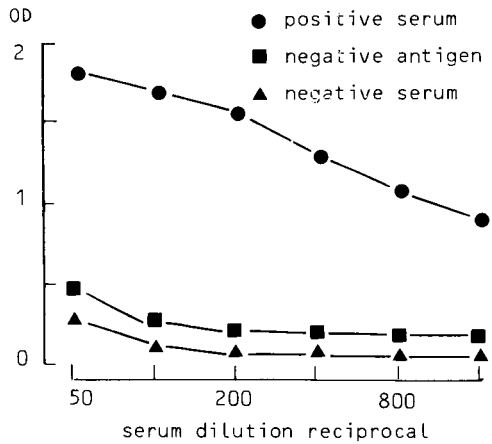


Fig. 4. — Elisa titration of anti Marek's disease adsorbed rabbit serum. The serum dilutions after 1/100 gave no non-specific reactions against negative antigen and negative serum.

Mercaptoethanol treatment of chicken serum diminishes its IgG activity (Higgins, 1975). In our studies with 2-mercaptoethanol treatment, we have observed that after mild reduction of chicken serum, as described earlier, the IgG activity of the chicken serum was not completely lost. In Elisa it gave a complete elimination of non-specific reaction. But this technique does not seem useful in relation with dilution of sera and also the immediate use when this serum was used 2 days later in Elisa almost all its activity was found to have been lost.

Goodman *et al.* (1952) observed that the increased precipitation observed in control serum was eliminated by using 1.5 M NaCl in the presence of 0.15 M NaI. This was contradicted by Higgins (1975). Our trials to determine the possible influence of these salts in eliminating non-specific reactions in Elisa were negative.

An attempt to eliminate the background binding of chicken serum was made by treating the serum with kaolin. As a result of this treatment, it was observed that the activity of both normal and positive sera was increased and there was no longer any significant difference between the two sera. However, kaolin treatment could be of use as regards of serum which has lost its activity on storage. This confirms the results of Shillitoe (1982).

Hyperimmune sera against Marek's disease virus were raised in Balb/c mice. The non-specific binding of mouse serum to the plastic surface was prevented by precoating the wells with poly-L-lysine. However, even after this, the results were

not completely satisfactory since a slight background binding still persisted.

In order to obtain highly specific sera, we raised hyperimmune sera in rabbits, as normal rabbit serum has the advantage of not developing non-specific reactions with plastic surfaces. This hyperimmune serum, after absorption to remove reactions with normal antigens was found to react specifically in the Elisa. Therefore for our studies involving Marek's disease viral antigens and

employing Elisa test, rabbit serum was found to give the best results.

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### Summary

A rapid indirect enzyme linked immunosorbent assay for the detection of antibodies against Marek's disease virus in mouse, chicken and rabbit-sera has been established. Optimal sensitivity was obtained by using 10 µg/ml of partially purified viral proteins. Various experimental procedures performed to eliminate nonspecific reactions are described. Best results were obtained by the introduction of NP 40 at a concentration of 0.1 % in sera. With mouse sera, the pretreatment of plaques with poly-L-lysine before antigen fixation substantially reduced the occurrence of nonspecific reactions.

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