Sandwich enzyme immunoassay of mouse mammary tumor virus

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Summary. A highly sensitive sandwich enzyme immunoassay for the mouse mammary tumor virus (MMTV) is described. The assay can detect 3 ng/ml of MMTV. The enzyme used is β-D-galactosidase from *Escherichia coli* and the solid phase used is a piece of silicon rubber.

Mouse mammary tumor virus (MMTV) can be determined quantitatively by radioimmunoassay[1,2], RNA-dependent DNA polymerase assay[3], and particle counting by electron microscopy[3,4]. Although the radioimmunoassay is reported to provide the most sensitive measurements of the viral antigens, it has some disadvantages: the hazard of radioactivity, the short life of the radionuclides used and so on. To overcome these disadvantages, we tried to standardize the enzyme immunoassay system for the measurement of MMTV. As many other investigators have reported, the enzyme immunoassay system is interfered with by serum factors present in the samples to be analyzed[5,6]. In our experiment, we examined the degree of interference by tissue culture medium to see if this technique is applicable to the study of the kinetics of MMTV production in a tissue culture system.

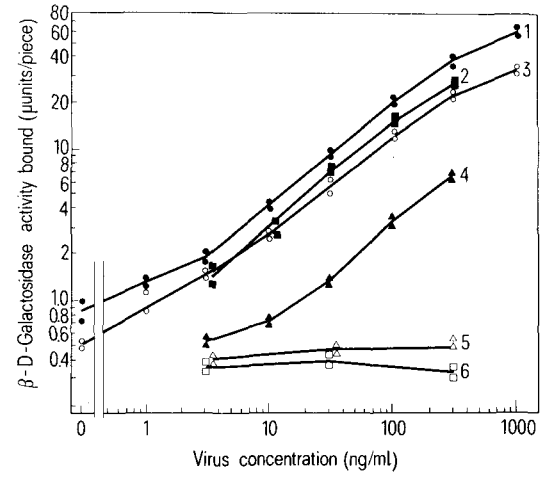
The method used in this experiment was the enzyme-linked sandwich immunoassay system using the antibody Fab'-β-D-galactosidase complex and antibody-bound silicon rubber pieces as the solid phase[6-8]. In brief, Fab' fragments of IgG fractions from rabbit anti-MMTV serum were coupled to β-D-galactosidase from *E. coli* (Boehringer, Mannheim) by use of N,N'-o-phenylenedimaleimide (Aldrich Chem. Co.) as described by Ishikawa and Kato[6]. The amounts of the complex are expressed as units of enzyme activity; 1 unit of activity is defined as that which hydrolyses 1 μmole of 4-methylumbelliferyl-β-D-galactoside per min under the conditions described below[9]. Small pieces of silicon rubber string (Sanko Plastic Co., Osaka, Japan), 3 mm in diameter and 4 mm in length, were coated with the $F(ab')_2$ from the anti-MMTV serum by physical adsorption[6]. The pieces were stored in buffer A (0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% NaN_3) at 4 °C until use.

The samples used were MMTV preparations purified from RIII mouse milk[10]. The standard MMTV preparation was estimated to contain 1.2×10^{11} particles/ml by particle counting with an electron microscope and 300 μg protein/ml as determined by the method of Lowry et al.[11]. The antiserum directed against MMTV was prepared by the immunization of rabbits with an ether-disrupted MMTV preparation. IgG fractions from the antiserum were prepared by fractionation with $(NH_4)_2SO_4$[12] followed by passage through a DEAE-cellulose column[13], and the $F(ab')_2$ and Fab' fragments were prepared by the method of Nisonoff and Rivers[14].

Enzyme immunoassay was performed by the sandwich procedure. A piece of the antibody-bound silicon rubber was incubated with various amounts of MMTV suspended in a final volume of 0.5 ml of buffer A or a tissue culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum) with shaking at 37 °C for 4 h. Each piece was washed twice with 1 ml of buffer A and incubated at 4 °C overnight with 3000 μunits of the (anti-MMTV) Fab'-β-D-galactosidase complex in 0.2 ml of buffer A. Each piece was then washed twice with 1 ml of buffer A and transferred to another tube. Then, the enzyme activity bound to the silicon pieces was assayed. Each piece was preincubated in 0.1 ml of buffer A at 30 °C for 5 min and the enzyme reaction was started by adding 50 μl of 0.3 mM 4-methylumbelliferyl-β-D-galactoside. After incubation for 20 min at 30 °C with shaking, the reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3, and the 4-methylumbelliferone formed was determined fluorometrically. The wavelengths used were 360 nm for excitation and 450 nm for emission analysis.

Standard curves for the sandwich immunoassay of MMTV suspended in buffer A or in the tissue culture medium are shown in the figure (curves 1 and 3, respectively). A linear dose-response of the enzyme activity bound to the solid phase is observed with between 3 and 300 ng/ml of MMTV in both. The enzyme activity bound in the case of the MMTV samples suspended in the tissue culture medium is 50–90% of that in the case of the samples suspended in buffer A. Therefore, when the viral samples containing the culture medium are assayed, it is necessary to add the same volume of the culture medium, free of viral antigens, to the samples to obtain standard curves. The assay is less sensitive when the IgG-solid phase, instead of the $F(ab')_2$-solid phase, is used (curve 4).

To test the specificity of the enzyme immunoassay, MMTV was suspended in 0.5 ml final volume of buffer A containing 10 μl/ml of the anti-MMTV serum and normal rabbit serum respectively. After incubation at 37 °C for 2 h in glass test tubes, each sample was subjected to enzyme immunoassay as described above. When the samples containing the anti-MMTV were assayed, β-D-galactosidase activity bound did not increase with the increase in concentration of MMTV (curve 6). In the samples containing normal rabbit serum, the enzyme activity decreased slightly (curve 2). The decrease is mainly due to serum interference. Furthermore, in order to determine whether the anti-



Calibration curves for sandwich enzyme immunoassay. Curves 1 and 3 are standard curves for MMTV suspended in buffer A and in the tissue culture medium, respectively. Curves 2 and 6 show the assay of MMTV preincubated in buffer A containing 10 μl/ml of normal rabbit serum and anti-MMTV serum, respectively. Curve 4 shows the assay of MMTV suspended in buffer A using the IgG-coated silicon rubber pieces. Curve 5 illustrates the assay of murine leukemia virus from AKR mice.

MMTV serum used in our experiment contained antibodies against C-type viruses, a murine leukemia virus preparation derived from AKR mice (provided by Dr Iwai of the Radiation Center of Osaka Prefecture) was similarly assayed. As shown in curve 5, no increase in the enzyme activity bound was observed.

Enzyme immunoassay, as described here, is highly specific for MMTV and reproducible. The assay can detect as little as 3 ng/ml of MMTV and makes it possible to monitor easily the kinetics of MMTV production in tissue culture systems. Sheffield et al.[2] reported that radioimmunoassay can measure 0.05 ng/assay tube of the purified gs antigen (gp 55) from MMTV and 0.5 ng/assay tube of whole virus. The lowest value to be determined by our method is converted into 1.5 ng/assay tube of whole virus. This means that enzyme immunoassay is almost as sensitive as radioimmunoassay. In addition, it was reported that enzyme immunoassay became more sensitive when the IgG possessing antibody activity was concentrated from the antiserum by immunoaffinity chromatography[15]. The use of concentrated monospecific antibodies in the assay system of each polypeptide of MMTV is currently under development.

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Histochemical localization of alkaline and acid phosphatase activities in the skin of *Mystus (Mystus) vittatus* Bl. (Siluriformes)

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Summary. An attempt has been made to localize alkaline and acid phosphatase activities in the skin of *Mystus vittatus* by using histochemical techniques. The alkaline phosphatase activity is found in metabolically active cells such as basal columnar cells, mucous cells and polygonal support cells. The acid phosphatase activity is intense in the outermost squamous support cells and in the basal columnar cells. These activities have been correlated with some physiological functions of the epidermis.

There have been several histochemical studies to establish the correlation between alkaline and acid phosphatase activities and the functional state of the skin of fishes[2-5]. These hydrolytic enzymes play a significant role in the metabolic activities of various cellular components of fish skin. The present attempt is intended to correlate alkaline and acid phosphatase activities with the functions of various cellular components in the skin of *Mystus vittatus*, the structural organization of which has been described by the present authors[6].

Material and methods. The fish specimens were collected locally and kept in aquaria for acclimatization. Skin fragments of 4×8 mm were taken from a lateral site, fixed in cold acetone and cut using 52-54 Paraffin wax. The alkaline phosphatase activity was localized by the calcium cobalt method of Gomori[7] and the modified coupling azo dye method of Pearse[8], and the acid phosphatase activity by the lead nitrate test and the standard azo dye method described by Pearse[8]. Control sections were incubated without using the substrates.

Results. The calcium cobalt method gave a strong brownish black colour reaction with basement membrane, basal columnar cells, small mucous cells, inner polygonal support cells and the *subcutis*, thereby showing alkaline phosphatase activity. These results were confirmed by the modified azo dye technique of Pearse[8] using salt 9, which gave a reddish brown colour reaction. The lead nitrate test of Gomori[7] for acid phosphatase activity gave a strong black colour reaction in basal columnar cells and outermost squamous support cells, and a weak reaction in small mucous cells. The standard coupling azo dye technique of Pearse[8] confirmed acid phosphatase activity in basal columnar and squamous support cells by a strong reddish brown colour in the cytoplasm and a deep blue colour in the nuclei.

Discussion. Alkaline phosphatase activity has been considered to indicate various functions for example, active cell division[9-11]; sites of active transport across the cell membrane[12], and it is closely associated with the osseous layer of the scale as well as sulphated acid mucopolysaccharides and calcium which play and active role in calcification[3] and in the synthesis of mucopolysaccharides[3,13,14]. In *Mystus vittatus* the alkaline phosphatase activity is found in metabolically active cells and extends from the basal layer of the epidermis to the surface, as found in whiting[5]. This activity is possibly connected with active cell division and synthesis of mucopolysaccharides. The replacement of lost cells of the epidermis and secretion of mucopolysaccharides in non-scaly fishes is important for protective functions. Intense activity of alkaline phosphatases in basal columnar