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PREPARATION OF ANTIGEN FOR THE COUNTERIMMUNOELECTROPHORETIC TEST FOR PLASMACYTOSIS IN MINK*

By

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BRUMMERSTEDT, E.: *Preparation of antigen for the counterimmunoelectrophoretic test for plasmacytosis in mink.* Acta vet. scand. 1976, *17*, 395—402. — Counterimmunoelectrophoresis as a test method for making the diagnosis of plasmacytosis in mink demands the specific virus antigen. The method for preparation of the antigen according to *Cho & Ingram* (1972 a, b) with minor modifications is described in details, and results obtained at 62 antigen preparations are presented. In addition an ultrafiltration method is outlined which may be useful as a replacement for ultracentrifugation procedures used in the technique described by *Cho & Ingram* (1974).

plasmacytosis; mink; antigen; counterimmunoelectrophoresis.

The fight against plasmacytosis in mink is strictly dependent on a satisfactory method for making the diagnosis as early as possible after the infection. Since the beginning of the 1950'ies Mallén's test (I.A.T.) (*Mallén et al.* 1950) has been applied leading to a decrease in the frequency of the disease. This method is, however, unspecific the principle being that any increase in the immunoglobulin concentration causes a precipitation, if iodine solution is added to the serum of the test animal. Since the immunoglobulin concentration is increased in plasmacytosis a precipitation reaction will occur. Two facts are, however, essential in this connection. The first one being that it may be impossible to point out sera having only a slight increase in immunoglobulin. The second one being that increase in the antibody content fol-

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lows several diseases other than plasmacytosis. Possibilities therefore exist for both false negative and false positive reactions. The introduction by *Cho & Ingram* (1972 a, b) of the counterimmunoelectrophoretic test (CIEP) for making the diagnosis using the specific virus antigen as reacting component together with the mink serum opened up new possibilities to combat plasmacytosis, as both the specificity and the sensitivity are improved compared with the Mallén's test.

This report deals with the practical experiences obtained during 62 antigen preparations according to the method described by *Cho & Ingram* (1974) but with some modifications. Furthermore an ultrafiltration technique is outlined. This technique may be useful in case ultracentrifugation procedures are to be avoided.

MATERIALS AND METHODS

Infection of animals

For the antigen production 237 minks of different types have been infected. Before inoculation the animals were examined and found to be clinically healthy and to give a negative reaction for plasmacytosis by the CIEP method. The organ material, liver, spleen, and mesenteric lymph nodes to be used for infection were removed aseptically from animals suffering from plasmacytosis. After addition of equal parts of phosphate buffered saline (PBS) (w/w) (*Herbert* 1973) the mixture was ground in an Ultra-turrax homogenizer $2 \times 2\frac{1}{2}$ min., followed by centrifugation at $10{,}500 \times g$/2 hrs. The supernatant was stored at —25°C until use. The animals were inoculated subcutaneously with varying doses ranging from 3.5 to 10 ml of the infective suspension. At the same time Endoxan-Asta®, 30 mg/kg, was injected as immunosuppressive agent in order to inhibit the production of antibodies being able to combine with the virus particles. This combination is supposed to decrease the value of the antigen preparation. The immunosuppressive drug was given at intervals corresponding to 5 mg/kg/day, until the animals were killed 9—10 days after infection, at which time the virus content according to *Larsen* (1969) is at its maximum.

Preparation of virus antigen

Spleen, liver and mesenteric lymph nodes are removed aseptically and stored at —25°C until use. After thawing, equal parts of PBS (w/w) are added, and the mixture homogenized 90 sec.

using the Ultra-turrax equipment. The homogenate is frozen and thawed 5 × at —25°C followed by addition of 1 part of fluorocarbon (1,1,2-Trichlortrifluoräthan Uvasol® Merck) per 2 parts of homogenate. This mixture is homogenized for 2×2½ min. with Ultra-turrax, and the mixture is centrifuged at 7000×g/1 hr. The tissue phase obtained at the centrifugation is extracted once more after addition of equal parts of PBS, and the supernatant added to the supernatant obtained at the first centrifugation. The pooled supernatants are concentrated by centrifugation at 105,000×g/2 hrs. To the pellet obtained after centrifugation of 9 ml is added 1 ml PBS, and the pellet is suspended by means of a Rotamixer. In the method described by *Cho & Ingram* (1974) 3 possibilities are mentioned for the activation: treatment of the material with a glycine-HCl buffer at pH 2.4—3.0, heating at 65—80°C up to 2 hrs. and repeated fluorocarbon treatment. All methods have been tried and the glycine-HCl buffer treatment found to be the most satisfactory. At this procedure 2 vol. of glycine-HCl buffer (0.2 M glycine 100 ml, 0.2 M-HCl 22.8 ml and distilled water ad 200 ml) are added to 1 vol. of suspension. The mixture is treated until homogeneity with a Potter S homogenizer and left for 30 min. before centrifugation at 136,000×g/1 hr. The supernatant is removed very carefully, the pellet resuspended with the Potter S equipment, and the centrifugation is repeated. Supernatant is removed once more, still very carefully, and 1 ml PBS added per pellet. The final product is obtained after Potter S homogenization followed by centrifugation at 400×g/10 min. The supernatant from this centrifugation represents the antigen preparation. Sodium azide 0.01 % is added to the PBS as a bactericide. The final product is tested in CIEP against positive and negative control sera. In case the preparation is inactive, the glycine-HCl treatment is repeated once or twice. If then still unreactive, the preparation will be discarded.

Ultrafiltration

Ultrafiltrations are to some degree laborious and expensive, and not all laboratories are in possession of this equipment. It is therefore tempting to provide a technique, which may replace the ultracentrifugation. For this purpose Amicon ultrafiltration equipment is applied using filter XM 300 and XM 100 with a nitrogen pressure 1.0—1.5 kg/cm² over night at 4°C. The concentrations and the washing may be carried out in the same cell.

Counterimmunoelectrophoresis

The antigen-antibody reaction is carried out in 1 % agarose (Litex) using barbital buffer pH 8.6, μ 0.02 with addition of 0.01 % NaN_3. This buffer system has recently been replaced by the Gelman high resolution buffer pH 8.8, μ 0.05 in the electrophoretic vessels and 0.025 in the gel. The diameter of the reservoir is 2.5 mm, the distance between reservoirs 5.0 mm, and the time for electrophoresis 30 min. at 3 v/cm. The precipitates are read after electrophoresis and again after the slides have been washed, dried, and stained with Coomassie Brilliant Blue R 250.

Electron microscopy

The final antigen preparation has been examined electron microscopically by B. Bloch at this department. The antigen preparation was concentrated through ultracentrifugation. One ml of antigen plus 8 ml distilled water were centrifuged at 136,000× g/1 hr. The supernatant was removed, the pellet resuspended in 9 ml distilled water and the centrifugation repeated. The water was removed and the pellet resuspended in 1 ml of distilled water, and the carbon coated grid was floated on the top of this solution. After 10 min. of absorption the material was stained in 1.3 % phosphotungstic acid at neutral pH and examined in a JEM 100B electron microscope.

RESULTS

The results obtained from the 62 antigen preparations are presented in Table 1 and Table 2 representing preparations based upon organs from 28 individual animals and from 34 pools of organs, respectively. The total of 7 negative preparations which

Table 1. Antigen preparation from organs deriving from 28 individual animals.

Number of animals	Glycine-HCl buffer treatment		
	1st	2nd	3rd
5	neg.	not done	not done
10	pos.		
13	neg*	weakly pos.	pos.

* The negative preparations were combined to 1 pool after which the second buffer treatment was carried out.

Table 2. Antigen preparation from 34 pools of organs. Each pool consists of organs from 3 to 6 animals.

Number of pools	Glycine-HCl buffer treatment		
	1st	2nd	3rd
2	neg.	not done	not done
1	weakly pos.	pos.	
19	pos.		
11	neg.	pos.	
1	neg.	neg.	pos.

have not been treated further, originate from the very beginning of the studies on the antigen preparation. Nothing is known of their reaction capability after repeated glycine-HCl buffer treatment. It appears from the tables that apart from the above 7 preparations, virus antigen was detected in all preparations. Thirty preparations reacted positively after the first buffer treatment, and only 1 preparation needed 2 buffer treatments to become positive. In 2 cases, it was possible to improve the antigen by repeated buffer treatment. The antigen preparations are normally used undiluted for the routine testing. Positively reacting antigen preparations may still be found after dilution 1:16. In a few cases a double precipitate has been registered. This may be a precipitation of 2 antigens Ag-1 and Ag-2 recently described by *Notani et al.* (1976).

The initial experiments where the ultracentrifugation procedures have been replaced by ultrafiltration technique showed that an antigen has been isolated in 7 out of 13 ultrafiltration experiments. Positively reacting preparations have been obtained with both types of filters. The 13 separations were made from 11 organ pools previously all found to contain antigen by the conventional isolation technique.

At electron microscopy 3 different particles were recognized as indicated in Fig. 1, a, b and c. Fig. 1a represents an icosahedral virus particle of about 23 nm, Fig. 1b an "empty" particle the same size and Fig. 1c a ring-like structure of about 11 nm supposed to be ferritin molecules of mink origin.

DISCUSSION

Introduction of the CIEP test based upon the ability to isolate the specific antigen as originally done by *Cho & Ingram* (1972a).

will most probably turn out to be a major advance in the plasmacytosis research. The most critical and expensive point is preparation of the virus antigen. From several laboratories it is known that not even isolation of the antigen, but also the stability of the ready-made antigen is a very delicate matter (Personal communication 1975, 1976). The reactivity of the antigen may be very short, even few hours. We have had the same experience particularly during work-up of the isolation. This is in contradiction to *Cho & Ingram* (1974) who found that over a 180 day-period the antigen preparation kept the titre both at 5°C and at 22°C. This was observed in a preparation based upon repeated fluorocarbon extractions and not using the glycine-HCl activation as used in this report. Whether this difference is essential is unknown. What is really happening at the final so-called activation is also unknown. *Cho & Ingram* (1974) believe that the activation consists of a dissociation of antigen-antibody complexes and thereby specific antibody-binding sites on the virus particles are exposed. If this is true, it must be essential to be extremely careful when separating the sedimented antibody-free virus particles from the antibody-containing supernatant after the final ultracentrifugation. Becoming aware of that the technique was changed, e.g. removal of supernatants was performed very carefully by suction with a fine pasteur pipette instead of decanting. This improved the stability to a satisfactory level. There may, however, be other factors responsible for destruction of the antigen reactivity.

Provided the above theory for the activation is correct it may be possible to separate the antibody-free virus particles from the antibodies by a filtration technique, since plasmacytosis virus is supposed to be a parvovirus (*Cho & Ingram* 1974), mol. wt. above 2×10^6, and the antibodies mostly IgG (*Larsen* 1969), mol. wt. about 0.15×10^6. Accordingly, a filter permitting antibodies to pass will be suitable. This demand is fulfilled by the Amicon filter XM 300 separating around mol. wt. 0.3×10^6. This has in fact been found to be suitable for preparation of the antigen without using the ultracentrifuge. The application of this method is, however, only in the preliminary phase. Most interestingly, in contradiction to the above it has been found that the filter Amicon XM 100 with separation around mol. wt. 0.1×10^6, which means below the molecular weight of the antibody molecules, is also applicable. It may, however, be recalled that it is still unknown what is going on under the activation procedure.

The electron micrograph of the concentrated final preparation corresponds to that obtained by *Cho & Ingram* (1973) by electron microscopy of the antigen-antibody precipitate and also that published recently by *Notani et al.*, obtained through purification on a CsCl gradient. The latter authors believe that the ring structures, corresponding in morphology and size to Fig. 1c, comprise the antigen Ag-1, while *Cho & Ingram* (1974) suggest that they represent ferritin of mink tissue origin. It is impossible on basis of the present investigation to determine whether the ring structure is ferritin or not. It should, however, be mentioned that morphologically the structure is exactly like the ultrastructure of the protein portion of ferritin presented by *Bessis* (1973).

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REFERENCES

Bessis, M.: Living Blood Cells and their Ultrastructure. Springer-Verlag, Berlin, Heidelberg, New York 1973, p. 91.

Cho, H. J. & D. G. Ingram: Antigen and antibody in Aleutian disease in mink. I. Precipitation reaction by agar-gel electrophoresis. J. Immunol. 1972a, *108*, 555—557.

Cho, H. J. & D. G. Ingram: Aleutian disease (AD) of mink: Activation of AD antigen and detection by immunoelectro-osmophoresis. Proc. Canad. Fed. Biol. Soc. 1972b, *15*, abstr. 299.

Cho, H. J. & D. G. Ingram: Isolation, purification and structure of Aleutian disease virus by immunological techniques. Nature New Biol. 1973, *243*, 174—176.

Cho, H. J. & D. G. Ingram: The antigen and virus of Aleutian disease in mink. J. immunol. Meth. 1974, *4*, 217—228.

Herbert, W. J.: Passive haemagglutination with special reference to the tanned cell technique. *In* D. M. Weir, ed.: Handbook of Experimental Immunology. Vol. I. Blackwell Sci. Publ., Oxford, London, Edinburgh, Melbourne 1973, p. 20.6.

Larsen, A.: Immunological and viral studies of Aleutian disease in mink. Thesis, Univ. of Utah 1969.

Mallén, M. S., E. L. Ugalde, M. R. Balcazar, J. I. Bolivar & S. Meyran: Precipitation of abnormal serums by Lugol's solution. Amer. J. clin. Path. 1950, *20*, 39—47.

Notani, G. W., E. C. Hahn, N. H. Sarkar & A. J. Kenyon: Characterisation of Aleutian disease antigens. Nature (Lond.) 1976, *261*, 56—58.

SAMMENDRAG

Fremstilling af antigen til brug ved undersøgelse for plasmacytose hos mink ved hjælp af modstrømselektroforese.

Malléns test har i en årrække været anvendt til testning af minkserum med henblik på forekomst af plasmacytose. Denne metode savner imidlertid specificitet og er ikke tilstrækkeligt følsom. *Cho & Ingram* beskrev i 1972 (a, b) en precipitationstest, som muliggør en forbedret diagnostik af plasmacytose, idet metoden er specifik og mere følsom end Malléns test. Fremstilling af det nødvendige, specifikke virusantigen til testen kan imidlertid være vanskelig. I foreliggende arbejde er beskrevet de modifikationer af originalmetoden, som er indført, herunder en ultrafiltreringsteknik, samt de praktiske erfaringer, der er opnået ved fremstilling af 62 antigenpræparater. Ved elektronmikroskopi af det færdige antigenpræparat er fundet ikosaederformede, ca. 23 nm store viruspartikler, „tomme" viruspartikler og ringformede ca. 11 nm store partikler, der formodes at være ferritinmolekyler.

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