Localization of Transplantation Antigens in Tissue Sections: Effects of Various Fixatives and Use of Tissue Preparations other than Frozen Sections

Research in transplantation biology can benefit of qualitative information on the precise histological distribution of transplantation antigens in solid tissues. For this type of study, immunofluorescence appears as one of the most efficient technique. However, preservation of tissue antigenicity can impose limitation on the choice of methods used to prepare satisfactory sections for the immunofluorescent test. Detection of transplantation or histocompatibility antigens in unfixed cryostat sections of normal mouse tissues has been reported using an indirect fluorescent antibody technique[1]. The use of unfixed frozen sections in immunofluorescence has major disadvantages. Tissue morphology is frequently distorted in thin unfixed cryostat sections. Furthermore, some loss or displacement of antigenic material can result from the thawing of the section on the slide or the incubation in aqueous media. To see whether it was possible to avoid the latter problem by fixation, the effects of various fixatives currently used in immunofluorescence were investigated. In addition, because the morphology is much better preserved in sections cut from tissue blocks embedded in paraffin after cold ethanol fixation, freeze-substitution or freeze-drying, than in cryostat frozen sections, tissues prepared by these methods were also used. The biological system, reagents and the immunofluorescent technique employed in this work have been described in detail[1].

1. Effects of fixatives. A series of air-dried cryostat sections were fixed respectively in 70% alcohol, absolute ethanol, methanol, 2-octanol and in 5,10 and 40% formaldehyde at room temperature for 2 h. The slides were then dried at 37°C for 15 min and rinsed in phosphate buffered saline before being used in immunofluorescent tests. In all cases, these fixation procedures resulted in the loss of specific fluorescence. Although the staining was preserved after fixation in acetone for 30 min at room temperature, the improvement was slight, with the exception of nervous tissues in which the fluorescent pattern was better defined[2].

Because the choice of fixatives in immunofluorescence is still empirical, other fixing agents used in general histochemistry were tried. Susa's, Zenker's, Carnoy's and Bouin's solutions were all found to have deleterious effects on histocompatibility antigens.

2. Use of tissue preparations other than cryostat sections. A) Cold ethanol fixation and paraffin embedding. SAINTE-MARIE[3] has described a method of preparing tissues for immunofluorescence in which small tissue blocks are prefixed in ethanol at 4°C, dehydrated in pre-cooled alcohol, cleared in xylene and finally embedded in paraffin at 56°C. 5 blocks of tissues (spleen, liver, kidney, cerebral cortex and sub-maxillary gland) were processed according to this method. It was found that specific staining could no longer be detected. The use of cold methanol instead of ethanol to prefix and dehydrate the tissue blocks gave similar results.

B) Freeze-substitution. This method[4] has been applied to immunofluorescent studies[5] and found to provide sections of better morphology than cryostat preparations. The aim of this technique is to preserve antigenic material in its normal position by fixation, and to minimize the cellular distortion produced by ice crystals. For this purpose, small tissue blocks are snap-frozen at low temperature (−70°C) and dehydrated by successive changes of precooled (−70°C) ethanol over a period of 3 to 4 days. Tissue blocks are then passed over several hours through successive baths of alcohol at increasing temperatures and embedded in an alcohol-soluble wax.

Five blocks of tissues (spleen, liver, kidney, cerebral cortex and sub-maxillary gland) were prepared by this method[5] with the exception that paraffin at 56°C was used for embedding instead of polyester wax at 37°C, and this necessitated previous clearing in xylene. The sections were washed in phosphate buffered saline. The loss of specific staining in sections from these blocks showed that histocompatibility antigens were adversely affected by the procedures involved.

C) Freeze-drying. This method provides tissue sections in which the morphology is especially well preserved and is therefore very suitable for the study of cytological details. Furthermore, no fixation is required and the displacement or destruction of antigenic material should theoretically be avoided by this procedure. After snap-freezing of small tissue blocks, at a temperature of approximately −160°C to avoid the formation of ice crystals, the frozen water is removed by sublimation in vacuo at −40°C. The dried blocks are then directly embedded in polyester wax or in paraffin. 5 very small blocks of tissues (using the same organs as above) were snap-frozen in a liquid nitrogen-isopentane mixture and dried in vacuo at low temperature using a 'Pearse tissue freeze-drier' apparatus. After complete drying, the blocks were vacuum-embedded in paraffin at 56°C. The sections cut from these blocks showed bright blue-green autofluorescence to such an extent that specific staining could no longer be distinguished with certainty.

In the last 3 methods, petroleum, ether and chloroform were used in addition to xylene for the deparaffinization of sections but no difference was noted in the results.

Discussion. The present results show that histocompatibility antigens are destroyed in cryostat sections treated with various fixatives. Because of the limited knowledge on the chemical nature of histocompatibility antigens, the mode of action of these fixatives in the present work is not understood. In SAINTE-MARIE's technique, the deleterious effect of alcohol fixatives found with frozen sections is likely to be responsible for the inactivation of histocompatibility antigens. However, these antigens could also have been denatured by heat since the tissue blocks were embedded in paraffin at 56°C.

In the freeze-substitution method, the denaturation of antigens probably occurred late in the procedure (i.e. when the tissue blocks were immersed in alcohol at increasing temperatures) because fixation by ethanol or methanol at low temperature (−70°C) is negligible. As with the previous technique, denaturation by heat may have taken place during embedding. However, it remains possible that denaturation of transplantation antigens by heat resulting from embedding at 56°C might be avoided by using an embedding medium of a lower melting point. Investigations along these lines are in progress.

The failure to obtain satisfactory preparations with the freeze-drying method may be due to excessive drying; the optimal degree of drying can only be found empirically and it has not been possible to investigate this during the work reported here. A considerable increase of auto-

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fluorescence in freeze-dried material has been reported[6] but no explanation has been proposed for this effect.

Our results indicate that in spite of the disadvantages of unfixed cryostat sections, these appear as the only suitable preparations for the localization of transplantation antigens in solid tissues by means of immunofluorescence.

Résumé. Plusieurs fixateurs produisent des effets délétères sur les antigènes de transplantation de la souris. La fixation à froid, la congélation-substitution et la cryodessication ne donnent pas de résultats satisfaisants.

A. G. GERVAIS[7,8]

Département d'Anatomie, Université de Montréal, Case postale 6128, Montréal (Canada), 1 September 1971.

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[7] The author is greatly indebted to Professor H. von MAYERSBACH for his help and advise throughout this investigation.

[8] This work was performed during tenure of a Research Fellowship from the Medical Research Council of Canada.

Effect of Blood Sampling Methodology on Plasma Levels of Corticosterone, Inorganic Phosphorus and Serum 5-Hydroxytryptamine Concentrations[1]

A wide variety of experimental factors including time of day[2,3] sex[2], environmental change[4-6] and level of anesthesia[7] have been reported to influence plasma corticosterone concentrations. In addition, it has been suggested that the methodology used in obtaining blood samples may also affect plasma levels of corticosterone[8]. Therefore, the present experiments were undertaken to explore the possible influence of blood sampling methodology on serum 5-hydroxytryptamine (serotonin) and plasma inorganic phosphorus as well as plasma corticosterone concentrations.

Materials and methods. Animals used in the present study were female Sprague-Dawley rats (Charles-River) that were housed 2 per cage for at least 3 weeks under conditions of controlled lighting (fluorescent illumination from 0400–1800) and temperature (24 ± 2°C). Purina laboratory chow and tap water were available ad libitum. 3 days prior to the experiment rats were transferred to individual cages.

In all experiments rats were taken individually from the animal quarters to the adjoining preparation room were they were subjected to one of the following methods for obtaining blood samples: Decapitation (DC), rats were rapidly decapitated (< 20 sec following initial handling) and 2 ml of trunk blood collected in a centrifuge tube to which 0.25 ml ascorbic acid had been added; the remaining trunk blood (3 ml) was collected in a heparinized centrifuge tube. Cardiac tap (CT), each rat was rapidly weighed and injected with sodium pentobarbital (35 mg/kg, i.p.). Exactly 10 min following pentobarbital injection, 5.0 to 6.0 ml of blood was withdrawn within 1 min from the heart into a 10 ml saline-rinsed syringe (1 inch 21 gauge needle). 2 ml of heart blood was then injected into a centrifuge tube to which 0.25 ml of ascorbic acid had been added; the remaining blood was deposited in a heparinized centrifuge tube. Jugular vein tap (JV), rats were rapidly anesthetized with ether, the external jugular vein exposed and 2 ml of blood collected in a saline-rinsed syringe (1 inch 21 gauge needle) following which 3.5 to 5.0 ml of blood was collected in a separate heparinized syringe; both samples were collected within 3 min following time of cage opening. In all cases, non-heparinized blood was mixed gently with 0.25 ml of ascorbic acid, centrifuged following clot formation and serum collected for serotonin determinations. Heparinized blood was centrifuged immediately and plasma collected for corticosterone and inorganic phosphorus determinations. Sampling periods, which began at 08.00 or 16.30 h of the same day, were approximately 90 min in duration.

Plasma levels of corticosterone and inorganic phosphorus were determined by the fluorometric method of GUILLEMIN et al.[9] and colorimetric method of FISKE and SUBBAROW[10] respectively. The fluorometric method of WEISSBACH et al.[11] was used to determine serum levels of serotonin. The amount of hemolysis was subjectively evaluated and correlated with the method of sampling blood by using an arbitrary scale of 0 = no hemolysis, + = slight hemolysis, ++ = considerable hemolysis and +++ = near maximal hemolysis. Statistical probabilities were derived from analysis of variance or Student's *t*-test.

Results. Analysis of variance revealed a significant difference between the corticosterone values of the 3 groups during morning but not afternoon experiments (Figure 1). Individual comparisons showed AM corticosterone values of the CT group to be higher ($P<0.05$) than those of DC or JV groups; DC and JV groups were not different. AM-PM differences ($P<0.01$) in plasma corticosterone were observed in DC and JV but not CT animals.

As summarized in Figure 2, plasma inorganic phosphorus was highest in DC rats and lowest in CT animals at both time points; concentrations in JV rats were intermediate and different ($P<0.01$) from CT and DC bled rats in the AM but not PM. AM-PM differences in inorganic phosphorus were not observed in any of the experimental groups.

As with the corticosterone and inorganic phosphorus, marked differences in serotonin concentration were observed (Figure 3). Individual comparisons indicated that serotonin values in JV rats were higher at both time points

[1] Supported by Louisiana Heart Association W. C. Feagel Research Award and U.S. Public Health Service grants No. NS08929 and No. AM12389-03.

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