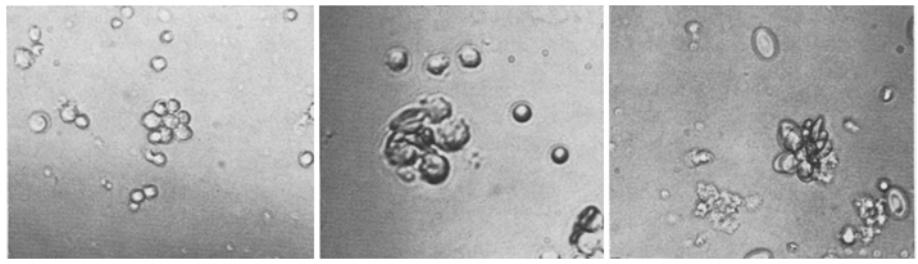

RFC from immunized rabbits with CRC (magnification × 80).

of CRC. The volume was adjusted to 1 ml with TC 199 Medium. The tubes were covered with thin folia and incubated for 24 h at 4 °C. Rosette forming cells (RFC) to which 4 or more erythrocytes were attached were counted in volume samples of 2 mm³. Spherical clumps of erythrocytes which showed a lymphoid cell in their centre after treatment with 2% acetic acid were counted as RFC if there were more than 3 erythrocytes per clump.

Results. Results of prolongation of skinallograft survival using different batches of ALS are listed in Table I. The mean values of 4 VW × A, each with two skinallografts from CH, are plotted. 3 days after immunization practically no RFC could be observed in any cell suspension. From day 14 on a marked increase of rosette formation with spleen cells was demonstrated, which was much less pronounced with lymphnode cells and peripheral blood lymphocytes.

The capacity of ALS to inhibit rosette formation was tested in dilution up to 1:100. Spleen cells from rabbits of the 28th day after reimmunization were used for this purpose. The results are given in Table III.

PLDK-3A caused no rosette inhibition formation, PLDK-3D and PLDK-3C showed a moderate rosette inhibition effect. Marked RFC inhibition was observed with ALS batches PLDK-3B in a dilution up to 1:50 (p 0.025) and ALS PLDK-3E 1:40 (p 0.04).

Discussion. We have confirmed in rabbits that the immunosuppressive activity of ALS determined with prolongation of skinallograft survival correlates well with in vitro rosette inhibition[5]. Thorough documentation of the immunosuppressive effect of ALS is of utmost importance before clinical use. Lymphocytetoxicity and opsonization have correlated poorly with the immunosuppressive potency[5,6], and in addition considerable difficulties may be encountered with the interpretation of skinallograft survival. Therefore the inhibition of rosette formation presents a very valuable additional parameter in the efficacy of ALS. The possibility of rosette formation without previous sensitization has to be borne in mind and must be carefully worked out because it may introduce errors in the interpretation of the results[7].

Zusammenfassung. Fünf Pferde Anti-Kaninchen-Lymphozyten Sera wurden in vivo mit Hautabstossungszeit und in vitro mit Rosettenformation auf immunosuppressive Aktivität am Kaninchen getestet, wobei sich zwei der fünf geprüften Sera als sehr wirksam erwiesen.

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The Primary Tissue Culture of Rat Adult Decapsulated Adrenal Glands: Problems of Methodology and Applications

The cultivation of mammalian adrenocortical cells always met considerable difficulties, due to the poor survival of zona fasciculata and reticularis cells in vitro[1,2], unless of foetal-newborn origin[2,3]. The only method previously reported to preserve in vitro adult zona fasciculata cells was poorly employed for experimental purposes[4].

We recently found that a technique previously used to cultivate human adult liver cells[5,6] works also with the decapsulated adrenocortical tissue of the adult rat[7]. In this paper some relevant methodologic difficulties, findings and applications are briefly reviewed and discussed.

Critical steps in the culture method. In each culture session the adrenals of 10–16 Wistar rats are carefully decapsulated[8] in order to take off their zona glomerulosa[9] and processed as already reported for the human liver[5,6]. A mixture of trypsinized cells and tissue microexplants is eventually implanted onto polythene discs after the method of FULTON[10]. The most critical step is trypsinization, as discussed elsewhere[7]. It should be emphasized here that the adrenal tissue is highly affected by the trauma of chopping: good cell outgrowths are obtained only if fragments of 1–2 mm in size are employed. Smaller explants contain mostly dead cells.

Cultivation after storage of adrenocortical cells in liquid nitrogen. Tissue explants and trypsin dispersed rat adrenocortical cells grew excellently in vitro even after a prolonged storage in liquid nitrogen (–196 °C), a feature never, as far as we are aware, previously reported. 10 stocks, each of 20–32 adrenals, were tested. The glands were stirred in trypsin solution, chopped as usual[7] and then resuspended in 1.0 ml Eagle's MEM[11] added with 20% (v/v) inactivated foetal bovine serum and 15% (v/v) glycerol. The suspension was transferred into a 2 ml

pyrex glass ampoule, whose neck was sealed by flame. All the ampoules were first stored for 10 min at 4°C and afterwards put into a RCB12 liquid nitrogen container (SIO, Padua) endowed with a Biogel cap. The freezing rate was first –0.7°C/min until the ampoules arrived at –25°C, then it was hightened to –4.9°C/min until the temperature of –70°C was reached. At this point the ampoules were dipped into liquid nitrogen. 6 months later they were recovered, and soon thawed in a 37°C water bath. Adrenal cells and explants were washed free of glycerol and implanted onto polythene discs. The success rate of these cultures was 8/10. It may be advisable to establish whether even the human adrenocortical tissue might survive the prolonged storage in liquid nitrogen. If this were the case, human adrenals could be stored at –196°C as soon as they are obtained and recovered for cultivation only when devised.

Cytomorphology. Examination by light and electron microscopy of the cultures showed that at least 8 different cell types grow in vitro[7], among which are dedifferentiated adrenocortical cells. Details on the basic morphology of the cells in culture have been given elsewhere[7]. The daily addition of β_{1-24}-ACTH (10 µg/ml) to the growth medium has been shown to induce, within 3 days, a full differentiation of the cortical elements into zona fasciculata cells like those found in the in vivo gland. A morphometric examination of this process has been carried out[7].

Histochemistry. Histochemical methods of any kind may be applied to rat adult adrenal cultures, provided that they do not destroy the clotted mouse plasma which allows the cells to stick to the polythene discs. Preliminary work has shown that in adrenocortical cells: a) glycogen, stained by the PA/S method[12] is scanty, as it is in the in vivo gland[13] and in rat liver cells cultured by a similar technique[14]; b) diastase-resistant, PA/S-positive material is also poor, as it is in vivo[13]; c) neutral fat droplets, evidenced by Sudan III and IV staining, increase their number in the cytoplasm after treatment with β_{1-24}-ACTH or 3′, 5′-cyclic adenosinemonophosphate (c-AMP); d) the cytoplasmic amount of RNA, as revealed under UV-light after staining with acridine orange[15] augments also after exposure to β_{1-24}-ACTH or c-AMP; e) acid phosphatase activity, revealed by RUTENBERG's method[16] is rather weak, as in vivo[17], whereas it is very intense in adrenal macrophages and giant cells.

Radioautography. The dip-coating method of LEBLOND and KOPRIWA[18] is easily applied to adrenal cultures. It has thus been possible for the first time to investigate the short-term and long-term direct metabolic effects of β_{1-24}-ACTH[19] and c-AMP[20] on in vitro rat adult adrenal tissue.

Biochemistry. Albeit not yet attempted, biochemical methods should also be of use in studies with adrenal cultures, due to the great number of separates (up to 48) which can be set up in a single culture session.

Attempts to develop cloned differentiated adrenal cell lines. Adult adrenocortical cells of rat have been found to survive and grow in vitro for as long as 40–60 days, maintaining their initial morphological features and the monolayer arrangement. Subcultures on glass of the primary cells were therefore attempted. Negative results were obtained if too young cultured cells (up to 15 days) in vitro were detached with an EDTA-(1:5000) solution[21] and then seeded into 50 ml prescription bottles. Older cultures gave rise to rapidly growing fibroblast-like cell lines, in which also macrophages are present. The same also happened if the adrenal subcultures were treated with daily doses of 10 µg/ml β_{1-24}-ACTH. In contrast, the prolonged exposure of subcultures to 50 µg/ml c-AMP 3 times per week brought about cell strains that even after 100 days in vitro contain islets of 'cortical'-like cells, as detected by light microscopy, intermingled with mesenchymal elements. Attempts to clone such 'cortical' cells are now under course with the hope of obtaining a pure line of differentiated rat adrenocortical cells.

Riassunto. Le colture primarie di surrene di Ratto adulto si prestano molto bene a studi morfologici e funzionali mediante tecniche di indagine diverse (microscopia ottica ed elettronica, istochimica, autoradiografia, biochimica). Sono stati effettuati studi originali sulla coltura di tessuto surrenale dopo permanenza prolungata in azoto liquido e sull'isolamento di linee cellulari differenziate di cellule corticali.

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[22] Acknowledgment. Authors thank Professor V. MENEGHELLI, Director of this Institute, for his encouragement and interest in this work. Miss LUCIA BOLOGNANI and Dr. ANNA BELLONI provided skilful technical assistance.