Evaluation of Antilymphocyteserum: Comparison of Skinallograft Survival and Rosette Inhibition

Prolongation of skinallograft survival is still considered to be the most reliable parameter to evaluate the therapeutic efficacy of ALS. Recently data have been presented which indicate that the inhibition of rosette formation may yield comparable information. It is the purpose of this paper to compare these two parameters in 5 batches of ALS.

Materials and methods. Rabbits of a cross between Vienna White and Alaska (VW × A) and Chinchillas (CH) 2.5–3 kg of both sexes were used throughout the experiment.

a) *Production of antilymphocyteserum (ALS).* Thoracic duct lymphocytes were gained from rabbits according to the method described by SANDERS et al.[1]. 10^9 lymphocytes were injected to a horse subcutaneously on 3 subsequent days and 10 days later ALS batch PLDK-3A was produced. 3 months later, 10^9 lymphocytes, in 5 ml Hanks B.S.S. solution, were injected with 5 ml incomplete Freunds adjuvans (DIFCO). Horse serum was tapped 10 days later (batch PLDK-3B). In further intervals of approximately 1 month, 10^9 lymphocytes gained by the same technique from rabbits, were injected and ALS batches PLDK-3C and 3E were produced.

b) *Testing of ALS with skinallograft survival.* Skingrafting was performed aseptically under general anesthesia. Donor and recipient were shaved and the skin desinfected with Hibitane 2% in 70% alcohol. Essentially the method of BALNER[2] was adopted. 2 skin pieces of 2 cm diameter from Chinchilla (CH) rabbits were placed on the backs of VW × A and fixed with 8–10 silk sutures. The grafts were covered with Carbonet (Smith and Nephews, England) and a few drops of aqueous pencillin were added. The Carbonet was fixed with scotch tape and the area padded with several layers of sterile gauze. The skingrafts were inspected on day 7 and every other day thereafter. The most important criteria of the graft survival were: thinness, pliability and blanching on pressure. The degree of skinallograft rejection was quantitatively expressed in points: 0 indicating no rejection at all, 1 minimal-, 2 moderate-, 3 marked-, 4 complete rejection. 2 days before skingrafting and during the whole experiment ALS was administered 3 times weekly in a dose of 2 ml/kg/day s.c. to groups of 4 rabbits.

A control group was given normal horse serum (NHS) (Table I).

c) *Testing of ALS with rosette inhibition.* Rabbits were immunized with a single i.v. injection of 2.0 ml chicken red cells (CRC) which were washed 3 times in Hanks buffered salt solution (BSS). 7 days later reimmunization was performed 3 more times; 1.0 ml washed CRC were administered every other day i.c. with 0.5 ml complete Freunds adjuvans (DIFCO) on multiple locations on the back. 28 days after completion of the immunization with CRC, the inhibiting effect on rosette formation by the 5 batches of ALS was tested with lymphocytes of these rabbits on day 3, 7, 14, 28 and 56. ALS was diluted from 1:10 to 1:100 and it was added in amounts of 0.1 ml per test. The rosette formation test was done according to the method of ZAALBERG, VAN DER MEUL and VAN TWISK[3] with minimal modifications. Spleen and lymphnodes were removed aseptically, cut into small pieces, passed through sterile nylon tissue and suspended in TC 199 Medium. The suspensions were washed twice with the same medium. Blood samples were obtained by heartpuncture and lymphocytesuspensions were prepared as described elsewhere[4]. 1.5×10^6 lymphoid cells were mixed with 0.1 ml of a 1% solution

Table I. Mean skinallograft rejection of 4 rabbits during treatment with normal horse serum and with different batches of ALS (PLDK-3A to -SE)

Serum batch	day 8	day 10	day 12	day 14	day 17
NHS	2.75	3.75	4.0	–	–
PLDK-3-A	3.25	4.00	–	–	–
PLDK-3-B	2.00	2.25	3.00	3.25	3.50
PLDK-3-C	1.50	2.50	2.75	3.75	–
PLDK-3-D	2.00	2.60	3.00	4.00	–
PLDK-3-E	1.50	2.00	2.00	2.50	3.75

Note that rejection is most markedly delayed by batches 3B and 3E. PLDK-3A is not active at all. Rosette inhibition: unimmunized rabbits showed very low RFC with CRC.

Table II. Mean results of RFC from 5 animals immunized with CRC on different days after sensitation

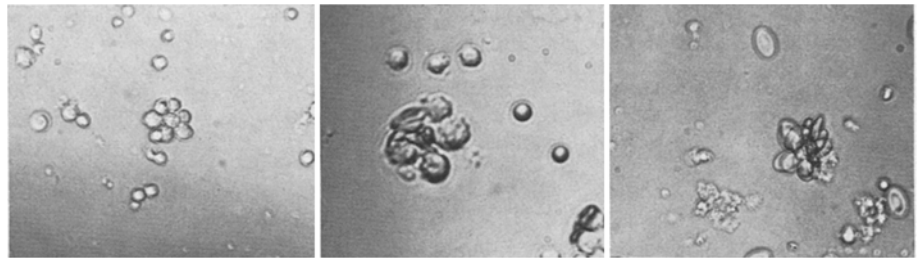
Day – after reimmunization with CRC	RFC per 1 mm³ suspension		
	spleen cells	lymph node cells	peripheral lymphocytes
3	20	–	–
7	190	100	100
14	608	274	150
28	704	194	307
56	695	284	201

Table III.

Dilution ALS	NHS	PLDK-3-A	PLDK-3-B	PLDK-3-C	PLDK-3-D	PLDK-3-E
1:0	575	527	–	225	82	–
1:10	695	607	–	284	299	72
1:20	633	600	102	489	307	77
1:30	621	582	297	407	522	370
1:40	554	594	284	594	627	398
1:50	597	622	305	584	632	429
1:60	531	575	384	575	784	500
1:70	590	654	507	579	622	629
1:80	627	702	680	622	622	650
1:90	630	690	680	590	607	607

Rosettes per mm³

[1] A. G. SANDERS, H. W. FLOREY and J. M. BARNES, J. exp. Med. *45*, 254 (1940).
[2] H. BALNER, Transplantation *8*, 206 (1969).
[3] O. B. ZAALBERG, V. A. VAN DER MEUL and J. M. VAN TWISK, Nature, Lond. *210*, 544 (1964).
[4] M. KISSLING and B. SPECK, Vox Sanguinis *21*, 261 (1971).



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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J. A. Cohen Instituut voor Radiopathologie en Stralenbescherming, Academisch Ziekenuis, Leiden (Nederland), 5 July 1972.

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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