Effect of Phytohaemagglutinin and Concanavalin A on Human Rosette-Forming Cells

The spontaneous human rosette formation is attributed mainly to T cells[1,2]. After the statements of different authors, 4–81% of the blood lymphocytes are rosette-forming cells (RFC). It has been supposed that RFC represent a special subpopulation of T cells[3]. We have found that the majority if not all T lymphocytes are able to bind sheep red blood cell (SRBC) antigens, but only a distinct proportion of the cells bind the SRBC tightly enough to be detected by conventional rosette techniques[4]. Here we present evidence that mitogens stimulate the rosette formation of human lymphocytes.

Material and methods. Peripheral lymphocytes were obtained from normal adults and patients with autoimmune diseases. The cells of heparinized blood were separated on Ficoll-Triosil gradient[5]. To detect the minimal time required for stimulation of rosette formation, the lymphocytes (10^6 cells/ml) were incubated with 12.5 µg/ml Con A (Calbiochem) or 25 µg/ml PHA (Difco) at 37 °C for various periods. After incubation, the lymphocytes were carefully washed with Parker medium 199 to eliminate unbound mitogens. First we used various concentrations of Con A and PHA to determine the optimal concentrations. In the second part of experiments, constant concentrations (12.5 µg/ml Con A and 25 µg/ml PHA) were used. Rosette formation was carried out as follows: 10^6 washed lymphocytes were suspended in 0.2 ml medium supplemented with 10% calf serum (inactivated and absorbed against SRBC) and mixed with 2×10^7 SRBC in equal volume. The samples were centrifuged for 10 min at 200 *g* at room temperature. They were then left at 37 °C for 15 min. The cells were thoroughly resuspended with repeated suctions. 1000 cells were counted to evaluate the percentage of rosettes. The rosettes were defined as a lymphocyte surrounded by at least 3 SRBC.

Long-term stimulation was made in sterile conditions. In plastic tubes in Parker medium 199 (containing 10% calf serum, 100 µg/ml penicillin and 50 µg/ml streptomycin) 10^6 lymphocytes were cultured. The samples were incubated for 24 and 48 h at 37 °C with 95% air and 5% CO_2. The rosette formation was performed as described above. Lymphocyte blast transformation was carried out under similar conditions. ^{3}H-thymidine was added 16 h before harvesting.

Results and discussion. The minimal time required for stimulation of rosette formation was found to be 15–30 min. No significant increase of RFC was observed after 30 min of stimulation. A rather narrow range of concentration of Con A (5–25 µg/ml) caused maximal stimulation. The stimulatory effect of PHA was more pronounced at higher concentrations; however, agglutination of lymphocytes occured more frequently. Agglutination of cells makes the exact estimation of RFC difficult. With 25 µg/ml PHA no significant agglutination occurred and the stimulation was nearly maximal.

The number of RFC varied among normal individuals. These findings are consistent with data in the literature. All the cases studied showed a significant increase after a 30 min mitogen stimulation (Table I). Further incubation did not raise the percentage of RFC significantly. PHA caused a more striking increase in the number of RFC than did Con A, in the majority of cases. In some cases, the percentage of RFC was near to 100%. The stimulation of the lymphocytes to rosette formation ran parallel with the stimulation to blast transformation. PHA caused a greater ^{3}H-thymidine uptake than Con A, and this difference was observed by rosette stimulation too. The results obtained with lymphocytes of autoimmune patients are interesting. There were cases with no apparent blast transformation capacity, and no stimulation of rosette formation (Table II). These findings are consistent with the low values of RFC stimulation obtained in Hodgkin's disease and in chronic lymphocytic leukaemia (in preparation).

The RFC stimulatory effect of different mitogens may be a consequence of several events. Lymphocytes having only a few receptors cannot bind the SRBC tightly enough to be detectable by conventional rosette techniques. PHA and Con A binding to the lymphocyte membranes can alter their surfaces[6] and facilitate the production of uropods[7]. The increased number of RFC after mitogen exposure may be due to the facilitation of loose rosette formation. We suppose that the majority if not all of T cells having SRBC receptors can be stimulated by PHA or Con A. We cannot exclude the possibility that bound mitogens attract red blood cells, and this force is added perhaps to that of the special SRBC receptors promoting rosette formation.

Table I. 30 min, 24 h and 48 h stimulation of RFC (Mean values ± SDM of 40 control cases)

Stimulation of RFC	30 min	24 h	48 h
No mitogen	31.3[a] ± 28.5	28.2 ± 30.0	27.5 ± 22.9
25 µg/ml PHA	66.2 ± 11.7	68.5 ± 9.5	63.9 ± 17.3
12.5 µg/ml Con A	45.7 ± 21.5	50.7 ± 16.8	49.8 ± 11.4

[a] Percent.

Table II. Response of lymphocytes of autoimmune patients to PHA and Con A

Patients	^{3}H-thymidine uptake[a]			30 min RFC stimulation[b]		
	Control	Con A	PHA	Control	Con A	PHA
1	314	455	633	45	45	50
2	218	545	3538	60	72	90
3	127	5452	15629	20	53	41
4	141	6631	4971	18	70	32
5	632	ND[c]	681	12	25	15
6	415	ND	524	26	20	26
7	350	ND	14380	46	45	95
8	279	ND	2764	15	50	60
9	330	ND	1940	25	25	85
10	180	ND	1390	28	40	70

[a] cpm. [b] Percent. [c] Not done.

[1] J. WYBRAN, M. C. CARR and H. H. FUDENBERG, J. clin. Invest. *51*, 2537 (1972).
[2] S. S. FRÖLAND, Scand. J. Immun. *1*, 269 (1972).
[3] R. L. DAWKINS and P. J. ZILKO, Lancet *1*, 368 (1973).
[4] P. GERGELY, G. SZEGEDI, B. FEKETE, G. SZABO and G. PETRÁNY Lancet, *1*, 883 (1973).
[5] R. HARRIS and E. O. UKAEIJOFO, Lancet, *2*, 327 (1969).
[6] R. E. SCOTT and V. T. MARCHESI, Cell. Immun. *3*, 301 (1972).
[7] P. BIBERFELD, Expl Cell. Res. *66*, 433 (1971).

The parallelism observed between RFC stimulation and lymphoblast transformation suggest that the rapid and simple RFC test could substitute the more laborious investigation of the mitogen-induced lymphoblast transformation for some purposes.

Zusammenfassung. Nachweis, dass Phytohaemagglutinin und Concanavalin A die spontane Rosettenbildung der menschlichen Lymphozyten anregt. Die Stimulation der Rosettenbildung und Lymphoblast-Transformation ergibt parallele Ergebnisse.

P. Gergely, G. Szabó, B. Fekete, Gy. Szegedi and Gy. Petrányi

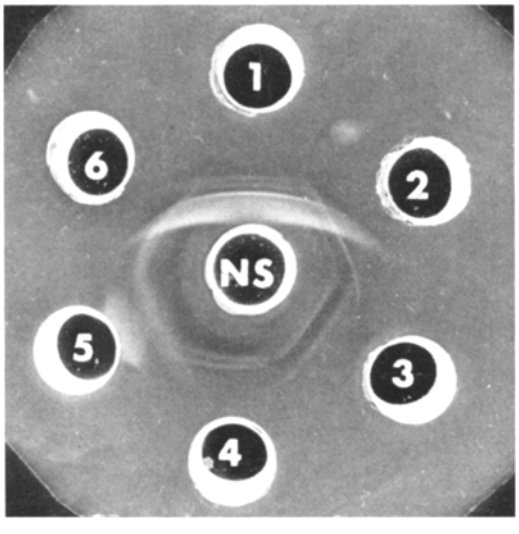
Department of Medicine, University of Debrecen, 4012 Debrecen (Hungary), 16 July 1973.

Tissue- and Species-Specific Antigens of the Rat Submandibular Gland

The presence of tissue-specific antigens have been detected in submandibular glands of rabbit[1], cattle[2], and man[3,4]. The object of the present study was to investigate the tissue- and species-specificity of the rat submandibular gland as a part of an effort to evaluate the antigenic constituents of normal salivary glands and their tumors.

Method. The submandibular glands of adult male Long-Evans rats were separated, and homogenized in saline. The homogenate was centrifuged at 10,000 rpm for 10 min and the supernatant was collected. Extracts of other rat tissues as well as submandibular glands of other species were prepared in the same manner. The protein concentration of the saline extracts was determined by the method of Lowry et al.[5]. Antisera were prepared by injecting saline extracts of rat submandibular gland subcutaneously into rabbits. The extract was incorporated into Freund's complete adjuvant for the first injection and into incomplete adjuvant for subsequent injections. Antiserum was absorbed with lyophilized rat serum in a concentration of 80 mg/ml, which was found to be a proper concentration for neutralization. When necessary, further absorption was carried out using lyophilized extracts of other rat tissues.

Two-dimensional immunodiffusion was performed according to the method of Ouchterlony[6]. Immunoelectrophoresis was carried out according to the method of Grabar and Burtin[7] as modified by Scheidegger[8] using 1% agarose in 0.05 *M* barbital buffer at pH 8.6. Cryostat sections of quick frozen rat submandibular gland were fixed in acetone and stained directly with fluorescein isothiocyanate conjugate of antiserum globulin according to the technique of Coons and Kaplan[9].



Immunodiffusion in agar gel. Antiserum to rat submandibular gland extract absorbed with rat serum and kidney extract (NS). Rat glandular extracts: 1. submandibular; 2. parotid; 3. extraorbital lacrimal; 4. intraorbital lacrimal; 5. pancreas; 6. sublingual.

Results and discussion. Immunodiffusion studies of antisera to submandibular gland exhibited several lines of precipitation with extracts of rat submandibular gland and other rat tissues, as well as with rat serum. Following absorption of the antisera with lyophilized rat serum, some precipitation lines with extracts of several rat tissues still remained. Further absorption of the antisera with extract of rat kidney abolished the reaction with the majority of rat tissue extracts and demonstrated that rat submandibular gland contained at least 5 antigenic components as well as other antigens shared with blood serum and tissues of the rat. Cross-reaction was still present with extracts of related exocrine glands: parotid, sublingual, pancreas, extraorbital and intraorbital lacrimal (Figure). Further absorption by these glands revealed that 2–3 of these antigens were specific for the submandibular gland and the remaining antigens were shared to varying degrees with the other glands. The immunologic relationship between these glands is substantiated by previous studies using antiserum to rat parotid gland[10].

It was also noticed that, of the 5 lines of precipitation exhibited on reacting the antisera with extracts of submandibular glands of male rats, only 4 lines could be detected with glands of adult female rats. It is interesting that this antigenic sexual dimorphism of the rat submandibular gland is associated with the presence of morphological differences between the glands of male and female[11].

Studies of glandular extracts of other species demonstrated high degree of species-specificity of the rat submandibular gland. Comparisons were made with extract preparations of submandibular glands of man, monkey, dog, guinea-pig and mouse and no cross-reaction was detected. Also, no cross-reaction was detected when the

[1] E. H. Beutner, A. Y. Djanian, R. C. Geckler and E. Witebsky, Proc. Soc. exp. Biol. Med. *107*, 486 (1961).
[2] S. P. Kent, J. Histochem. Cytochem. *9*, 491 (1961).
[3] S. P. Kent, Ann. N.Y. Acad. Sci. *106*, 389 (1963).
[4] U. Bertram and P. Halberg, Acta allerg. *19*, 485 (1964).
[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. *193*, 265 (1951).
[6] O. Ouchterlony, Microbiologia scand. *25*, 186 (1948).
[7] P. Grabar and P. Burtin, *Analyse Immuno-Electrophoretique* Masson and Cie, Paris (1960).
[8] J. J. Scheidegger, Int. Arch. Allergy *7*, 103 (1955).
[9] A. H. Coons and M. H. Kaplan, J. exp. Med. *91*, 1 (1950).
[10] S. El-Mofty, Ala. J. med. Sci. *9*, 437 (1972).
[11] B. Grad and C. P. Leblond, J. Endocr. *45*, 252 (1949).