Induction of binding of sheep red blood cells with the surface of mouse thymocytes by means of phytohaemagglutinin and concanavalin A

A. MYŚLIWSKI, JOLANTA MYŚLIWSKA and J. WITKOWSKI[1]

Department of Histology, Medical School, PL-80-210 Gdańsk (Poland), 5 May 1976

Summary. Incubation of mouse thymocytes with mitogens PHA and Con A enhances rosette formation by these cells. This phenomenon is dependent on the character of the cell surface at the moment when the cell makes contact with mitogen and on the presence of the mitogen bound to the cell surface.

GERGELY et al.[2] have shown that the mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) stimulate rosette formation on human lymphocytes. These authors suggest that the lymphocytes which form rosettes belong to the mitogen-responsive fraction and that the binding of sheep red blood cells (SRBC) by lymphocytes is a result of the alteration of their surfaces. These suggestions inspired us to investigate the effect of PHA and Con A on the binding of SRBC by mouse thymocytes, whose ability to form spontaneous rosettes with SRBC is very limited[3].

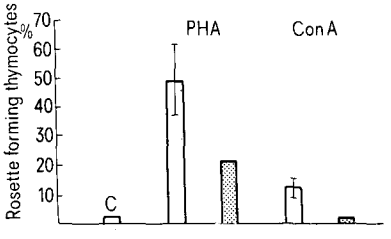

Fig. 1. Effect of PHA and Con A on SRBC binding by mouse thymocytes. White column C, the control samples; white columns, the thymocytes incubated with PHA or Con A; black columns, the thymocytes incubated with PHA or Con A and washed, respectively, with *N*-acetyl-D-galactosamine or α-methyl-D-mannoside. Columns represent arithmetic means, vertical lines represent standard errors.

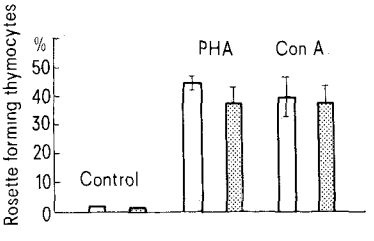

Fig. 2. Influence of db cAMP (10^{-5} *M* 1 ml^{-1}) on rosette formation by mouse thymocytes with SRBC after preincubation with PHA and Con A. The incubation medium was without calf serum. Black columns, samples with db cAMP.

Material and methods. Fifty 6-week-old male mice of Balb/c strain were used. The thymocytes were obtained by mechanical dissociation of the thymus and passing the cells through a nylon mesh. After threefold washing with phosphate-buffer saline (PBS) the thymocytes were resuspensed in TC 199 culture medium supplemented with 15% calf serum. Then an equal volume of thymocytes (1×10^7 1 ml^{-1}) and a 0.5% suspension of SRBC were mixed. The tubes containing the mixture were incubated at room temperature for 15 min, centrifuged for 5 min at 200 *g* and next left at 4 °C for 12 h. After that time, the pellets were gently resuspensed and 1000 cells were examined with a view to evaluating the percentage of rosettes. Before the addition of SRBC, some of the cells were preincubated with PHA (Wellcome) at a final concentration of 2 µl 1 ml^{-1} or Con A (Sigma) at a concentration 30 µg 1 ml^{-1} for 60 min. Next the cells were 3-fold washed with PBS. The applied concentration of mitogens gave the optimal stimulation of DNA synthesis. Another part of the thymocytes, after preincubation with mitogens, was washed with 0.05 *M* *N*-acetyl-D-galactosamine (Sigma) solution to remove PHA from the cell surface or with 0.05 *M* α-methyl-D-mannoside (Sigma) solution to remove Con A.

Results and discussion. We found that incubation with mitogens markedly enhanced rosette formation by mouse thymocytes (Figure 1). We also found, like GERGELY et al.[2], that PHA was a much better inducer than Con A. Since mouse thymocytes are much more responsive to Con A than PHA, the suggestions mentioned above, that rosette formation depends on cell activation, seemed to us to be doubtful. Therefore we decided to examine the activity of rosette-forming and non-rosette-forming thymocytes by means of the Rigler method[4,5], which enables us to determine the activity of a single cell by cytofluorometry. We found a statistically significant (Table) difference between 2 groups of mouse thymocytes. The rosette-forming cells appeared to be less active than non-rosette-forming cells. The result of these examinations suggests that activation interfers in the reaction between SRBC and thymocytes. In order to test this, the follow-

Comparison of fluorescence intensity of rosette-forming and non-rosette-forming thymocytes stained with Acridine orange

Cell fraction	No. of cells	Fluorescence intensity at λ = 530 nm (arbitrary units, $\bar{x} \pm$ SE)	*t*-test	Fluorescence intensity at λ = 590 nm (arbitrary units, $\bar{x} \pm$ SE)	*t*-test
Rosette forming thymocytes	100	6.57 ± 0.96	$p < 0.01$	1.15 ± 0.24	$p < 0.01$
Non-rosette-forming thymocytes	100	10.81 ± 0.81		2.12 ± 0.15	

The measurements were performed after 24 h of incubation of the thymocytes with SRBC at 4 °C; the thymocytes were previously treated with Con A for 60 min. The cells were fixed with a mixture of methanol-acetic acid (3:1 v/v) and dry smears were prepared. The smears were acetylated with 40% acetic acid anhydride in pyridine and stained with an Acridine orange solution (10^{-4} *M* in citric acid –Na_2HPO_4 buffer pH = 4.1) for 15 min. The fluorescence was excited by light at λ = 365 nm. The measurements were made with a Reichert microphotometer mounted on a Reichert 'Zetopan' microscope.

ing experiment was set up: together with the mitogen, dibutyryl cyclic AMP (db cAMP) was added to the incubation medium in a concentration which is able to inhibit thymocyte transformation[6]. In a separate experiment, it was found that db cAMP, at the concentration used (1×10^{-5} *M* 1 ml^{-1} of medium), decreased the incorporation of ^{3}H-thymidine to 9% of the control (Con A without db cAMP), which corresponds to the value for the non-stimulated thymocyte population. The results obtained in these experiments (Figure 2) indicate that inhibition of the cell activation does not change the proportion of rosette-forming thymocytes. Therefore we can conclude that rosette formation by mouse thymocytes after incubation with mitogens is not dependent on the changes in the cell membrane linked to cell activation, but on the character of the cell surface at the moment when the cell makes contact with a mitogen, and on the presence of the mitogen bound to the cell surface. Since this conclusion contradicts that of POLITIS et al.[7], we examined the effect on rosette formation of specific inhibitors[8] of the binding of mitogen to the cell surface. We found (Figure 1) that washing with these inhibitors thymocytes previously exposed to a mitogen caused a significant decrease in the number of rosette-forming thymocytes, which showed that our conclusion was correct.

The difference between the mouse thymocytes in ability to form rosettes could, however, be connected with a different distribution of the surface receptors for PHA and Con A because all the thymocytes bind these mitogens[9]. Perhaps the different distribution of the surface receptors is also responsible for the difference among thymocytes in responsiveness to the mitogens[10,11].

[1] We are grateful to Dr. J. M. N. Boss, University of Bristol, for reading the manuscript and comments.

[2] P. GERGELY, G. SZABO, G. SZEGEDI and G. PERTRANYI, Experientia *30*, 300 (1974).
[3] J. F. BACH, in *Contemporary Topics in Immunobiology* (Eds. A. J. S. DAVIES and R. L. CARTER; (Plenum Press, New York and London 1973), vol. 32, p. 189.
[4] R. RIGLER, Acta physiol. scand., Suppl. *67* (1966).
[5] R. RIGLER and D. KILLANDER, Expl. Cell Res. *54*, 171 (1969).
[6] R. HIRSCHHORN, in *Cyclic AMP* (Eds. W. BRAUN, L. LICHTENSTEIN and CH. W. PARKER; Springer-Verlag, Berlin, Heidelberg, New York 1974), p. 45.
[7] G. POLITIS, M. PLASSARA and H. TKOMAN-POLITI, Nature, Lond. *257*, 485 (1975).
[8] N. SHARON and H. LIS, Science *177*, 949 (1972).
[9] J. D. STOBO, A. S. ROSENTHAL and W. E. PAUL, J. Immun. *108*, 1 (1972).
[10] J. MYŚLIWSKA, A. MYŚLIWSKI and J. WITKOWSKI, Cell Tiss. Res. *166*, 553 (1976).
[11] J. D. STOBO, Transplantation Rev. *11*, 60 (1972).

Lack of correlation between structural features and function of synthetic agents tested for leukocyte chemotaxis[1]

T. W. Jungi[2]

Schweiz. Forschungsinstitut, Med. Abteilung, CH-7270 Davos (Switzerland), 6 September 1976

Summary. A number of proteins, poly-L-amino acids, oligopeptides and lipids were tested for neutrophil, eosinophil and macrophage chemotactic activity. One myoglobin preparation was active. Based on the negative findings for all other substances, primary structure, secondary structure, degree of hydrophobicity, size and charge of a molecule, could be ruled out as structural features recognized by chemotactically responding phagocytes.

Numerous leukocyte chemotactic factors have been described recently[3] but it nonetheless remains unclear how phagocytes are able to discriminate chemotactic agents and by what mechanisms they respond to a vectorial signal. Phagocytes could carry substrate-specific receptors for chemotactic recognition or they could recognize various factors by a basic common perception mechanism. The great diversity of cytotaxins has thus far precluded establishment of the characteristics which specify a molecule as a leukocyte attractant.

In the present work, this problem was explored by using an array of compounds of known structure for evaluating their chemotactic activity in vitro. Agents were selected on the basis of criteria which might contribute to chemotactic recognition:

a) specific amino-acid forming the N-terminal or C-terminal end of an active peptide, b) defined primary structure (sequence) forming the active center of a cytotaxin, c) defined secondary structural conformation (e.g. random coil[4], d) the overall charge of a molecule as expressed by its isoelectric point[5], e) the degree of hydrophobicity of a molecule[3,6].

Material and methods. Most of the 28 compounds tested were synthetic di-, tri- and poly-L-aminoacids and lipids obtained from Sigma, St. Louis, Mo., USA. Myoglobin was obtained from Sigma (M 1882) and Serva, Heidelberg, BRD (29895). Test materials were dissolved in Gey's balanced salt solution to which 0.1% ethanol and 0.01% $NaHCO_3$ were added for dissolving lipids.

Test cells were rabbit neutrophils, guinea-pig neutrophils, rabbit macrophages and guinea-pig eosinophils. Tests on lipids were made only with rabbit neutrophils. Preparation of cell suspensions, chemotaxis chambers and other details of methodology were as described previously[7]. Positive controls were immune complex-activated heated (30 min, 56 °C) rabbit serum (5% v/v) and casein (1% w/v) for rabbit neutrophils, casein (1–0.1%) for rabbit macrophages, dextran-activated heated pig serum (10 and 5%) for guinea-pig eosinophils and the latter as well as casein (1% and 0.5%) for guinea-pig neutrophils.

1 I thank Dr J. H. Wissler for suggesting the reported experiments and Dr M. Landy and Prof. E. Sorkin for advice and criticism during preparation of this article. The excellent assistance by Mrs J. Kerschbaumer-Bech and Miss Margrit Bühlmann is gratefully acknowledged. This work was supported by Swiss National Foundation for Scientific Research, grant 3.8750.72, and by Sandoz-Stiftung, Basel.

2 Present address: James A. Baker Institute for Animal Health, New York State College for Veterinary Medicine, Cornell University, Ithaca, N. Y. 14853, USA.

3 P. C. Wilkinson, in: Chemotaxis and inflammation. Churchill-Livingstone, Edinburgh 1974.
4 P. C. Wilkinson, Experientia *28*, 1051 (1972).
5 M. Frimmer and B. Zweidler, Arch. exp. Path. Pharmak. *251*, 315 (1965).
6 P. C. Wilkinson and I. C. McKay, Eur. J. Immun. *2*, 570 (1972).
7 T. W. Jungi, Int. Archs Allergy *48*, 341 (1975).