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Inhibition of Phytohaemagglutinin-Induced Transformation of Lymphocytes by Human Spleen Extracts *

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The presence in human spleen extracts of a substance capable of inhibiting the phytohaemagglutinin (PHA)-induced transformation of lymphocytes into lymphoblasts was demonstrated by analysing the nuclear size of cultured human lymphocytes. In this way the artefacts produced by the methods based on the uptake of ^{3}H-thymidine were avoided. The inhibitor did not compete with PHA for the same membrane receptor-site, since adding the extract 24 h after the PHA-stimulation still produced a significant inhibition. The lack of cytotoxicity of the extract was demonstrated by the fact that cultures that lasted for six days, instead of three days, resulted in a pattern similar to that of control cultures. An attempt to partially purify the inhibitor by means of ammonium sulphate fractionation showed that the highest activity precipitated between 0.5 and 0.6 saturation although, due to the characteristics of the assay, it was difficult to assess the actual degree of purification.

The presence, in porcine lymph nodes, of an activity capable of inhibiting the transformation of human lymphocytes into lymphoblasts was first described by MOORHEAD *et al.* (18). Subsequently, several groups found similar activities in the thymus and spleen of cows, pigs and rats (3, 8, 10, 13, 14, 17). Although many attempts have been made to purify this activity, tentatively identified as a chalone, none has yielded a homogeneous product. How-

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ever, it appears that the lymphocyte chalone is a peptide with a molecular weight of about 1,400 (11), of a highly basic character as it is strongly bound to tRNA (15) and containing very likely mannose and perhaps some other carbohydrate because it interacts with concanavalin A-Sepharose (15). ALLEN *et al.* (1) pointed out that the chalone activity obtained from spleen extracts following some published methods could be accounted for by the presence of a protein-spermine complex. This result was rebuked by LENFANT *et al.* (16).

One of the main problems in the study of a chalone is the artefacts that appear in the activity assay, specially when the rate of tritiated thymidine uptake into DNA is measured (2). Another important factor that has to be taken into account and that would mask the results is the possible cytotoxicity, specially when crude extracts are assayed (12).

In order to avoid these problems the inhibition of the PHA-induced transformation of human lymphocytes into lymphoblasts by human spleen extracts was studied by means of a visual method.

Materials and Methods

Human spleens were obtained from the Department of Pathological Anatomy from the Santa Cruz y Sant Pablo Hospital (Barcelona, Spain). They were collected from corpses not afflicted from haemathological pathology (ictus, infarction) and stored at —20° C. Spleen extracts were obtained by grinding the glands in acetone previously cooled to —20° C (0.75 l/100 g tissue). The suspension was filtered under vacuum and the cake was extracted several times with acetone (—20° C) until removal of lipids was complete as checked by means of the Chabrol-Charonnet test (Cromatest, Knickerbocker, Barcelona, Spain). The final powder was dried under vacuum over silica gel.

Samples for the study of the chalone-like activity were prepared by dissolving the powder in 0.9 % sodium chloride (2 mg/ml). The insoluble material was removed by filtration and the solution was adjusted to a total protein concentration of 0.8-1.0 mg/ml as measured by means of a modified LOWRY assay (9).

For the chalone activity assay, human blood was obtained from healthy individuals by venopuncture and was maintained heparinized until use. Lymphocytes were separated according to the method of BÖYUM (4) using a Ficoll (9 % in water) — Urografin 76 % gradient until a final density of 1.080. The blood, which had been previously diluted with an equal volume of phosphate buffer saline (PBS) was carefully added to the gradient and was centrifuged at $800 \times g$ for 20 min. The layer of mononuclear cells was pipeted out, washed twice with PBS and then resuspended in autologous serum at a mean concentration of $1\text{-}2 \times 10^7$ cells/ml. The yield was 80-95 % of lymphocytes. Lymphocyte cultures were carried out in a culture medium type 167 with PHA or 199 with PHA (both from GIBCO, Grand Island, N.Y. USA). Test tubes with a threaded cap containing 2 ml of culture medium were used. The culture was started by adding 10^6 lymphocytes per ml of culture medium plus 1 ml of spleen extract in the samples or 1 ml of PBS in the controls. The tubes were incubated at 38 ± 0.1° C. After 70-72 h incubation the cultures were fixed and dyed according to the method of EGOZCUE (7) except that the samples were not subjected to hypotonic shock. During the whole procedure special care was taken in order to avoid bacterial or

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Abbreviations:

PHA, phytohaemagglutinin.

PBS, phosphate buffered saline (NaCl, 8.0 g; KCl, 0.2 g; $Na_2HPO_4 \cdot 7H_2O$, 1.15 g; KH_2PO_4, 0.2 g; H_2O, dist. up to 1 l).

fungal contamination; for this reason the spleen extract solution and the saline were added after filtration through 0.2 μm disposable sterile Millipore filters (Millipore, Bedford, Ma. USA). Contaminated cultures were discarded. Counting of lymphocytes was done with the aid of a grating (scale 0.1 mm = 50 divisions) attached to the microscope. Nuclei were classified into five different groups according to size: Group 1: 2-4 μm; group 2: 4-6 μm; group 3: 6-8 μm; group 4: 8-10 μm; group 5: 10-12 μm. A minimum of 200 nuclei were counted in each preparation.

The partial purification of the extract was carried out by adding solid ammonium sulphate (Merck) to the aqueous extract. The amounts of ammonium sulphate needed to reach a given saturation was calculated by means of the nomogram of DIXON and WEBB (6). The precipitates were recovered by centrifugation at 10,000 $\times$ g for 30 min at 4° C and were dialysed against distilled water until a conductivity in ammonium sulphate of 4 mS was obtained. They were made isotonic by adding the necessary amounts of NaCl.

Results

Table I lists the mean values, together with the standard deviations of 13 problems and 13 controls classified in five different groups according to nucleus size. It can be seen that the presence of the

Table I. *Nucleus-size distribution of lymphocyte cultures stimulated with PHA after 72 h incubation.*

In experiments 1 and 2 the PBS or spleen extract was added at the start of the culture. In experiments 3 and 4 they were added 24 h after the cultures were started. The number of experiments are given in parenthesis. In both sets of experiments differences between controls and problems were significant to $P < 0.05$.

Experiment	Medium	Nucleus-size (percent of total)				
		Group 1	Group 2	Group 3	Group 4	Group 5
1. Control (13)	167 [a]	7 ± 3	37 ± 11	33 ± 6	20 ± 7	5 ± 3
2. Spleen extract (13)	167 [a]	17 ± 6	50 ± 10	23 ± 2	8 ± 6	2 ± 1
3. Control (3)	199 [b]	6 ± 2	25 ± 6	23 ± 3	28 ± 5	18 ± 3
4. Spleen extract (3)	199 [b]	10 ± 2	33 ± 8	44 ± 7	10 ± 3	3 ± 1

a) GIBCO 167 with PHA. b) GIBCO 199 with PHA.

Table. II. *Ammonium sulphate fractionation of the lymphocyte-transformation inhibitor found in human spleen extracts.*

Figures correspond to the mean of 3 cultures. The fraction precipitated at 0.6 saturation is significantly inhibited ($P < 0.05$) with respect to the other fractions.

Fraction ($[NH]_4SO_4$ saturation)	Nucleus-size (percent of total)				
	Group 1	Group 2	Group 3	Group 4	Group 5
Control *	13 ± 2	37 ± 4	32 ± 2	15 ± 2	3 ± 2
0.3	18 ± 3	44 ± 4	30 ± 2	8 ± 3	0 ± 0
0.4	17 ± 3	38 ± 3	34 ± 3	10 ± 3	1 ± 1
0.5	11 ± 2	42 ± 5	32 ± 3	13 ± 5	2 ± 2
0.6	11 ± 2	55 ± 5	28 ± 3	6 ± 3	0 ± 0
0.8	17 ± 2	36 ± 2	37 ± 2	8 ± 4	2 ± 1
Supernatant	23 ± 2	37 ± 3	25 ± 2	14 ± 3	1 ± 1

* The conductivity had been previously adjusted to a conductance of 4 ms by the addition of ammonium sulphate. Isotonicity was obtained by adding the corresponding amount of NaCl.

spleen extract causes an increase in the number of the smaller sizes (groups 1 and 2) and a decrease in the number of transformed cells (groups 3-5). If these latter groups are considered as representing transformed cells, a 43 % inhibition, with respect to controls, is found in the conditions used. If only groups 4 and 5 are considered, a 60 % inhibition is attained. Table I also shows the values found for another set of cultures in which the spleen extract was added to the medium 24 h after the culture was started. In this way it is possible to distinguish whether the inhibitory effect found with the extract was due either to a specific inhibition of the cell-cycle or to a competition with the PHA for the same site. It can be seen a dramatic decrease of the larger nuclei (groups 4 and 5), a slight increase in the smaller nuclei (groups 1 and 2) and a large increase of group 3.

Table II shows the distribution of nuclei sizes in cultures in which several fractions precipitated by the addition of ammonium sulphate have been added. In this case the controls had been obtained by adding a solution with ammonium sulphate (corresponding to a conductance of 4 mS) that had been made isotonic by adding the required amount of NaCl. In this way it was possible to ascertain the effect due to the inhibitor from that due to the ammonium sulphate. It is seen that only in the fraction precipitated between 0.5 and 0.6 $(NH_4)SO_4$ saturation an increase in the number of small —size nuclei (groups 1 and 2) and a corresponding decrease in the other three groups appears. A slight inhibition of the lymphocyte transformation also appears in the final supernatant.

Discussion

In the study of a putative chalone many problems of a methodological nature appear. One of the main problems lies in the assay chosen to quantify the inhibition of mitosis. The most widely used assay, the incorporation of tritiated thymidine to DNA, is subject to many artefacts (2, 12) apart from the perturbing influence of ^{3}H-thymidine on the cell-cycle (19). It is for these reasons that the method chosen in the present work, although much more laborious, is of a more reliable nature. On the other hand, as a strict division between lymphocytes and lymphoblasts is difficult, due to the subjective appreciation of the experimenter, the division in 5 groups according to nucleus size allows an objective classification. A shift in the distribution pattern towards the smaller sizes will be indicative of an inhibition of the transformation. Unfortunately, this method does not allow an absolute quantification of the degree of inhibition and, therefore, its main usefulness is the demonstration, without experimental artefacts, of the presence of a substance in human spleen extracts (probably a chalone) that inhibits the transformation of lymphocytes.

The chalone concept, as stated by BULLOUGH (5) requires the demonstration of a lack of cytotoxicity. Although the exclusion of vital dyes had been used as a proof of the viability of cells, this is a concept under strong criticism (2, 12). In the system used in the present work the lack of cytotoxicity is shown by the fact that extending the duration of the culture to six days — after washing and resuspending in fresh medium at the third day — results in the disappearance of the inhibitory effect because the same pattern is found both in problems and controls.

There is, *a priori*, the possibility that the inhibitory effect found with spleen extracts might be due to some factor that either competes with PHA for the same receptor-site in the cell membrane or binds to PHA producing a conformational change in the protein with the concomitant loss of affinity for the corre-

sponding cell receptor-site. In this case the inhibition would not be caused by the action of an inhibitor of the mitosis (chalone) but because of the lack of effective stimulating agent (PHA). This latter possibility was ruled out with the experiments in which the extract was added 24 h after the initiation of the culture. By then a number of cells would be already stimulated by the action of PHA and would have entered from G_0 into the cell-cycle. It is not surprising, then, to find a lack of full-size lymphoblasts and a large number of cells of an intermediate size (group 3). This might be indicative of cells already in the cycle and then arrested by the action of the inhibitor. It should be noticed that PHA-stimulated lymphocytes are fully committed to division after 18 h from the start of stimulation (20).

The ammonium sulphate fractionation shows that the inhibiting activity is found in the fraction precipitating between 0.5 and 0.6 saturation. The already stated difficulty of the assay method to quantify the degree of inhibition makes it impossible to have a clearer idea of the extent and yield of purification. For this reason no more attempts to purify this chalone activity are being carried out until a method for the reliable quantification of the results is found.

In order to demonstrate the chalone nature of the substance found in human spleen extracts it would be necessary to demonstrate that it is tissue-specific and that it is not species-specific. However, the main goal of this work was to demonstrate the presence of a non-cytotoxic mitosis-inhibitor in human spleen extracts. The use of extracts from other sources would be informative in the sense of a lack of species-specificity, but the aspects of non-cytotoxicity could be masked, at the extract level, by the presence of heterologous elements. The aspect of non species-specificity is, however, of the utmost importance for the preparation of large amounts of inhibitor, necessary to study its molecular properties and the mechanism of action, as well as its possible use as an antimitotic drug.

At present studies are in progress to authomatise the cell-counting and size-distribution in order to avoid the present method which is of a very tedious and laborious nature.

Resumen

Mediante el análisis del tamaño nuclear de linfocitos humanos cultivados, se demuestra la presencia en bazo humano, de una substancia inhibidora de la transformación de linfocitos en linfoblastos inducida por la fitohemaglutinina (PHA). De este modo se han evitado los artefactos experimentales producidos en los métodos basados en la incorporación de H^3-timidina. El inhibidor no compite con la PHA por el mismo receptor de membrana ya que si se adiciona el extracto 24 h después de la estimulación por PHA se observa aún una inhibición significativa. La no citotoxicidad del extracto se demuestra por el hecho de que cultivos cuya duración se prolonga durante seis días, en lugar de tres, dan un porcentaje de transformación igual al de los cultivos control. Finalmente, una purificación parcial con sulfato amónico muestra que la actividad inhibidora más potente se encuentra entre 0,5 y 0,6 de saturación. No obstante, debido a las características del método de análisis, es difícil evaluar el grado de purificación alcanzado.

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