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A SIMPLIFIED PROCEDURE FOR THE
USE OF THE "BLAST-LIKE TRANSFORMATION"
OF LYMPHOCYTES IN
CULTURE AS A ROUTINE CLINICAL TEST
RESULTS IN A GROUP OF ALLERGIC CHILDREN

By

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It is well known that human cultured lymphocytes transform into "blast-like" cells in response to a variety of stimuli: phytohaemagglutinin (17, 15), antigens against which the subject has been immunized (18, 6), allergens (including several common drugs) (13, 14, 5) to which he is sensitized, and to cells of different genetic constitution (2, 4). This provides the basis for a very useful clinical test to replace others, implying the administration to patients of potentially harmful substances (11). However, this test has not been used extensively as counting transformed cells on smears is time consuming, and the interpretation is complicated by the fact that control cultures show some degree of transformation (10, 9). The amount of blood necessary for the test is also a draw-back, if it is to be used for young children.

The present study describes a procedure that eliminates these difficulties. Only small samples are necessary: finger cutaneous puncture gives enough blood. The technique can be performed entirely by a skilled technician, is not time consuming, and ends up by a measure of radioactivity giving clear quantitative data.

The method is based on the following well-known facts. During transformation, the lymphocytes increase in size, synthesize DNA, and later tend to divide. If the cells receive tritiated thymidine during DNA synthesis, they will incorporate it into their nuclei which become radioactive. No other element of the normal peripheral blood will do so under the circumstances: red blood cells have no nuclei and most other leukocytes degenerate rapidly in cultures. Therefore, there is no need to separate lymphocytes from other blood elements to measure radioactivity in their DNA.

PROCEDURE

Six (or more) venous sterile blood samples are diluted directly, each in 3 ml of TC medium 199 supplemented by heparin (15 I.U. per ml) in conical centrifuge tubes. The same number of blood drops (usually 10) from finger puncture in children or identical venous blood sample in adults (usually 0.5 ml) are added to every tube. The substance to be tested is dissolved in 0.5 ml of Hank's balanced salt solution (BSS) and added to several tubes. At least two concentrations are used. Control tubes receive 0.5 ml of BSS only. Penicillin or other antibiotics may be added if there is doubt about the sterility of sampling, provided the patient is *not allergic to these drugs*.

Tubes are stoppered and incubated at 37°C on an inclined rack. Two to five days later, ^3H -thymidine (4 or 5 μC) is added, and the cultures are incubated further for 15 to 24 hours. The culture period before addition of the radioactive precursor depends on the substance to be tested. Two periods are used in each case.

At the end of the cultivation period, DNA is extracted by a procedure indicated by *Feinendegen et al.* (7). The cells are washed twice with ice cold BSS, treated for one hour with 1 per cent cold acetic acid to lyse red cells, and extracted for two hours with ice cold 2 per cent perchloric acid. After each step of the procedure, the tubes are centrifuged and the supernatant discarded. The pellet is each time dispersed in the following solution. DNA is then extracted by 10 per cent perchloric acid at 60°C for 2 hours.

Aliquots of the supernatants are neutralized by N NaOH and the radioactivity is measured with a scintillation counter. An extraction of RNA with 10 per cent perchloric acid at room temperature before the treatment at 60° is optional but not necessary. It has been confirmed that all radioactivity not bound to DNA is eliminated. To ensure minimal losses, the whole procedure is carried out in the same tubes in which the cells were incubated. With the above procedure of sampling, a 20 per cent standard deviation is found when a group of sister samples are treated together. This scattering is mainly due

to volume errors. It has now been reduced by a more accurate determination of the amount of blood per tube (see discussion).

RESULTS

The technique has so far been used in 51 cases representing a variety of clinical disorders: agammaglobulinemia, allergy to several drugs, and control of genetic compatibility between prospective donors and receivers of graft.

From this material we present the results in a homogeneous group. In order to investigate the reliability of the method, a systematic study was performed in 20 children, 5 to 16 years old, by determining the reaction of their lymphocytes to pollen allergens. In all but four control patients, asthmatic attacks and or dyspnea episodes had been observed for the last one to ten years.

The etiologic factors were evaluated in the following way. From the case history and the clinical signs, allergic influences were suspected when a contact with an allergen and an asthmatic attacks were observed simultaneously. The infectious factors were considered dominant when the asthma attacks were more frequent in the winter, and when signs of infection were observed (cough, fever, leucocytosis, RCSR, purulent sputum).

In asthmatic children, skin tests were systematically performed with grass pollens extracts and, at least, twelve common pneumallergens. The cutaneous reactions following the prick test and the intradermal injections were considered "doubtful" ($\pm$) if the whealing was small (3 to 5 mm diameter) and circumscribed by erythema. The reaction was "positive" (+) when the wheal and the surrounding erythema was larger than 5 mm, and "highly positive" (++) when pseudopodium-like branches extended from the wheal. The threshold doses of house dust and animal epithelial allergens had previously been determined in studies on groups of healthy, non-allergic children (16, 1).

For many years, large discrepancies have been observed between case histories, skin tests and the results of pulmo-

nary provocation tests (8). Therefore aerosols of allergens were systematically given to the patient if, from the case history and the skin tests, an allergen was suspected to be the ethiologic factor of the asthma syndrome. During inhalation of the allergen total pulmonary flow resistance was continuously calculated from measures of intra-eosophageal pressure changes and from the air flow. The reaction was "positive" (+) when the resistance was double or more that of the starting value.

All the intradermal injections and respiratory challenges were performed with allergen extracts prepared by the Institut Pasteur (Paris)*. Skin tests, pulmonary provocation tests and blood cultures were done twice, at 6 months interval, in one patient (LIB.). All the blood cultures were performed as blind tests. Pollen aqueous extract (prepared from dry pollen supplied by the Institut Pasteur) was added at the beginning of the culture so that the final concentrations of protein nitrogen in the medium were 3 and 0.3×10^{-6} g/ml. The lymphocytes were tested for radioactivity on the 4th and 5th days of culture.

All blood culture positive cases had at least one (and usually two) samples for which the ratio of radioactivity uptake compared to the controls was above 2.5, and some had ratios up to 70. The mean of negative results was equal to the mean of the control. Ninety-five per cent of them were 1.5 time below the relevant control. Only two tubes from negative cases go up to ratios of 1.9 and 2.1. This can be considered satisfactory owing to the technique of blood sampling (finger cutaneous puncture), which has a volume standard deviation of up to 20 per cent.

The results are summarized in Table 1.

1. For grass pollen allergens, the blast-like transformation of lymphocytes in vitro occurs when the respiratory test is positive (group A) but not in others. There is, on the con-

* This an aqueous extract of equal amounts of pollen from *Lolium perenne* L., *Dactylis glomerata* L., *Secale cereale* L. and *Phleum pratense* L.

TABLE I
Correlation Between Lymphocyte Cultures and Other Tests in Children.

Group	Patient	Clinical signs		Skin tests		Pulmonary provocation tests			Radioactivity of lymphocytes in contact with pollen Cultures Test ≥ 2.5 controls
		Allergy	Infection	Pollen	Other Allergens	Pollen	Allergens	Other	
A	DUJ.	+	o	+	+	+	+	+	+
	NOT.	$\pm$	$\pm$	+	+	+	+	o	+
	PIJ.	+	o	+	+	+	+	o	+
	PIA.	+	$\pm$	+	+	+	+	o	+
B	VAD.	o	+	+	+	+	o	o	o
	HAM.	+	+	+	+	+	o	$\pm$	o
C	GER.	o	+	o	+	+	o	+	o
	TIL.	$\pm$	+	o	+	+	o	+	o
	LIB.	+	+	o(2x)	+	+	o(2x)	+	+
	COL.	$\pm$	+	o	+	+	o	+	+
D	ZIG.	$\pm$	+	o	$\pm$	+	o	o	o
	VAW.	o	+	o	+	+	o	o	o
	BER.	$\pm$	+	o	+	+	o	o	o
	CHA.	$\pm$	+	o	+	+	o	o	o
	MAT.	o	+	o	+	+	o	o	o
	BUS.	o	+	o	+	+	o	o	o
E	KEL.	o	o	+	+	+	+	+	o
	HOU.	o	+	o	+	+	+	+	o
	DEK.	o	o	+	+	+	+	+	o
	DRO.	o	o	+	+	+	+	+	o

trary, some discrepancy between the pulmonary provocation test (4 positive cases) and the skin test (6 positive cases) (group A and B).

2. As previously quoted, for pneumallergens other than pollens, the discrepancy between the skin test reactions (15 "positive") and the respiratory challenge (5 "positive") is large (groups A, B, C, D). Except for *one* case with conflicting results, the pollen test is negative in cultures.

3. The specificity of the blood test is also demonstrated by negative results in four non allergic patients (group E).

DISCUSSION

The results indicate a clear cut separation between individuals, whose lymphocyte transformation is stimulated by the allergen tested, and those who do not show this phenomenon. In the group chosen it fits well with the results of the pulmonary provocation test, and is more specific than skin reactions. We have one exceptional case (LIB.) who was tested a second time and showed a weaker but still marginal positive reaction.

The procedure described above is simplified to make it a routine test with small samples of blood. It uses for the "transformation" a radioactivity test which tends to be equally useful in mixed lymphocyte cultures (3, 19) in relation to grafting compatibility problems. But the use of whole blood (not possible in mixed cultures) simplifies the procedure and gives at the same time a high survival rate and a small spontaneous transformation rate for the cells, as they do not suffer from damage due to centrifugations or other manipulations. The presence of other leukocytes, such as the monocytes may be beneficial as it has been claimed they must be present to ensure a full response of the lymphocytes (12).

The test can be made more accurate by more precise countings of the lymphocytes in each tube. We are now weighing the amount of blood added instead of counting drops. If more

blood can be used, a preliminary separation of lymphocytes may increase the precision of the measure, but not necessarily the sensitivity of the test.

The only possible gross cause of error is unrecognized bacteriological contamination of some of the cultures which could give a high radioactivity count. Actual radioactivity counts depend on several factors. The number of lymphocytes (about 1 to 3×10^6) in each case may vary and also that of other nucleated cells present. These degenerate soon, and fragments of their DNA may be used later for synthesis in the lymphocytes, thus "diluting" the radioactive precursor added. All these factors, however, are the same in the control and test cultures of the same individual.

The rate at which lymphocytes take up thymidine may be maximal at different moments in different patients, even in response to the same antigen. It is thus useful to test the radioactivity during several periods of time. The concentration of the allergen necessary for a positive response may also vary and too high a dose can be inhibitory or toxic. We use routinely two or three doses of allergen extract with a tenfold or fivefold difference in concentrations. As the increase of work incurred by including larger numbers of cultures in the procedure is practically nil (compared to the slow reading of smears), the different variables tested (e.g. days, concentrations, number of substances) can be increased deliberately without much trouble.

The only possible drawback of the technique is that it is more expensive in terms of reagents used and implies the use of a scintillation counter.

SUMMARY

A measure of lymphocyte transformation to blast cells in cultures of small samples of whole blood is obtained by the amount of radioactivity in DNA after addition of tritiated thymidine. The procedure, described in detail, is simplified so as to make it a routine clinical test. The specificity of

response to allergens is better than that of other tests, as shown by a study of 20 allergic and control children.

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