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Cellular immunity in newborn infants and children: stimulation of lymphocyte protein synthesis as a measure of immune competence

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SUMMARY

An assay based on the early stimulation of protein synthesis in lymphocytes has been used as an *in vitro* measure of cellular immune competence. ^{3}H-labelled leucine incorporation into human peripheral lymphocytes (PBL) stimulated by the mitogens phytohaemagglutinin (PHA), wax bean agglutinin (WBA) and Concanavalin A (Con A) was measured after one day in culture. This assay offers a technical advantage over the analogous ^{3}H-labelled thymidine incorporation assay, because of the short incubation time required and the absence of homologous serum in the assay system. Newborn infants and patients with Down's syndrome as a group had normal responses, whereas those suffering from recurrent infections demonstrated normal or hyper-reactive responses. Patients with lymphoproliferative disorders, ataxia telangiectasia, and some patients under steroid therapy had diminished immune proliferative reactions. These results are in agreement with most previously reported studies using other assay systems.

INTRODUCTION

Competent lymphocytes, when stimulated by mitogens and specific antigens in tissue culture, transform into proliferative lymphoblasts. This property of lymphocytes has been extensively used as a diagnostic tool to detect cellular immune deficiency in humans (Oppenheim *et al.*, 1975; Wells & Fudenberg, 1974; Kersey, Gajl-Pezalska & Nesbit, 1974). Ability to proliferate, and thereby competence of lymphocytes can be evaluated by measuring the increase of DNA synthesis in stimulated cells following 3 to 4 days of incubation in cell culture with the mitogen. However, it has been shown that the interaction between the lymphocyte membrane and a mitogen such as PHA takes place within minutes of contact (Mendelson, Skinner & Kornfeld, 1971), setting loose a series of early events, of which the increase in protein is one (Hirschhorn *et al.*, 1963; Levy & Rosenberg, 1972). Based upon this early stimulation of protein synthesis (SPS), a rapid semimicroassay in animals was developed (Rosenberg *et al.*, 1972) and later adapted for use in humans (Adkinson, Rosenberg & Terry, 1974). The assay can be completed within 1 day, thus having an advantage over other culture techniques. The present study was performed in order to evaluate this test for the investigation of the immune system in newborn infants as well as in healthy and immunodeficient children.

MATERIALS AND METHODS

Patients. Healthy children aged 1–15 years, who were undergoing venipuncture for diagnostic tests, mainly prior to elective surgery, were used as normal controls. Such paediatric patients were a cross-section of hospitalized and ambulatory children with possible immunodeficiencies, seen at the Kaplan Hospital. Cord blood was used as the source of lymphocytes from newborn infants. The SPS assay was part of a wider investigation of the immune system in these subjects (Levin *et al.*, 1974; Levin, Nir & Mogilner, 1975; Hahn, Levin & Handzel, 1976).

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Cell preparation. Blood was drawn into heparinized tubes and lymphocytes were separated by the Ficoll–Hypaque gradient technique (Böyum, 1965). In general, 5×10^6 lymphocytes obtained after separation from 4–5 ml blood were sufficient for the study in duplicate of three T-cell mitogens at three dosage levels.

Mitogens. Three mitogens were used in the study: Phytohaemagglutinin (PHA, Wellcome Research Laboratories, Beckenham) and concanavalin A (Con A, Miles-Yeda, Rehovot, Israel) were diluted in phosphate-buffered saline pH 7·4 (PBS) and stored in a freezer. Wax bean agglutinin (WBA), like PHA, is prepared from the plant *Phaseolus vulgaris*, and has similar mitogenic properties (Schumann, Schnebli & Dukor, 1973). WBA was kindly donated by Professor N. Sharon from the Department of Biophysics of the Weizmann Institute of Science and was dissolved and stored as described for PHA and Con A.

Stimulation of protein synthesis by mitogens. The lymphocytes and monocytes obtained by Ficoll–Hypaque sedimentation were suspended at a concentration of $2{\cdot}5 \times 10^6$ cells/ml in Eagle's medium lacking leucine (obtainable from Microbiological Associates, Baltimore, Maryland), supplemented with penicillin and streptomycin. The technique for the SPS assay has been described in detail previously (Rosenberg *et al.*, 1972; Adkinson *et al.*, 1974). Briefly, cells were cultured in microtitre plates containing 96 V-shaped wells (Cooke Microtiter System) in volumes of 0·1 ml, containing $2{\cdot}5 \times 10^5$ cells per well, followed by addition of 0·01 ml mitogen solution in at least three concentrations between 0·5 and 10 μg/ml (the range which yields optimal response). PBS alone was added to control cultures. The plates were then mixed on a microshaker (Cooke Microtiter System), covered and incubated for 27 hr at 37°C, in a humidified atmosphere of 10% CO_2 in air. Three hours before termination of the culture, 0·01 ml containing 0·25 μCi ^{14}C-labelled leucine (350 mCi/mmole) or 1 μCi ^{3}H-labelled leucine (1 Ci/mmole, both obtainable from the Radiochemical Centre, Amersham) were added to each well. The pulse was stopped by the addition of 0·02 ml of 50% trichloroacetic acid. The rate of cellular protein synthesis was estimated from the measurement of the incorporation of the radioactive label into acid precipitable material as previously described. Experiments were performed in duplicate or triplicate, depending upon the cell yield. Stimulation rate (SR) was calculated as follows:

$$\text{SR} = \frac{\text{ct/min with mitogen}}{\text{ct/min with PBS (control)}}.$$

In each series of mitogen concentrations, the highest SR obtained was chosen for comparison of the tested subject with a normal control.

RESULTS

The effect of total cell number on stimulation

The effect of varying the cell concentration between $0{\cdot}125 \times 10^6$ and $1{\cdot}5 \times 10^6$ on the SR in cells stimulated by a single dose of PHA is shown in Fig. 1. The increase in cell number resulted in a proportional increase in the rate of incorporation of the radioactive leucine in both control and stimulated cells, reaching a plateau at $1\text{–}2 \times 10^6$ cells per well. The stimulation ratio is therefore not affected or affected only slightly by the change in cell number. This observation was of importance since cell supply in children is usually limited.

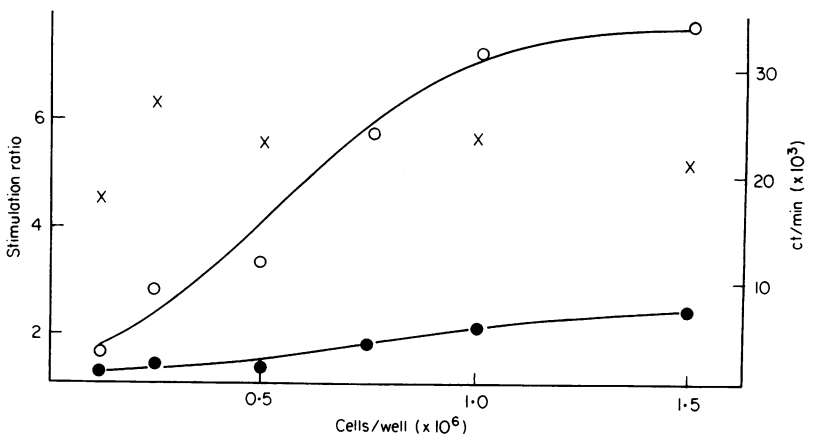

FIG. 1. Effect of increasing cell concentration of normal PBL on the ^{3}H-labelled leucine uptake in response to 1·2 μg/ml PHA. The incorporation by unstimulated control cells (●) and PHA-stimulated cells (○), as well as the stimulation ratio (×) are shown.

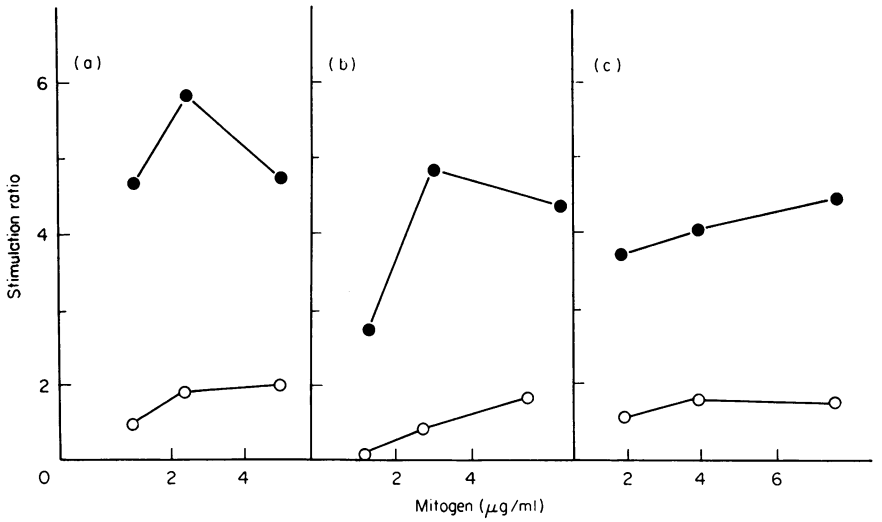

FIG. 2. The response (SR) of normal PBL ($2{\cdot}5 \times 10^5$ cells/well) to PHA (a), WBA (b) and Con A (c) after treatment with normal goat serum (●) or with goat anti-human-T-cell antiserum (○).

The effect of treatment of lymphocytes with anti-human T-cell antiserum on the stimulation by mitogen

In order to test the effect of T-cell elimination on the stimulation by PHA, WBA and Con A, PBL from a normal adult were treated with goat anti-human T-cell antiserum obtained following immunization of the animal with foetal thymus cells (Levin *et al.*, 1975). T cells were diminished from 65–4% by the anti-T-cell antiserum, as determined by a cytotoxicity assay. The stimulation pattern with PHA, WBA and Con A of the T cell-depleted population ($2{\cdot}5 \times 10^5$ cells/well), compared with control PBL treated with normal goat serum, is demonstrated in Fig. 2. Stimulation in the depleted cells by all three mitogens was reduced to 20–30% of normal, indicating that these mitogens are primarily T-cell stimulators.

Stimulation in normal children and newborns

PBL from normal children were tested with PHA, WBA and Con A. As reported in previous studies

TABLE 1. Mitogenic stimulation of lymphocytes from normal children and newborn infants*

Test number	Normal children			Newborns		
	PHA	WBA	Con A	PHA	WBA	Con A
1	(a) 4·82	4·3	3·43			
	(b) 5·07	4·92	4·15			
2	(a) 3·86	4·8	4·25			
	(b) 6·4	5·95	4·9			
3	(a) 4·6	—	3·9			
	(b) 6·07	—	4·97			
4	5·55	5·35	3·65	4·05	3·22	3·18
3	1·9	1·9	2·24	2·25	2·2	2·0
5	4·9	—	5·8	5·8	—	5·4

* SRs of PBL from two normal children tested on the same day are shown in tests 1–3. SRs of newborn cord blood lymphocytes compared to PBL from a normal child are shown in tests 4–5. SR = ct/min with mitogen/ct/min with PBS.

TABLE 2. Stimulation of lymphocytes in suspected immunodeficient children

Subjects	Mitogen		
	PHA	WBA	Con A
Normals	1·09±0·74 (20)*	1·08±0·86 (12)	1·05±0·62 (18)
Down's syndrome	1·01±0·72 (9)	1·00±1·00 (4)	1·12±0·82 (7)
Ataxia telangiectasia	0·21±0·05 (3)	0·056±0·06 (2)	0·15±0·20 (5)
Lymphoproliferative disorders			
Hodgkin's disease	0·17 (1)	0 (1)	0·07 (1)
Chronic lymphatic leukemia	0·33 (1)	0·40 (1)	0·47 (1)
Acute lymphatic leukemia	0·19 (1)	0·16 (1)	0·50 (1)
Acute lymphoma	0·05 (1)	0·07 (1)	0 (1)
Autoimmune disorders			
Henoch Shoenlein syndr.	1·56±0·72 (3)	0·9±0·61 (3)	0·83±0·57 (3)
SLE	1·17 (1)	1·15 (1)	0·68 (1)
Myasthenia gravis	0·92 (1)	1·0 (1)	0·8 (1)
Myasthenia gravis thymectomized	0·69 (1)	0·81 (1)	0·67 (1)
Nephrotic syndrome†	0·48 (1)	0·24 (1)	0·14 (1)
	0·53 (1)	—	0·16 (1)
Nephrotic syndrome‡	0·32 (1)	0·31 (1)	0·18 (1)
Rheumatoid arthritis‡	1·54 (1)	1·04 (1)	1·36 (1)
Rheumatic fever	1·14 (2)	—	0·85 (2)
Miscellaneous recurrent infections			
Bronchopneumonia	1·08 (1)	—	1·24 (1)
Mucopolysaccharidosis + bronchopneumonia	2·49 (1)	2·4 (1)	3·16 (1)
Pseudomonas sepsis	2·61 (1)	2·87 (1)	3·53 (1)
Furunculosis	1·18 (1)	0·89 (1)	0·75 (1)
Respiratory infections	2·00 (1)	1·01 (1)	1·17 (1)
Recurrent respiratory infection	1·67 (1)	2·11 (1)	1·35 (1)

* Pairs of normals were stimulated with PHA and the ratios of (SR normal 1/SR normal 2) were calculated. The mean±2 s.d. of ratios of twenty such pairs is close to 1 (1·09±0·74), indicating that two normals tested at the same time have similar proliferative reactivities. Patients whose reactivity fell below the 95% confidence limits of the response (e.g., ataxia telangiectasia, lymphoproliferative disorders) were considered to be significantly hyporeactive.

† The patient, who was under prolonged steroid treatment, was tested twice within a period of 5 months.

‡ Treated with steroids for a prolonged period.

(Adkinson *et al.*, 1974; Levy & Kaplan, 1974), a variable range of SR values (1·4–8·6) was obtained in these normal children tested over a period of 2 years. However, in any particular test, similar rates of stimulation were observed for all three mitogens. In addition, SR values of two normal individuals tested on the same day fell into a narrow range (Table 1). When twenty pairs of normal individuals were tested with PHA, the mean ratio ±s.d. of SR normal 1/SR normal 2 was 1·09±0·37 (Table 2). Therefore, the stimulation ratio of a tested subject was always compared to a concomitantly tested normal subject. As shown in Table 1, when cord blood lymphocytes were compared in the same assay to PBL from a normal subject, no significant difference could be detected between the two cell populations.

Stimulation of PBL in children with suspected immunodeficiency

Here, too, patients' lymphocytes were routinely compared in the same assay with lymphocytes from a normal subject. The patient's reactivity (SR patient/SR normal) was assessed relative to the 95% confidence limits (means ±2 s.d.) of the reactivity of the normal pairs, i.e., SR normal 1/SR normal 2. Since the mean ratio ±2 s.d. of twenty normal pairs stimulated by PHA was 1·09±0·74 (Table 2), patients'

lymphocytes were evaluated as being significantly hyporeactive if the ratio of SR patient/SR normal was below 0·35 (1·09–0·74) or significantly hyperreactive if above 1·83 (1·09±0·74). This approach is more demanding than that using Student's *t*-test.

The results summarized in Table 2 show that patients with lymphoproliferative disorders and ataxia telangiectasia (AT) were hyporeactive, whereas patients with Down's syndrome as a group were in the normal range, although some individual patients showed hyporeactivity. Most cases with autoimmune disorders were normal, except for two patients with nephrotic syndrome that had been treated with steroids for a prolonged period and were markedly hyporeactive. One case of rheumatoid arthritis treated with steroids was in the normal range. None of the patients suffering from various recurrent infections had a diminished response. On the contrary, several patients with recurrent respiratory infections were significantly hyperreactive.

DISCUSSION

With the accumulation of knowledge on the diversity of the immune system, it becomes important for the clinician to have a battery of immunological tests in order to define the immune status of patients. It is essential that these tests be simple and reliable, and because many of the patients with immune deficiencies are infants or children, the tests should require minimal amounts of blood or lymphocytes for analysis. Evaluation of the ability of lymphocytes to undergo blast transformation by mitogens such as PHA is used extensively as a measure of immunological competence. Lymphocyte transformation provides an appropriate means of assessing both genetic and acquired immunological deficiency states as well as for monitoring the effects of various immunoenhancing and immunosuppressive therapies. In such studies, degree of blast transformation can be determined by tritiated thymidine incorporation which requires at least 3 days in culture. Such a test can be cumbersome for routine clinical use because of the rigorous sterility requirements and the delay in obtaining results. Therefore, it seemed that measuring early stimulation of protein synthesis in activated lymphocytes after only one day in culture offers many advantages, including rapidity in obtaining results and low incidence of contamination. In addition, no serum is needed in the culture medium, thus avoiding the introduction of undesirable variables.

The early changes in protein synthesis following lymphocyte activation seem to represent the early stages of cellular events that ultimately culminate in DNA synthesis and cell division. This assumption is based on the correlation between the stimulation of protein and DNA synthesis in various systems. Gorczynski & Rittenberg (1974) have demonstrated that similarity to the stimulation of DNA synthesis, that of protein synthesis as a response to PHA and alloantigens was defined as T cell-dependent, while the response to lipopolysaccharide endotoxin was determined to be of B-cell origin. We have been studying various stimulatory as well as nonstimulatory plant lectins in this system and in all cases the patterns and extent of protein synthesis stimulation correlated well with previous reports in which stimulation of DNA synthesis was measured (Rosenberg *et al.*, 1972; Schechter *et al.*, 1976). The SPS assay which was first developed in laboratory animals was later adapted for use in humans, demonstrating that protein synthesis in human PBL can be evaluated in one-day cultures following stimulation by antigens, mitogens or allogeneic cells (Adkinson *et al.*, 1974). In a recent study we compared protein and DNA synthesis in the same individual using stimulation by PHA, Con A, PPD and allogeneic cells (Oppenheim & Schechter, 1976). The results indicated that while reaching the same incorporation (3×10^4 ct/min) with both ^{3}H-labelled thymidine and ^{3}H-labelled leucine, the incorporation background for DNA synthesis was 220 ct/min while that for protein synthesis was 5×10^3 cpm. The reason for the high background of radiolabelled leucine incorporation is that protein synthesis, unlike DNA synthesis, is an on-going process in resting lymphocytes. Since these background levels are reproducible and because of the small variations between replicate cultures, small increases in protein synthesis by stimulated cultures can be significant (for statistical analysis in stimulated human PBL, see Adkinson *et al.*, 1974).

Down's syndrome patients (trisomy 21) are known to suffer from increased susceptibility to infections and from a higher incidence of lymphatic leukaemia. We and others have shown that these individuals

have a partial T-system deficiency with thymic displasia (Schlesinger *et al.*, 1976), reduced percentage of T cells in the PBL (Levin *et al.*, 1975) and reduced leucocyte migration inhibition factor production by PHA-stimulated cells (Hahn *et al.*, 1976). In previously reported studies, the proliferative response in Down's patients as measured by thymidine incorporation was found to be contradictory, since low responsiveness was reported in some studies (Agarwal *et al.*, 1970; Hsia *et al.*, 1971), while in others the response was shown to be normal (Szigeti, Revesz & Schuler, 1974; Burgio *et al.*, 1975) or increased with suboptimal PHA concentration (Mayakawa *et al.*, 1971). With the SPS assay we found that the pattern and extent of stimulation in Down's patients was similar to their normal controls, although some patients had hyporeactive responses. As shown in Table 2, the variability as a group was not different than in testing pairs of normal subjects.

Ataxia telangiectasia is a familial disease associated with progressive cerebral ataxia and cutaneous or conjunctival telangiectasia. The frequent severe sinopulmonary infections in AT patients is due to a partial deficiency of the immune system, manifested by lymphopenia, impaired delayed hypersensitivity and absent or deficient immunoglobulin A (Petersen, Kelly & Good, 1964; Eisen *et al.*, 1965). An impaired lymphocyte response to PHA in this condition was found by Oppenheim *et al.* to be in part due to a plasma inhibitory factor (Oppenheim *et al.*, 1966; McFarlin & Oppenheim, 1969).

AT patients have a diminished number of PBL, although the percentage of T cells is usually normal. Therefore, in the test system equivalent numbers of lymphocytes from patients and normal controls included comparable proportions of T cells. We found that AT patients had diminished stimulatory response to all three mitogens, indicating that in addition to lymphopenia, the mitogenic response of the lymphocytes was impaired. Since in our study the use of serum was omitted, the low reactivity of the PBL appears to be due to an intrinsic defect. These results confirm a previous report using the same assay system (Adkinson *et al.*, 1974).

Four patients with acute lymphoproliferative disorders had markedly depressed stimulatory responses, more so than any other group. Patients with autoimmune disorders included a group of heterogenous disease states, their common denominator being a suspected autoimmune etiopathogenesis. Stimulation ratios in these patients were within the normal range, except for two nephrotic syndrome patients receiving prolonged steroids therapy who demonstrated a reduced response. However, one patient suffering from rheumatoid arthritis, who was also under steroid treatment, had a normal response. No diminution of stimulatory capacity could be shown in patients suffering from various recurrent infections. On the contrary, many were hyper-reactive, probably indicating a vigorous anti-infectious immune response.

In conclusion, SPS was found to be an additional convenient and reliable *in vitro* test of cellular immune reactivity. It has certain advantages over other assays of lymphocyte proliferation and has been shown to be useful in the evaluation of the cellular immune status in humans.

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