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In vitro T- and B-cell reactivity in cartilage hair hypoplasia

ANNAMARI RANKI, J. PERHEENTUPA, L. C. ANDERSSON & P. HÄYRY *Transplantation Laboratory, Department IV of Surgery, and Children's Hospital, University of Helsinki, Helsinki, Finland*

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SUMMARY

We have investigated *in vivo* and *in vitro* parameters of cellular immunity in ten patients with cartilage hair hypoplasia, CHH. All ten patients displayed a negative skin test to 1 Tu tuberculin, eight patients did not respond to 10 Tu and seven not even to 100 Tu. Six patients were skin test-negative to 1:50 oidiomycin; and three were negative to even 1:10 oidiomycin. The absolute and relative distribution of blood T lymphocytes was normal. The absolute distribution of blood B lymphocytes was slightly decreased in three patients, but all patients had normal levels of IgM, IgG and IgA. Major changes were observed in the proliferative responses of blood leucocytes to mitogens and antigens. Five patients responded suboptimally to phytohaemagglutinin (PHA), two to concanavalin A, three patients were hyporesponsive to *Staphylococcus aureus* strain Cowan I bacteria, six to tuberculin (PPD) and six to oidiomycin. The *in vitro* responses of two patients were entirely normal. Except for three patients responding suboptimally to PPD or oidiomycin only, the reduced responses were all confined to the five patients responding suboptimally to PHA. The suboptimally responding patients displayed normal dose–response profiles to both PHA and Con A.

The *in vitro* hyporeactivity was a persistent phenomenon, as the pattern of PHA and Con A reactivity was essentially similar when the same patients had been tested to the same mitogens 5 years earlier. Except for the skin test hyporeactivity, none of these patients displayed any detectable clinical handicap, suggesting that the Finnish variant of the syndrome is dissimilar from the Amish variant, where the *in vitro* deficiency to mitogens and antigens is related to susceptibility to severe and often fatal infections, especially to varicella and vaccinia.

INTRODUCTION

Cartilage hair hypoplasia (CHH) is a rare autosomal resessive syndrome with short-limbed dwarfism and often abnormally fine and sparse hair (McKusik *et al.*, 1965). The subjects are exceptionally susceptible to viral infections (Lux *et al.*, 1970; McKusick *et al.*, 1965). Lymphopenia, neutropenia, diminished delayed skin test reactivity to several antigens and decreased *in vitro* responsiveness of blood lymphocytes to phytohaemagglutinin (PHA) and in the mixed lymphocyte culture (MLC) have been reported by Lux *et al.* (1970) and Hong *et al.* (1972) in patients of Amish origin.

This condition is exceptionally common in the Finnish population (Norio, Nevanlinna & Perheentupa, 1973). In an earlier study from this hospital, Virolainen *et al.* (1977) investigated twenty-eight unselected cases of CHH. None of the patients had severe infections, and all of them were immunized at birth with BCG and most with vaccinia, without complications. All patients were negative in skin test reactivity to 1 Tu PPD. *In vitro* PHA transformation was significantly depressed in twenty-five cases, and abnormally low concanavalin A (Con A) reactivities were also observed.

In this study we have investigated the persistence of the mitogen hyporeactivity, by comparing current

Correspondence: Dr P. Häyry, Transplantation Laboratory, University of Helsinki, Haartmaninkatu 3, SF 00290 Helsinki 29, Finland.

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PHA and tuberculin (PPD) responses of ten patients to corresponding responses of the same patients 5 years earlier. We have also quantified the absolute and relative distribution of the major T- and B-lymphocyte subclasses in the blood of these patients, and tested the reactivity of their blood leucocytes to pokeweed mitogen and to the exclusive B-cell mitogen *Staphylococcus aureus* strain Cowan I bacteria (Forsgren, Svedjelund & Wigzell, 1976). The results demonstrate a persistent hyporeactivity of selected patients to PHA and PPD. The same patients that display depressed *in vitro* responses to PHA often respond also suboptimally to Con A, *S. aureus*, PPD and oidiomycin.

MATERIALS AND METHODS

Subjects. Ten subjects with CHH were investigated (Table 1). The age range was 6–27 years. The clinical features of these patients were characteristic of the syndrome. The height of all the subjects was more than 3·0 s.d. below the mean for their age and sex, and their limbs were abnormally short in relation to the length of the trunk. The usual changes of the metaphyses of the tubular bones were always present and ranged from mild irregularities to scalloped, sclerotic and cystic areas observed in radiography. In only one subject (J.T.) the hair was normal, but the brother of this subject (K.T.) was also affected, and in other respects the subject represented the typical clinical picture. The intelligence level of all these subjects was normal. Congenital megacolon was present in one case. Intestinal malabsorption was present in none. The history of past infections, as well as physical signs of chronic infections, were recorded. None of the subjects represented any clearly increased proneness to infections.

TABLE 1. Age, sex and skin test reactivity of patients with CHH

Subject	Sex and age	Skin tests*	
		Tuberculin (Tu)	Oidiomycin (dilution)
K.T.	M, 18	10(+)	1:50(+)
J.S.	M, 7	100(−)	1:50(+)
J.T.	M, 17	100(+)	1:500(+)
R.M.-K.	M, 27	10(+)	1:10(−)
L.S.	M, 6	100(−)	1:10(+)
M.M.	M, 14	100(−)	1:10(+)
K.N.	F, 8	100(−)	1:10(−)
H.S.	M, 13	100(−)	1:10(+)
M.E.	F, 8	100(−)	1:10(−)
P.K.	M, 21	100(−)	1:50(+)

* The figures represent highest test concentration employed. Concentrations lower than those indicated in the Tables gave a negative result. (+) Positive, (−) negative result.

Immunoglobulin determinations. The serum levels of IgG, IgA and IgM were measured by single radial immunodiffusion and expressed as g/l (Immonen, 1967).

Skin testing. Serial intracutaneous tuberculin testing was performed, starting from 0·1 Tu of purified tuberculin (PPD, Statens Seruminstitut, Copenhagen). Eight subjects were tested up to 100 Tu, or until a positive reaction was observed. Oidiomycin (dermatophytin O, Hollister–Stier Laboratories, Spokane, Washington, USA) was tested at dilutions of 1:500, 1:50 and 1:10.

Preparation of lymphocytes. Venous blood (2–5 ml) was obtained by venipuncture into a heparinized syringe (1:100 heparin–Medica, Helsinki, no preservative added). Blood films were made for differential counts and for ANAE staining, and the white cell count was performed with acetic acid. The blood was diluted 1:3 in PBS, layered on Ficoll–Isopaque, density 1·087 g/ml (Böyum, 1968), and was centrifuged for 30 min at 300 *g*. The lymphocyte-enriched population was recovered at the interphase. It contained approximately 70–80% lymphocytes, 15–25% monocytes and few neutrophilic leucocytes. We have earlier tested and found that no subclass-specific lymphocyte depletion takes place during the centrifugation procedure (Häyry, Tötterman & Ranki, 1977).

Distribution of T and B lymphocytes. The sheep erythrocyte rosette-forming lymphocytes (SRBC-RFL). These were quantified by using the 2-aminoethyl-isothiouronium bromide-hydrobromide (AET) method of Pellegrino *et al.* (1975) as reported in

detail elsewhere (Ranki, Tötterman & Häyry, 1976). The rosettes were prepared by incubating 50–100 μl of 1% SRBC suspension with 50–100 μl of lymphocyte suspension containing 2–4 $\times 10^6$ cells per ml in RPMI 1640 medium. The cells were immediately centrifuged in round-bottom Cooke microtitre plates for 1 min at 200 *g*. After centrifugation, the rosettes were gently suspended, and the cells were cytocentrifuged onto microscope slides (300 *g* for 5 min). The slides were stained with May–Gruenwald–Giemsa (MCG). Lymphocytes binding five or more erythrocytes were counted as rosette-forming lymphocytes.

The Fc receptor-carrying lymphocytes (EA–RFL). These were detected by antibody-coated human O red cell indicator cells. Human red cells were washed with PBS, incubated for 30 min at 37°C in the presence of the first subagglutinating dilution (1:600) of rabbit anti-human red cell IgG, washed three times in PBS and finally diluted into a 1% suspension in Dulbecco's balanced salt solution. 50–100 μl of red cell suspension and 50–100 μl of lymphocyte suspension containing 2–4 $\times 10^6$ lymphocytes per ml were distributed onto the microtitre trays and incubated for 15 min at room temperature. After centrifugation (1 min at 200 *g*) the cells were incubated for another 15 min at 4°C, the rosettes were disrupted and the cells were centrifuged onto microscope slides. The slides were stained with MGG. Lymphocytes binding four or more erythrocytes were regarded as rosette-forming.

Surface Ig-carrying lymphocytes (S. aureus RFL). These were identified by rosetting anti Ig-coated lymphocytes with *Staphylococcus aureus* strain Cowan 1 bacteria (Ghetie, Nilsson & Jöquist, 1974; Ranki *et al.*, 1976). 100 μl of lymphocyte suspension (4 $\times 10^6$ cells per ml) in HEPES-buffered RPMI 1640 medium containing 0·5% BSA and 0·02% sodium azide were distributed in round-bottom microtitre trays, incubated with 100 μl of 1:25 diluted polyvalent rabbit anti-human Ig (anti-IgM, -IgG and -IgA) for 30 min at 4°C, and were washed three times. Thereafter, 50 $\times 10^6$ formalin-treated, heat-inactivated *S. aureus* bacteria (a gift from Professor H. Wigzell, University of Uppsala, Sweden) were added in 100 μl volumes per well. The tray was centrifuged for 5 min at 250 *g*, and incubated for 30 min at 4°C. The rosettes were gently suspended and cytocentrifuged onto microscope slides. The slides were stained with May–Gruenwald–Giemsa. Lymphocytes binding approximately twenty or more bacteria on their surface were scored positive.

Demonstration of ANAE activity. The demonstration of esterase activity was performed in conditions described by Mueller *et al.* (1975) with minor modifications (Ranki *et al.*, 1976). Blood films or cytocentrifuged cell smears were incubated in a medium consisting of 40 ml of 0·067 M phosphate buffer, pH 5·0, 2·4 ml of hexazotized pararosaniline and 10 mg of α-naphthyl acetate (Sigma, St Louis, Missouri, USA.) in 0·4 ml of acetone. The mixture was adjusted to 5·8 using 1 N NaOH. Smears were incubated at room temperature for 8–17 hr, washed in distilled water for 10 min, dried and counter-stained in 1% aqueous toluidine blue solution for 60 min. This counter-staining gave an excellent morphology to the preparations, and lymphocytes were easily distinguished from monocytes or from other white blood cells. The quantification of ANAE marker-carrying lymphocytes was performed on cytocentrifuged preparations of Ficoll–Isopaque-purified white blood cells, or directly from blood smears. As reported before (Hayry *et al.*, 1977), the results did not significantly differ from each other. A lymphocyte was scored as ANAE-positive if its cytoplasm contained a single or a few distinct spots of the reddish brown reaction product.

Lymphocyte cultures. The lymphocytes were washed twice with Hanks' balanced salt solution (HBSS) and suspended in a 4 $\times 10^6$ cells per ml concentration of RPMI 1640 medium (Orion Pharmaceuticals, Helsinki, Finland) without serum. The lymphocyte cultures were performed in Falcon Microtest II (Falcon Plastics, Los Angeles, California, USA.) tissue culture trays by employing 4 $\times 10^5$ responder cells per well in 200 μl of culture medium.

For the stimulation of T cells, a 1:150 final dilution of phytohaemagglutinin (PHA-M, Difco Laboratories, Detroit, Michigan, USA.) or 2·5 μg/ml of Con A (a gift from Professor H. Wigzell) was added to the cultures. In the dose–response experiments, purified leukoagglutinin (LA, Pharmacia Chemicals, Uppsala, Sweden) was substituted for PHA. Pokeweed mitogen (PWM, Difco Laboratories) stimulating both T and B cells was used in the optimal dilution of 1:160. The culture medium was RPMI 1640 supplemented with freshly added glutamine, penicillin (50,000 iu/100 ml) and streptomycin (50 mg/100 ml), plus 3% heat-inactivated human AB serum.

For the stimulation of B cells we used 10^7 heat-killed *S. aureus* per well. The B-cell stimulation was performed under serum-free conditions (Forsgren *et al.*, 1976).

For the stimulation with the two antigens, purified PPD was added to cultures at 625 Tu/ml concentration and Oid. (dialysed extensively against water) at a concentration 1:60. These concentrations were tested and found to give maximal proliferative responses in our conditions.

All cultures were incubated in a humidified atmosphere of 5% CO_2 in air. The peak responses were recorded on day 3 (PHA-, Con A-, and PWM-stimulated cultures), day 4 (*S. aureus*-stimulated cultures) and days 5–6 (antigen-stimulated cultures), as verified by also testing the proliferation on the days immediately before and after the expected maximal response. The responses were quantified by the [^{3}H] incorporation after 18 hr pulse-labelling with 20 μCi of [^{3}H]thymidine (sp. act. 1 mCi/ml, New England Nuclear, Boston, Massachusetts, USA.) and harvesting of the culture with the Scatron (Flow Laboratories Inc., Irvine, Scotland) semi-automatic cell harvester.

RESULTS

Serum immunoglobulins and skin test reactivity

The serum immunoglobulin levels of CHH patients were normal: IgM 1·1 $\pm$ 0·4 (0·9 $\pm$ 0·2) g/l; IgG

12·1±1·9 (12·1±2·2) g/l; and IgA 0·7±0·3 (0·9±0·3) g/l as compared to age-matched healthy control subjects (indicated in parentheses). Both the relative and absolute distribution of blood neutrophils, 50±14% (44±11%) and 2·6±0·8 (2·6±0·9)×10^9 g/l, were equally normal.

All patients were skin test-negative to 1 Tu PPD, two responded to 10 Tu and one to 100 Tu. The remaining seven patients did not respond to even 100 Tu PPD. One patient gave a positive skin test reaction to a 1:500 dilution of oidiomycin three to 1:50 and three to 1:10. Three patients did not respond to any oidiomycin dilution tested (Table 1).

Distribution of the major lymphocyte subclasses in the blood

Both the mean percentage and absolute distribution of ANAE marker carrying lymphocytes and lymphocytes forming rosettes with AET-sensitized SRBC (T lymphocytes) were within normal limits compared to age-matched controls (Table 2). The mean distribution of surface Ig-carrying lymphocytes (*S. aureus*) was, however, slightly though significantly decreased. No significant differences were observed in the mean number of lymphocytes expressing the surface Fc receptor for IgG. Only one individual had a decreased relative number of ANAE marker-carrying lymphocytes (within 95% confidence limits; and two individuals demonstrated decreased absolute numbers of ANAE-, Fc receptor- and surface Ig-carrying lymphocytes.

In vitro responses of the lymphocytes to mitogens and antigens

The mean reactivity of blood lymphocytes to the mitogens PHA and PWM was significantly depressed (Table 3). The reactivity to *S. aureus* was on the normal control level. The mean *in vitro* reactivity of the blood lymphocytes to the antigens PPD and oidiomycin was also depressed. The mean reactivity to Con A and PWM was lower than in the controls, although the difference was significant only with PWM.

Five of the patients displayed a significantly (more than 95% confidence limits) depressed response to PHA, and two to Con A. Only one patient was significantly hyporeactive to PWM. Three of the patients were significantly hyporeactive to the B-cell mitogen *S. aureus*, six patients were hyporeactive to PPD and six to oidiomycin. As seen in Table 3, the patients who did not respond to the PHA were usually also hyporeactive to the rest of the mitogens and antigens, whereas patients responding well to PHA usually also responded to the rest of the mitogens.

Persistence of hyporeactivity to PHA and PPD

All patients reported in this study were tested for blood lymphocyte distribution and for mitogen or antigen reactivity at least twice during a period of 1 year. No major changes were observed. All ten patients reported here had also been tested for *in vitro* responses to PHA and PPD 5 years earlier. In order to elucidate how persistent the *in vitro* hyporeactivity was to the mitogens, the responses were compared. The results of these comparisons are given in Table 4.

The five patients demonstrating a significant hyporeactivity, to PHA now had also been hyporeactive 5 years earlier, while four out of five patients demonstrating a normal response now had also responded normally before. All six patients who were significantly hyporeactive to PPD today had also been weakly responsive 5 years earlier. The two patients giving strongest PPD responses now had also been the strongest responders in 1972.

Dose–response to PHA and Con-A

To exclude the possibility that the lymphocytes of mitogen-hyporeactive patients would respond optimally in higher or lower mitogen concentrations than normal individuals, the dose–response profiles were determined for two patients (R.M.-K., M.E.) and for three control subjects. Both the strongly hyporeactive patient (M.E.) and the weakly hyporeactive patient (R.M.-K.) demonstrated (Fig. 1) dose–response curves parallel to those of healthy subjects, with lower responses throughout the mitogen concentration scale of 0·1–300 μg/ml.

TABLE 2. Mean relative (%) and absolute ($\times 10^9$/l) distribution of four lymphocyte subclasses in CHH and in normal control subjects

Distribution	Patients	Marker				
			T lymphocytes			
		Lymphocytes	ANAE	AET–RFC	Fc (EA)	B lymphocytes, SIg (*S. aureus*)
Relative distribution of lymphocyte subclasses (%)	Patients ($n = 10$)	42±11	63±6	64±7	17±3	9±2
	Controls ($n = 8$)	48±11	70±8	68±9	21±5	12±5
	(P)	(n.s.)	(n.s.)	(n.s.)	(n.s.)	(0·01)
Absolute distribution of lymphocyte subclasses ($\times 10^9$/l)	Patients ($n = 10$)	2·4±1·1	1·5±0·8	1·5±0·6	0·4±0·2	0·2±0·1
	Controls ($n = 8$)	2·8±1·2	2·0±0·7	1·9±0·8	0·6±0·2	0·4±0·1
	(P)	(n.s.)	(n.s.)	(n.s.)	(< 0·1)	(< 0·001)

TABLE 3. T and B mitogen and antigen-induced lymphocyte proliferation

	Response ($log_{10} \times 10^3$/min)						
	PHA (3)*	Con A (3)	PWM (3)	*S. aureus* (4)	PPD (6)	Oidiomycin (6)	BP† (4)
K.T.	1·97	1·81	1·60	1·18	1·43	0·78	0
J.S.	2·32	1·99	1·83	1·54	**0·60**	0·9	0·45
J.T.	2·16	1·86	1·74	1·53	0·90	1·15	0
R.M.-K.	1·86	1·46	1·70	1·38	2·05	**0·30**	0
L.S.	1·94	1·90	1·65	1·54	0·78	**0·30**	0·15
M.M.	**1·08**‡	1·43	1·68	**−1·00**	**0·3**	**−0·52**	0
K.N.	**0·95**	**0·9**	1·96	**−0·40**	**0·35**	**0·50**	−0·52
H.S.	**1·15**	1·34	1·53	0·90	**0·6**	**−0·52**	0·90
M.E.	**0·48**	**0·00**	1·46	n.d.	**0·0**	**−1·00**	−0·30
P.K.	**1·40**	1·58	**1·20**	**0·25**	**−0·30**	1·04	−0·42
Mean ± s.d. (n = 10)	1·53 ± 0·57	1·42 ± 0·56	1·63 ± 0·19	0·76 ± 0·8	0·67 ± 0·64	0·20 ± 0·70	0·03 ± 0·39
(P)	< 0·01	n.s.	< 0·01	n.s.	< 0·002	< 0·02	n.s.
Controls (n = 12)	2·18 ± 0·19	1·74 ± 0·28	1·98 ± 0·27	0·97 ± 0·27	1·76 ± 0·27	1·04 ± 0·28	0·02 ± 0·26

* Figures in parentheses indicate the day the response was quantitated.
† Background proliferation in non-stimulated control cultures.
‡ Abnormally low values with 95% confidence limit given in bold.

TABLE 4. PHA and PPD reactivity in ten patients with CHH in 1972 and 1977

Subject	*In vitro proliferative response**			
	PHA		PPD†	
	1972	1977	1972	1977
K.T.	71	51	10	34
J.S.	90	114	6	5
J.T.	84	79	2	10
R.M.-K.	**37**	39	68	140
L.S.	65	48	6	8
M.M.	**30**	**7**	0	**3**
K.N.	**47**	**7**	0	**3**
H.S.	**25**	**8**	2	**5**
M.E.	**11**	**2**	0	**1**
P.K.	**53**	**11**	0	**1**
Abnormally low responses	6/10	5/10	0/10	6/10

* Percentage of normal control responses; figures in bold represent significantly suppressed responses with >95% confidence limits.

† In 1972 blast cell differential counts were used to quantify the response. Hence the comparisons are given as a percentage of the normal control. Due to the large variation in the blast cell differential count method, none of the PPD responses were significantly (95% confidence limit) depressed in 1972 (see Virolainen *et al.*, 1977).

DISCUSSION

The diagnosis of CHH is based on clinical findings. Short-limbed dwarfism with methaphyseal dysplasia was present in all ten patients, and only one of the patients did not have the abnormally thin hair characteristic of the syndrome (Lux *et al.*, 1970; Virolainen *et al.*, 1977).

While the absolute and relative distribution of T and B lymphocytes was grossly normal, the *in vitro* blood lymphocyte reactivity of these patients was clearly abnormal. Two groups of patients were easily distinguished: one group (five patients) with entirely normal *in vitro* mitogen responses, but occasionally depressed responses to one test antigen only; and the other (five patients), with overall depressed responses to most test mitogens and antigens. The inability to mount a 'normal' *in vitro* response to these substances was clearly long-lasting, as the pattern of reactivity was essentially similar in tests performed 5 years earlier. As the stimulations were performed in serum-free conditions or in the presence of pooled human AB plasma, 'serum blocking' factors cannot be suggested as the cause of the inhibition. In contrast to the mitogen reactivity, all patients—including those with depressed B-cell responses—displayed a normal level of immunoglobulins in their circulation.

Considering the depressed *in vitro* responses to mitogens, and the inability of these patients to mount a normal skin test to PPD, one would expect these patients to have an immunological handicap. We could not find such a handicap. None of the hyporeactive patients, nor the normally responding subjects, had a history of susceptibility to infections. In a radiological examination of the lungs, performed regularly every second year, no evidence of tuberculosis was found. Neither was a history of candidiasis or complicated varicella recorded in any one of these patients.

The notable absence of clinically manifest infections in the Finnish patients is in contrast to the CHH patients of Old Order Amish origin (Lux *et al.*, 1970) and other reported cases from the United States

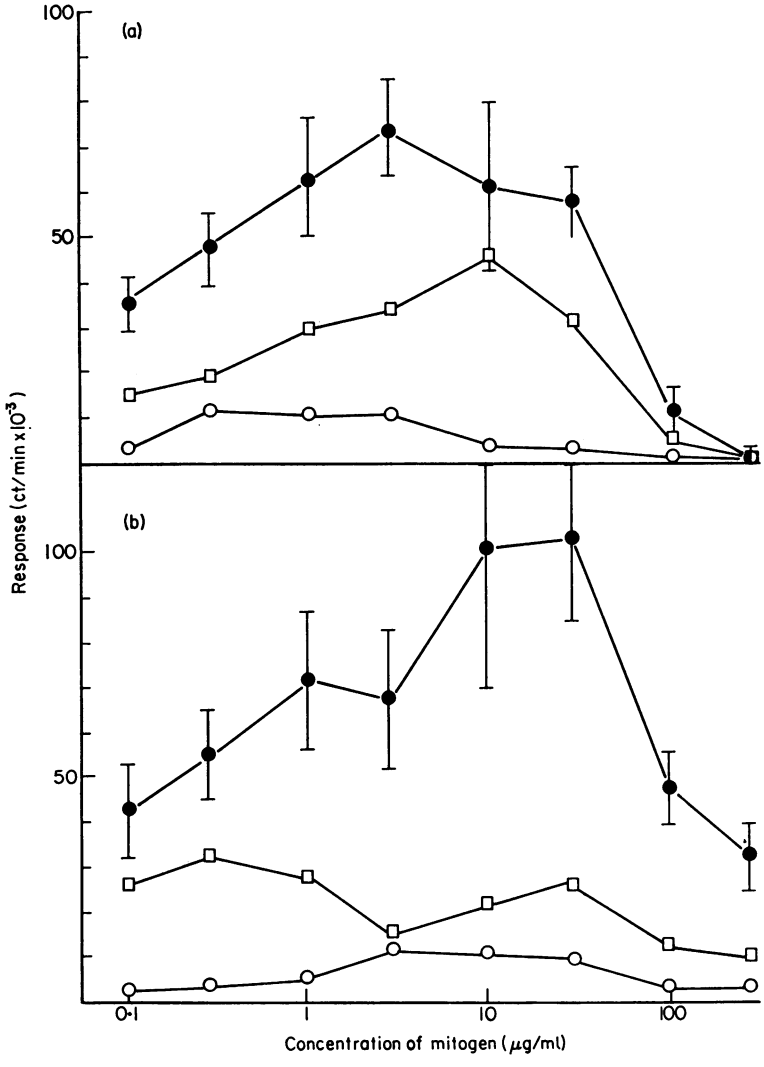

FIG. 1. Dose–response profiles of blood lymphocytes of two patients, (□) R.M.-K. and (○) M.E., and (●) three healthy control subjects to (a) Con A and (b) leukoagglutinin. Each value represents mean value of three determinations; the bars represent standard deviations between the control determinations.

(Hong *et al.*, 1972). It seems apparent that reduced or deficient *in vitro* reactivity of T and B lymphocytes to mitogens or antigens is not necessarily linked to a reduced resistance to infectious organisms, but that other factors must also be present. One such factor may be neutropenia and/or an impairment in neutrophilic function, as none of our patients showed reduced numbers of neutrophilic granulocytes, but at least some of the Amish cases suffered neutropenia. The other factors contributing to increased susceptibility to infections are presently unknown.

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