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In vitro Lymphocyte Response in Some Immunity Deficiency Diseases and in Intrauterine Virus Infections

Immunity deficiency diseases may be classified as humoral immunity deficiency, cellular immunity deficiency or combined forms (Soothill 1968). Such cases are being recognized more frequently in infants and children due to an increased awareness of the clinical syndromes by pædiatricians, a realization of their frequency (Berry 1968, Berry & Thompson 1968) and the development of laboratory tests for immunological function. Tests for cellular immunity function are far less satisfactory than those for humoral immunity function.

In this communication preliminary data are presented on *in vitro* lymphocyte responses to stimulation with phytohæmagglutinin (PHA) as a test of cellular immune function as applied to the investigation of patients with some immunity deficiency syndromes and with intrauterine virus infections.

Materials and Methods

Peripheral lymphocytes were obtained from 5–10 ml of venous blood and defibrinated in siliconized universal containers with 4 mm glass beads. They were separated from red cells and polymorphs by mixing with methyl cellulose and carbonyl iron and allowing to stand. Over 90% of cells in the supernate were lymphocytes. The tissue culture medium used was medium 199, to which 20% fresh human serum from a pool of two young healthy adult donors (or occasionally autologous serum) was added. $0{\cdot}5 \times 10^6$ lymphocytes were suspended in 0·5 ml of this medium and incubated at 37°C for three days in an upright position in 12×100 mm Pyrex tubes stoppered with white rubber bungs. 0·05 ml of a 1:25 dilution of phytohæmagglutinin (Difco PHA-M) was added at the onset of the culture period, to triplicate or duplicate tubes. 0·5 μCi of tritiated thymidine (^{3}HT) (specific activity 5 Ci/mmol) was added to each culture two hours before termination by the addition of ice cold saline. The cells were then washed three times in ice cold saline (0·4%) and the tritium-labelled DNA protein precipitated by ice cold 5% trichloracetic acid. The precipitates were dissolved in Nuclear Chicago solubilizer and prepared for counting by addition of toluene scintillator fluid. The results were expressed as counts per min per $0{\cdot}5 \times 10^6$ lymphocytes after correction for quenching. Control cultures of lymphocytes from young healthy adult volunteers were run in parallel with the test cultures.

Immunoglobulins were estimated by a radial gel diffusion method (Mancini *et al.* 1965); results are expressed as percentage of MRC reference standard serum for immunoglobulins G, A and M.

Patients

The patients studied were: (1) Infants and children with severe combined immunity deficiency syndrome (CIDS). (2) Patients with sex-linked hypogammaglobulinæmia, ataxia telangiectasia, or Wiskott-Aldrich syndrome. (3) Patients with congenital rubella and cytomegalovirus infection.

(1) *Severe combined immunity deficiency syndrome:* Seven of 9 patients (Cases 1–7) comprising 5 males and 2 females (Table 1) presented with very similar clinical features. They were only a few months old and in those who were not first-born there was a history of a sibling who had died in infancy of a similar disorder. Infections, bacterial, viral, fungal and protozoal (there were three instances of *Pneumocystis carinii* pneumonia) started within the first few weeks or months of life. These infections were persistent or recurrent. Diarrhœa and failure to thrive were very frequent. Lymphocyte counts eventually were very low ($<1{,}000/mm^3$) but earlier in the illness, and intermittently throughout, higher counts were

Table 1
Combined immunity deficiency syndrome

Case No.	Sex	Family history	Age at onset of symptoms	Recurrent infections	Diarrhœa	Failure to thrive	Lympho-penia	Hypogamma-globulinæmia	
1	M	+	2 months	+	−	+	+	+	Died 8 months
2	F	−●	5 months	+	+	+	+	+	Died 20 months
3	M	+	2 weeks	−	+	+	+	±	Died 7 weeks
4	M	−■	10 weeks	+	+	+	+	+	Died 11 months
5	M	−■	6 weeks	+	+	+	+	+	Died 6 months
6	F	+	3 months	+	−	+	+	+	Alive 6 months
7	M	−	2 months	+	+	+	+	+	Alive 9 months
8	M	−■	3 years	+	+	−	+	+	Died 6 years
9	F	+	3 years	+	−	−	+	+	Died 14 years

●Adopted ■First child

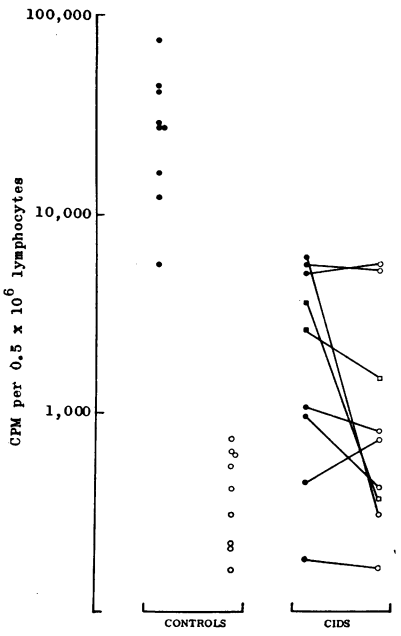

Fig 1 In vitro *lymphocyte stimulation in combined immunity deficiency syndrome.* ●, ■, *PHA.* ○, □, *unstimulated.* ●, ○, *Cases 1–7.* ■, □, *Cases 8 and 9*

noted. Serum immunoglobulins were sometimes severely depressed though the interpretation of the IgG values was difficult because of passively transferred maternal IgG and in some cases injected IgG.

Replacement therapy with γ-globulin, antibiotics and antifungal agents resulted in some control of the infections, and grafting of immunologically competent cells was attempted in some cases without permanent success. There was generally a remorseless progression of the disease process, leading to death in the first year of life.

Two of the patients (Cases 8 and 9) differed in that the onset of infections did not occur until they were 3 years of age and they survived for several years. In one a sibling had died in infancy of a syndrome similar to the first group of patients. Lymphopenia, hypogammaglobulinæmia and negative skin tests to candida, tuberculin and dinitrofluorobenzene (DNFB) were noted in these 2 patients.

The responses of the lymphocytes from these 9 patients to *in vitro* stimulation with PHA (Fig 1) were lower than in the young healthy adult controls tested in parallel but there was a wide range of activity. There was also a wide range of activity of cultures of unstimulated lymphocytes which in some cases was considerably greater than that of unstimulated cultures from the healthy adults. In the comparison of the activity of PHA-stimulated cultures and unstimulated cultures from individual patients three types of response were noted. In some, the activity in both sets of cultures was very low. In some there was a higher level of activity, which was similar in both stimulated and unstimulated cultures. In these cases activity occurring in PHA-stimulated cultures is therefore probably due to the spontaneous activity and is not an effect of PHA. In others, two of whom were Cases 8 and 9, some activity occurred after PHA stimulation which was in excess of that which occurred in the unstimulated cultures. These different responses were not related to the total lymphocyte counts in individuals at the time of testing but were consistent in each patient on repeated testing.

There is no simple way of expressing these results. In Fig 2 sequential results from patients who were tested more than once are expressed as PHA-stimulated count/unstimulated count, which eliminates the effects of high spontaneous activity. There is an impression in some patients that the lymphocyte response to PHA decreases with time.

(2) *Patients with sex-linked hypogammaglobulinæmia, ataxia telangiectasia, or Wiskott-Aldrich syndrome:* Six patients with sex-linked hypogammaglobulinæmia – two sets of brothers and a boy with an affected first cousin (Table 2) – were studied. In spite of profound immunoglobulin deficiency they have remained remarkably well on γ-globulin replacement therapy.

The two boys (brothers) with ataxia telangiectasia (Table 2) are well except for the neurological manifestations of the syndrome. They do not experience recurrent sinopulmonary infections and the levels of IgA are normal. All 3 boys with the Wiskott-Aldrich syndrome had recurrent infections; 2 have affected siblings. In one some

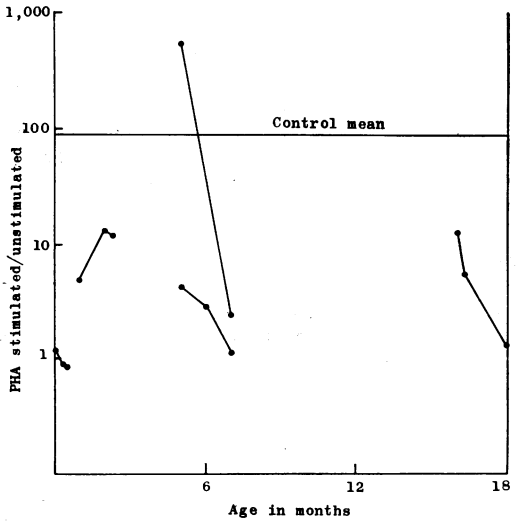

Fig 2 *PHA responses in CIDS*

Table 2
Immunoglobulin levels and lymphocyte counts in patients with sex-linked hypogammaglobulinæmia, ataxia telangiectasia and Wiskott-Aldrich syndrome

Case No.	Age (years)	Immunoglobulins % IgG	IgA	IgM	Lymphocyte count/mm³
Sex-linked hypogammaglobulinæmia					
1	5●	6	1	75	
	18	48	2·5	2	2,350
2	11●	1·5	0	5	
	23	33	2	2	—
3	4●	10	25	175	
	12	40	2	50	3,640
4	8/12●	8	5·5	35	
	7½	12	2·5	5	3,150
5	7●	12	25	12	
	15½	41	4	5	—
6	6½●	13·6	2·5	16	5,625
Ataxia telangiectasia					
1	10	1,680	156	172	1,480
2	7	2,040	132	188	1,924
Wiskott-Aldrich syndrome					
1	8/12	200	200	200	2,100–5,700■
2	1½	156	132	200	740–2,400■
	2	40	144	180	
3	8/12	92	56	60	1,150–2,800■
	1	54	88	20	

●Immunoglobulin levels before treatment with γ-globulin
■Range of lymphocyte counts

fall in IgG concentration and lymphocyte count has occurred.

The response to PHA stimulation of these 3 groups of patients fell within the range of the control subjects (Fig 3). The similar findings in hypogammaglobulinæmia are consistent with previous reports (Ling & Soothill 1964, Cooperband *et al.* 1966, Gotoff 1968). In ataxia telangiectasia a range of response to PHA stimulation has been reported (Oppenheim *et al.* 1966, Naspitz *et al.* 1968, Gotoff 1968) possibly reflecting the degree of the cellular immunity deficiency, which in some cases may be progressive. Serial studies on these patients will therefore be important. Immunological defects which occur in Wiskott-Aldrich syndrome are complex, variable and not clearly defined. Deterioration may occur with age (Cooper *et al.* 1968) so serial studies will also be of importance in this group.

Unstimulated activity in these three states was within the range of the controls but there was a trend for higher levels in the patients with Wiskott-Aldrich syndrome.

(3) *Congenital rubella and cytomegalovirus infection:* Intrauterine infection with these viruses, besides producing multisystem disease, results in persistent infection for varying periods after birth, together with the development of a specific antibody response. A further complexity is the occasional association with IgG deficiency (Soothill *et al.* 1966, Plotkin *et al.* 1966, Hancock *et al.* 1968) and abnormal susceptibility to infections in spite of raised IgM levels. *Pneumocystis carinii* pneumonia, which is thought to be associated with deficiency of cellular immunity, has been reported in patients with congenital rubella (Lingeman *et al.* 1967). Recurrent or persistent diarrhœa may also occur (Miller & Thorburn 1966). Abnormalities of the thymus have been reported, ranging from absence of Hassall's corpuscles to severe lymphocyte depletion (Berry & Thompson 1968). Thymic weight may be considerably reduced (Naeye & Blanc 1965, Thorburn & Miller 1967). Absence of epithelial elements and Hassall's corpuscles have been noted in rubella-infected fœtuses at a stage of development where they are usually present. Lymphocyte reactivity has consequently received some attention. Failure of lymphocytes to respond to PHA was observed by Olson *et al.* (1967) in some congenital rubella babies and this was attributed to virus being present in the lymphocytes (Montgomery *et al.* 1967). Simons & Fitzgerald (1968) have also reported decreased responses to PHA in younger patients, but have stressed the importance of the dose dependency of the response to PHA in different individuals.

Our patients with congenital rubella have been divided into two groups: (*a*) Those aged 6 months to 11 years from whom virus was not isolated from the nasopharynx or urine. (*b*) A group aged 2 days to 7 weeks from the majority of whom virus was isolated. The activity of PHA-stimulated lymphocytes was not depressed in any of these

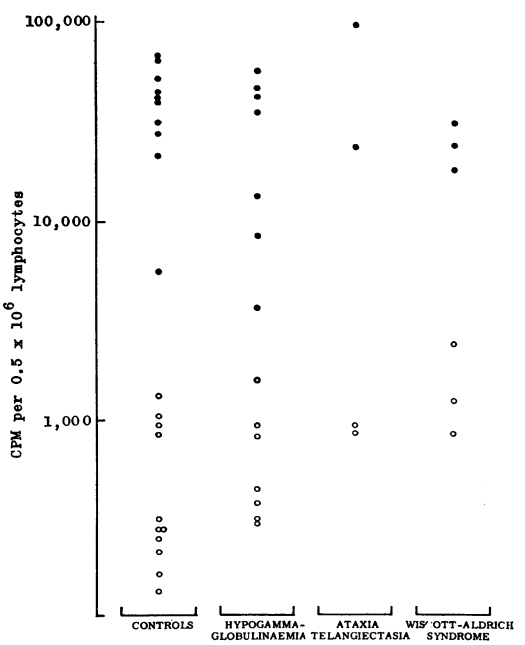

Fig 3 In vitro *lymphocyte stimulation in sex-linked hypogammaglobulinæmia, ataxia telangiectasia and Wiskott-Aldrich syndrome* ●, *PHA.* ○, *unstimulated*

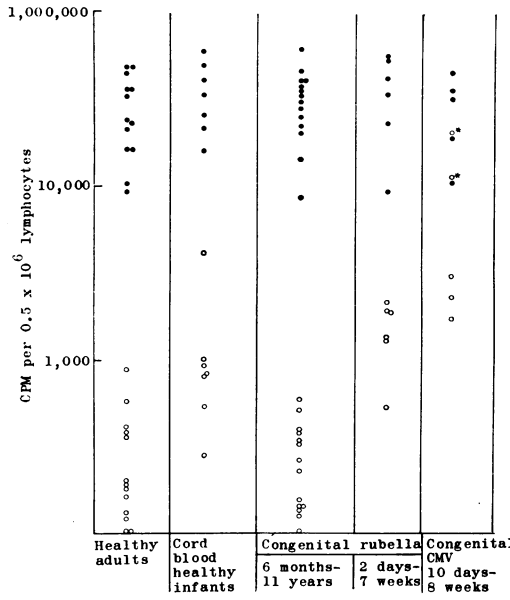

Fig 4 In vitro *lymphocyte stimulation in congenital rubella and cytomegalovirus infection.* ●, *PHA.* ○, *unstimulated.* ★, *blood transfusion 1 and 3 weeks before test (on same patient).* CMV, *cytomegalovirus*

patients (Fig 4). Of particular interest were 2 patients, one of whom, aged 9 days, is included in Fig 4 (the other, aged 3 weeks, has been seen since), from whose lymphocytes the rubella virus was isolated; the same lymphocyte preparation showed a normal activity following PHA stimulation (counts per minute, PHA-stimulated, 42,042 and 15,019; counts per minute, unstimulated, 1,311 and 948). There was a higher level of activity of the unstimulated lymphocytes in most of the younger patients compared with the older ones or the controls. The findings in the patients with congenital cytomegalovirus infection were similar; virus was isolated from all of them, the response to PHA stimulation was not depressed and the unstimulated cultures showed increased activity. In one patient unstimulated activity was remarkably high when tested on two occasions, but lymphocytes in this case were obtained one and three weeks after a transfusion of whole blood for anæmia. We do not know whether this can explain such activity.

Only a few cultures of lymphocytes from cord bloods of normal infants have so far been examined, and there is no difference in the PHA response from that of the adult controls. The unstimulated activity was in the upper range of that found in the controls. Pulvertaft & Pulvertaft (1966) and Leikin *et al.* (1968), using counts of blast cells or of labelled cells, found increased spontaneous transformation in cord blood lymphocytes from healthy infants. Although the numbers are too small for analysis, the unstimulated activity in young infants congenitally infected with rubella and cytomegalovirus is generally higher than that in normal cord blood lymphocytes.

Increased unstimulated activity in some patients with congenital rubella, cytomegalovirus infection or combined immunity deficiency syndrome (including some over 6 months of age) requires explanation. Possible explanations are:

(*a*) Cells in the peripheral blood recognized morphologically as small lymphocytes may be heterogeneous, with different rates of metabolic activity, and there may be differential deficiency in these different populations in the combined immunity deficiency syndrome.

(*b*) *In vivo* lymphocyte stimulation may have occurred in these patients as an immunological reaction to the infecting bacteria, fungi or viruses.

(*c*) Persistence of maternal lymphocytes, stimulated by the infant's lymphocytes or *vice versa*, may be responsible for increased activity. This may particularly occur in congenital rubella and cytomegalovirus infection, as a result of placental damage by these viruses.

Deficiency of PHA response in immunity deficiency appears to be related to the phenomenon we regard as cellular immunity deficiency and it therefore provides a useful quantitative test in this field. Technical and interpretative problems remain, however, and it is likely that the response to specific antigens will provide a useful further measurement for investigating these complex problems.

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