T-Lymphocyte Differentiation In Vitro in Severe Combined Immunodeficiency

DEFECTS OF STEM CELLS

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ABSTRACT A study of T-lymphocyte differentiation was made on fractionated bone marrow cells from normal volunteers and from 11 patients with severe combined immunodeficiency (SCID) using normal thymic epithelial monolayers and their culture supernates as inducing agents. Normal marrow cells could regularly be induced to bear the human T-lymphocyte antigen (HTLA), to form rosettes with sheep erythrocytes (E rosettes), and to respond to the mitogen concanavalin A (Con A) after coculture with the thymic epithelial monolayers or their culture supernates. In contrast, studies of T-cell differentiation on the marrow cells of patients with SCID revealed varying defects, ranging from a complete "absence" of definable T-cell precursors to partial differentiation resulting in acquisition of one (HTLA) or two (HTLA and E rosettes) markers for T lymphocytes. Only in one patient was there induction of all three T-cell markers, namely, HTLA, E rosettes, and responsiveness to Con A. These observations indicate that SCID is a heterogeneous disorder in which defects of differentiation can occur at one or more multiple sites of differentiation leading to the clinical expression of T- and B-cell dysfunction. Further, our studies indicate that in T-cell differentiation, HTLA probably appears before the capacity to form E-rosettes, and development of the latter capacity is followed by a state of responsiveness to mitogens. A scheme of normal differentiation along with the defects of precursor T cells seen in SCID is presented.

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INTRODUCTION

Children with severe combined immunodeficiency (SCID)[1] lack both T- and B-cell immunity systems. These patients have varying genetic backgrounds; the immunodeficiency may be X-linked, autosomal recessive, or sporadic. A significant proportion of patients with SCID have associated adenosine deaminase (ADA) deficiency (1). Occasionally, SCID has been associated with the syndrome of short-limbed dwarfism (2) or cartilage hair hypoplasia (3).

Although the ultimate immunologic expression of these disorders is quite similar, the defect of lymphoid development may vary. Studies using thymic extracts, thymic epithelial monolayers, and thymic hormones have revealed that the differentiation of precursor cells may be abnormal in SCID (4–6). Additionally, the experience of Hong et al. (7, 8) and studies of Pyke et al. (9) and Gelfand et al. (10) suggest that in certain variants of SCID, a principal defect may lie in the thymus; to this view, stem cells of these patients are considered normal.

Most of the previously reported studies on human T-lymphocyte differentiation have employed mainly rosette formation with sheep erythrocytes (E rosettes) as a differentiation marker for T cells. Other criteria, such as induction of human T-lymphocyte antigen

[1] *Abbreviations used in this paper*: ADA, adenosine deaminase; Con A, concanavalin A; E rosettes, cells forming spontaneous rosettes with sheep erythrocytes; HTLA, human T-lymphocyte antigen; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SCID, severe combined immunodeficiency; TCS, thymic epithelial culture supernates; TEM, thymic epithelial monolayer.

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(HTLA) or of functional competence, have been used only rarely. In this communication we are reporting the induction of three markers characterizing T lymphocytes, namely, HTLA, capacity to form E rosettes, and responsiveness to mitogens in fractionated normal bone marrow cells after incubation on normal thymic epithelial monolayers and their culture supernates. Using this system, we have investigated marrow differentiation in 11 patients with SCID and have focused on the defects of "stem" cells in this disorder.

METHODS

Patients

The patients under study, all under 15 mo of age, were diagnosed as having SCID on clinical and laboratory bases (Table I). All patients were lymphopenic, with lymphocyte counts <1,500/cu mm. Numbers of T lymphocytes were decreased in all except for patient K who had T cells of maternal origin in his circulation. B lymphocytes ranged from complete absence to markedly increased proportions. Proliferative responses to mitogens and antigens were severely depressed in all patients. With the exception of patient A, who had a weak response in the mixed lymphocyte culture reaction (Fireman syndrome [11]), all other patients failed to respond to allogeneic cells in vitro. Patient A in addition had low titers of diphtheria and tetanus antibodies after immunization. Minimal antibody production was also seen in patient D, who had normal levels of immunoglobulins. Three patients (C, D, and I) had deficiency of the enzyme ADA and treatment had been attempted with erythrocyte transfusion in patients D and I to replace ADA; although there was apparent clinical improvement, no change in immunologic parameters occurred. Patients E and F had been given fetal liver transplantations and were studied for T-cell differentiation at a time when donor cell chimerism was present but immunologic reconstitution could not be demonstrated. All other patients were studied before immunologic manipulations. Patient E was the subject of a previous report (5).

TABLE I
Features of Test Patients with SCID

Patient	ADA	Lymphocytes		Immunoglobulins		
		T*	B‡	IgG	IgA	IgM
		%		mg/dl		
A	+	3	18	150	0	31
B	+	2	84.5	180	4	16
C	−	1	7	310	0	7
D	−	34	10	1,100	40	145
E	+	2.5	85	71	0	21
F	+	9.5	22.5	100	2	30
G	+	9.5	0	119	0	0
H	+	3.5	97.5	248	0	0
I	−	3	12	200	10	30
J	+	30	72	203	0	24
K	+	71§	14	2	0	6

* Forming spontaneous rosettes with sheep erythrocytes (normal±1 SD, 72±12).
‡ Surface Ig-bearing cells (normal±1 SD, 17±7) tested with fluorescein-conjugated polyvalent rabbit antihuman immunoglobulin antisera.
§ Of maternal origin, as determined by sex karyotype.

Cultures of thymic epithelium

Thymic epithelial monolayers (TEM) were established as described previously (5, 12). Briefly, normal thymic tissues obtained from children undergoing cardiac surgery were cut into small pieces, teased with forceps, and then cultured in small Petri dishes in RPMI 1640 medium supplemented with 30% heat-inactivated fetal calf serum, gentamycin (4 μg/ml), and amphotericin B (1 μg/ml). The cultures were incubated in an atmosphere of 5% CO_2–95% air at 100% relative humidity. The culture media were changed weekly, and the supernates from the cultures were collected, centrifuged, filtered through millipores, and stored at −20°C. These were designated thymic epithelial culture supernates (TCS). Cells from all cultures of epithelial monolayers were tested to ensure that they did not contain any E-rosetting cells before using these monolayers as inducers of differentiation. Satisfactory epithelial monolayers were established after a culture period of 3–5 wk. Monolayers from fetal kidney or skin and their supernates (also stored at −20°C at weekly intervals) were used as controls.

Precursor cell isolation

Small volumes (0.5–0.7 ml) of bone marrow from normal volunteers aged 20–30 yr and from patients were aspirated from several sites on the iliac crest into 10-ml heparinized glass syringes. Mononuclear cells from the marrow were initially separated by flotation on standard sodium metrizoate/Ficoll gradients (Lymphoprep, Nyegaard and Co., Oslo, Norway) and centrifuged at 400 *g* for 30 min at 22°C. Cells at the interface were washed twice with RPMI 1640 medium (containing 50 U/ml penicillin and 50 μg/ml streptomycin) and further fractionated either on a discontinuous density gradient or by velocity sedimentation at unit gravity.

Discontinuous density gradient. A maximum of 10^8 cells, in 2-ml vol were layered on a discontinuous density gradient consisting of Ficoll concentrations of 13, 15, 17, 20, and 25%, and centrifuged at 10°C for 30 min in the SW 40 rotor of the L5-65 model Beckman Ultracentrifuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) at 22,000 *g* as described by Incefy et al. (13). This resulted in formation of five layers of cells, which were harvested, washed, and counted. This method was employed for isolation of bone marrow cells of patients A through E, and of seven normal volunteers.

Velocity sedimentation. Marrow mononuclear cells from patients G through H and from two normal volunteers were separated predominantly on the basis of size by sedimentation at unit gravity, using a step gradient of bovine serum albumin in phosphate-buffered saline, according to the method of Miller and Phillips (14, 15). After sedimentation at 4°C for 4 h, 35-ml fractions were collected at a flow rate of 30 ml/min after an initial discard of 250 ml. Approximately 30 fractions were collected. Cells in each fraction were washed, counted, and aliquots from these were pelleted into smears in a Shandon cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). Fractions were subsequently pooled as follows: pool I, fractions 1–10; pool II, fractions 11–16; and pool III, fractions 17-25. Pool I consisted mainly

of the faster-sedimenting myeloid cells, pool II was comprised of large- to medium-sized lymphocytes, and pool III consisted of medium- to small-sized lymphocytes.

Induction of markers

Cells from each gradient layer or from each pool of velocity-sedimented cells were adjusted to a concentration of 3×10^6 ml in RPMI 1640 medium with antibiotics and cocultured with the TEM or their supernate for 15 h at 37°C in a humidified 5% CO_2–95% air incubator at 100% humidity. After incubation, cells were washed three times, counted, and then their viability assessed by trypan blue exclusion. The cells were adjusted to appropriate concentrations of viable cells and tested for surface markers and functional characteristics of T cells. HTLA was determined by a microcytotoxicity test in the presence of specific antihuman T-cell serum (Institut Merieux, Lyon, France) and rabbit complement as described by Touraine et al. (16). 200 cells were counted in each well of the microtiter plates, and the percentage of HTLA-positive cells was determined by means of a cytotoxic index:

$$\text{Cytotoxic index for \% HTLA-positive cells} = \left(\frac{[\%\text{ cells alive with normal rabbit serum} + C] - [\%\text{ cells alive with anti-T-cell serum} + C]}{\%\text{ cells alive with normal rabbit serum} + C} \right) \times 100.$$

Spontaneous rosettes with sheep erythrocytes were made as described by Bentwich et al. (17). The ability of the cells to respond to mitogens was tested as described by Cunningham-Rundles et al. (18) using phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). A wide dose range was used for each mitogen: PHA, 0.5–50 μg/culture; Con A, 0.1–50 μg/culture; and PWM, 1–25 μg/culture. Each culture contained 150 μl of cell suspension (5×10^4 cells) and 25 μl of the appropriate mitogen.

Statistical analysis

Results observed after incubation of normal marrow with medium alone were compared with those obtained after incubation with TEM or with TCS, respectively, in paired *t* test analyses. Significance was determined by Student's *t* test. Range of "normal" induction was established by determining the mean and SD of increases observed in each marker over control values after incubation with TEM or TCS. Results of patient samples were compared with those obtained with normal marrow.

RESULTS

Precursor cell isolation

Normal marrow. Characteristics of marrow cells fractionated on discontinuous density gradients or by velocity sedimentation have been described in detail by several investigators (6, 13–16, 19). In experiments reported herein, ~60–80% of cells initially applied to the fractionation procedure were recovered with a viability of >95% as determined by trypan blue dye exclusion.

Of the total cells recovered from discontinuous density gradients, the percentages and absolute numbers of cells recovered in each layer are shown in Fig. 1, as is the distribution of HTLA-bearing and E-rosette-forming cells in each of these layers. HTLA and E-rosette-forming cells were maximally concentrated in layers III and IV by this method. The profile of cells recovered by velocity sedimentation and the content of HTLA and E-rosette-forming cells in each fraction are depicted in Fig. 2. A majority of HTLA-positive and E-rosette-forming cells separated out in fractions 16–19 which consisted of medium- and small-sized lymphocytes. Mitogen-responsive cells resided predominantly in layer III, and to a lesser extent in layer IV, of the discontinuous density gradient (see below). In the velocity-sedimented cells, proliferative responses to mitogens were seen predominantly in pool III.

Patient marrow. Distribution of marrow cells

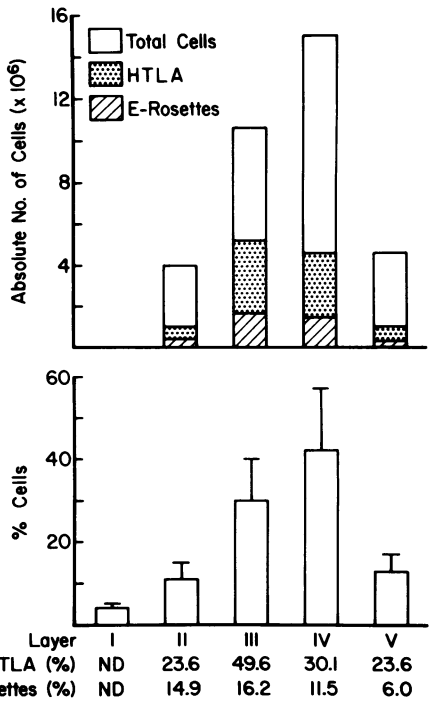

FIGURE 1 Profile of normal bone marrow cells in layers obtained by fractionation on a discontinuous density gradient, showing distribution of HTLA-positive and E-rosette-forming cells in each layer. Bottom: cells in individual layers (expressed as percentage of total cells recovered), and the percentage of HTLA-positive and E-rosette-forming cells in each layer. Top: absolute cell numbers, determined on the basis of percentages (bottom) and total cells recovered. Results represent mean±SD of six normal marrows.

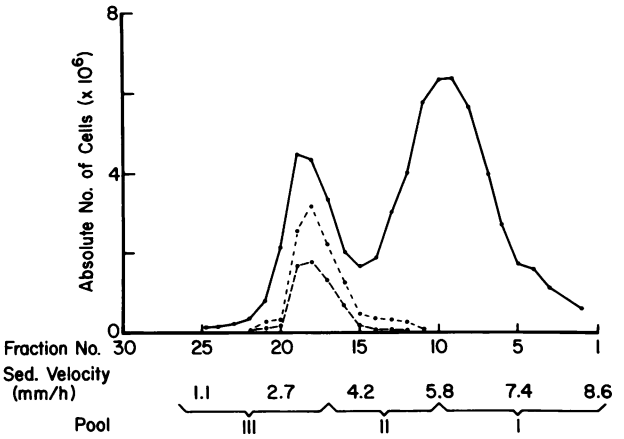

FIGURE 2 Profile of normal bone marrow cells recovered by velocity sedimentation (——), and content of HTLA-positive (- - -) and E-rosette-forming cells (— —) in individual fractions.

separated on the discontinuous density gradient varied among patients and usually differed from normal marrow both in terms of the percentage of cells constituting each layer and in the content of HTLA-bearing cells in the various layers. However, no characteristic pattern of cell distribution was noted in patient marrow. Upon fractionation of marrow by velocity sedimentation, in the majority of patients a paucity of small lymphocytes (velocity 2.5–3.5 mm/h) was observed. A marked deficit in E-rosette-forming cells and absence of proliferative responses to mitogens were noted in all patients irrespective of the method of fractionation of their marrow.

Induction of T-cell markers in normal marrow

A constant number of cells from each gradient layer (discontinuous density gradient) or pool (velocity sedimentation) were cocultured with TEM, TCS, and medium as control for 15 h. Recovery of cells was comparable (80–90%) after each of these incubations for a given experiment, and therefore it did not make any difference in interpretation whether results of HTLA and E-rosettes were expressed in percentages or in absolute numbers; for simplicity and convenience, results are expressed only in percentages. After incubation with TEM or TCS, induction of T-cell markers was noted in the fractionated marrow cells, as described below.

HTLA and E rosettes

Table II depicts results of HTLA and E rosettes in density gradient-separated marrow layers and in the velocity-sedimented pools after incubation of marrow cells with medium (control), TEM, and TCS. Maximum induction of HTLA was observed in layer IV and in pool III; frequent smaller inductions were noticeable in layers II or III and in pool II. Maximum induction of E rosettes was observed in layer III and in pool III; occasionally, induction of a lesser degree was appreciated in layer IV and in pool II. Increases in percentages of HTLA and E rosettes were determined by subtracting control values from those obtained after incubation of marrow cells with TEM or TCS; results of maximum increases in individual experiments are plotted in Figs. 3 and 4. Mean maximum increase in HTLA (Fig. 3) was 18.67±7.8% with TEM (P = <0.005)

TABLE II
Induction of HTLA and E Rosettes in Fractioned Bone Marrow of Normal Volunteers*

	Incubation	HTLA: cytotoxic index in gradient layers‡ II	III	IV	V	Percent large E-rosette in gradient layers§‖ II	III	IV	V
(*a*)	Medium	23.6±4.8	49.6±9.5	**30.1±11.7**	23.3±8.3	14.9±11.2	**16.3±8.8**	11.5±7.0	6.0±4.8
	TEM	29.0±6.9	55.6±5.7	**48.8±9.7**	21.0±1.4	20.6±6.3	**35.0±5.0**	13.3±8.9	8.6±7.2
	TCS	33.0±8.3	53.6±8.5	**54.3±9.6**	24.5±1.2	23.5±6.9	**36.3±4.7**	14.2±8.2	10.9±5.5
		HTLA: cytotoxic index in pool**				% Large E-rosette in pool§**			
		I	II	III		I	II	III	
(*b*)	Medium	4.6±0.7	24.0±1.7	**27.5±5.1**		1.0±0.5	0.3±0.6	**17.7±4.1**	
	TEM	3.7±2.1	33.2±5.0	**54.2±3.7**		2.0±1.1	0	**39.0±5.1**	
	TCS	2.6±2.6	42.4±6.8	**46.0±10.3**		0	0.6±0.3	**31.3±2.5**	

Only layers or pools in which maximum induction was observed are shown in bold face type.
* Marrow fractionated by (*a*) discontinuous density gradient or (*b*) velocity sedimentation.
‡ Mean of six normal marrow samples±SD.
§ Lymphocytes with four or more sheep erythrocytes attached.
‖ Mean of seven normal marrow samples±SD.
** Representative experiment.

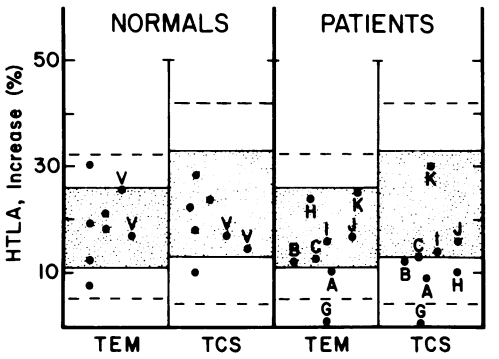

FIGURE 3 Induction of HTLA in individual normal and patient bone marrows. Range of normal increase represented by shaded area (67% confidence limits) and by broken lines (95% confidence limits). Marrows were fractionated on discontinuous density gradients and some (V, and patients G through K) by velocity sedimentation.

and 24.1±10.4% with TCS (P = <0.005). Mean maximum increase in E rosettes (Fig. 4) was 18.7±8.3% with TEM (P = <0.005) and 20.1±7.0% with TCS (P = <0.001).

Responses to mitogens

When marrow cells were studied for the induction of mitogen responses to Con A, PHA, and PWM, it was found that in most instances a significant PHA response was already present in the marrow with a smaller Con A response and a low response to PWM. Coculture of marrow cells with TEM and TCS sometimes resulted in a slight to moderate degree of proliferation so that the background counts, i.e., [^{14}C]thymidine incorporation was higher in these cells as compared with those cultured in medium alone (i.e., the control cells). Results of peak proliferative responses obtained in induction experiments performed in four normal marrows upon stimulation with mitogens Con A, PHA, and PWM are depicted in Fig. 5. Con A responses were regularly induced in normal bone marrow after coculture with TEM or TCS (P = <0.05 for both). However, with PHA the induction was significant only in a few instances when the existing PHA response was not too high. No significant induction of PWM response was observed. Similar magnitude of induction of responses to Con A was obtained in velocity-sedimented cells, predominantly in pool III. Representative experiments showing induction of proliferative responses to various doses of Con A in layer III (discontinuous density gradient) and pool III (velocity sedimentation) are depicted in Fig. 6 and 7. Background counts were much lower in lymphoid cells after velocity sedimentation in comparison with density gradient-separated cells, most probably because of elimination of myeloid cell series in the former test sample.

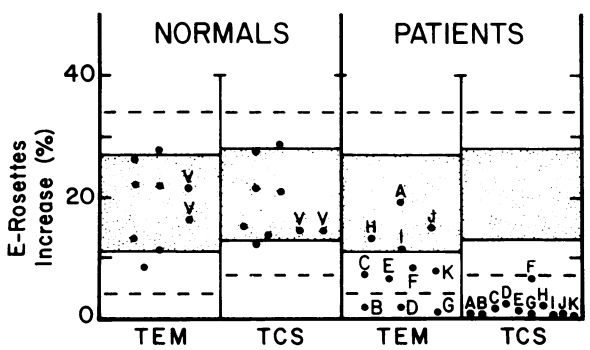

FIGURE 4 Induction of E-rosette-forming capacity in individual normal and patient bone marrows. Range of normal increase represented by shaded area (67% confidence limits) and by broken lines (95% confidence limits). Marrows were fractionated on discontinuous density gradients, and some (V, and patients G through K) by velocity sedimentation.

Induction of T-cell markers in SCID marrow

Results of induction of HTLA and E rosettes in fractionated marrow cells of patients with SCID are shown in Table III. Increases in the perecentages of HTLA-positive cells and of E rosettes after incubation of marrow cells with TEM and TCS are plotted in Figs. 3 and 4, respectively, along with results obtained with normal marrow.

HTLA. Of eight patients studied for induction of HTLA, increases within normal range were observed in four patients (C, I, J, K) after incubation of marrow cells with TEM or TCS. In two patients (B and H) induction of HTLA within normal range was observed only with TEM, this being suboptimal with TCS. Decreased induction with both TEM and TCS was noted in patient A. In the last patient (G), there was no induction of HTLA with either TEM or TCS. Patient G

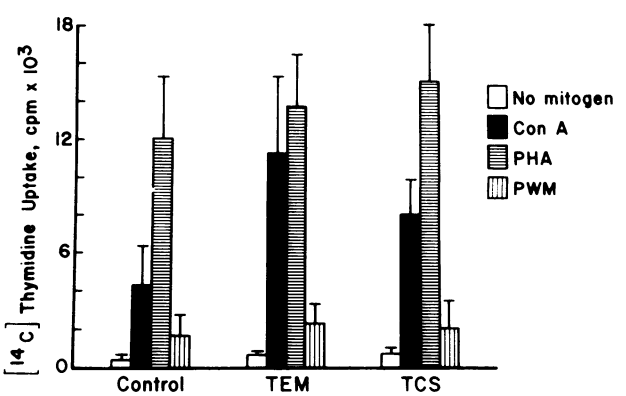

FIGURE 5 Mitogen responses in normal fractionated bone marrow cells (discontinuous density gradient) after coculture with TEM, TCS, and medium as control (mean±1 SD, n = 6).

was also found to be the only patient who lacked completely HTLA-bearing cells in his marrow.

E rosettes. Normal induction of E rosettes was observed in marrow of only 4 of the 11 patients (A, H, I, J) after incubation with TEM; suboptimal induction, below 1 SD of normal mean, was observed in 4 others (C, E, F, K). No induction of E rosettes was achieved in three patients (B, D, G). TCS could not induce E-rosette-forming capacity in the marrow of any patient.

Mitogen responses. In only one patient (J) was there an induction of proliferative responses to Con A after incubation with TEM. This increase in response was suboptimal, but significant (Fig. 8).

Monolayers from fetal kidney and normal skin and their supernates did not induce any surface characteristics of T cells or functional markers of differentiation in normal or patient marrow cells.

DISCUSSION

Observations reported herein indicate that bone marrow can effectively be used as a source of precursor cells to study T-lymphocyte differentiation in humans. These precursors of T lymphocytes can be enriched by fractionation of marrow either based on cell density or cell size. After incubation with TEM or TCS, induction of HTLA, E-rosette-forming capacity, and responsiveness to the mitogen Con A are regularly observed in normal marrow. These observations confirm and extend findings previously reported on the subject of normal human T-lymphocyte differentiation (9, 10, 13, 16, 19, 20), using induction systems both similar to and different from those reported here.

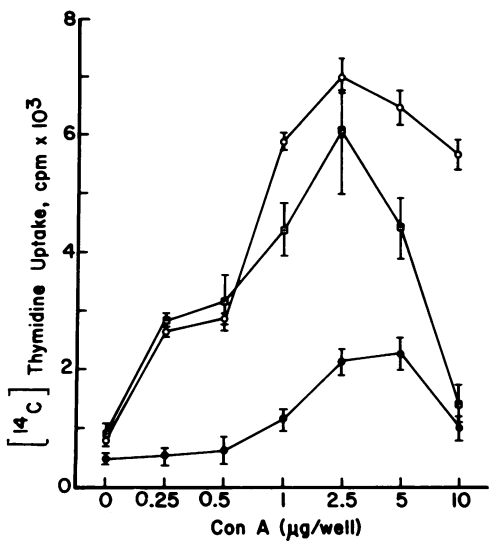

FIGURE 6 Proliferative responses to ConA in layer III (discontinuous density gradient) of normal bone marrow after coculture for 15 h with TEM (□), TCS (○), and medium as control (●).

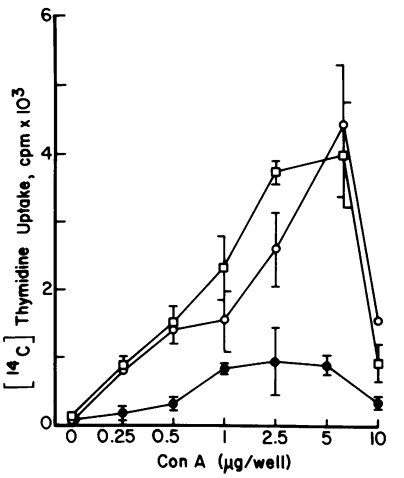

FIGURE 7 Proliferative responses to ConA in pool III (velocity sedimentation) of normal bone marrow after coculture for 15 h with TEM (□), TCS (○), and medium as control (●).

Evidence is increasing that defects of T-lymphocyte differentiation can be very heterogenous in SCID. With thymic hormones or thymopoietin as inducing agents, it has been shown that induction may occur only as far as the development of the surface marker HTLA (6) or not at all (4, 21) in marrow of patients with SCID, implicating a stem cell defect. However, some patients have been shown to attain complete T-cell differentiation in vitro with TEM (10), and in at least a few patients' immune functions have been reconstituted simply by implanting cultured thymic epithelium (7, 8). These observations suggest that some patients with SCID may have intact stem cells but their thymus is nonfunctional in vivo.

In the patient population described here, a wide spectrum of T-cell differentiation defects was noted when the marrow was studied both for surface characteristics and for functional competence. These observations are summarized in Table IV. One patient (G) appeared to lack definable precursors of T cells, based on the criteria that his marrow lacked HTLA-positive, E-rosette-forming, or mitogen-responsive cells and also could not be induced to bear any of these markers by incubation with either TEM or TCS. Another patient (B) manifested induction restricted to HTLA, thereby suggesting that in this patient differentiation was blocked beyond HTLA positivity. In the majority of patients (A, C, E, F, H–K) satisfactory or suboptimal induction of HTLA and E-rosette-forming capacity was observed, without induction to develop cells responsive to phytomitogens. It appears that in this latter group of patients the block is located at another, most probably later step along the pathway of T-lymphocyte differentiation. Only in one patient (J) did we observe induction of HTLA, E-rosette-forming capacity, plus development of the ability to generate a proliferative

TABLE III

Induction of HTLA and E Rosettes in Fractioned Bone Marrow Cells of Patients with SCID*

(*a*)	Patient	Incubation	HTLA: cytotoxic index in marow gradient layer				Percent large E rosette§ in marrow gradient layer			
			II	III	IV	V	II	III	IV	V
					(IV + V)				(IV + V)	
	A	Medium	**18.0**	30.0	7.0		**2.0**	**2.0**	0.7	
		TEM	**28.0**	24.0	9.0		**22.0**	**22.3**	6.7	
		TCS	**27.0**	28.0	8.0		3.0	3.0	0	
	B	Medium	**5.2**	6.0	**33.0**	17.0	2.0	0.3	0.7	0
		TEM	**17.0**	4.0	**44.0**	23.0	2.7	0	0	0.3
		TCS	**18.0**	6.0	34.0	ND	0	1.0	0	0.7
	C	Medium	5.0	0	7.0	**25.0**	0.7	0.3	0	**0.3**
		TEM	1.0	**13.0**	4.0	**38.0**	0.7	0	0	**7.5**
		TCS	9.0	**12.0**	9.0	19.0	0	1.0	0	0.7
	D	Medium					3.3	2.3	0.3	0.3
		TEM		ND‡			5.0	2.0	1.7	0
		TCS					5.7	2.3	0.3	1.0
	E	Medium					0.3	0	0	**0.3**
		TEM		ND			1.3	0.7	5.3	**8.0**
		TCS					1.0	0	0	0
	F	Medium					**1.3**	1.0	0.3	0
		TEM		ND			**10.3**	5.3	1.0	1.0
		TCS					1.3	7.0	0.3	0

(*b*)			HTLA: cytotoxic index in velocity sediment pools			Percent large E-rosette§ in velocity sediment pools		
			I	II	III	I	II	III
	G	Medium	4.2	2.0	2.0	0	0.3	0.3
		TEM	6.2	1.3	2.6	0	0.3	2.0
		TCS	4.0	1.5	1.0	0	1.5	0
			(I + II)			(I + II)		
	H	Medium	**18.5**		**20.0**	**19.7**		0.6
		TEM	**35.0**		**46.4**	**32.0**		1.7
		TCS	**28.5**		**28.2**	18.0		0
	I	Medium	**21.3**		**27.6**	0.5		**1.6**
		TEM	**33.8**		**42.9**	1.0		**15.0**
		TCS	18.5		**43.6**	0.5		1.0
				(II + III)			(II + III)	
	J	Medium	4.2	**22.0**		1.0	**7.0**	
		TEM	6.6	**39.0**		1.0	**18.3**	
		TCS	4.7	**35.5**		1.3	9.0	
	K	Medium	**20.7**	**25.7**		1.3	**14.0**	
		TEM	**42.1**	**50.3**		2.3	**22.3**	
		TCS	24.7	**56.3**		1.5	13.0	

Certain layers or pools were mixed in instances when cells were insufficient in individual layers or pools. Results of layers or pools in which induction was noted are shown in bold face type.

* Marrow cells fractionated by (*a*) discontinuous density gradient or (*b*) velocity sedimentation.

‡ Not done.

§ Lymphocytes with four or more sheep erythrocytes attached.

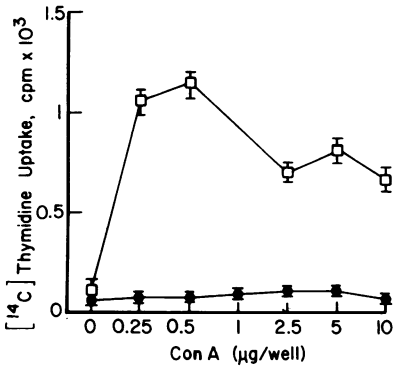

FIGURE 8 Proliferative responses to Con A in fractionated marrow cells of patient J after incubation with TEM (□) and with medium as control (●).

response to Con A. The response generated, however, was suboptimal. Although one cannot state with certainty that stem cells of patient J were intact, it is quite possible that his defect may have resided primarily in the thymus.

The significance of partial induction (below 1 SD of normal) for HTLA or E-rosette-forming capacity in some patients is unclear. This finding may indicate a partial deficiency of or an intrinsic abnormality in precursors of the T cells. It is unlikely that the observed differences in induction of T-cell markers among patients and normal volunteers are age related. Two patients, one aged 1 yr with congenital agammaglobulinemia and another with SCID (patient C) who was successfully reconstituted by marrow transplant from a 4-yr-old matched sibling, were studied for T-cell differentiation, and results obtained for induction of HTLA, E rosettes, and mitogen responses were of the same magnitude as those observed for the older normal volunteers.

The observed blocks in T-lymphocyte differentiation in patients with SCID provide insights into possible sequential stages of normal T-cell differentiation. It seems probable that in the ontogeny of T-lymphocyte differentiation HTLA appears earlier than the capacity to form E rosettes, and this capacity probably precedes the development of mitogen responses. This scheme of T-cell differentiation is in agreement with that proposed by Touraine et al. (22), who showed that in normal marrow the induction of E-rosettes and of mitogen responses was abolished by elimination of HTLA-positive cells, and that the induction of mitogen responses was abolished by elimination of E-rosette-forming cells.

Contact of marrow cells with TEM appears to be essential for induction of E-rosette-forming capacity or responsiveness to mitogens in patients with SCID. TCS by itself could induce only the HTLA marker in certain patients in whom TEM induced HTLA and E-rosette-forming capacity with or without induction of responsiveness to mitogens. Similarly, thymic extracts or thymopoietin also could induce only HTLA in two patients from this group (A and C) in whom TEM induced both HTLA and E-rosette-forming capacity. These findings are in accord with previous reports of a few other cases (6, 9, 10). Normal marrow on the other hand is differentiated in vitro as effectively and completely with TCS or soluble thymic mediators (9, 10) as with TEM.

In trying to define the mechanisms involved in T-cell differentiation, one can speculate that thymic hormones can readily mediate an initial processing of precursor cells from $HTLA^-$ to $HTLA^+$ but are incapable of driving the differentiation of T cells any further. It is highly likely that precursor cells (which may be $HTLA^+$ or $HTLA^-$) have to be exposed to thymic stroma (namely, TEM) before they can be driven any further by the thymic hormones. Normal marrow has been shown by Stutman et al. (23, 24) to contain early postthymic immunoincompetent cells in addition to the true precursor cells. It would appear that it is these early postthymic cells, putatively lacking in SCID, which in mice and in humans, differentiate into mature immunocompetent cells under the influence of secreted thymic hormones. Thus, the thymic culture supernate, thymic extracts, and purified peptides of thymic origin like thymopoietin were all found to be incapable of differentiating cells from SCID patients to E-rosette-forming capacity, whereas the epithelial

TABLE IV
Summary of T-Cell Differentiation In Vitro in Patients with SCID

	Induction* of					
	HTLA		E rosettes		Mitogen responses	
Patient	TEM	TCS	TEM	TCS	TEM	TCS
A	+	+	++	−	−	−
B	++	+	−	−	−	−
C	++	++	+	−	−	−
D	ND	ND	−	−	ND	ND
E‡	ND	ND	+	−	−	−
F‡	ND	ND	+	±	−	−
G	−	−	−	−	−	−
H	++	+	++	−	−	−
I	++	++	++	−	−	−
J	++	++	++	−	+	ND
K	++	++	+	−	−	−

* Indicates: ++, induction within 1 SD of normal mean; +, within 2 SD of normal mean; ±, borderline; and −, below 2 SD; ND, not done.
‡ Patients studied after fetal liver transplantation when they were chimeric but without immunologic reconstitution.

monolayer could do so. Alternatively, it may be that TEM and TCS or thymic hormones act on different populations of precursor cells, and this hypothesis remains to be tested.

Based on the proposed steps of T-cell differentiation, one can postulate that in SCID the possible defects of the stem cells might involve the following: (*a*) absence of stem cells (or of inducible precursor cells); (*b*) defective precursors of T cells, whose differentiation is arrested at any step along the T-cell pathway, such as: (*i*) $HTLA^- \not\rightarrow HTLA^+$, (*ii*) $HTLA^- \rightarrow HTLA^+ \not\rightarrow$ E rosettes, (*iii*) $HTLA^- \rightarrow HTLA^+ \rightarrow$ E rosettes $\not\rightarrow$ mitogen responses.

The vase majority of patients having demonstrable defects of stem cell differentiation most likely also have defects of the thymus. These thymic defects may be primary or secondary to the abnormality of stem cells: thymic secretory activity, as assessed by the measurement of the serum thymic hormones facteur thymique serique (25) or thymopoietin (26), which were abnormal in many patients with SCID became normal in patients who were successfully reconstituted by bone marrow transplantation alone. Thus the thymic secretory defect in these cases was reversed when normal stem cells were provided to the patients. Recent studies done on the cultured thymuses from two patients with SCID have indicated that the thymic defect may involve the initial processing of cells, a step which may be a prerequisite for the subsequent differentiation events in T-cell development (27).

An assessment of the possible site of defect in SCID with the type of in vitro experiments described herein helps to provide better understanding of the steps involved in normal T-lymphocyte differentiation. Such studies in patients with SCID should lead to a clearer understanding of the pathogenesis of each disorder encountered clinically, and assist in arriving at a rationale for effective reconstruction of patients with these otherwise fatal disorders.

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