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Severe combined immunodeficiency disease: a model of T-cell dysfunction

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SUMMARY

Studies in three patients with severe combined immunodeficiency disease (SCID) and normal adenosine deaminase demonstrated that the combined defect of both T- and B-lymphocyte function may reflect the lack of normal maturation of thymic epithelial cells. This results in the failure of initiation of T-cell differentiation and consequent failure of T-cell dependent maturation of B-lymphocytes to an antibody-secreting stage. SCID B lymphocytes were shown to be capable of generating a specific IgM-antibody response to two T-cell-dependent antigens *in vitro* under either of the following conditions: (a) provision of autologous T-helper cells which were induced following incubation of precursor cells on monolayers of cultured human thymic epithelium or (b) in the presence of allogeneic T-helper cells. Specific IgM anti-ovalbumin (OA) responses were also generated in the absence of provided T-helper cells when the antigen was insolubilized (Sepharose–OA). The antibody-secreting cells and their circulating precursors carried surface IgM, HLA and Ia-like determinants and proliferated in response to antigen. Identification of this form of SCID may be important when considering therapy and provides an excellent model for the study of the T-cell-dependent acquisition and expression of B-cell immunity.

INTRODUCTION

Severe combined immunodeficiency disease (SCID) is characterized by the profound impairment of both humoral and cell-mediated immunity. Heterogeneity of this condition has been previously suspected because of the varied inheritance, the presence or absence of lymphopenia and some variation in severity; these patients all die within the first two years of life if untreated (Gelfand, Biggar & Orange, 1974). Heterogeneity has also been documented at the biochemical level as approximately 15–25% of reported infants with SCID lack adenosine deaminase (ADA) (Giblett *et al.*, 1972).

In 1975 we demonstrated that cells from a patient with normal ADA (ADA^+) acquired the ability to form E rosettes and T-helper-cell function following short-term incubation on monolayer cultures of normal human thymic epithelium (Pyke *et al.*, 1975). In contrast to their similar effects on normal T-precursor cells, thymic factor(s) (thymic epithelium conditioned medium) and soluble thymus extracts (thymosin) were ineffective in this patient. We therefore proposed that in some SCID patients, an early block in T-cell differentiation, secondary to a failure of normal maturation of thymic epithelium, could underlie the combined humoral and cellular immunodeficiency (Pyke *et al.*, 1975; Gelfand, Dosch & Shore, 1978). This hypothesis is in contrast to the earlier suggestion that these patients manifest abnormalities of lymphoid stem cells and require bone-marrow transplantation to circumvent their defect (Good *et al.*, 1968).

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In the present report, the responsiveness of circulating SCID B lymphocytes has been investigated. Some SCID B lymphocytes may be induced to release immunoglobulin by mitogen-stimulated normal T-cells (Buckley *et al.*, 1976; Seeger *et al.*, 1976). Our studies of specific immune responsiveness now suggest inherently normal B-cell function with the production of specific (IgM) antibody in response to antigen when autologous or allogeneic T-helper lymphocytes are provided. These data support the hypothesis that the B-cell deficiency documented in certain patients with SCID may reflect the absence of functional T lymphocytes alone.

MATERIALS AND METHODS

Patients. The diagnosis of SCID was established by severe hypogammaglobulinaemia, absence of antibody responses *in vivo*, absence of mitogen-induced lymphocyte proliferation, negative delayed hypersensitivity skin tests to a battery of antigens and failure to reject an allogeneic skin graft (Pyke *et al.*, 1975). E-rosette-forming T cells were few (Table 1) but the absolute and relative numbers of circulating sIgM-positive B lymphocytes were normal or elevated. Peripheral blood lymphocytes (PBL) from a patient with agammaglobulinaemia (Aγ) and no detectable circulating B lymphocytes were used as a source of allogeneic helper cells. This patient has been shown by radioimmunochemical analysis (Percy, Dosch & Gelfand, 1977) to have a defect in immunoglobulin biosynthesis.

Cell preparations. PBL from heparinized venous blood were obtained by floatation on Ficoll-hypaque gradients. E-rosette depletion on Ficoll-hypaque gradients was used for the enrichment (> 90%) of T lymphocytes and E-rosette-negative, non-T-lymphocytes (Dosch & Gelfand, 1977). Confluent monolayer cultures of human thymic epithelium (HThy-ML) or their supernates (human thymus- conditioned medium, HTCM) were used for the induction of T-cell differentiation (Pyke *et al.*, 1975; Pyke & Gelfand, 1974). Fibroblast monolayers (HFib-ML) or their supernates (HF-CM) served as controls. Isolated mononuclear cells were incubated for 2 hr at 37°C either directly on monolayers (HThy-ML or HFib-ML) or in dilutions of the conditioned media. Usually about 90% of cells were recovered following treatment. The effect of these treatment procedures was assessed in parallel by monitoring the appearance of E-rosette-forming cells and the induction of antigen reactive T-helper cells (Pyke *et al.*, 1975; Gelfand *et al.*, 1978).

Plaque-forming cell (PFC) response. Direct PFC towards the T-cell-dependent antigens OA and sheep red blood cells (SRBC) were induced as described (Dosch & Gelfand, 1976; 1977). Briefly, $3–5 \times 10^6$ mononuclear cells were incubated in 10 ml RPMI-1640 supplemented with antibiotics, glutamine, 5×10^{-5}M 2-mercaptoethanol (2-ME) (Gibco, Grand Island, N.Y.) and 10% inactivated IgM-free human serum unable to transfer cell-free plaques (Muchmore *et al.*, 1976). Insolubilized OA was prepared by coupling the soluble protein to CNBr-activated Sepharose (Pharmacia Ltd., Montreal, Quebec) following the producers' instructions. Graded amounts of antigen ($10^2–10^5$ washed, OA-coupled or uncoupled Sepharose beads, or varying concentrations of soluble OA (0·1–100 μg) or SRBC ($10^5–10^7$)) were added at the beginning of culture. After 5–7 days of incubation direct haemolytic plaque-forming cells were enumerated on poly-L-lysine coupled monolayers (Microtest-II plates, Falcon Plastics, Oxnard, California) of either SRBC- or OA-coated red cells (Dosch & Gelfand, 1977). Controls were monolayers of unrelated (anti-SRBC response) or uncoated (anti-OA response) red cells. Results (triplicates) were normalized to express PFC/culture of 10^7 cells $\pm$ standard deviation.

Interactions of allogeneic cells. Equal numbers of SCID–PBL and cells from either normals or the Aγ patient (ON) were cultured together with OA. Where indicated, cells from one donor were treated with mitomycin C (40 μg/ml), puromycin (10 μg/ml)or various antisera to cell surface components. Cells were treated with these antisera for 2 hr at 37°C in the presence of absorbed guinea-pig complement as described earlier (Dosch & Gelfand, 1977). The monospecific goat anti-human immunoglobulin reagents have been described previously (Dosch & Gelfand, 1977). Following histocompatibility testing of cell donors (Roy *et al.*, 1976), two monospecific alloantisera detecting HLA-A2 or B12 were selected. The family study for B-cell typing utilized a panel of 187 mono- and oligo-specific alloantisera in conjunction with freeze-stored B-cell standards (Seventh International Workshop on Histocompatibility, 1978). The alloantiserum to Ia-like determinants on lymphocytes from one patient with SCID (DH) delineated a paternal locus and was designated WIa4x7.

RESULTS

Short-term contact with thymic epithelial cells reconstitutes PFC response of SCID–PBL.

Following direct contact with HThy-ML, SCID–PBL were able to generate PFC in parallel to the appearance of E-rosetting (T) lymphocytes (Table 1). Treatment of SCID cells with HT-CM or fibroblast cultures had no effect. In contrast, HT-CM treatment of normal bone-marrow cells did result in increased numbers of E-rosetting cells and a greater PFC response. We have previously demonstrated that both OA and SRBC are T-dependent antigens in man (Dosch & Gelfand, 1977). Antigen was required for the induction of PFC and in all cases the induced PFC responses were specific;

TABLE 1. Induction of E-rosette formation and reconstitution of the PFC response.

	Antigen	RPMI 1640 E	RPMI 1640 PFC/culture	HT-CM E	HT-CM PFC/culture	HF-CM E	HF-CM PFC/culture	HThy-ML E	HThy-ML PFC/culture	HFib-ML E	HFib-ML PFC/culture
Normal bone marrow	OA	2·2%	510±120	8·6%	920±100	3·2%	480±70	9·8%	1240±170	2·9%	460±90
	SRBC		720±110		1310±140		790±90		1350±120		660±210
Normal PBL	OA	54%	3160±430	55%	2980±320	55%	3290±360	52%	3350±280	51%	3910±440
	SRBC		5730±710		5350±660		5820±780		5200±610		5520±540
DH (SCID)	OA	< 1%	}< 200	< 1%	}< 200	< 1%	}< 200	4·7%	570±160	< 1%	200
	SRBC								960±150		250±30
SK (SCID)	OA	< 1%	n.d.	< 1%	n.d.	< 1%	n.d.	11·6%	n.d.	< 1%	n.d.
	SRBC		< 200		< 200		< 200		630±120		< 200
DK (SCID	OA	< 1%	}< 200	< 1%	}< 200	< 1%	< 200	5·3%	640±110	< 1%	}< 200
	SRBC								1130±180		
ON (Aγ)	OA										
	SRBC	72%	}< 200	70%	}< 200	69%	}< 200	71%	}< 200	68%	}< 200

Ficoll-hypaque separated mononuclear cells from PBL or normal marrow were incubated with medium (RPMI 1640), thymus epithelium conditioned medium (HT-CM), fibroblast conditioned medium (HF-CM), thymic epithelial monolayers (HThy-ML) or fibroblast monolayers (HFib-ML) and assayed for E-rosette formation and PFC response to ovalbumin (OA) or sheep red blood cells (SRBC). E-rosettes are expressed as % mononuclear cells (at least 1000 cells counted) and PFC as PFC/culture of 10^7 cells± 1s.d. Numbers of SIgM-positive circulating B lymphocytes (identified by immunofluorescence) were: normal PBL = 8% DH = 60%, SK = 30%, DK = 22%, ON = < 1%. n.d. = not determined.

less than 200 PFC/culture were observed when ox red blood cells were used instead of SRBC; free OA (100 μg/well) but not BSA, inhibited anti-OA–PFC by at least 90% (see Fig. 2). This precluded the possibility that polyclonal B-cell activation (by thymic epithelial cells) was responsible for the observed reconstitution of the SCID–PFC response. PBL from normals were unaffected by the treatments confirming that substantial cell losses did not occur during either treatment regimen. This failure to manifest thymopoietic effects in normal PBL has been reported earlier and likely reflects the small size of the circulating precursor cell pool (Pyke & Gelfand, 1974). PBL from the agammaglobulinaemic patient (ON) failed to generate a PFC response following any treatment.

Direct activation of SCID B lymphocytes and the effect of polyclonal stimulation

Pokeweed mitogen (PWM) induces PFC in a T-cell-dependent reaction in man (Fauci & Pratt, 1976; Insel & Merler, 1977) and may amplify the antigen-dependent generation of PFC (Dosch *et al.*, 1977). The effect of PWM on the anti-OA-response is shown in Table 2. In addition to soluble OA, insolubilized OA (OA–Sepharose) was used as antigen. All PFC were assayed in parallel using the same batch of OA-coupled red cells. Both soluble and insoluble OA were able to induce the generation of OA-specific PFC in normal PBL. PWM (1 μg/ml) enhanced the response of normal cells, particularly to soluble OA. PBL from both SCID patients generated a subnormal but significant PFC response only to OA–Sepharose. PWM was without effect on patient cells. A PFC response was not observed in cultures of PBL from the agammaglobulinaemic donor (ON) in more than fifteen experiments. These results indicated that SCID B-cells could be directly activated to respond to specific antigen presented as a protein-Sepharose complex.

TABLE 2. Effect of PWM on the PFC response to soluble and insoluble OA

	Control	DK (SCID)	DH (SCID)	ON (Aγ)
OA	3300±420	< 200	< 200	< 200
OA+PWM	5040±460	< 200	< 200	< 200
PWM	280±110	< 200	< 200	< 200
OA–Seph	2200±340	1760±350	1170±270	< 200
OA–Seph+PWM	2740±280	n.d.	980±180	< 200

The concentration of PWM used (1 μg/ml) provided optimal enhancement of normal PFC (Dosch *et al.*, 1977). Several antigen concentrations were tested and optimal responses (PFC/culture of 10^7 PBL± 1 s.d.) are shown. Similar results were obtained on three or more occasions. n.d. = not determined; OA = soluble OA; OA–Seph = Sepharose–OA.

Normal co-operation with allogeneic T-helper cells

Allogeneic cell-mixing experiments were designed to analyse the ability of SCID B cells to interact with T-helper cells and to study the characteristics of the interacting cells, especially the PFC and direct PFC precursors. Table 3 illustrates one such experiment; cells from the Aγ patient (ON) served as a source of differentiated T lymphocytes devoid of functional B lymphocytes. SCID–PBL were cultured either alone, or mixed with an equal number of allogeneic PBL, plus antigen. Neither patient alone could generate PFC and mixtures of SCID cells with Aγ–PBL consistently showed good anti-OA responses. Furthermore, addition of SCID but not Aγ cells to normal control cultures resulted in considerable enhancement of the PFC response without evidence for the manifestation of suppressor cell activity in either combination.

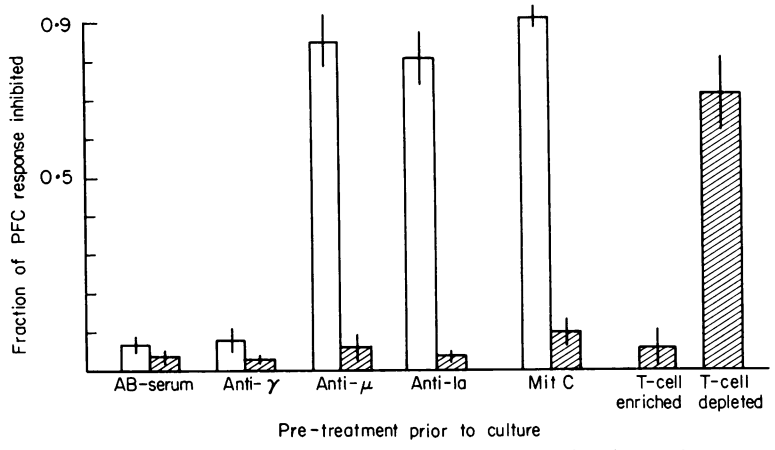


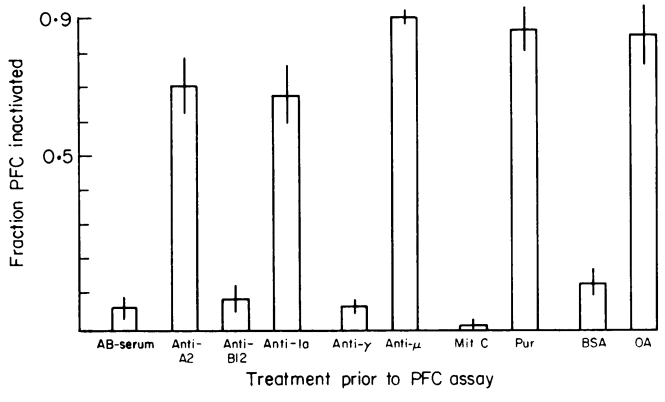
FIG. 1. Effect of various pre-treatments on the co-operating cells in allogeneic cultures of SCID–PBL and Aγ-lymphocytes. Only one partner in the allogenic mixtures was treated prior to co-culture; treatment of SCID–PBL (DH), open columns, treatment of Aγ-cells (ON), hatched columns. Following treatment, cells were washed and incubated together as described. AB-serum treatment refers to the complement control which was carried out in the presence of AB serum. Enrichment and depletion of T cells was possible only with ON–PBL and two separate experiments were performed. Results were calculated as the fraction of the response inhibited by the various regimens.

In the second set of experiments cells from one donor (SCID or Aγ) were treated as indicated in Figs 1 and 2 prior to co-culture with antigen or the cultured mixture was treated just prior to the haemolytic PFC assay. In Fig. 1 data from several replicate experiments are expressed as the fraction of the PFC response inhibited by a given treatment. Maximal responses in the untreated control DH–ON mixtures, normalized for standard cultures of 10^7 cells, varied considerably (1460 ± 270 to 5360 ± 450 PFC) in seven different experiments. Pre-treatment of SCID cells with the anti-μ and anti-Ia reagents resulted in significant inhibition of the PFC response. Similar results were observed following mitomycin C treatment. In contrast, pre-treatment of the Aγ cells with anti-μ, anti-Ia or mitomycin C was ineffective. Treatment with anti-γ or complement alone were without effect on both cell populations. The helper cell effect provided by the Aγ cells was found in the T-cell enriched population.

TABLE 3. Reconstition of the anti OA-response in allogeneic cultures

Donor A	Donor B	PFC/culture
DH (SCID)	—	< 200
DK (SCID)	—	< 200
ON (Aγ)	—	< 200
DH	DK	< 200
DH	ON	2660 ± 200
DK	ON	1460 ± 270
Normal PBL	—	3540 ± 120
Normal PBL	DH	5380 ± 760
Normal PBL	DK	4750 ± 390
Normal PBL	ON	3830 ± 540

Three $\times 10^6$ PBL from each donor with SCID (DH, DK), agammaglobulinaemia (ON) or normals were mixed as indicated and co-cultured for 5–6 days in the presence of soluble OA and then assayed for direct anti-OA-PFC. Similar results were obtained in three separate experiments.



	HLA-A2	HLA-B12	Anti-Ia
DH (SCID)	+	−	+
ON (Aγ)	−	+	−

FIG. 2. Effect of various treatments on PFC generated in allogeneic cultures of SCID–PBL (DH) and lymphocytes from a B-cell deficient, agammaglobulinaemic donor (ON). Following treatment, cells were washed and assayed for residual PFC activity. Mitomycin C (40 μg/ml) or puromycin (10 μg/ml) were added 4–6 hours prior to harvesting. In studies of specificity, free bovine serum albumin (BSA) or OA were added directly to the plaque assays. The ordinate indicates the fraction of PFC inactivated by the treatment shown along the X-axis.

The PFC generated in cultures containing both SCID and Aγ–PBL were characterized using similar treatment protocols on cultured cells just prior to the plaque assay (Fig. 2). When aliquots of the generated PFC were treated with alloantisera recognizing HLA determinants, only the anti-HLA-A2 but not the anti-B12 antiserum resulted in inactivation, establishing that PFC were derived from precursors present in the SCID–PBL (DH). Similarly, treatment of SCID but not Aγ cells with the anti-Ia antiserum and complement inhibited PFC activity. Treatment of DH-maternal PFC with this Ia antiserum did not affect generated PFC whereas it led to inactivation of paternal PFC (data not shown) delineating a paternal locus on DH cells. PFC were also shown to carry accessible surface IgM as demonstrated by the effect of treatment with anti-immunoglobulin reagents. Cell division appeared not to be required at this stage (i.e., after culture with antigen), since mitomycin C left the PFC activity unaffected. However, in agreement with earlier reports, PFC activity required *de novo* protein synthesis and was specific for the inducing antigen, since excess free OA but not bovine serum albumin (BSA) inhibited plaque formation (Dosch & Gelfand, 1977).

DISCUSSION

We have attempted to characterize the stage of B-cell differentiation and function achieved in patients with an intrathymic form of SCID* using three approaches: (a) the assessment of the influence of cultured thymic epithelium on the ability of patient cells to generate specific PFC, (b) the induction of specific PFC following complexing of the protein antigen OA with Sepharose, in an attempt to alter T-cell requirement(s) for this response, and (c) the generation of specific PFC in cultures containing combinations of allogeneic cells from SCID patients and a donor with an isolated defect of B lymphocytes but apparently normal T-cell function.

The induction of specific immune responsiveness in PBL from these patients in parallel to the appearance of E-rosette-forming lymphocytes following incubation with thymic epithelium may be interpreted in two ways. Firstly, functional B-cell precursors, in addition to T-cell precursors, could be induced to differentiate to a more mature, immunoresponsive stage. Indeed, Hämmerling has reported the presence of factor(s) in extracts of thymus tissue which are capable of triggering B-lymphocyte differentiation

* Where a thymic epithelial cell defect has been defined.

(Hämmerling *et al.*, 1975). Alternatively, the results of these experiments may suggest that the acquisition and expression of B-cell function *in vitro* and *in vivo* is dependent on the presence of sufficiently differentiated (post thymic epithelial) T lymphocytes. The two additional types of experiments were designed to resolve these questions.

Direct activation of B lymphocytes has been observed utilizing polyclonal activators (mitogens) (Fauci & Pratt, 1976; Insel & Merler, 1977) as well as antigens, chemically altered to provide a rigid and repetitive backbone structure (Möller, 1975). Although the PFC response to SRBC and OA requires T lymphocytes, sonicates of SRBC show a lesser, if any, T-cell dependency (Dosch & Gelfand, 1977). This suggested that in man, as in other species, the physico-chemical presentation of antigen may be important. Indeed, only OA–Sepharose complexes triggered the generation of OA-specific PFC in cultures of untreated SCID–PBL whereas soluble OA did not. Since SCID B cells generate specific PFC *in vitro* in response to antigen appropriately presented, it is likely that their circulating B lymphocytes are functionally effective. The failure of PWM to induce (soluble OA) or enhance (OA–Sepharose) the response of SCID–PBL, emphasizes the T-cell dependence of polyclonal B-cell activation in the presence of PWM (Insel & Merler, 1977).

We also investigated whether the patients' circulating B lymphocytes could interact with allogeneic T-helper cells. In inbred mice there is increasing histocompatibility restriction for lymphocyte co-operation with increasing maturity of the immune response assayed; identity at the major histocompatibility loci is not required in a primary (IgM) response (Swain & Dutton, 1977; Katz, 1972). Studies in man suggest less stringent complementarity requirements for interacting cells (Bergholtz & Thorsby, 1977). In our experiments we combined SCID–PBL with those from a B-cell deficient patient (ON). Cells from patient ON alone did not make a PFC response and did not suppress normal PFC responses. These cells, however, could reconstitute the PFC response of sIgM-bearing SCID B cells and this helper effect was mediated by E-rosetting T lymphocytes. Thus, these patients appeared to have complementary cellular deficiencies at the T-cell (SCID) and B-cell (Aγ) levels. This conclusion was supported by inhibition by an Ia alloantiserum, detecting a paternal determinant on the SCID-cells, an HLA antiserum (anti-HLA-A2), and an anti-μ reagent. Treatment with these reagents affected both PFC and their precursors but left T-helper-cell activity (derived from the Aγ patient) intact. The residual PFC activity left following treatment with the alloantisera, as opposed to the almost complete ($>90\%$) effect of the heterologous anti-μ reagent may reflect the lower cytotoxic titres of the alloantisera and different densities or accessibilities of the antigenic determinants on the small population of PFC ($\leqq 0{\cdot}1\%$) present in the cultures (Klein, 1973).

The data obtained in these experiments permit several conclusions to be drawn concerning the functional capacity of circulating SCID B-cells and the inducing effect of cultured normal thymic epithelium. The latter effect appears to be restricted to the induction of T-cell differentiation, although additional effects such as non-specific factors released in epithelial cultures, cannot be ruled out entirely at present. The functional integrity of SCID B cells to mount a specific antibody response was substantiated in experiments circumventing obligatory T-helper-cell requirements by complexing OA with Sepharose, by demonstrating effective co-operation with thymic epithelial monolayer-induced autologous T cells or with allogeneic T-helper cells. Our data are consistent with reports of polyclonal immunoglobulin secretion by SCID lymphocytes co-cultured with normal PWM-stimulated T lymphocytes (Seeger *et al.*, 1976; Buckley *et al.*, 1976). The finding of sIgM and Ia-like determinants on the surface of specific (and reactive) PFC precursors characterizes the stage of B-cell differentiation achievable in the absence of functional T lymphocytes. The inherent functional integrity of SCID B lymphocytes is in contrast to the absence of *in vivo* antibody formation in these patients. This dichotomy emphasizes the importance of thymus function in man and suggests that thymus-independent antigens are not important in the elicitation of humoral immunity in man. This contrasts with findings in athymic nude mice where immunoglobulins and antibodies are regularly found in the absence of easily recognizable T cells or T-cell function (Pantelouris, 1971).

In summary, the failure to express humoral immunity in certain SCID patients with normal levels of adenosine deaminase can be linked to a failure of terminal differentiation of B lymphocytes secondary to

the absence of differentiated T cells. This in turn is the consequence of absent or impaired thymic epithelial cell function as the initiator of T-cell differentiation. These findings not only emphasize the thymic dependence of specific B-cell differentiation in man but reveal the importance of identifying this group of SCID patients whose combined immunodeficiency may be reversed by implantation of thymic epithelium alone. Recent reconstitution *in vivo* of both humoral and cellular branches of host immunity following implantation of cultured thymic epithelium in such patients supports this hypothesis (Hong *et al.*, 1976; Gelfand *et al.*, 1977).

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