

Development of population-based newborn screening for severe combined immunodeficiency

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Background: Severe combined immunodeficiency (SCID) is a treatable, inherited lack of cellular and humoral immunity caused by diverse mutations in several different genes and leading to death in infancy unless immune reconstitution is provided. Currently no population screening exists for SCID, but early diagnosis would improve outcome.

Objective: Because all patients with SCID make few or no T cells, we asked whether the absence of T-cell receptor excision circles (TRECs), DNA episomes in newly formed T cells, could identify SCID regardless of genotype.

Methods: DNA isolated from dried blood spots was assayed by real-time PCR to quantitate TRECs. Control PCR was performed on a segment of the β -actin gene. After pilot studies with adult and cord blood control subjects, blood from SCID patients was spotted onto filters and tested, followed by screening of actual blood spots from the Maryland Newborn Screening Program. Finally, newborn blood spots were recovered and tested from 2 infants after their diagnosis of SCID.

Results: In contrast to filters from the newborn screening program, which had a mean of 1020 TRECs in two 3-mm punches, samples from 23 infants with SCID had <30 TRECs. The newborn screening filter was retrieved from a state laboratory for one of these infants plus another infant who had died of SCID previously; although both samples had detectable β -actin DNA, neither had TRECs.

Conclusion: TRECs are a stable analyte that can identify T-cell lymphopenia in newborn dried blood spots so that infants with SCID can receive early, life-saving treatment. (*J Allergy Clin Immunol* 2005;115:391-8.)

Key words: SCID, newborn screening, TREC, T-cell receptor excision circle, T-cell maturation, early intervention, primary immunodeficiency, cost-effectiveness, bone marrow transplant, dried blood spots

Severe combined immunodeficiency (SCID) represents a spectrum of genetic disorders characterized by profound

Abbreviations used

BMT:	Bone marrow transplant
DHMH:	Department of Health and Mental Hygiene
NEMO:	Nuclear factor kappa B essential modulator
PKU:	Phenylketonuria
SCID:	Severe combined immunodeficiency
TCR:	T-cell receptor
TREC:	T-cell receptor excision circle

defects in both cellular and humoral immunity.¹⁻⁴ Infants with SCID die of infections in the first year of life unless immunity is reconstituted by bone marrow transplantation (BMT),^{5,6} enzyme replacement,⁷ or, in some recent cases, gene therapy.⁸⁻¹¹ There is no current screening program for SCID, which is caused by diverse mutations in any of at least 11 known genes and others not presently known, as summarized in Table I.¹²⁻²⁸

Screening newborns for treatable genetic disorders allows early intervention.^{29,30} After the introduction by Guthrie of filter paper spotted with heel-stick blood to screen for phenylketonuria (PKU),³¹ all states in the United States test newborns for PKU, hypothyroidism, and galactosemia.^{29,32} New tests use tandem mass spectrometry to detect many additional metabolic disorders^{30,33} and DNA analysis to diagnose specific mutations causing cystic fibrosis, hemoglobinopathies, and biotinidase deficiency.³⁴⁻³⁷ Screening programs are evolving to meet the growing number of conditions that can be identified and treated.^{29,30,38}

A recent report on primary immunodeficiencies from the Centers for Disease Control and Prevention noted that SCID meets many of the accepted criteria for newborn screening.³⁹ SCID is asymptomatic at birth, but it becomes fatal without treatment; effective treatment is available; and early intervention improves outcome. Infants receiving BMT before developing devastating infections have better survival at lower cost than those whose treatment is delayed.^{5,6,40} Indeed, Myers et al⁴⁰ reported 95% survival among infants undergoing transplantation in the first month of life compared with only 70% for those treated after 3 months.

Arguments against newborn screening for SCID include its low incidence and the lack of a practical test. The true incidence of SCID is unknown; estimates of

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TABLE I. Multiple genotypes of SCID

Gene defect	Molecular pathogenesis	% of SCID cases*	Characteristic lymphocyte subpopulations		
			T cells†	B cells	Natural killer cells
<i>IL2RG</i> (X-linked)	Failure of IL-2 receptor (common) γ -chain signaling by cytokines IL-2, -4, -7, -9, -15, -21 ¹⁶	45-50	Low/absent	Present	Absent
<i>ADA</i>	Adenosine deaminase deficiency ⁷	16	Low/absent	Absent	Absent
<i>IL7R</i>	Failure of signaling through IL-7 receptor ¹⁸	9	Low/absent	Present	Present
<i>JAK3</i>	Failure of Janus kinase 3 activation by common γ -chain ¹⁷	6	Low/absent	Present	Absent
<i>DCLRE1C</i> ‡	Failure of antigen receptor rearrangement ¹⁹	<5	Absent	Absent	Present
<i>RAG1</i> ‡	Failure of antigen receptor rearrangement ¹⁹	<2	Absent	Absent	Present
<i>RAG2</i> ‡	Failure of antigen receptor rearrangement ¹⁹	<2	Absent	Absent	Present
<i>CD45</i>	Lack of a cell surface protein tyrosine phosphatase receptor (PTPRC), required for T- and B-cell activation by antigen ^{20,21}	Rare	Low/absent	Present	Low, maybe variable
<i>TCRD</i>	T-cell receptor δ chain deficiency ²²	Very rare	Low/absent	Present	Present
<i>LCK</i>	Lack of lymphocyte tyrosine kinase p56lck, required for T-cell development and activation ²³	Very rare	Low/absent	Present	Present
<i>FOXN1</i>	Lack of forkhead box N1 transcription factor, required for thymus and hair follicle development (ortholog of nude mouse) ²⁴	Very rare	Low/absent	Present	Present
Deletion of Chr 22q11	Complete DiGeorge syndrome with thymus aplasia; part of DiGeorge-Velocardiofacial syndrome ²⁵	Rare	Absent	Variable	Present
Currently unknown	Unknown, including reticular dysgenesis ²⁶ and congenital anomaly syndromes with SCID ^{27,28}	Each one rare	Low/absent	Variable	Variable

*Based on Buckley,¹⁵ Antoine et al,⁶ and unpublished estimates (J Puck).

†Some patients have substantial numbers of maternally derived T cells at time of diagnosis.

‡T- and B-cell antigen receptor genes include the DNA cross-link repair protein 1C (*DCLRE1C*, or *ARTEMIS*) and the recombinase activating genes (*RAG*).

1/100,000 births³⁹ exclude patients who die before their immunodeficiency is recognized. The incidences of galactosemia and biotinidase deficiency, included in many current screening programs, are comparable at 1/60,000 and 1/80,000, respectively.^{29,32} An inexpensive, high-throughput screening method for SCID is needed. Such a test should identify SCID caused by defects in known disease genes as well as SCID with unknown genotype. Characteristic lymphocyte phenotypes are associated with the different genotypes of SCID (Table I). Patients with *IL2RG* and *JAK3* defects, for example, can make B cells and are designated T⁻B⁺ SCID, whereas those with *RAG1* defects are T⁻B⁻. Natural killer cells are typically absent in patients with *IL2RG* SCID but present in *IL7R* SCID. In some patients, regardless of genotype, maternal T cells are present at diagnosis in numbers substantial enough to cause spontaneous graft-versus-host disease. However, a very low number of patient T cells is a feature common to all SCID genotypes. Thus, attributes of infant T cells could provide the basis for a universal SCID screening test.

Normal thymic maturation of T cells requires cutting and splicing of the DNA encoding the T-cell antigen receptor (TCR) genes to produce T cells with diverse antigen specificities. As a byproduct of this recombination, excised DNA forms episomal T-cell receptor excision circles (TRECs). One species, the δ Rec- ϕ J α TREC, is produced late in maturation by 70% of developing human T cells that express $\alpha\beta$ TCRs.⁴¹⁻⁴³ Quantitative PCR amplification across the joined ends of the δ Rec- ϕ J α

TREC reflects the number of recently formed T cells in peripheral blood. We have developed a newborn screening method for SCID based on measurement of TRECs from dried blood spots.

METHODS

Samples

Blood samples from healthy adults and from infants newly diagnosed with SCID were donated with consent under approved research protocols at the National Institutes of Health. SCID molecular diagnosis was performed by sequencing *IL2RG* in male subjects as described¹⁶ and obtaining *JAK3* sequence (GeneDx, Rockville, Md) where indicated. Umbilical cord blood was obtained from routine deliveries via the National Disease Research Interchange (Philadelphia, Pa). Simulated blood spots were made by applying 50 μ L of blood to the 1.3-cm circles on standard filter paper cards provided by the Maryland State Department of Health and Mental Hygiene (DHMH) (Baltimore, Md). The cards were air-dried and stored desiccated away from light. Actual, outdated newborn blood spots (stored >6 months desiccated at -20°C), for which screening had been completed, were provided by Dr Susan Panny and colleagues at the Maryland State DHMH after approval by the DHMH Institutional Review Board. Samples had been obtained in routine care nurseries from babies at 1 to 2 days of age or in premature and special care nurseries at 2 to 3 days of age.⁴⁴ Identifying numbers were removed from the filters.

T-Cell depleted simulated Guthrie cards

To model blood from SCID patients, adult blood was separated, depleted of T cells, and reconstituted as follows: after centrifugation,

the buffy coat was aspirated and spun over Ficoll (MP Biomedicals, Irvine, Calif). The granulocyte/erythrocyte pellet was retained. Mononuclear cells were treated with anti-CD3 coated microbeads and passed through a magnetic separation system (MACS; Miltenyi Biotech, Auburn, Calif). T-cell depleted mononuclear and granulocyte fractions were added back to the platelet-rich plasma and erythrocytes from the original centrifugation. Immunophenotyping showed 1% of the lymphocytes of this mixture were T cells. Aliquots were spotted onto filter circles as above.

DNA extraction

Disks 3 mm in diameter were punched out of the dried spots. DNA was extracted by using a Gentra Systems kit (Minneapolis, Minn). Glycogen 1 μL , 20 mg/mL (Invitrogen, Carlsbad, Calif), was added before precipitation. DNA was resuspended in 40 μL H_2O and heated at 65°C for 1 hour. Quality and yield were assessed by spectrophotometry and visualization in agarose gels. Preliminary DNA isolations showed optimal yields from 2 punches, corresponding to about 6 μL of blood.

Quantitative real-time PCR

Real-time PCR for $\delta\text{Rec-}\phi\text{J}\alpha$ TRECs used primer, probe, and control plasmid sequences of Douek et al.⁴⁵ Each triplicate reaction of 20 μL contained 4 μL DNA, TaqMan 2 \times PCR Master Mix (Applied Biosystems, Foster City, Calif), 500 nmol/L forward and reverse primers (F, 5'-cacatcccttcaacctgct; R, 5'-gccagctcagggtttagg), and 150 nmol/L probe (FAM-5'-acacctctggtttttaaaggtgcccaact-TAMRA). Samples were heated at 95°C for 5 minutes and then given 40 cycles of 95°C for 30 seconds, 60°C for 1 minute (7900HT Prism Sequence Detection System; Applied Biosystems). PCR for β -actin used identical cycles with 1 μL of DNA, 375 nmol/L primers (F, 5'-tcaccacactgtgccatctacgag; R, 5'-cagcgaaccgctcattgccatgg), and 250 nmol/L probe (FAM-5'-atgccctccccatgccatctcgcg-TAMRA). TREC and β -actin copy number were calculated relative to standard curves generated from serially diluted plasmids.

Statistical analysis

Relationships between DNA concentrations, TRECs, and β -actin copies were determined by linear regression. Analysis of the variance was used to compare samples from routine versus special care nurseries. A test based on simulating the Kolmogorov-Smirnov statistic, adjusting for having to fit mean and variance, was used to establish whether values were normally distributed.

RESULTS

Although simple to collect, dried blood spots vary widely in cellular content and extractable material, as illustrated by the 10-fold variation in DNA yields in our samples from 0.12 to 1.35 μg (mean, 0.46 μg) per 2 punches. Nonetheless, preliminary experiments showed significant positive correlations between TRECs and DNA content; β -actin copies and DNA content; and TRECs and β -actin copies (not shown). Multiple independent DNA preparations and PCR reactions were performed on a series of spots made from cord and adult blood, demonstrating that TREC number was reproducible for a given individual's sample (Fig 1, A). Mean TREC values for cord and adult blood were 740 and 164, respectively, consistent with published observations that cord blood has more newly formed T cells and TRECs.^{41,46} To assure that adequate DNA for PCR had been extracted, TREC values

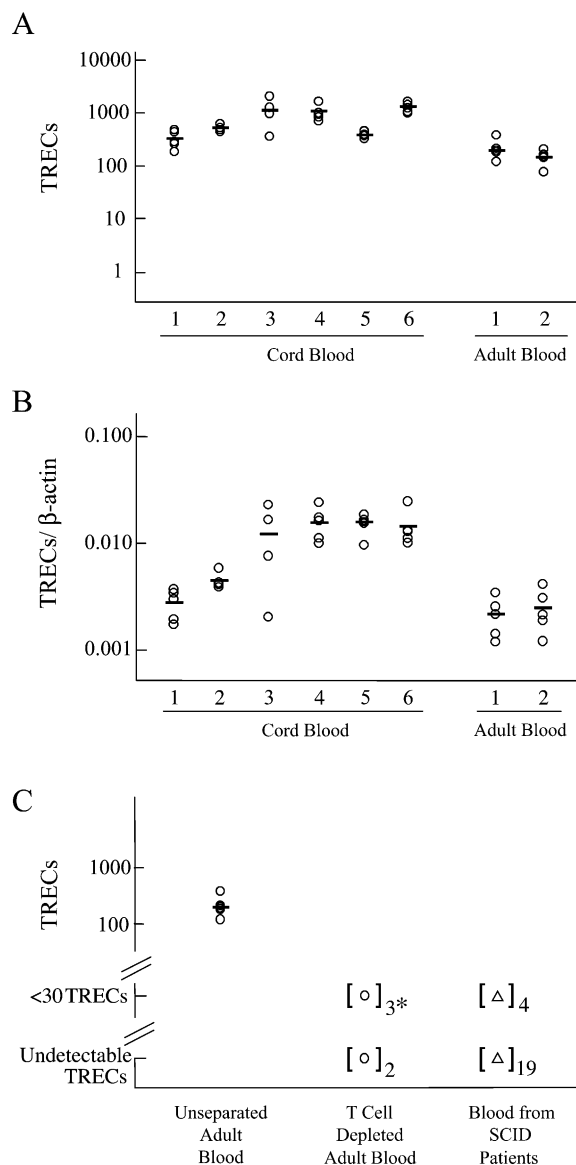


FIG 1. Cord blood and adult blood TRECs (A) and TREC/ β -actin (B) in replicate DNA isolations from 2 punches representing 6 μL of blood dried on filters. C, TRECs from filters with unseparated vs T-cell-depleted adult blood (circles) and from SCID infant blood (triangles). Bars represent mean of replicate samples from the same subject; *numbers of samples with the same value are in brackets.

were normalized to copies of β -actin genomic DNA (Fig 1, B). The mean cord blood TREC/ β -actin was 0.0109 (median, 0.0125; range, 0.0028-0.0158; SEM, 0.0014), 4-fold higher than the adult mean of 0.0024 (median, 0.0022; range, 0.0013-0.0042; SEM, 0.0003).

To model SCID blood, an aliquot of adult blood was depleted of T cells, spotted onto filters, and used for 5 DNA extractions and PCR quantitations of TREC and β -actin (Fig 1, C, circles). Two of the T-cell-depleted DNA preparations had no amplification, whereas 3 had minimal amplification, corresponding to <30 copies, the lower limit of detection in standard serial dilutions.

TABLE II. Clinical and molecular data and TREC analysis at time of diagnosis in 23 patients

Case code	Age diagnosed	Family		Clinical history	Total lymphocytes†	T cells‡	B cells§	NK cells	SCID genotype	PCR from blood spot¶	
		Sex	history*							TRECs	β-actin
340	2 d	M	+	Healthy	801	15	136	68	<i>IL2RG</i>	—**	+
126	1 wk	M	+	Healthy	Not available	0	444	3	<i>JAK3</i>	—	+
220	2 wk	M	+	27-wk gestation, respiratory distress	<u>2820</u>	24	448	NA	<i>IL2RG</i>	—	+
140	1 mo	M	+	Healthy	441	9	361	13	<i>IL2RG</i>	—	+
339	3 mo	F	+	Thrush, pneumonia, adenovirus, FTT†† (died in post-BMT period)	1740	0	1672	0	<i>IL2RG</i>	—	+
344	3 mo	M		Diarrhea, thrush, Parainfluenza 3, sepsis	<u>2780</u>	0	2724	0	<i>IL2RG</i>	—	+
345A	4 mo	M		FTT, chronic upper respiratory infections, rash	446	101	96	167	Not <i>IL2RG</i> or <i>JAK3</i>	—	+
345B	4 mo	M		FTT, chronic upper respiratory infections, rash	623	204	146	158	Not <i>IL2RG</i> or <i>JAK3</i>	—	+
346	4 mo	M		Diarrhea, PJP‡‡	987	0	809	138	Not <i>IL2RG</i>	—	+
318-2	4 mo	M	+	Thrush (brother of 318-1)	<u>2160</u>	22	1805	NA	<i>IL2RG</i>	—	+
336	4 mo	M	+	Cyanosis, pneumonia (maternal uncles died of infections and immune insufficiency)	395	16	371	0	<i>IL2RG</i>	—	+
349	4 mo	M		PJP, rash eczema	1210	35	236	0	<i>IL2RG</i>	—	+
317	6 mo	M		FTT, Rotavirus, PJP	1000	160	800	0	<i>IL2RG</i>	—	+
348	6 mo	F		FTT, Rotavirus, PJP	1020	10	950	70	AR, not <i>JAK3</i>	Trace§§	+
352	6 mo	F		FTT, PJP	<u>2390</u>	93	2008	NA	AR	—	+
320	7 mo	M		PJP	<u>3348</u>	10	3367	34	<i>IL2RG</i>	Trace	+
321	7 mo	M		PJP, absent radii and thumbs	396	40	350	0	SCID with anomalies	Trace	+
353	7 mo	F		PJP (died before BMT)	1300	28	1057	285	AR	—	+
354	7 mo	M		FTT, diarrhea, PJP	1080	0	921	38	Not <i>IL2RG</i>	—	+
326	8 mo	M		FTT, diarrhea, PJP	710	24	670	14	<i>JAK3</i>	—	+
342	9 mo	M		FTT, diarrhea, dehydration	2750	82	2336	385	<i>IL2RG</i>	—	+
318-1	12 mo	M		FTT, diarrhea, CMV, bronchitis	800	260	420	80	<i>IL2RG</i>	Trace	+
335	13 mo	M		Eczema, elevated IgE, HSV, Rotavirus, Adenovirus, pneumonia, sepsis	1000- <u>2448</u>	268 (DR+, RO+)	558	285	Not <i>IL2RG</i> or <i>JAK3</i>	—	+
350	3 mo	F		Rash, PJP, mother with incontinentia pigmenti	4550	4573	939	NA	Not SCID; NEMO mutation	596	+
329	5 mo	M		PJP	1400	506	458	60	Not SCID; T cells normal at 1 y	624	+
343	7 mo	M		FTT, 3 episodes of pneumonia, died after 2 BMT attempts	<u>2226</u>	140	4	171	Not <i>IL2RG</i>	—	+

Abnormal values are in bold type. Values approaching normal range are underlined.

NK, Natural killer; AR, autosomal recessive; CMV, cytomegalovirus; HSV, herpes simplex virus.

*Known sibling(s) and/or maternal male uncle(s) with SCID or severe immune compromise in infancy.

†Total white blood counts for the patients ranged from 5,110 to 15,300/μL; total lymphocytes/μL, 10th to 90th percentiles⁴⁷: 0-3 mo, 3400-7600/μL; 3-6 mo, 3900-9000/μL; 6-12 mo, 3400-9000/μL; 1-2 y, 3600-8900/μL. Below 2000/μL considered abnormal in infants.^{15,48}

‡10th to 90th percentiles for T cells⁴⁷: 0-3 mo, 2500-5500/μL; 3-6 mo, 2500-5600/μL; 6-12 mo, 1900-5900/μL; 1-2 y, 2100-6200/μL.

§10th to 90th percentiles for B cells⁴⁷: 0-3 mo, 300-2000/μL; 3-6 mo, 430-3000/μL; 6-12 mo, 610-2600/μL; 1-2 y, 720-2600/μL.

||10th to 90th percentiles for NK cells⁴⁷: 0-3 mo, 170-1100/μL; 3-6 mo, 170-830/μL; 6-12 mo, 160-950/μL; 1-2 y, 180-920/μL.

¶2 determinations performed for each sample.

**No PCR product detectable.

††Failure to thrive (weight <5% and falling behind on growth curve).

‡‡Pneumonia due to *Pneumocystis jirovecii* (formerly *P carinii*).

§§Trace PCR detected below threshold level of 30 TRECs/2 punches.

|||DNA from newborn screening filter spot; no other sample available.

Our laboratory receives samples for mutation testing for SCID. In 2002, 34 newly diagnosed infants with confirmed SCID who were born in the US had blood, cell lines, or DNA submitted for mutation diagnosis; 19 (56%), had disease-causing mutations in *IL2RG*. Beginning in 2003, blood submitted in excess of the amount needed for diagnostic studies was spotted onto a filter and dried for TREC analysis. Twenty-three spots were analyzed from newly diagnosed SCID patients, whose characteristics are summarized in Table II. The 3 female and 20 male patients included 2 affected brothers in family 318 and twins in family 345. Four patients were diagnosed in the first month of life, all recognized to be at risk for SCID because of a positive family history. Although one of these infants had respiratory difficulties associated with prematurity, none had infections or failure to thrive as a result of SCID. Three other infants, 339, 318-2, and 336, had known immunodeficiency in siblings or maternal uncles, but they were not investigated by medical providers before onset of infectious complications of SCID. All patients were referred to BMT centers for treatment. Clinical features (including 2 deaths caused by infections with onset before BMT) and lymphocyte phenotypes are indicated. Twelve of the 23 cases (52%) had defects in *IL2RG*.

Duplicate DNA isolations from blood spots of all 23 infants had detectable β -actin. In contrast, 19 had no detectable TRECs, and 4 had <30 TRECs (Table II and Fig 1, C, triangles). The patients with trace amounts of TREC amplification were 6 months of age or older at diagnosis. Of the latter, patient 348, diagnosed at 6 months, had autosomal recessive T^-B^+ SCID with some natural killer cells; patients 320 and 321, diagnosed at 7 months, had X-linked SCID and SCID with congenital anomalies, respectively; and patient 318-1, diagnosed at 12 months with X-linked SCID, had a younger affected brother with no detectable TRECs when diagnosed at 4 months of age.

Blood spots were also made from 2 additional immunodeficient patients (Table II, 329 and 350) for whom SCID was considered but later ruled out. TRECs were positive in samples from both patients. Patient 329 had intermittent lymphocytopenia and pneumocystis pneumonia at 5 months, but by 1 year his lymphocytopenia had resolved without a definite diagnosis. Patient 350 and her mother, who had incontinentia pigmenti, were found to have a previously reported, disease-associated missense mutation of the nuclear factor kappa B essential modulator (NEMO), a condition in which naïve T cells are produced normally, but memory T cells are not.^{49,50}

To measure performance of the TREC assay on actual newborn screening spots, we collected an arbitrary number of sequential, outdated, anonymous Guthrie filters being discarded after testing. Enough saturated blood area for our assay was present on 242 filters. Repeated DNA isolation and PCR were done on samples that had either <1000 β -actin copies or <30 TRECs. Three were rejected for failure to yield DNA that could be amplified for β -actin, leaving 140 cards from routine and 99 from special care nurseries. Mean TREC number for all 239 samples was 1020; the mean (median) ratio of TRECs to β -actin copies

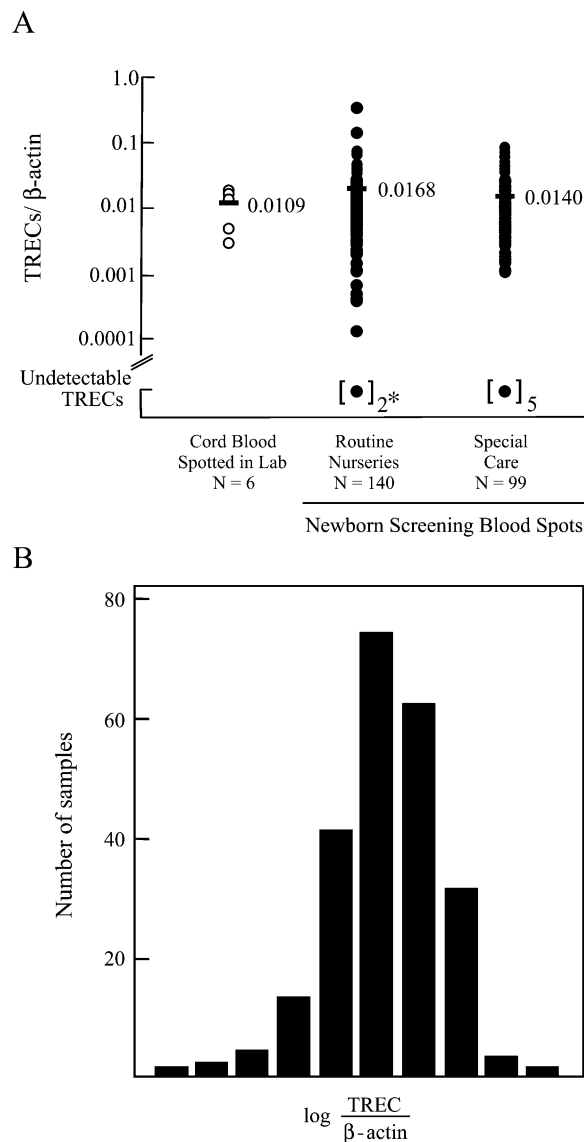


FIG 2. A, TREC/ β -actin from filters made in lab (open circles) and actual Guthrie cards (filled circles) from newborns in routine (center) and special care nurseries (right). N, Total samples/group; bar, mean; *numbers of samples with undetectable TRECs. **B,** Distribution of $\log[\text{TREC}/(\beta\text{-actin})]$ values in 232 Guthrie cards from all nurseries.

was 0.0168 (0.0120) for samples from routine nurseries and 0.0140 (0.0086) from special care nurseries, comparable to filters spotted in the laboratory from cord blood (Fig 2, A). Two spots from routine and 5 from special care nurseries had no detectable TRECs, but they had β -actin amplification. Although blood from a SCID patient would have this profile, these results were almost certainly false positives, given our small sample size. The function $\log[\text{TRECs}/(\beta\text{-actin})]$ evaluated for all samples with non-zero values of TREC and β -actin was normally distributed (Fig 2, B). Means and ranges for this function did not differ between routine versus special care nurseries, and a 1-day

analysis of variance showed no difference for log[TREC/ β -actin] between the nursery types ($P = .73$).

We were able to retrieve the actual newborn screening cards from 2 patients subsequently diagnosed with SCID, patients 126 and 343 (Table II). Copy numbers of β -actin, 17,173 and 38,026, respectively, confirmed that sufficiently pure DNA had been obtained for PCR, but TRECs were undetectable for both patients. Patient 343 had T⁻B⁻ SCID diagnosed with failure to thrive at 7 months; despite 2 BMTs, his immune function was not restored, and he died at the age of 2 years.

DISCUSSION

This study addresses previously unsatisfied criteria for newborn population screening for SCID: unavailability of incidence data and absence of an appropriate screening test. Paradoxically, the true incidence of SCID will be learned only through population screening. However, a minimum incidence can be estimated from our identification of 19 infants with XSCID mutations in a year and the annual US birthrate of 4,000,000.³² If blood from every XSCID infant born in the US were submitted to our laboratory for *IL2RG* mutation detection, the incidence of XSCID would be 1/210,000. Given that X-linked *IL2RG* defects account for about half of all SCID, there must be at least 38 SCID cases annually or at least 1/105,000 births. Other laboratories performing molecular diagnosis for XSCID make this an underestimate, and at least 2 additional lines of evidence indicate that SCID is actually more frequent. First, cases are recognized only in retrospect on diagnosis of a subsequent affected child in a family (Table II, patient 336). Furthermore, in a 1997 survey, parents ascertained through a subsidized SCID family conference were highly educated and had a median income nearly double the national average, suggesting that recognition of SCID is related to family resources.⁵¹ Although heritable, SCID is most often sporadic.¹⁶ Moreover, positive family history does not guarantee early diagnosis, as illustrated by 3 of 7 patients with positive family history in Table II not diagnosed before onset of infections. Immune evaluation is often omitted in newborns with congenital anomalies or incomplete manifestations of chromosome 22q11 deletion.

A total lymphocyte count has been suggested as a screen for SCID that could be performed on cord blood locally in nurseries. Although a pilot series identified most newborns with SCID, around 10% of SCID samples had lymphocyte counts approaching normal values as a result of the presence of high numbers of B cells or maternal lymphocytes.^{14,39} Similarly, of our 23 newly diagnosed SCID patients, 7 had >2000 lymphocytes/ μ L at the time of diagnosis (Table II), although no information is available for them as newborns. Universal screening by lymphocyte counts would also require costly liquid blood collection.

Mutation screening is possible with DNA extracted from dried blood spots, but for all SCID genes a wide variety of mutations occur. For example, hundreds of

mutations in the cytokine receptor gene *IL2RG* have been found to cause X-linked SCID.¹⁶ Mutation screening presents difficulties in interpretation of missense changes that have not previously been proven pathogenic, and only known SCID genes can be screened for mutations.

Screening dried blood spots for specific T-cell surface proteins has been suggested. An immunoassay for CD4 eluted from dried blood spots was claimed to be useful for monitoring patients with HIV infection.⁵² However, such assays have not been adapted for newborn screening, and both CD4 on monocytes and admixture of maternal T cells in circulating blood of newborns with SCID are potential sources of false normal results.

TRECs are a promising analyte for newborn screening because they represent newly formed T cells, they are stable in dried blood spots, and high-throughput screening can be implemented with low cost per test. Circulating T-cell numbers reflect thymic production of naïve cells and antigen-driven, peripheral expansion of cells in which TRECs become diluted. Healthy newborns have TREC numbers equal to 10% of T cells.⁴⁶ In contrast, adult T cells expand predominantly by proliferation and have around 5-fold fewer TRECs (Fig 1, A and B).⁴⁶ Thus, unlike T-cell surface proteins, TRECs in SCID newborn blood spots are unlikely to be derived from maternally transferred lymphocytes. Indeed, maternal T cells found in SCID patients have been reported to have a memory phenotype and to lack TRECs.^{15,53}

Analysis of blood spotted onto filters confirmed that at the time of diagnosis SCID patients lack TRECs. Samples from all of 23 patients with various forms of SCID had <30 TRECs in two 3-mm punches (equivalent to <5 TRECs per μ L of circulating blood). One patient with NEMO deficiency and one with an undefined, transient T-cell lymphopenia had normal TRECs. Further testing will establish the range of cellular immunodeficiency phenotypes that can be found on the basis of low numbers of TRECs.

Importantly, the TREC test was abnormal in 2 actual Guthrie cards obtained from newborns with SCID. The clinical course of these affected infants illustrates the benefit of early diagnosis. Patient 126 has never been ill; a previously diagnosed affected older brother triggered presymptomatic diagnosis and led to his successful treatment by early haploidentical T-cell-depleted BMT. However, patient 343 was not diagnosed for 7 months, during which he experienced chronic diarrhea, failure to thrive, and 3 episodes of pneumonia. Despite 2 attempted haploidentical BMTs during long hospitalizations his immune function remained poor, and he died at 2 years of a sudden, overwhelming infection.

As with other screening tests, failure to amplify TRECs would trigger further screening and/or follow-up for definitive diagnostic tests. Our rates of failure to amplify TRECs were 1.5% in routine and 5% in special care nurseries. Failures in samples from infants in special care (9% of infants⁵⁴) may reflect prematurity, stress, infection, or medications. Prospective screening with larger numbers of samples and follow-up of infants with low TRECs

will reveal both clinical and technical factors that give false positive results as well as identify infants in need of treatment for immunodeficiency.

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