STEM CELL DEFICIENCIES AND THYMIC ABNORMALITIES IN FETAL MOUSE TRISOMY 16

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In the search for models for studying the pathogenesis of Down syndrome in man, mouse trisomy 16 has been identified as an animal model for human trisomy 21 (1, 2). This identification is based on comparative genetic mapping studies demonstrating that three genes on the distal part of the long arm of human chromosome 21 are syntenic in the mouse and are located on mouse chromosome 16 (3–6). The existence of this synteny in both man and mouse indicates that a significant chromosomal segment has been evolutionarily conserved (7), and it is likely that many other genes, yet to be identified, are also conserved in this region. If this is the case, trisomy for mouse chromosome 16 will not only reproduce the genetic imbalance produced by human trisomy 21 of the three specifically mapped genes, but of many other homologous genes as well.

Mice with trisomy 16 can be readily bred, and initial descriptions of these animals have already been published (8, 9). When trisomy 16 ↔ 2n aggregation chimeras prepared by joining trisomic and diploid (2n) preimplantation embryos and then transferring them into foster mothers, were analyzed at the end of gestation, the proportion of trisomic cells in the thymus was significantly reduced (1). In chimeras investigated between 2 and 5 mo after birth, marked reductions (to as low as 10%) were noted in the thymus, spleen, blood, bone marrow, and coat (pigment cells). These findings are consonant with those of Herbst et al. (10) who observed that stem cells from nonchimeric fetal trisomy 16 livers were markedly impaired in their ability to restore hematopoiesis to lethally irradiated animals (radiation chimeras).

In view of the fact that hematological and, in particular, immunologic abnormalities are known to exist in Down syndrome (11–13), the finding of cellular defects in both types of chimeras was of considerable interest. A detailed study of the development and function of the lymphoid and hemopoietic systems in the mouse trisomy 16 fetus was therefore undertaken. We report here that the trisomic fetuses exhibit severe defects in many stem cell populations and devel-

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oping lymphoid and erythroid tissues, deficits out of proportion to the effects of the trisomic state on the development of other tissues and organs. Trisomy 16 in the fetal mouse thus results in a state of genetically caused, combined immunodeficiency and anemia, which differs from all of the other described hereditary mouse disorders of these types.

Materials and Methods

Generation and Identification of Trisomy 16 Mouse Fetuses. Trisomy 16 mouse fetuses were generated as previously described (1). Males doubly heterozygous for two metacentric chromosomes containing chromosome 16, *Rb(16.17)32Lub/Rb(11.16)2H*, were obtained from Professor Alfred Gropp and Dr. Heinz Winking, Medizinischen Hochschule Lübeck, Lübeck, Federal Republic of Germany, and mated with C57BL/6 females. The pregnant females were killed by cervical dislocation at different stages of gestation and the fetuses removed. The genetic status of each fetus, which could be judged from the phenotype (massive edema in midgestation and short, thick neck, flat snout, and open eyelids in late gestation), was verified by karyotypic analysis of tail cells (fibroblasts) cultured for 5–7 d. Between 15 and 20% of the progeny of a C57BL/6 × *Rb32Lub/Rb2H* cross will be trisomic for chromosome 16, and the remainder will be diploid. Monosomy 16 embryos die at the time of implantation.

Preparation and Enumeration of Cell Suspensions. For the enumeration of the cells in fetal livers, the livers were minced in a drop of Dulbecco's modified Eagle's medium (DME)[1] supplemented with 2% fetal calf serum (FCS), and a single-cell suspension was obtained by repeated passage of the suspension through a 25 gauge needle. Red cells were lysed with ice-cold hypotonic (0.085%) saline, and the liver cells were then washed in DME with 2% FCS and suspended in 1 ml of the medium. Total cell counts and viability were ascertained on 1:5 dilutions of the suspension in 0.1% erythrocin B (Aldrich Chemical Co., Milwaukee, WI) in phosphate-buffered saline (PBS). For the quantitation of spleen cells, the same procedure as for fetal liver was followed except that the medium was DME with 10% FCS. For quantitation of thymus cells, the same procedure was also followed, using DME with 2% FCS. Thy-1-positive thymocytes were identified after staining with fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 serum (Becton, Dickinson Monoclonal Center, Inc., Mountain View, CA).

Quantitation of Stem Cell Populations. CFU-S (colony-forming units–spleen) were assayed by the method of Till and McCulloch (14). C57BL/6 mice were x-irradiated with 800 rad. Within 4 h of irradiation, each animal received an injection through the tail vein of 0.2 ml of a suspension of day 16 fetal liver cells in PBS. The animals were killed 8–9 d after injection, the spleens removed and fixed in Bouin's solution, and the number of spleen colonies determined.

CFU-C (colony-forming units–culture) were assayed by a modification of the method of Moore and Metcalf (15). Briefly, for each 35 mm plate, 5×10^5 to 1×10^6 fetal liver cells were suspended in 1 ml of 0.5% Bacto-agar (Difco Laboratories, Inc., Detroit, MI) in RPMI 1640 (Gibco Laboratories, Grand Island, NY), to which was added 0.1 ml of giant cell tumor (GCT)-conditioned medium (Gibco Laboratories), a source of colony-stimulating activity. The plates were incubated for 6–7 d at 37°C in 5% CO_2 in air, and scored for colony formation. In some experiments, the GCT-conditioned medium was omitted and the liver cells were plated over a 24-h-old feeder layer of STO cells that had been treated with 10 μg/ml mitomycin C at 37°C for 2 h (16). The identities of the cells in the colonies were verified by staining for peroxidase (granulocytes) and nonspecific esterase (monocytes).

[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; BFU-E, burst-forming units–erythroid; CFU-B, colony-forming units–B lymphocyte; CFU-C, colony-forming units–culture; CFU-E, colony-forming units–erythroid CFU-S, colony-forming units–spleen; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GCT, giant cell tumor; IL-2, interleukin 2; PHA, phytohemagglutinin; PBS, phosphate-buffered saline; RITC, tetramethylrhodamine isothiocyanate; SRBC, sheep red blood cells.

CFU-B (colony-forming units–B lymphocyte) were enumerated in fetal livers as described by Paige (17). Briefly, 10^5 day-17 fetal liver cells were plated in 0.3% Bacto-agar over a normal, day-17 fetal liver–adherent cell layer covered with Bacto-agar containing 50 μg/ml lipopolysaccharide B. The cultures were incubated for 8 d at 37°C in 5% CO_2 in air, and a cell plaque assay performed as described by Paige and Skarvall (18).

BFU-E (burst-forming units–erythroid) and CFU-E (colony-forming units–erythroid) were assayed in day-14 fetal liver as described by Rich and Kubanek (19). To quantitate CFU-E, sheep plasma erythropoietin (Step III; Connaught Laboratories, Inc., Swiftwater, PA) was added at concentrations of 0.08 or 0.25 U/ml and the cultures were incubated in a 37°C humidified incubator with 5% CO_2 for 36–48 h. The plates were stained with benzidine hydrochloride (Sigma Chemical Co.) (20) and scored under an inverted microscope; CFU-E were identified as aggregates consisting of at least eight cells that stained blue after 5 min.

To quantitate BFU-E, sheep plasma erythropoietin was used at concentrations of 1 or 2 U/ml, and the cultures were incubated in a 37°C humidified incubator with 5% CO_2 for 10 d. Using an inverted microscope, the plates were scored either unstained or after staining with benzidine.

Detection of Pre-B and B Cells in Fetal Liver. For the quantitation of pre-B and B cells, liver cell suspensions were prepared as described above and stained by a procedure provided by Dr. John Kearney (University of Alabama, Birmingham, AL). One to two million liver cells were pelleted and stained for surface-bound IgM with FITC-conjugated goat anti–mouse μ chain antibody (a gift from Dr. John Kearney) used at a concentration of 0.5 mg/ml and a fluorescein/protein ratio of 2.2:1. Cytocentrifuge slide preparations of the stained cells were made using a Cytospin II (Shandon Southern Instruments, Inc. Sewickley, PA) and, after air drying, the slides were fixed and washed four times in PBS. At this stage, the slides can be kept refrigerated in PBS or used immediately for staining of cytoplasmic IgM.

To detect cytoplasmic bound IgM, tetramethylrhodamine isothiocyanate (RITC)-conjugated goat anti–mouse μ chain antibody (a gift from Dr. John Kearney) was used at a concentration of 0.2 mg/ml and a rhodamine/protein ratio of 1.0:1. After being washed in PBS, the slides were mounted with 75% glycerol (Mallinckrodt, Inc., St. Louis, MO) in PBS and covered with a glass coverslip; fluorescence was determined using a Zeiss Photomicroscope III. Surface-positive B cells were small and showed a characteristic B cell pattern of fine spotty staining. Under RITC illumination, pre-B cells showed a characteristic perinuclear staining, often Golgi associated, but were surface μ chain negative.

Transformation with Abelson Murine Leukemia VIrus (A-MuLV). Day-17 fetal liver cells were transformed with A-MuLV in semi-solid agarose as described by Rosenberg and Baltimore (21), using an A-MuLV stock prepared from 54/Cl_2 cells (kindly provided by Dr. Naomi Rosenberg, Massachusetts Institute of Technology, Cambridge, MA).

To assay for transformation with A-MuLV in liquid medium, 1 ml cell suspension containing 2×10^6 cells was mixed with 1 ml A-MuLV stock in a plastic tube. Polybrene was added at a concentration of 4 μg/ml, and the tubes were incubated in a humidified 37°C incubator in 5% CO_2 for 2.5 h, with agitation every 15 min. After virus adsorption, the cell suspensions were diluted 1:20, 1:10, or 1:2 with RPMI 1640 containing 20% FCS, 50 μM 2-mercaptoethanol, and 2 μg/ml polybrene, and 0.1 ml aliquots of the cell suspension were dispensed into wells of a 96-well microplate (Micro Test III: Falcon Labware, Oxnard, CA). The cultures were refed with 0.1 ml complete RPMI 1640 medium on day 4, and visible colonies were scored on day 12 using an inverted microscope.

Fetal Thymus Organ Culture and Thymocyte Stimulation. Fetal thymuses taken from day-14 or day-17 mouse fetuses were cultured in vitro by the method of Robinson and Owen (22). For mitogen stimulation experiments, individual lobes of thymuses were removed from culture and teased apart with fine-nosed forceps in 0.1 ml RPMI 1640 medium with 15% FCS in a flat-bottomed, 96-well microtiter plate (Falcon Labware). An aliquot of the cell suspension was diluted with 0.1% erythrosin in PBS and counted. The thymus cell suspension was then adjusted to a concentration of 6×10^5 viable cells/ml, and 0.05-ml

aliquots (3×10^4 cells) were dispensed into wells of round-bottomed, 96-well microtiter plates. Reagent grade phytohemagglutinin (PHA) (Wellcome Reagents Ltd., Beckenham, England) and concanavalin A type IV-S (Con A) (Sigma Chemical Co.) were each diluted in PBS to concentrations of 4.2 or 8.3 μg/ml, and 0.01 ml of either was added to the test wells. For control cells, an equivalent volume of PBS was added. After 48 h in culture in 5% CO_2 in air, 1 μCi of [^{3}H]thymidine in 0.01 ml PBS was added to each well, and the plates were incubated an additional 18–24 h. The cultured cells were then harvested onto glass fiber filters wtih an automatic cell harvester (MASH; Otto Hiller Co., Madison, WI) as described by Goldblatt et al. (23). After drying, the filters were placed into scintillation fluid and counted.

Assay for Interleukin 2 (IL-2) Production. The IL-2 activity of the supernatant fluids from the Con A–stimulated fetal thymocytes was determined by the T cell growth assay described by Gillis et al. (24), using CTLL-A2 cells (a cloned, H-2D^d-specific, cytotoxic, IL-2-dependent T cell line) as the responding cell. The IL-2 activities of the supernatant fluids are expressed relative to the activity of a standard rat IL-2 preparation.

Enumeration of Germ Cells. Day-12 fetuses were fixed in 10% neutral buffered formalin, infiltrated in glycol methacrylate (25), cut at 2 μm on a JB-4 microtome (DuPont Co., Sorvall Instruments Div., Newtown, CT) with glass knives, and every fifth section air dried to a slide. To identify germ cells, the sections were stained for alkaline phosphatase as described by Sheehan and Hrapchak (26), using a 2 h incubation at 37°C. The nuclei were then stained with 0.1% aqueous neutral red, the slides air dried, and coverslips applied with Permount (Fisher Scientific Co., Pittsburgh, PA).

Statistical Calculations. Except as noted otherwise, all statistical comparisons were done using the nonparametric Wilcoxon signed rank test.

Results

Deficiencies of Cell Populations in Trisomic Organs. Overall, the trisomy 16 mouse fetuses were modestly retarded in development, with total fetal weights reduced ~25% compared with diploid siblings over the last week (days 14–19) of gestation. For this reason, comparisons of the numbers of cells in trisomic and diploid fetal thymuses, spleens, and livers were carried out on a weight rather than length-of-gestation basis. Profound deficiencies of cells occurred in both the thymus (Fig. 1) and spleen (Fig. 2), with the trisomic organs showing >80% decreases in total cell number at all stages of development. By contrast, the number of cells in the livers of trisomic fetuses were decreased only ~50% throughout the period studied. Circulating red cells were also decreased, with the mean red cell count of trisomic day-17 fetuses reduced by 36% (5.45 vs. 8.56×10^6 cells/ml). The mean hematocrit of trisomic day-18 fetuses was 52% lower than that of diploid controls: 14.3 ± 1.6% (SEM) and 30.0 ± 1.2%, respectively.

In addition to its small size, the trisomic thymus was abnormally located. Late in gestation, rather than anteriorly overlying the heart, it was high in the thorax near the great vessels and trachea. Furthermore, the trisomic thymocytes were somewhat retarded in the expression of the Thy-1 antigen. On day 17, the proportions of Thy-1-positive cells in trisomic and diploid fetal thymuses were, respectively, 88.7 ± 4.5% and 91.4 ± 2.1%. However, on day 18, the values were 79.3 ± 6.8% and 95.3 ± 0.9%, respectively, a difference significant at $P < 0.005$.

Myeloid Granulocytic, and Erythroid Stem Cell Deficiencies in the Trisomic Fetal Liver. In addition to decreases in overall organ cell numbers, specific stem cell populations were also found to be markedly decreased in the trisomic liver. The

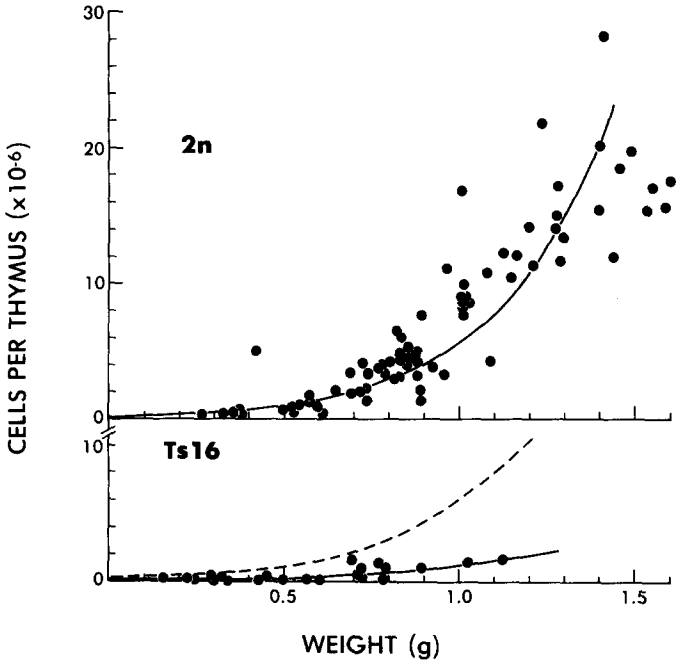

FIGURE 1. Number of cells per thymus in trisomic (*Ts16*) and diploid (*2n*) fetuses, expressed as a function of fetal weight. Each point represents an individual fetus. The curve for fetuses is shown on top and is reproduced with a dashed line in the lower panel, which shows the data for trisomic fetuses. The curves were obtained by fitting the data to the best exponential function by the method of least squares.

myeloid stem cell population defined by the CFU-S assay was studied on day 16 of fetal development using livers from sibling animals (Table I). In two independent experiments, the total numbers of CFU-S in the trisomic livers were 27 and 33% of the numbers in the diploid controls.

The granulocyte-macrophage stem cells identified by the CFU-C assay were also markedly diminished during days 14–18 of gestation (Table II). Using a GCT-derived colony-stimulating activity, the numbers of colonies per liver were decreased in trisomic livers by >90% on days 14 and 16 of gestation and by ~80% on day 18. When an STO cell feeder layer was used as the source of colony-stimulating activity, total colony numbers were greater than with GCT-conditioned medium. Nevertheless, although the deficiency was less severe on day 16, the same profound decrease in CFU-C in trisomic livers was noted on day 14.

Two erythroid stem cells, the more primitive BFU-E and the later CFU-E, were assayed in day-14 fetal livers (Table III). Like the stem cells described previously, the number of trisomic BFU-E was severely reduced. Relative to the number of cells used in the assay, reduction was 60–80%. However, on a total liver basis, the reduction was even greater, 80–90%. In contrast, the decrease in CFU-E was considerably less, with normal relative numbers and only an ~50% decrease in total number per liver, as would be expected from the reduced size of the trisomic liver.

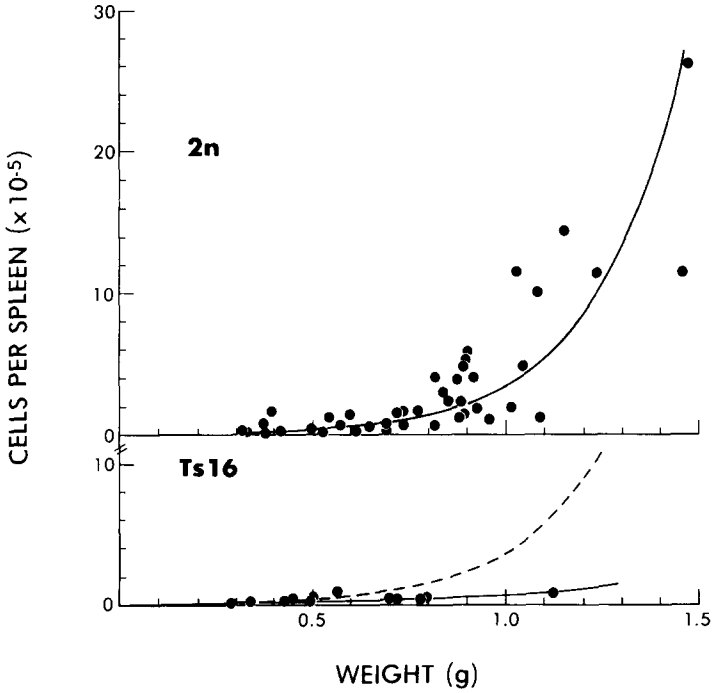

FIGURE 2. Number of cells per spleen in trisomic (*Ts16*) and diploid (*2n*) fetuses, expressed as a function of fetal weight. See legend to Fig. 1 for further details.

TABLE I
CFU-S in Day-16 Trisomy 16 Fetal Livers

	CFU-S			
	Experiment 1		Experiment 2	
	Ts16	2n	Ts16	2n
Number of cells injected				
4.0×10^5	15.6	11.6	9.8	15.6
2.4×10^5	10.5	6.8	6.5	8.6
0.8×10^5	2.8	3.2	1.8	4.2
Mean per 10^6 cells	39.3	32.4	24.7	43.9
Cells per liver $\times 10^{-6}$	(6.2)	(28.0)	(22.7)	(39.3)
CFU-S per liver	243	908	561	1723
$\frac{\text{Ts16}}{\text{2n}}$	0.27		0.33	

Data represent the mean spleen colony count of three (occasionally two) animals receiving the same number of fetal liver cells.

Pre-B and B Lymphocyte Deficiencies in Trisomic Liver. Two methods were used to assess the number of pre-B and B lymphocytes in fetal liver (Table IV). The first, based on immunofluorescent staining of pre-B and B lymphocytes with antibodies against μ chains, revealed a <20% decrease in the relative number of these cells in day 17 and 18 trisomic livers. The other, using a colony-forming assay for CFU-B, revealed a statistically nonsignificant 15% decrease in colonies

TABLE II

CFU-C in Trisomy 16 Fetal Livers

Day of gestation	Colonies per 10^6 fetal liver cells						Colonies per liver					
	GCT growth factor			STO cell feeder layer			GCT growth factor			STO cell feeder layer		
	Ts16	2n	Ts16/2n	Ts16	2n	Ts16/2n	Ts/16	2n	Ts16/2n	Ts16	2n	Ts16/2n
14	2.0	52.0	0.04	18	143	0.13	16	829	0.02	140	2,239	0.06
	0	15.0	0	14	107	0.13	0	204	0	142	1,556	0.09
	4.4	28.4	0.15				44	588	0.07			
16	2.7	30.8	0.08	64	74	0.86	21	302	0.07	431	700	0.62
	3.8	31.5	0.12	31	74	0.42	55	707	0.08	357	1,611	0.22
17	3.9	12.5	0.31									
18	6.3	12.6	0.50				114	511	0.21			

Each point represents the mean of three (occasionally two) replicate plates; the results of individual experiments are placed in separate rows. The GCT growth factor and STO cell feeder layer experiments were carried out on the same cell preparations.

TABLE III

Erythroid Stem Cells (BFU-E and CFU-E) in Day-14 Trisomy 16 Fetal Livers

Cells per plate	Erythropoietin concentration	Colonies per plate				Colonies per liver × 10^{-3}			
		Ts16	2n	P*	$\frac{Ts16}{2n}$	Ts16	2n	P	$\frac{Ts16}{2n}$
	U/ml								
BFU-E									
4×10^4	1	1.4	3.7	<0.05	0.38	0.19	1.02		0.18
7.5×10^4	2	1.8	9.8	<0.005	0.18				
		1.6	7.7	<0.005	0.20	0.11	1.09		0.10
CFU-E									
4×10^4	0.08	1,040	980	—		142	362	—	0.39
	0.25	1,190	1,040	NS		166	328	NS	0.51
		1,060	860	—		234	403	—	0.58
	Combined	1,150	990	NS		186	349	0.05	0.53

For BFU-E, each value represents the mean of two or four replicate plates; for CFU-E, the mean of two to five assays from individual animals, each performed in duplicate.

* Calculated by Wilcoxon rank sum test. NS, not significant.

TABLE IV

Pre-B and B Lymphocytes in Trisomy 16 Fetal Livers

Day of gestation	Genotype	Immunofluorescence				CFU-B			
		Proportion (percent of total cells)			$\frac{Ts16}{2n}$	Colonies per 10^5 liver cells	$\frac{Ts}{2n}$	Colonies per liver × 10^{-4}	$\frac{Ts16}{2n}$
		B	Pre-B	B + Pre-B					
17	Ts16	0.43 ± 0.19	2.68 ± 1.10	3.12 ± 0.74	0.83	22.4 ± 3.9	0.85	2.12 ± 0.43	0.39
	2n	0.24 ± 0.10	3.53 ± 0.69	3.77 ± 0.65		26.5 ± 3.1		5.37 ± 0.87	
18	Ts16	2.46 ± 1.02	3.84 ± 0.90	6.50 ± 0.42	0.93				
	2n	2.15 ± 0.42	4.97 ± 0.43	7.01 ± 0.61					

The immunofluorescence data are expressed as mean ± SEM of 5–15 individual animals and represent proportions of cells expressing surface (B) and cytoplasmic only (pre-B) μ chains; the Ts16/2n ratio is expressed for the proportions of B + pre-B cells. The CFU-B data are expressed as mean ± SEM of 9 and 20 determinations for Ts16 and 2n livers, respectively.

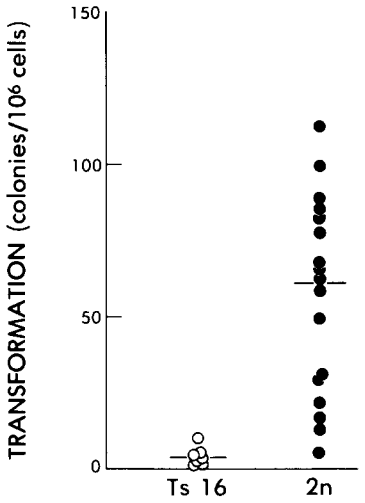

FIGURE 3. Transformation in agar of trisomic (*Ts16*) and diploid (*2n*) fetal liver cells by A-MuLV. Each point represents the mean of three replicate plates from a single liver preparation.

per 10^5 liver cells on day 17, but, when expressed on a total liver basis, the mean value in trisomic livers was decreased by 61% ($P < 0.005$).

Insensitivity of Fetal Liver Cells to Transformation by A-MuLV. Because of our interest in a possibly enhanced sensitivity of trisomy 16 pre-B and B cells to leukemogenic transformation (see Discussion), we exposed trisomic and control liver cells from day-17 or day-18 fetuses to A-MuLV in vitro and scored the appearance of transformed colonies. When the assay was carried out in agar culture, there was a profound decrease (94%, $P < 0.005$) in the number of colonies. The means for trisomic and diploid liver cells were 3.7 ± 1.2 and 61.4 ± 9.0 colonies per 10^6 cells, respectively (Fig. 3). When the transformation occurred in liquid culture, the respective means were 4.7 ± 1.3 and 127 ± 16 colonies per 10^6 cells, a decrease of 96%. These decreases could not be attributed to equivalent decreases in the proportion of pre-B and B lymphocytes, for which results were presented in Table IV.

Normal Rates of Proliferation But Deficiency in Cell Numbers in Trisomic Thymuses Grown in Organ Culture. To ascertain whether the reduction in the number of lymphocytes in trisomic thymuses is the result of a decreased rate of lymphocyte proliferation, a decreased number of early thymocytes, or both, we removed day-14 thymuses and cultured one lobe of each in vitro for 7 d. The other lobe was analyzed at the time of initiation of the cultures. Two experiments are illustrated in Fig. 4. During the week of culture, the increase in cell number was 10–12-fold, with the rates for trisomic and diploid thymuses being indistinguishable. As a result, the 78% reduction in cell number in trisomic thymuses at the time of explant was matched by an 82% reduction after 7 d in culture. Furthermore, despite these large differences in cell numbers, the proportion of lymphoid cells in the thymuses, both before and after culture, and of large lymphocytes after culture, was not significantly different between the trisomic and diploid

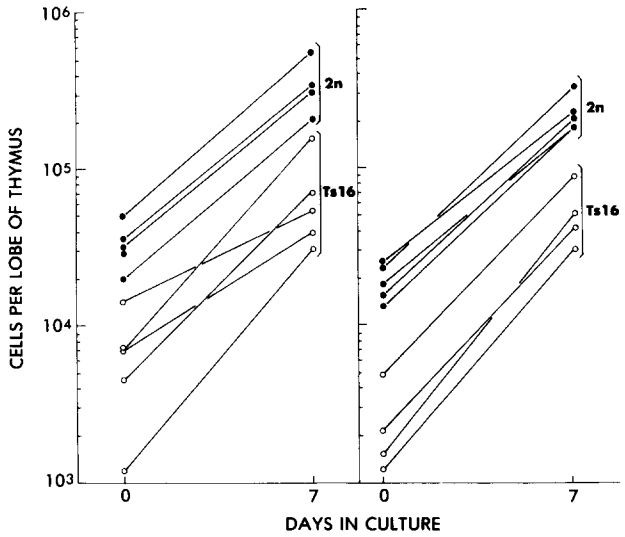

FIGURE 4. Proliferation of thymocytes in trisomic (*Ts16*) and diploid (*2n*) fetal thymic lobes cultured in vitro. For each pair of points connected with a line, the day 0 count was obtained from one lobe of a thymus and the day 7 count from the other lobe after 7 d of culture in vitro. Data from two different experiments are shown.

thymuses. Both sets of results suggest that abnormalities in the proliferation of thymocytes are not the principal problem in the trisomic thymus.

Delayed Functional Maturation of Thymic Lymphocytes in Trisomic Thymuses Grown in Organ Culture. To study the functional capacities of trisomic T lymphocytes, as manifested by proliferative and IL-2 production responses to mitogens, fetal thymuses were explanted at day 17 and cultured in vitro for up to 17 additional days. As expected, the initial cell count was much higher in the diploid than in the trisomic thymuses, 1.54 (±0.41) × 10^6 vs. 0.15 (±0.03) × 10^6 cells per thymic lobe (Fig. 5). However, while the number of trisomic cells increased, with a somewhat wavelike pattern, during the period of culture, the diploid thymuses demonstrated an early precipitous drop in cell number before demonstrating a level quite close (within a factor of two) to that in the trisomic thymuses. This drop in cell number was also reflected in the decrease in the viability of the cells in the diploid thymuses during the first week of organ culture, from a mean cell viability of 84% in the trisomic thymuses to 63% in the diploid organs (Fig. 5).

When the proliferative response of the cultured fetal T lymphocytes to mitogenic stimulation was assessed, it was found that PHA was a poor mitogen. All further experiments were therefore carried out with Con A, and the results of a large number of determinations are shown in Fig. 6. For diploid fetal lymphocytes, the maximal response occurred after 9–10 d in culture. In contrast, the trisomic cells, although showing an early peak in the stimulation index after 10 d in culture, did not reach their maximal responsiveness until after 15–16 d. At that time the trisomic lymphocytes showed a response seven to eight times greater than that of the diploid cells.

In general terms, the curves for IL-2 production are similar to those for proliferation, with the trisomic cells showing a somewhat lower response than

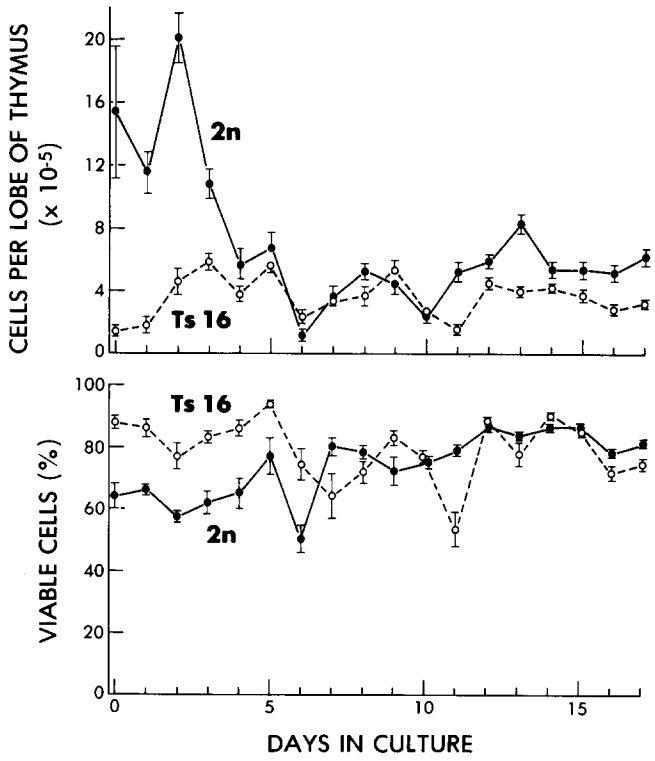

FIGURE 5. Mean cell counts (*top*) and viabilities (*bottom*) of trisomic (*Ts16*) and diploid (*2n*) thymocytes from day-17 fetal thymuses cultured in vitro. Each point represents the mean ± SEM of 12–41 thymic lobes for the diploid fetuses and 8–22 lobes for the trisomics.

the diploid cells for the first 13 d of culture and then a much higher response after 15–17 d (Fig. 6). However, when IL-2 production was plotted as a function of the mitogenic response to Con A, there was only a weak correlation between the two: $r = 0.50$ for the trisomic and diploid data combined, and 0.42 and 0.55, respectively, for the diploid and trisomic data separately.

Normal Numbers of Germ Cells in the Genital Ridges. Because of the frequent association of germ cell deficiencies or sterility with hereditary anemias, we undertook to determine whether trisomy 16, with its multiple stem cell deficiencies, also had a germ cell defect. The mean total embryo germ cell counts obtained in this manner were 3,585 ± 450 ($n = 8$) and 2,476 ± 514 ($n = 5$) for diploid and trisomic embryos, respectively. Although the overall mean for the trisomic embryos was 31% lower than for the normal ones, the difference was not statistically significant.

Discussion

Trisomy for chromosome 16 in the mouse had profound effects on all aspects of hematopoietic stem cell differentiation that have been studied. There were marked deficiencies of several stem cell and precursor populations, ranging, on an absolute (per fetus or liver) basis, from 40 to 60% in late erythroid stem cells

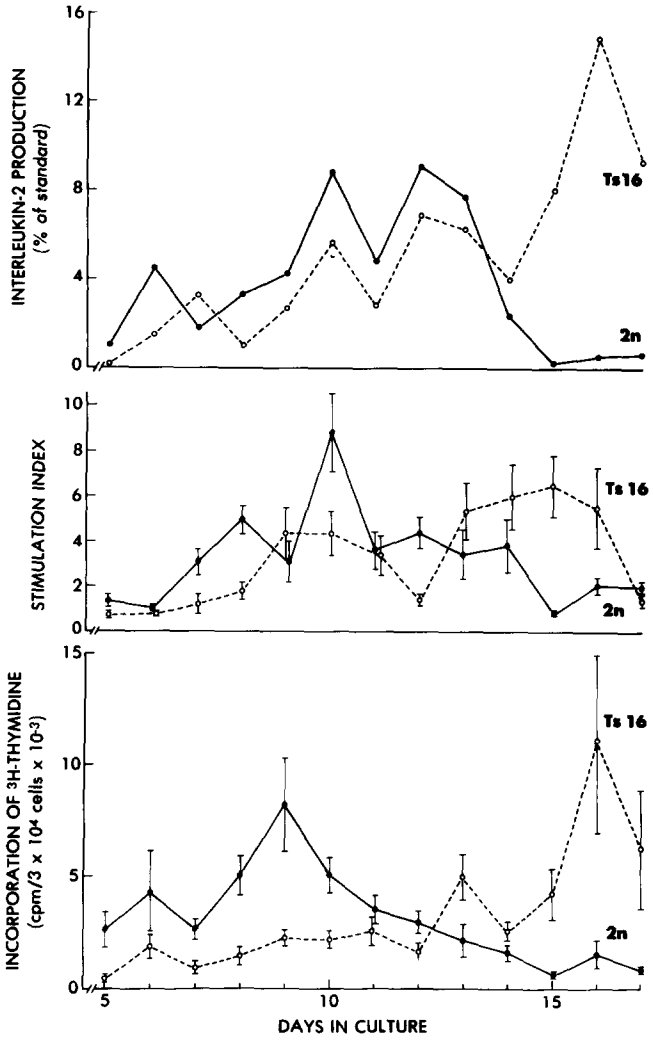

FIGURE 6. The proliferative responses and production of IL-2, after mitogenic stimulation with Con A, of lymphocytes obtained from day-17 fetal thymuses cultured in vitro for the time specified on the abscissa. After release from the cultured thymus, the lymphocytes were cultured with Con A for 72 h; the medium was changed and [^{3}H]thymidine added 48 h after initiation of the culture. The results are expressed as mean incorporation ± SEM of [^{3}H]thymidine by the stimulated cells (*bottom*); mean ± SEM stimulation index (cpm in stimulated cultures/cpm in unstimulated cultures) (*middle*); and IL-2 content of the 48 h culture medium relative to a rat IL-2 activity standard (*top*).

(CFU-E) and pre-B lymphocytes and CFU-B to 70–90% decreases in multipotential (CFU-S), granulocyte-macrophage (CFU-C), and early erythroid (BFU-E) stem cells. These decreases were coupled with severe reductions, on the order of 50 to ≥80%, in the number of differentiated cells derived from these precursors, reductions that were reflected in a reduced hematocrit and in decreased sizes and cellularity of the late fetal thymus, spleen, and liver. These

reductions and decreases are out of proportion to the moderate retardation in growth and development that characterizes the trisomic condition.

Although all of the hematopoietic cell lineages appear to be affected, the abnormality of the thymus is particularly striking and T lymphocytes appear much more severely affected than the B lymphocytes. Our results suggest that the principal problem in the growth of the thymus is not in the proliferation of the thymocytes per se. Rather, there appears to be a reduced number of thymocyte precursors settling in the primordial thymus, either because of a decrease in their number in the fetal liver or because of an inhospitable environment in the early embryonic gland.

Even though thymocyte proliferation occurs at a normal rate in the developing trisomic fetal thymus, the thymocytes present in trisomic animals late in gestation demonstrate distinct differences in functional maturation. The most striking feature is a delay in the maturation of the fetal thymocytes in vitro to a state of responsiveness to the mitogenic effect on Con A. It is noteworthy, however, that the magnitude of the peak response was quite similar in trisomic and diploid thymocytes. The delay in achieving peak responsiveness was also reflected in IL-2 production, a marker of T helper cell function (27), although, in this instance, the trisomic cells were actually more responsive than normal cells (Fig. 6). Thus, although maturation is delayed for both functions, the ultimate degree of responsiveness is not reduced. However, the time lag does suggest that the maturational delays we observed with the trisomic thymuses in vitro may in part be related to retarded development of the trisomic thymus and thymocytes in vivo. In addition to the reduced overall growth and development of trisomic fetuses, the modest reductions in the proportion of Thy-1-positive cells on days 17 and 18 may be construed as evidence for such in vivo maturational delay.

Perhaps the most remarkable observation concerning the trisomic fetal cells is the very low susceptibility (<5% of control) of cells from the trisomy 16 liver to transformation by A-MuLV. This result was totally unexpected since it was felt, by analogy with the increased susceptibility of humans with trisomy 21 to the development of leukemia (28), if anything, the murine trisomic cells might be more susceptible to viral transformation. Available evidence suggests that undifferentiated cells of the B lymphocyte lineage are the targets for transformation by A-MuLV (29). However, we were unable, in our assays for both B and pre-B lymphocytes, to show a decrease in these populations of a degree anywhere near that required to explain the transformation results. Three other possibilities may therefore be considered. One is that the target cell for A-MuLV is a type of very primitive B cell that does not make a major contribution to the cell populations detected by the assays used here and whose almost complete absence would not be detected. Another possibility is that the target cell which is greatly reduced in number in the fetal liver is not really a B cell; in this regard, it is of interest that erythroid colonies have been obtained from A-MuLV-infected day 9–12 fetal liver. Finally, it is conceivable that the target cell population is not reduced at all and that the trisomic state alters the target cells, making them highly resistant to transformation. Further study is required to distinguish among these possibilities.

As was mentioned in the introduction, Herbst et al. (10) observed that stem

cells from fetal trisomy 16 livers were unable to restore normal hematopoiesis to irradiated normal hosts. They also found, as did we, that CFU-C and CFU-S were reduced in numbers, the former by ≥75% and the latter by 50%, on a relative basis. Moreover, similar to our findings, the relative reduction in trisomy 16 CFU-C was greatest at 14–16 d gestation, when it was >90%. These stem cell deficiencies, as well as the others defined in the present work, can explain the poor reconstitution and limited survival of the radiation chimeras. In addition, they explain the poor representation of the mature derivatives of these cells (lymphocytes and erythrocytes) in the trisomy 16 ↔ 2n aggregation chimeras that we prepared. Although stem cells from some of the other mouse trisomies may also be defective, serious hematopoietic abnormalities are not necessarily a feature of mouse trisomies in general (30). It seems fair to conclude, therefore, that the hematopoietic abnormalities in trisomy 16, while not necessarily unique, are characteristic of this particular form of genetic imbalance.

Although the genetic defect in trisomy 16 is the extra copy of a normal chromosome carrying normal genes, and not a mutation or set of mutations, our striking findings in trisomy 16 invite comparison with the other genetically caused immunodeficiencies and anemias in the mouse. These comparisons are shown in Table V and include those conditions that appear to result from abnormalities in cellular development; autoimmune disorders are omitted. The marked deficiencies in lymphoid and granulocytic cell precursors and the absence of germ cell deficiency in trisomy 16 distinguish it from the W/W^v and Sl/Sl^d forms of heterditary anemia (31, 32). In this regard, it most closely resembles the immunodeficiencies with combined T and B lymphocyte defects (33–35). However, the anemia and erythroid stem cell abnormalities in trisomy 16 indicate that the defects go beyond the lymphoid system and presumably involve very early hematopoietic stem cells or components common to several different cell types. Therefore, although complete information is not available on all mouse immunodeficiencies, trisomy 16 does not appear to be identical to any of them and produces more extensive hematopoietic aberrations. However, of all of the genetic immunodeficiencies and anemias thus far examined, only *nu/nu xid*/Y, a combination of two independent mutations affecting both T and B lymphocytes, demonstrates a decrease in Abelson virus transformation similar to that found in trisomy 16 (29).

We come, finally, to a consideration of the relationship between our observations in trisomy 16 mice and the immunologic and hematologic findings in humans with trisomy 21, since it was the genetic relationship between these two trisomies that was originally responsible for the interest in mouse trisomy 16. Although there is considerable disagreement in the literature, a picture of immunologic impairment associated with human trisomy 21 has emerged (for review see reference 13). T lymphocytes have often been reported as decreased in number (36), with decreased ratios of helper to suppressor cells as defined with monoclonal antibodies (12).[2] The proliferative response of lymphocytes to

[2] Philip, R., A. C. Berger, N. H. McManus, N. H. Warner, M. A. Peacock, and L. B. Epstein. Numerical alterations in lymphoid subpopulations and abnormalities of the in vitro cellular and humoral responses to bacterial and viral antigens in Down syndrome (trisomy 21). Submitted for publication.

TABLE V

Comparison of Trisomy 16 with Other Genetic Mouse Immunodeficiencies and Anemias

	Ts 16 (fetal)	Immunodeficiencies							Anemias	
		nu/nu	*Dh/+*	*xid/Y*	*nu/nu Dh/+*	*scid/scid*	*wst/wst*	*nu/nu xid/Y*	*W/W^v*	*Sl/Sl^d*
Chromosome location of gene	(Ts16)	11	1	X	1, 11			11, X	5	10
Lymphocyte class affected		T	?T/?B	B	T/?B	T + B	T + B	T + B		
Thymus	↓↓	0	↑		0	↓↓	↓		nl Immune response	nl Immune response: ↓ thymus in Sl^{Wahi}/Sl^{Wahi}
Spleen	↓↓	nl/↑	0	↓	0	↓↓	↓ (T + B)		↓ Erythropoiesis and leukopoiesis	
Liver	↓								↓ (fetal)	↓ (fetal)
Response to:										
T cell mitogens	Delayed response in thymus culture	0	↑	nl	↓↓↓	↓↓	↓↓ (SRBC in vivo)	↓↓	↓ θ-Sensitive regulatory cell	
B cell mitogens		nl	nl	↓↓	sl ↓	↓↓		↓↓		
Erythrocytes										
Number	↓	nl	nl		nl				↓↓	↓↓
Size	nl/↑								↑	↑
Stem cells										
BFU-E	↓↓								↓↓ (Adult)	↑ (Fetal) ↓ (Adult)
CFU-E	↓								nl/↓ (Adult)	↓ (Fetal + adult)
CFU-S	↓			nl					↓↓	nl
CFU-C	↓↓	sl ↑	nl		sl ↑				nl	↓
CFU-B (pre-B)	↓			0		0 Pre-B in marrow			nl (Adult), ↓ (Fetal)	
Viral transformation										
Abelson	↓↓↓	↑		nl				↓↓	nl	nl
Friend									↓↓	↓↓
Germ cells	nl								↓↓	↓↓

0, Absent; nl, normal; sl, slightly. See text for references.

mitogens such as PHA and Con A has also been frequently reported to be decreased (37, 38), as has in vitro response to several antigens (11).[2] Of particular relevance to the observations reported here are the reports of anatomic and functional abnormalities of the thymus, including marked lymphoid depletion in infants, enlargement and cystic changes of Hassall's corpuscles (36), and decreased levels of serum thymic factors (39). Burgio et al. (12) have suggested that all of the immunologic problems in trisomy 21 result from a primary defect in the thymus that interferes with the ultimate maturation of thymocytes into fully competent T lymphocytes. Although anemia is not characteristic of trisomy 21, abnormal activities of several erythrocyte enzymes suggestive of some impairment of erythropoiesis have been noted (40). Similarly, while leukopenia is not a feature of Down syndrome, disturbances of granulocyte kinetics have been reported (41) and a reduced number of circulating stem cells (CFU-C) has been described (42).

It thus appears that, while the hematopoietic abnormalities in the two trisomic conditions are not identical, those in the mouse being much more severe than those in man, interesting parallels can be drawn, particularly regarding the thymus. The evidence for thymic deficiency in Down syndrome, coupled with the finding of dramatic thymic hypoplasia in the trisomy mouse fetus, suggests that the same or a similar mechanism might be operating to produce the T lymphocyte abnormalities in both species. If this should turn out to be the case, it might be expected that finer genetic dissection of the trisomic state in the mouse would reveal a relationship between the hematopoietic defect and the specific unbalanced gene or genes that causes it. However, even if the defects in the trisomy 16 mouse should prove dissimilar to those in humans with trisomy 21, the trisomic mouse will still be worthy of consideration on its own merits and will provide valuable information about the genetic control of development of the hematopoietic system. As with the other genetic abnormalities of hematopoiesis, the precise mechanisms giving rise to the defects in trisomy 16 remain to be elucidated. Although many mechanisms might be suggested, the relationships between the presence of an extra chromosome and the developmental abnormalities are still unknown (13).

Summary

Mouse fetuses with trisomy 16 have severe abnormalities of several hematopoietic stem cell and precursor populations. The thymus is extremely hypoplastic, with a ≥80% reduction in the number of thymocytes. This cellular deficiency appears to be the result of a deficiency in the number of precursor cells in the early thymus, since the rate of proliferation of thymocytes in explanted day-14 thymuses was normal. However, the functional maturation of thymocytes was delayed in vitro in day-17 organ explants, although the maximal response to the mitogenic and interleukin 2–stimulating effects of concanavalin A are quantitatively normal. B cells and pre-B cells in the fetal liver were moderately decreased, but the ability of fetal liver cells to be transformed by Abelson murine leukemia virus was nearly totally lost. There were also significant relative and absolute decreases in the number of spleen, culture, and erythroid colony-forming units (CFU-S, CFU-C, CFU-E) and of erythroid burst-forming units (BFU-E) in the

trisomic liver, and the trisomic animals were anemic with small spleens and livers. However, unlike other genetically caused anemias, there was no reduction in the number of germ cells. The hematopoietic abnormalities in the trisomy 16 mouse, involving the lymphoid, myeloid, and erythroid cell lineages, are much more generalized than the abnormalities in any of the other described genetically caused immunodeficiencies or anemias in the mouse. They are also more severe than those in human trisomy 21 (Down syndrome), for which mouse trisomy 16 is a genetic model, but there does exist an interesting parallel between the thymic abnormalities in the two species.

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