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Chronic exposure to benzene results in a unique form of dysplasia

Richard D. Irons [a,b,c,d,*], Ling Lv [a,e], Sherilyn A. Gross [a,c], Xibiao Ye [a,b], Liming Bao [a,f], Xiao Qin Wang [a,e], John Ryder [a,d], Thomas W. Armstrong [g], Yimei Zhou [g], Lizhaung Miao [b], Anh T. Le [c], Patrick J. Kerzic [c], Weimin Ni [h], Hua Fu [a,b]

[a] *Sino-US Joint Clinical and Molecular Laboratory (JCML), 138 Yi Xue Yuan Road, Fudan University, Shanghai 200032, China*
[b] *School of Public Health, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, China*
[c] *Department of Pharmaceutical Sciences, School of Pharmacy, 4200 East 9th Avenue/Box C238, University of Colorado Health Sciences Center, Denver CO 20262, USA*
[d] *Department of Pathology, School of Medicine, AIP Bldg./Box F768, University of Colorado Health Sciences Center, Denver CO 80262, USA*
[e] *Huashan Hospital, Shanghai Medical University, No. 12 Wulumuqi Zhong Road, Shanghai 200040, China*
[f] *Division of Human Genetics, University of Cincinnati Children's Hospital Medical Center, 3333 Burnett Avenue, Cincinnati OH 45229-3039, USA*
[g] *ExxonMobil Biomedical Sciences Inc., 1545 Route 22 East Room LF294, Annandale NJ 08801-0971, USA*
[h] *Yangpu District Central Hospital, 450 Tengyue Road, Shanghai 200090, China*

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Abstract

Hematotoxicity following chronic benzene exposure has been recognized for over a century, although the mechanism remains unknown. We describe a novel form of bone marrow dysplasia in 23 workers exposed to high concentrations of benzene. Distinguishing features of benzene-induced dysplasia include: marked dyserythropoiesis, eosinophilic dysplasia and abnormal cytoplasmic granulation of neutrophilic precursors. Hematophagocytosis, stromal degeneration and bone marrow hypoplasia are also seen. Severe bone marrow dysplasia is frequently accompanied by clonal T cell expansion and alterations in T lymphocyte subsets. No clonal cytogenetic abnormalities were observed. These results suggest that autoimmune-mediated bone marrow injury is an early or predisposing event in the pathogenesis of benzene-induced persistent hematopoietic disease.

Keywords: Myelodysplastic syndrome; Hematotoxicity; Benzene; Immunopathology

1. Introduction

Myelodysplastic syndrome (MDS) is a heterogeneous group of diseases characterized by ineffective hematopoiesis, dysplasia in one or more hematopoietic lineages, clonal evolution and a tendency to progress on to bone marrow (BM) failure or acute myelogenous leukemia (AML). The etiology and pathogenesis of MDS are unknown, with genetic, infectious and environmental influences variously suggested to play a role in conferring susceptibility to these diseases. Clonal cytogenetic abnormalities, abnormal cytokine production and immune activation all are prominent features of MDS, and it has been hypothesized that damage to hematopoietic stem or progenitor cells leads to immunologic response directed against antigens in the hematopoietic environment [1–4]. Nevertheless, neither the initial clonal origin of MDS nor the cellular or molecular targets of activated immune cells, or even the order of events in the evolution of the disease have been identified, and a unifying hypothesis for the pathogenesis of MDS in general or for individual subtypes remains elusive.

Chronic exposure to benzene is known to result in bone marrow failure and increase the risk of AML [5–7]. Previous

* Corresponding author. Tel.: +1 303 315 7170; fax: +1 303 315 7237.
E-mail address: richard.irons@uchsc.edu (R.D. Irons).

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descriptions of AML associated with occupational exposure to pesticides or solvents that have contained benzene suggest a pattern of disease similar to therapy-related AML (tAML), although direct evidence linking benzene to tAML is lacking [8–11], and the mechanism of benzene-induced hematotoxicity remains unknown. Chronic exposure to high concentrations of benzene has long been associated with aplastic anemia (AA), diagnosed nearly always on the basis of pancytopenia, with hundreds of cases reported during the first half of the 20th century. However, direct evaluation of BM was almost never performed in these cases [12–14]. Although, AA and MDS are thought to be related conditions, MDS is distinguished from other forms of progressive BM failure by the presence of dysplasia and a tendency to progress or evolve into AML. MDS (non-specific) has previously been reported in a very small number of benzene workers who were generally described as having hypocellular bone marrow and dyserythropoietic changes [15]. In contrast, the majority of cases of de novo MDS present with hypercellular BM and abnormal hematopoietic cell morphology, while a hypocellular BM is observed only in a subset of de novo cases. In 2001, the World Health Organization (WHO) published new criteria for the diagnosis of MDS subtypes [16]. Dysplasia in one or more lineages is a central diagnostic feature of MDS, although cytopenia, anemia, or pancytopenia, together with BM hypercellularity and a blast cell count <20% are prominent characteristics of most subtypes of the disease.

We describe a novel pattern of dysplasia developing in 23 individuals who were previously exposed to high concentrations of benzene. The evaluation of these patients employing current diagnostic and molecular techniques has given us the opportunity to revisit and to further characterize the clinical paradigms associated with benzene-induced hematotoxicity and to examine early events in the pathogenesis of persistent benzene-induced blood disease. Most cases of benzene-induced dysplasia exhibited BM pathology that was characterized by: hypocellularity, multilineage dysplasia including megaloblastic changes, marked dyserythropoiesis and abnormal granulation in the cytoplasm of maturing neutrophilic precursors and neutrophils. A particular striking feature was a severe dysplasia in eosinophilic precursor cells. Additional findings were consistent with altered inflammatory and immune response, including prominent hematophagocytosis, increases in circulating large granular lymphocytes (LGLs) and altered distribution of CD4 and CD8 T cells which were often accompanied by evidence of clonal expansion of T cell subpopulations in BM. Significantly, the peripheral blood CBC was nearly normal in approximately 25% of cases. These findings suggest a distinct form of multilineage dysplasia in individuals chronically exposed to benzene, and provide evidence for a prominent role for altered immune response in the development of benzene-induced dysplasia. These findings further suggest that routine CBC may not be a reliable monitor for BM injury following chronic exposure to benzene.

2. Materials and methods

2.1. Patients

Patients were referred by physicians to Shanghai hospitals based on initial clinical presentation and/or a medical history of benzene intoxication. Participation was voluntary and the framework for consent was obtained according to the Declaration of Helsinki, 2004 and the NIH Common Rule (45 CFR 46). Informed consents were approved by both the Colorado Multiple Institutional Review Board and the Internal Review Board at Fudan University in Shanghai, China. All individuals were administered questionnaires requesting information on medical, occupational and environmental history. A total of 27 subjects employed in the rubber, petrochemical, pharmaceutical, manufacturing or painting industries were referred to our laboratory with a history of benzene poisoning. Previous occupational exposure to benzene was independently confirmed by: review of factory industrial hygiene monitoring records, real time quantitative industrial hygiene analysis, including personal samples ($n = 325$) and breathing zone analyses ($n = 225$), and/or previous evidence of hydrocarbon intoxication and anecdotal descriptions of solvent use and composition. Benzene from personal samples (3M 3500 organic vapor monitors) and area samples were measured according to the National Institute for Occupational Safety and Health (NIOSH) method with minor changes [17] and were performed using a Finnegan Trace Gas Chromatograph Ultra with a Flame Ionization Detector (FID), autosampler and SPB-1 capillary column (30 m × 0.25 mm × 0.25 m) (Agilent Technologies, Wilmington, DE). Individuals for which quantitative data was available ($n = 17$) were estimated to have full shift exposures averaging between 50 and 300 parts per million (ppm) benzene for varying periods of time ranging from 6 to 22 years and were removed from exposure an average of 2.7 years prior to evaluation in our laboratory (Table 1). Patients were evaluated for potential confounding factors, including: vitamin B12, folate or iron deficiencies and viral status (i.e. HCV, HIV). Two patients were excluded due to concurrent iron deficiency, and two patients were excluded because of inability to confirm exposure to benzene. The final study series consisted of 16 females and 7 males with a mean age of 44.4 year (S.D. = 7.8). This represents a small subset of individuals chronically exposed to high concentrations of benzene at the facilities studied.

2.2. Hematology and flow cytometry

Peripheral blood (PB), BM aspirates and core biopsies were collected in conjunction with diagnostic procedures and were evaluated in our laboratory. Peripheral blood smears were obtained by finger stick. Enumeration of LGL was performed by visual examination of Wright-Giemsa-stained smears. Blood samples were collected by venipuncture and processed for routine CBC (CellDyne 3700, Abbott, Park, IL), viral screen (HCV and HIV) (IMx®, Abbott

Table 1
Initial presentation of occupational benzene poisoning[a]

Case	Exposure duration (year)[b]	Time since last exposure (month)[c]	Signs/symptoms[d]	Laboratory findings[d]
1	17	48	Bleeding, fatigue	Pancytopenia
2	6	24	Bleeding, fatigue, dizziness	Thrombocytopenia/leukopenia
3	19	24	Bleeding, fatigue	Pancytopenia
4	13	48	ND	Granulocytopenia
5	ND	96	ND	Leukopenia
6	20	0	Bleeding, fatigue, dizziness	ND
7	8	3	Bleeding	Thrombocytopenia
8	22	36	Fatigue, dizziness	Pancytopenia
9	7	36	Bleeding, fatigue	Granulocytopenia/anemia
10	17	24	Bleeding, fatigue	Leukcopenia
11	ND	24	ND	ND
12	8	48	Bleeding, dizziness	Anemia
13	7	48	Bleeding, fatigue	Pancytopenia
14	16	27	ND	Thrombocytopenia
15	6	56	Bleeding, infection, fatigue	Pancytopenia
16	15	36	Fatigue, dizziness	Pancytopenia
17	10	30	Bleeding, fatigue, dizziness	Leukopenia/thrombocytopenia
18	9	36	Bleeding, fatigue, dizziness	Pancytopenia
19	15	24	ND	ND
20	13	13	Bleeding, dizziness	Leukopenia/thrombocytopenia
21	14	3	ND	Leukopenia/thrombocytopenia
22	18	9	Infection	Leukocytosis
23	16	48	ND	Leukopenia/thrombocytopenia

[a] All subjects were originally diagnosed with benzene poisoning according to Chinese occupational health criteria (i.e. a total WBC count <4000 ($\times 10^6$/l) or 4000–4500 ($\times 10^6$/l) and a platelet count <80,000 ($\times 10^6$/l, employment in a factory with documented benzene exposure for at least 6 months and exclusion of other causes for abnormal blood counts).
[b] Duration of chronic exposure to benzene prior to initial complaint.
[c] Time between cessation of benzene exposure and diagnosis of benzene-induced dysplasia in our laboratory.
[d] Signs, symptoms and laboratory findings consistent with hematologic injury documented at the time of exposure. ND indicates information that was not available.

Laboratories, Abbott Park, IL), clinical chemistry for liver enzymes (LDH, ALT and AST enzymes) (COBAS, Integra 400 plus, Roche Diagnostics, Shanghai, China) and serum analysis for nutritional factors. Vitamin B12 and folate were measured by chemical luminescence (Beckman Coulter Dxi800), and total iron binding capacity was measured using a Beckman Coulter LX20. BM aspirates and core biopsies were obtained by Jamshidi needle extraction from posterior iliac crest. Aspirates were stained with fluorochrome-conjugated antibodies for flow cytometric analysis of BM cellular subsets. Antibody panels included: anti-CD45, CD4, CD8, CD3 (Beckman Coulter, Hialeah, FL; Immunotech, Miami FL). Multiparameter analysis was performed on stained BM cells using a dual laser flow cytometer (FC-500, Beckman Coulter) equipped with compensation software (Software CXP, Beckman Coulter).

2.3. Bone marrow morphology

BM smears were prepared and evaluated using Wright-Giemsa stained preparations and special stains. Core biopsy sections were evaluated using sections stained with Hematoxylin-Eosin, Gomori trichrome, and immunoperoxidase-immunohistochemistry. Morphology was independently evaluated by two of us (R.D.I., J.R.). Dyserythropoiesis was scored on the basis of abnormal nuclear morphology, including internuclear bridging, abnormal budding, multiple nuclei and abnormal mitotic figures or megaloblastoid features. Myeloid or granulocytic dysplasia was defined by nuclear hypolobulation, (i.e. pseudo-Pelger Huet cells, hypersegmentation, abnormal mitotic forms) either hypo- or hypergranulation of the cytoplasm as well as the presence of large irregular granules. Megakaryocyte dysplasia was defined by non-, or hyper-lobulated nuclei, multiple individual nuclei or the presence of prematurely segmented or "shedding" cytoplasm. Eosinophilic dysplasia was defined by the presence of megaloblastoid features and abnormal cytoplasmic basophilic and eosinophilic hypergranulation. Microscopic analysis was performed using an Olympus BX51 bright field microscope (Olympus Optical, Ltd., Tokyo, Japan) equipped with a Sony EXwave HAD color video camera (Sony Ltd., Tokyo, Japan). Images were processed using software custom designed for the purpose (Vision Image Technology, Shanghai, China), and photomicrographs were cropped and edited using Adobe Photoshop (San Jose, CA). Cases of benzene-induced dysplasia were compared to MDS subtypes diagnosed according to the WHO classification system

2.4. Cytogenetic analysis

Fluorescence in situ hybridization (FISH) and cytogenetic analyses were performed on unstimulated BM cells

following 24–72 h of culture. Chromosomes were prepared and G-banding after trypsin was performed according to standard techniques. If possible, 29 metaphases were analyzed. Interphase or metaphase FISH analyses were performed on the short-term cultured cells or metaphases prepared from the BM or PB samples. All probes used in the FISH studies were purchased from Vysis (Downers Grove, IL). Sample preparations and hybridizations were performed according to the protocols provided by the manufacturer. Slides were viewed using an Olympus fluorescence microscope (Olympus Optical, Ltd., Tokyo, Japan) equipped with the appropriate filters and the PowerGene Macprobe image analysis system (Applied Imaging International Ltd., Newcastle, UK). Whenever possible, 1000–2000 nuclei were analyzed for each probe and scored by two readers. Signal patterns for each probe were compared against a reference range established from analysis of 100 samples from individuals with no evidence of clonal disease or previous benzene exposure.

2.5. Analysis of FLT3 mutations

FLT3 mutations are often encountered genetic abnormalities in AML, frequently involving an internal tandem duplication (ITD) or activation loop mutations in the tyrosine kinase domain (TKD) of the *FLT3* gene [18,19]. *FLT3* mutations were determined by PCR analysis and sequencing using DNA isolated from BM cells. Genomic DNA was isolated from blood and BM samples using A Qiagen QIAmp DNA mini Kit (Chatsworth, CA) according to the manufacturer's directions. Exons 14 and 15 and the intervening sequence intron of the *FLT3* gene were amplified by PCR to detect ITD. The PCR products were run on both 2% agarose gel and 5% polyacrylamide gel. Exon 20 of the *FLT3* gene was also amplified by PCR to detect TKD using primers as reported by Yamamoto et al. [20]. These PCR products were subjected to digestion by EcoRV and analyzed on a 3.5% agarose gel. Finally, PCR products were isolated from the gels using the DNA Recovery Kit (Biologic Technology Co., Ltd., Shanghai, China) and directly sequenced on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA).

2.6. T cell receptor (TCR) rearrangement analysis

Detection of clonal and oligoclonal expansion of T cell populations was determined by analysis of rearrangements of TCR beta (TCRβ), TCR gamma (TCRγ) and TCR delta (TCRδ) genes by multiplex PCR using BIOMED-2 kits (InVivoScribe Technologies, San Diego, CA) according to the manufacturer's protocol. For the *TCRβ* gene, the kit comprised of three individual master mix reactions, the *TCRγ* kit utilized two master mixes and *TCRδ* kit utilized a single PCR master mix. Amplification of *TCRβ, γ and δ* genes was performed in a thermocycler and the PCR samples were loaded unto a 6% non-denaturing polyacrylamide TBE gel, stained with ethidium bromide, visualized by UV illumination and documented by digital photography. Clonal rearrangements were determined according to published guidelines with exclusion of nonspecific bands [21].

3. Results

Patients diagnosed with benzene-induced dysplasia exhibited a distinct set of characteristic features that both overlap with and can be distinguished from standard classifications of MDS. At the time of diagnosis in our laboratory, PB findings in these cases included pancytopenia ($n = 1$), and cytopenias, singly or in combination ($n = 16$). The most consistent abnormal finding was lymphocytopenia ($n = 14$). Six subjects presented with normal or marginally normal blood counts (Table 2). All patients exhibited significant BM pathology including evidence of multilineage dysplasia which was characterized by macrocytic megaloblastic changes in granulocytic, eosinophilic as well as erythroid lineages and at all stages of development. Stromal degeneration was also present in 12/23 cases. Most cases of benzene-induced dysplasia were hypocellular (17/23) with residual hematopoietic cells unevenly distributed throughout the BM. Dyserythropoiesis was commonly found and often severe (Figs. 1 and 2). Several features distinguished benzene-induced dysplasia from other previously defined subtypes of MDS including therapy related (t)MDS [16,22,23]. Prominent among these were the presence of abnormal BM eosinophilic precursor cells (22/23 cases). These cells are reminiscent of those found in AML with the 16(p13;q22) chromosome abnormality [16] (Fig. 3). Hematophagocytosis, which is commonly associated with a poor prognosis in BM failure, was a particularly frequent and striking observation (16/23 cases) (Fig. 4). Maturing myeloid cells and granulocytes also typically exhibited megaloblastic alterations and abnormal cytoplasmic morphology (18/23 cases) (Fig. 5). Finally, for a significant subset of cases there was a lack of concordance between the severity of pathology observed in the BM and the relatively mild or moderate abnormalities encountered in the PB (6/23 cases). This suggests that monitoring of the peripheral blood CBC may not accurately reflect the progression of hematopoietic disease in patients previously exposed to benzene.

3.1. Clonal cytogenetic abnormalities in benzene-induced dysplasia

Reoccurring clonal genetic abnormalities are frequently encountered in MDS, and have been suggested to play an early or predisposing role in the development of the disease. Therefore, we analyzed BM cells from each subject for evidence of clonal hematopoietic lesions. In contrast to the overall frequency of cytogenetic abnormalities observed for MDS in our laboratory (26%; $n = 100$), cytogenetic analyses revealed no clonal abnormalities in any benzene poisoning case in this series. The most frequent cytogenetic abnormalities encountered in tMDS/tAML are −5, −7, del(5q)−, and del(7q−) which are found in greater than 70% of reported

Table 2
Characteristics of PB and BM in benzene-induced dysplasia

Case	ANC (10^6/l)	ALC (10^6/l)	LGL (%)	PLT (10^9/l)	Hgb (g/dl)	Bone marrow							
						Cellularity	CD4/CD8 Ratio	Stro	Dysplasia				HP
									Gran	Ery	Meg	Eo	
1	1240	730	27	93.7	11.5	Norm	0.24	N	+	ND	ND	+	+++
2	1920	1620	15	122	11.9	Hypo	1.62	N	ND	+	++	++	+++
3	1250	1390	28	176	13	Hypo	0.92	N	+++	+++	0	+	++
4	1520	980	20	200	13.5	Norm	0.76	N	+++	++	++	++	+++
5	930	1530	24	61.8	15.9	Hypo	2.42	N	++	+++	0	+++	ND
6	2980	190	25	77.8	13.9	Norm	0.60	SA	0	+++	0	+++	++
7	1760	340	32	124	8.3	Hyper	0.64	N	+++	+++	+++	+++	+++
8	1590	2220	40	146	13	Hypo	0.59	SA	ND	+	0	++	+
9	3390	1740	41	203	13.3	Hypo	0.71	SA	+++	+++	+++	+	+++
10	1650	1440	46	168	12.9	Hypo	0.45	FN	ND	+	+	++	+
11	1510	1160	30	122	12.7	Hypo	0.43	SA	+	+	0	ND	ND
12	3140	823	42	92.7	11	Hypo*	0.42	QNS	+++	0	0	+++	ND
13	2020	2300	41	133	11.8	Hypo*	0.97	QNS	0	0	0	+++	ND
14	2380	1600	28	174	15.2	Hypo	0.69	SA	+	+	0	+++	ND
15	2080	1710	30	65.9	12	Hypo	0.83	SA	+++	+	0	+++	ND
16	2270	1780	32	137	13.8	Hypo	0.71	N	+++	+++	0	+	+
17	2370	1350	28	71.1	11.8	Hypo	0.37	SA	+++	+	0	++	ND
18	2950	710	18	113	12.8	Hypo	0.91	SA	+++	+	0	+++	+
19	2240	870	18	33.5	11.4	Hypo	0.20	SA	+++	+++	0	+++	+
20	1400	600	32	84.1	12.9	Hypo	0.59	SA	+++	+++	0	++	+++
21	2090	810	22	99.8	11.9	Hypo	1.39	SA	+++	+	0	++	+
22	4000	2850	33	275	14.7	Hyper*	0.88	0	+++	+	0	++	+
23	1520	990	ND	147	13.2	Norm	0.50	SA	+	+	0	+	+

ANC, absolute neutrophil count; ALC, absolute lymphocyte count; LGL, large granular lymphocytes (%ALC); PLT, platelet count; Hgb, hemoglobin; bone marrow cellularity was estimated from the core biopsy or the aspirate* (e.g. hypo-hypoplastic [<40% cellularity], norm-normal [40–60% cellularity] and hyper-hyperplastic [>60% cellularity]; BM lymphocyte CD4/CD8 ratios as determined by flow cytometry; Stro, stroma; SA, focal serous atrophy; FN, fibrinoid necrosis, 0, stromal cells were absent or too rare to evaluate; dysplasia was estimated for granulocytic (Gran), erythroid (Ery), megakaryocytic (Meg) and eosinophilic (Eo) lineages and scored based on the percentage of lineage-specific cells involved, ND [none detected], + [10–50%], ++ [50–70%], or +++ [>70%]). 0, cells of individual lineage were absent or too rare to evaluate for dysplastic changes. HP, hematophagocytosis, was scored as not detected (ND) present (+), moderate (++) or severe (+++). Reference ranges: ANC (2000–7000 × 10^6/l); ALC (1600–6000 × 10^6/l); LGL (5–15%); PLT (100–300 × 10^9/l); Hgb (12–16 g/dl); CD4/CD8 (0.72–2.56) [55].

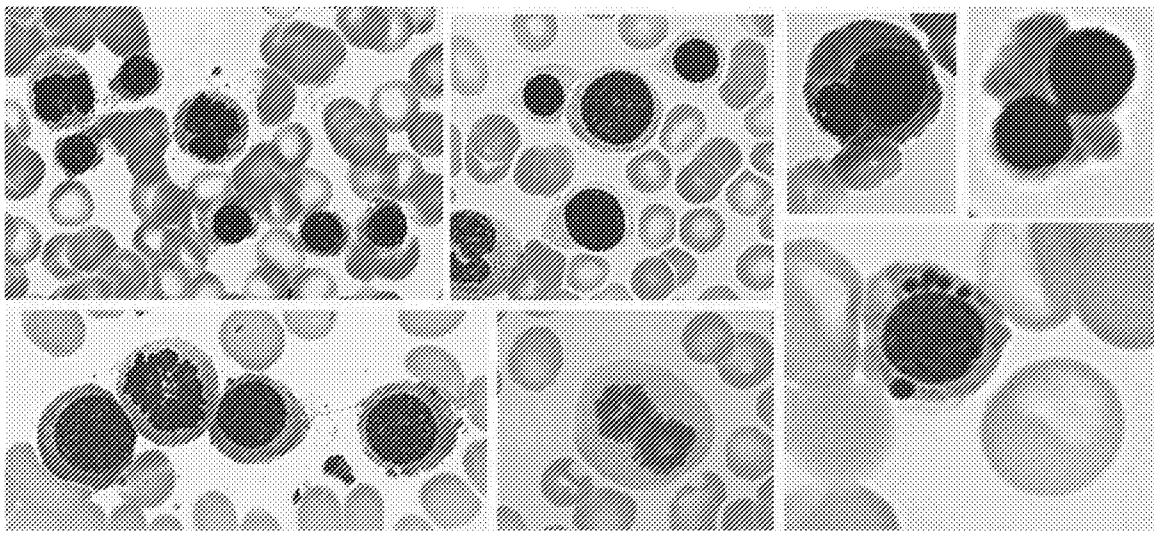

Fig. 1. Erythroid dysplasia and dyserythropoiesis in benzene-induced dysplasia. Abnormal erythroid cells exhibit megaloblastic abnormalities and abnormal nuclear morphology including nuclear bridging. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

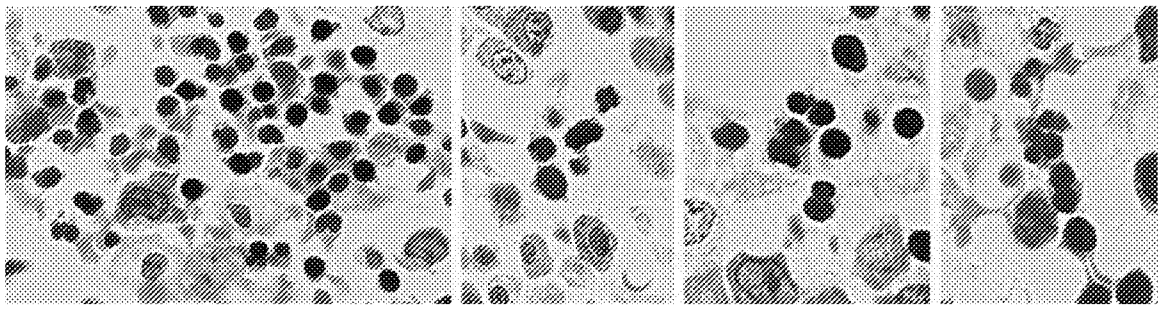

Fig. 2. Prominent dyserythropoiesis in a core biopsy from a representative patient with benzene-induced dysplasia. The most frequent dyserythropoietic changes in H&E stained sections are bizarre budding of nuclei in erythroid precursor cells. Core biopsy section stained with Hematoxylin-Eosin (original magnification 1000×).

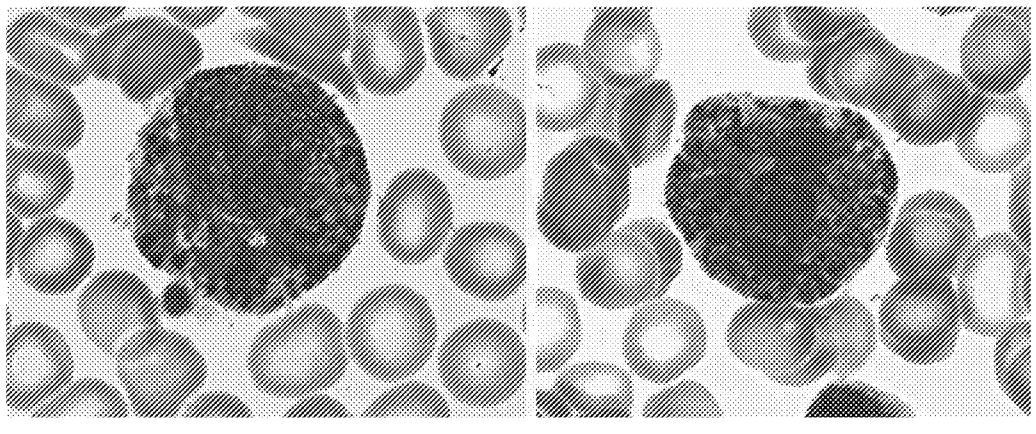

Fig. 3. Abnormal eosinophilic precursor cells in benzene-induced dysplasia. Abnormal eosinophils exhibit megaloblastic nuclear abnormalities, nuclear hypersegmentation and atypical giant basophilic and eosinophilic cytoplasmic granulation. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

cases [24,25], and some studies have suggested a similar pattern might exist for AML developing after chronic benzene exposure [10,26–28]. We also performed additional analyses for −7, 5q−, 11q23 and +8 structural abnormalities using FISH and observed no signals above background (data not shown). *FLT3* mutations are the most frequently encountered genetic abnormalities in AML, commonly involving ITD or activation loop mutations in the TKD of the *FLT3* gene [18,19,29]. However, no *FLT3* mutations were observed in this series. These findings suggest that acquisition of cytogenetic or molecular abnormalities in benzene-induced dysplasia may be relatively late events in the neoplastic progression of the disease.

3.2. Benzene-induced dysplasia may involve an autoimmune process

The frequency and severity of lymphocytopenia, hematophagocytosis and eosinophilic dysplasia in benzene-induced dysplasia led us to examine additional immune cell parameters. Patients with benzene-induced dysplasia exhibited relative increases in LGL (mean = 29.6%, $n = 22$) in PB and decreases in the ratio of CD4+/CD8+ lymphocytes in the BM (0.77 ± 0.48, $n = 23$) (Table 2). These observations coincided with demonstration of clonal and oligoclonal proliferations in BM T lymphocytes (14/23 cases), including clonal rearrangements in Vβ TCR gene segments ($n = 4$), Vδ TCR gene segments ($n = 6$) and both Vβ and Vδ TCR segments ($n = 4$).

4. Discussion

The role of autoimmune mechanisms in the development of MDS remains largely unexplored, although, evidence is accumulating to implicate immunologic abnormalities in the pathogenesis of the disease. Frequent findings in MDS include lymphocytopenia, inverted CD4/CD8 T cell ratios, increases in cytotoxic CD8+ T cells (CTL) and serum levels of inflammatory cytokines such as tumor necrosis factor α (TNF-α), and interferon γ (INF-γ) [2], abnormal expression of HLA-DR [30] and evidence of TCR gene rearrangements [31–34]. Positive clinical responses to immunosuppressive therapy with anti-thymocyte globulin (ATG) or cyclosporin A also have been reported in some patients with MDS [3,33,35], together with treatment-related decreases in both CD8+ T cell clones and T-LGL [32,36,37]. The role of environmental exposure in the evolution of MDS is unknown, although it has been hypothesized that the pathogenesis of MDS may involve

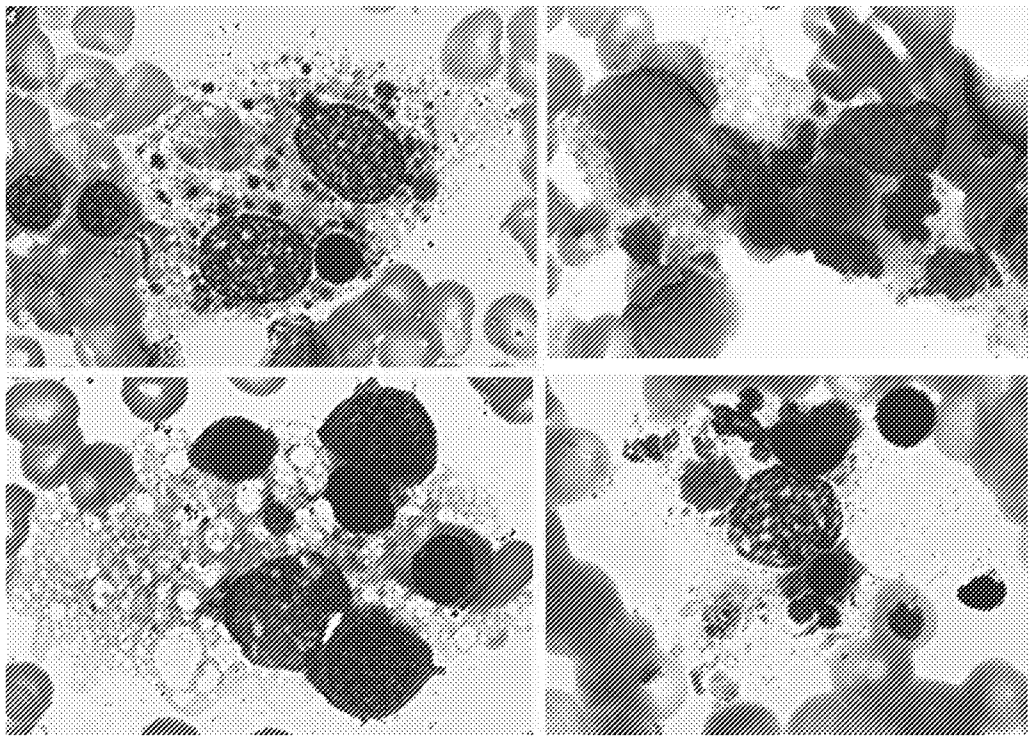

Fig. 4. Hematophagocytosis in benzene-induced dysplasia. Activated histiocytic cells within the BM exhibit prominent phagocytosis of degenerating erythroid and granulocytic cells which is indicative of an immuno-reactive inflammatory process. The histiocytes themselves do not appear atypical and generally exhibited a low nuclear cytoplasmic ratio with small nuclei containing condensed chromatin. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

damage to hematopoietic progenitor cells exposed to intrinsic or environmental toxic agents that might lead to immunologic suppression of cell growth and maturation [2,38]. Transient anemia, cytopenias and dysplasia frequently occur in BM toxicity during or immediately following exposure to toxic agents. This usually is not associated with persistent disease and therefore, is not generally considered to be MDS [39–41]. However, our findings show that prolonged chronic exposure to high concentrations of benzene results in the development of a distinct form of dysplasia that differs from commonly defined subtypes of MDS and that can persist for years after cessation of benzene exposure.

Previous proposals to explain benzene carcinogenesis, including those from our laboratory, have presumed a role for genetic injury in the origin of benzene-induced persistent toxicity and the development of AML [28,42–44]. Alternatively, our current results suggest the possibility that an autoimmune process may precede the acquisition of frank structural cytogenetic abnormalities and may be a predisposing event in the development of persistent BM disease following benzene exposure. Histological manifestations of benzene-induced dysplasia include multilineage dysplasia including severe dyserythropoiesis, stromal degeneration, alterations in BM T lymphocyte subsets, abnormal eosinophilic precursors and hematophagocytosis. Hematophagocytosis is associated with severe viral infection or inflammatory response and activation of immune cells [45]. Benzene-induced dysplasia is also accompanied by clonal and polyclonal proliferation of T

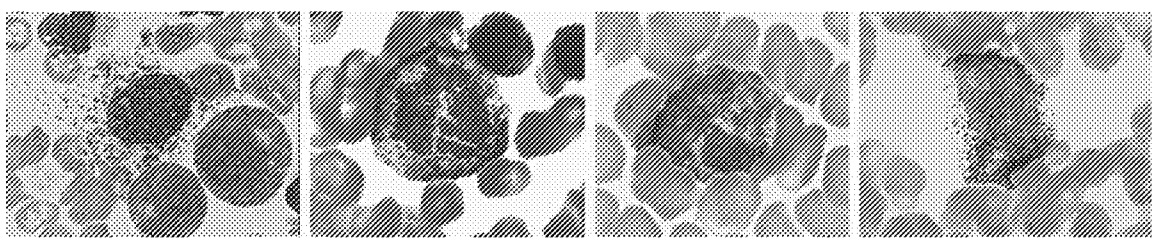

Fig. 5. Dysplastic changes in granulocytes in benzene-induced dysplasia. The BM and PB in the case series typically contain maturing myeloid cells with megaloblastic, hypo-segmented (pseudo Pelger-Huett) or hypersegmented nuclei. In some instances abnormal mitotic figures are present in granulocytes with mature cytoplasm. Cytoplasmic abnormalities include protruding pseudopodia which often trails cell bodies and abnormal cytoplasmic granulation in which large granules were unevenly distributed in clusters or marginated directly beneath the plasma membrane. BM aspirate slides stained with Wright-Giemsa (original magnification 1000×).

lymphocytes in the BM which is indicative of an active immune process and has been described in a variety of immune-related conditions involving suppression of hematopoiesis including MDS [4,46,47]. The function of γδ T cell subsets is largely unknown, although increases in γδ T cells also have been observed in patients with BM failure, have been shown to modulate eosinophilic inflammation in other tissues and are known to be activated by TNF-α [35,37,48,49]. In this study, *TCRδ* expansions were accompanied by evidence of corresponding expansion of *TCRγ* gene rearrangements. However, confirmation of γδ T cell subsets by heteroduplex analysis is required.

These observations indicate that previous chronic exposure to benzene is associated with the development of BM dysplasia, and suggest that a reactive inflammatory process may be involved in the suppression of hematopoiesis in persistent BM failure following chronic benzene exposure. These findings further suggest the possibility that markers of immune activation or inflammation may prove useful in monitoring the development or progression of benzene-induced dysplastic disease. Nevertheless, the role of altered immune regulation in the pathogenesis of benzene-induced dysplasia, as well as the identity of any putative antigens remain a mystery. The mechanisms of benzene-induced hematopoietic cell injury are not completely understood. However, a subpopulation of CD34+ BM cells that are responsive to granulocyte-macrophage colony-stimulating factor (GM-CSF) have been implicated as targets. Studies in our laboratory have shown that the benzene metabolite, hydroquinone, enhances cytokine-dependent clonal proliferation of a subpopulation of GM-CSF-responsive human CD34+ BM cells which appears to be mediated via the extracellular signal-regulated kinase/activation protein-1 signaling pathway (ERK/AP-1) [50–53]. Independently, hydroquinone also synergizes with TNF-α to produce apoptosis in human CD34+ hematopoietic progenitor cells (HPC) via a mechanism that involves the inhibition of NF-κB [54].

In recent years occupational exposure to benzene in China has been significantly reduced with the current Chinese occupational exposure limit being 1.9 ppm as a time weighted average (TWA) with a short-term exposure limit (STEL) of 3.2 ppm. Despite these changes, exposures to high concentrations of benzene continue to occur, and subjects diagnosed with persistent BM dysplasia in this report represent only a small fraction of the individuals exposed to high concentrations of benzene in the facilities studied. Previous reports have suggested that genetic polymorphisms in metabolizing and detoxification enzymes may play a role in conferring susceptibility to benzene toxicity. However, preliminary results in our laboratory do not suggest a prominent role for these genetic variants in the development of benzene-induced BM dysplasia. Evaluation of the influence of polymorphisms in other genes, such as TNF-α, on susceptibility to benzene-induced BM dysplasia are ongoing in our laboratory. Continued characterization and follow-up of these subjects with benzene-induced dysplasia may provide insights into early events associated with the pathogenesis, clonal selection and progression of persistent BM disease, including MDS and its potential progression to AML.

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University of Colorado Grant for the Shanghai Health Study
Draft Financial Review Plan

A review of the financial records related to the API grant to the University of Colorado, Dr. Richard Irons, principal investigator, to fund the Shanghai Health Study will be performed based on information provided by the University. This material will include items such as expenditures reports, purchase documents, ledgers, timesheets and contracts. Availability of this information and any other information related to the grant (contract number 0100004788) will be important to perform a quick and effective review. Report of the financial audit will be made available to the Benzene Health Research Consortium.

1. Review personnel costs
    a. Review documentation supporting personnel costs on expenditures reports (ledgers, time reports, timesheets, other documents noting allocation of time and/or expense to the project).
    b. Review accumulation of personnel charges for specific periods/tasks to verify roll up into expenditures reports.
    c. Review documents verifying personnel working on project.
    d. Review list of employees with titles who are allocating time to the project for reasonableness

2. Review operating expenses
    a. Review documentation supporting operating costs on expenditures reports (ledgers, subledgers, other documents noting allocation of expense to the project).
    b. Review original documentation for a sample of operating expenditures

3. Review subcontractor expenses
    a. Obtain and review a list of subcontractors
    b. Review documentation supporting subcontractor costs on expenditures reports (ledgers, subledgers, other documents noting allocation of expense to the project).
    c. Review subcontractor agreements for applicability to project
    d. Review documentation for payments to subcontractors
    e. Review reports from subcontractors (e.g. status, progress).

4. Review equipment and supplies
    a. Review documentation supporting equipment costs on expenditures reports (ledgers, subledgers, other documents noting allocation of expense to the project).
    b. Review original documentation for a sample of equipment expenditures
    c. Inspect a sample of equipment purchases

5. Review indirect costs
    a. Review development of overhead rate methodology
    b. Review application of overhead calculation to amounts on expenditures reports.

6. Review invoice approval process