local mast cell replacement (*7*). Triggering of mast cells could result in the release of preformed mediators, thereby increasing vascular permeability, activating complement, and stimulating the local adhesion and migration of neutrophils. Resident macrophages, dendritic cells, or neutrophils may serve a similar role in triggering a chemotactic cascade. FcR cross-linking by immune complexes on either of these cells may directly or in synergy with complement receptors result in the activation of complement components, along with known pro-inflammatory mediators, and thereby set off the cascade of events that culminates in the dramatic sequelae of the inflammatory response. In either case, inhibition of FcR cross-linking by immune complexes can be expected to attenuate the inflammatory response by targeting the initiation of the cascade rather than its propagation and amplification. Targeting of these receptors in autoimmune diseases represents another potentially potent therapeutic approach to preventing tissue injury by immune complexes.

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Generation of Lymphohematopoietic Cells from Embryonic Stem Cells in Culture

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An efficient system was developed that induced the differentiation of embryonic stem (ES) cells into blood cells of erythroid, myeloid, and B cell lineages by coculture with the stromal cell line OP9. This cell line does not express functional macrophage colony–stimulating factor (M-CSF). The presence of M-CSF had inhibitory effects on the differentiation of ES cells to blood cells other than macrophages. Embryoid body formation or addition of exogenous growth factors was not required, and differentiation was highly reproducible even after the selection of ES cells with the antibiotic G418. Combined with the ability to genetically manipulate ES cells, this system will facilitate the study of molecular mechanisms involved in development and differentiation of hematopoietic cells.

The mechanisms of determination and differentiation that lead to the formation of hematopoietic cells from the inner cell mass of blastocysts through mesodermal cells are still unelucidated in spite of identification of numerous hematopoietic growth factors. Hematopoietic differentiation of ES cells can be induced in vitro (*1–3*), but these systems require formation of complex embryoid structures or addition of exogenous growth factors, or both (*1–3*). Other limitations are the inability to dissect the developmental processes from ES cells to blood cells and the lack of simultaneous induction of both myeloid and lymphoid lineage cells. To overcome these limitations, we tested whether coculture of ES cells on stromal cells without exogenous growth factors might induce lymphohematopoietic differentiation. We initially tried the stromal cell lines ST2, PA6, and RP0.10 (*3*, *4*), but these cell lines gave rise almost exclusively to macrophages.

Because M-CSF might be responsible for the preferential differentiation of ES cells into the monocyte-macrophage lineage (*5*), we examined the differentiation-inducing activity of the OP9 stromal cell line. This line was established from newborn calvaria of the $(C57BL/6 \times C3H)F_2$-*op*/*op* mouse that lacks functional M-CSF because of a mutation in the M-CSF gene (*6*, *7*). D3 ES cells established from the 129/Sv mouse strain (*1*) produced two types of colonies 5 days after transfer onto OP9 cells; one was typical of an undifferentiated ES cell colony (Fig. 1A) and the other had features of differentiated mesoderm-like colonies which consisted of $\sim 10^3$ adherent blastic cells larger than D3 cells (Fig. 1B) (*8*). The whole culture was then trypsinized and passed onto fresh OP9 cells (*9*). Clusters consisting of round cells with homogeneous sizes developed within 1 day, and the numbers of cells increased rapidly (Fig. 1C). When individual day 5 colonies were analyzed, more than 95% of differentiated colonies produced the clusters, whereas less than 5% of undifferentiated colonies did (*10*). If the first passage was not done, hematopoietic cells were buried in the colonies and could not be observed. Cells were usually passed again 5 days after the first passage. This second passage, although not essential for dissecting hematopoiesis, eliminates residual undifferentiated or differentiated colonies. After the second passage, round cells of various sizes predominated

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(Fig. 1D), whereas undifferentiated or mesoderm-like colonies were virtually undetectable. After 14 days, round cells began to detach from the stromal cells, presumably because of complete differentiation.

The majority of cells at day 8 looked like immature hematopoietic cells (Fig. 1E). At day 14, 2- to 8 × 10^6 cells arose from 10^4 D3 cells, and more than 90% of the cells exhibited variable morphology of hematopoietic lineage cells (Fig. 1F) consisting of neutrophils, macrophages, erythroid cells, mast cells, megakaryocytes, and lymphoid cells. Differentiation into hematopoietic progenitor cells, erythroid lineage cells, and granulocyte-macrophage cells was confirmed by staining with monoclonal antibodies (mAbs) against c-kit (15%), TER-119 (25%), and Mac-1 (5%), respectively (Fig. 2A). A significant percentage (7%) of the day 14 cells expressed B lineage marker B220 on their surface, although no cells were detected that stained positive for surface immunoglobulin M (IgM). A linear relation was observed between the number of cells plated at day 5 and the clusters formed on OP9 cells 5 days later (Fig. 3), suggesting that the individual clusters are of clonal origin (*11*). The development of mesodermal cells and hematopoietic precursor (or stem) cells from ES cells appeared to occur within the first 5 days, with the proliferation and differentiation of specific hematopoietic lineages occurring later. These sequential processes could be observed under the microscope.

To determine the differentiation capacity of day 10 clusters, we separately picked clusters and transferred them to semisolid culture medium containing interleukin-3 (IL-3) and erythropoietin (Epo) under conditions that promote myeloid cell growth (*12*). Out of 20 clusters, 19 produced colonies consisting of various myeloid lineage cells. More than 75% of the clusters differentiated into mixed colonies (Table 1). Thus, most of the clusters contained hematopoietic progenitor cells that could differentiate along various myeloid lineages.

We also examined the lymphohematopoietic differentiation capacity of individual clusters. Well-separated day 10 clusters were picked, trypsinized, and divided into two aliquots. One aliquot was transferred to semisolid medium under the myeloid condition and the other to semisolid medium containing IL-7 as the growth factor for B lineage cells (*13*). Two of 45 clusters produced colonies under both conditions; the cells that appeared under the myeloid conditions and the B cell conditions consisted of myeloid cells and lymphoid cells, respectively (Fig. 1, G and H). Twenty-three clusters produced only myeloid colonies and 20 did not produce any. The addition of IL-7 and 2-mercaptoethanol (2-ME), which promotes the growth of immature B lineage cells on stromal cells (*14*), caused bursting proliferation of B220-positive and surface IgM–negative immature B lineage cells which completed immunoglobulin DJ gene rearrangement in about 10% of day 14 hematopoietic clusters (Fig. 2, B and D). The lymphoid cells in a small proportion of clusters (one per 500 to 1000 day 14 clusters) persisted and proliferated on OP9 cells after the detachment of the majority of the cells and differentiated in 40 days into surface IgM–positive cells that express the com-

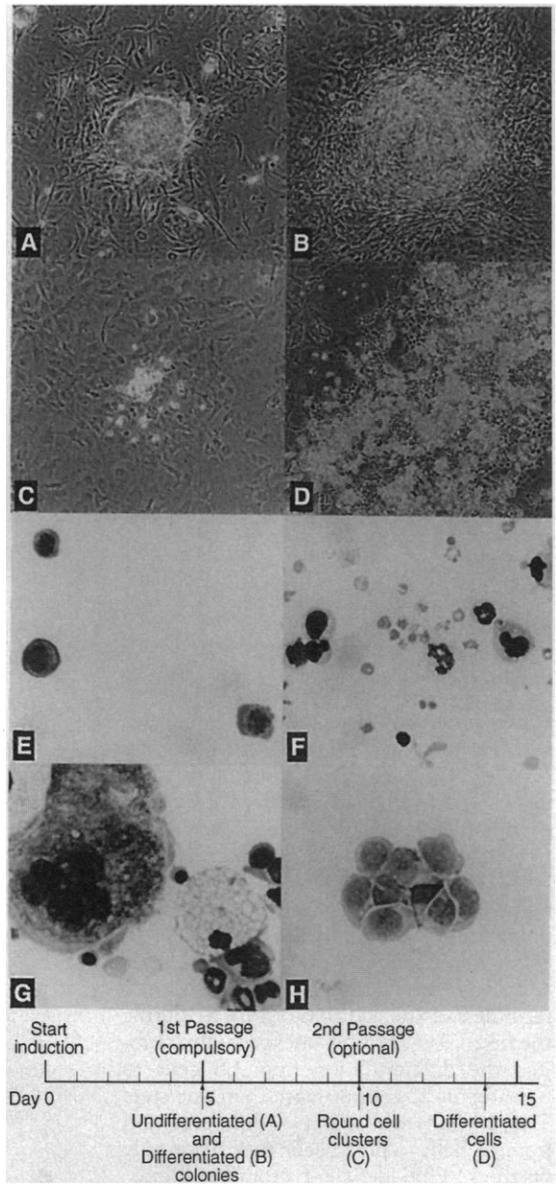

Fig. 1. Photographs of the cells after differentiation induction. Passage of cocultured ES cells was done 5 and 10 days after initiation. Time points of phase contrast microscope photographs (**A** to **D**) are indicated at the bottom of the figure: undifferentiated colonies (A), differentiated mesoderm-like colonies (B), hematopoietic cell clusters (C), and differentiated blood cells (D). Cells from clusters (**E**) 8 and (**F**) 14 days after the differentiation induction were prepared by cytocentrifugation (Shandon Southern Products, Sewickly, PA), fixed, and stained with May-Grunwald Giemsa. Day 10 hematopoietic cell clusters were picked up and cultured in semisolid media under (**G**) myeloid conditions or (**H**) B cell conditions. Original magnification, ×100 (A to D) and ×400 (E to H).

Table 1. Differentiation capacity of day 10 clusters in semisolid medium containing IL-3 and Epo. Colonies which appeared 8 days after the transfer of individual day 10 clusters to the semisolid media were picked and their cytospin specimens were stained with May-Grunwald Giemsa. Types of cells are n, neutrophil; m, macrophage; E, erythroid; mast, mast cell; M, megakaryocyte; and blast, blastic cell.

Colony						
Type						Number
n	m	E	mast	M	blast	2
n	m	E			blast	2
	m	E		M	blast	1
	m	E			blast	1
	m				blast	1
n	m	E	mast			1
	m	E		M		2
	m	E				5
n	m					2
	m					1
		E				1
None						1

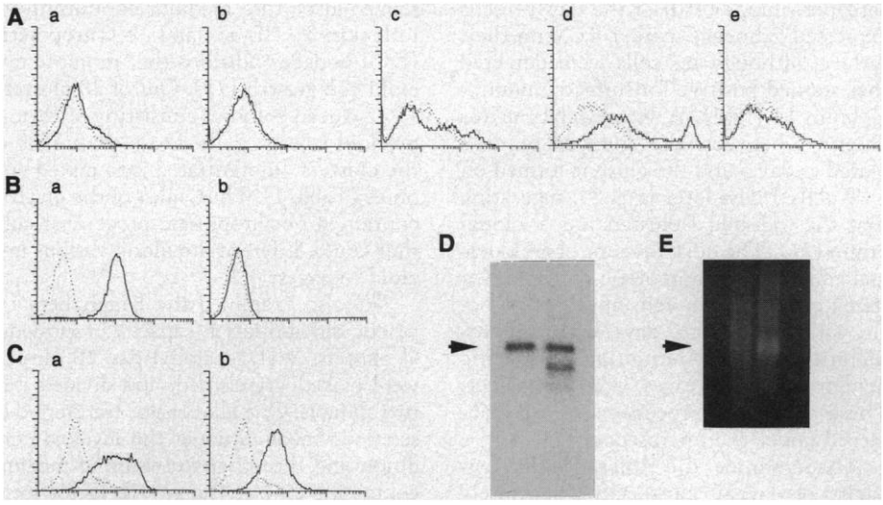

Fig. 2. Expression of lineage markers and immunoglobulin gene rearrangement. (**A** to **C**) Standard two-stage immunofluorescence was performed with optimal concentrations of primary mAb against (a) B220 (hybridoma 6B2) (*21*); (b) IgM [biotinylated goat antibody to mouse IgM $F(ab')_2$]; (c) c-kit, ACK-2 (*22*); (d) an erythroid lineage–specific antigen, TER-119 (*23*); and (e) Mac-1, M1/70.15.1 (*24*). The second stage was fluorescein isothiocyanate (FITC)–conjugated mAb to rat κ (Cosmo, Tokyo, Japan) or FITC-conjugated avidin (Cappel, West Chester, PA). Control samples of B220 were stained with isotype-matched rat IgG antibody and FITC-conjugated mAb to rat κ. The cells were analyzed on a FACScan. Day 14 cells (A) and day 40 cells (C) were maintained on OP9 cells without IL-7 and 2-ME. Day 20 cells (B) were obtained with stimulation of IL-7 and 2-ME after day 10. (**D**) Southern hybridization of DNA of undifferentiated D3 cells (left) and day 20 + IL-7 cells (right) with mouse JH4 probe (*18, 19, 25*). The arrowhead shows the size of the unrearranged JH band. (**E**) Reverse transcriptase PCR analysis of immunoglobulin VDJ transcript of undifferentiated D3 cells (left) and day 40 cells (right) (*20, 26*). The arrowhead shows the expected size of PCR products from VDJ-Cμ transcripts.

Table 2. Effect of M-CSF on the differentiation induction. The values show the cumulative number of clusters and cells yielded from 10^4 D3 ES cells 10 days after transfer to OP9 cells (*27*). Data are mean ± SE of four plates and the *P* value is <0.05 when any combination of data in each column is compared by *t* test. N.C., not countable because of a large number of macrophages diffusely located on dishes.

Addition of M-CSF (day)		Number of small round cell clusters	10^5 cells		
0–5	5–10		Total	Nonmacrophages	Macrophages
–	–	1240 ± 60	17.7 ± 1.6	13.2 ± 0.9	4.5 ± 0.8
–	+	N.C.	78.1 ± 2.3	7.0 ± 1.2	71.1 ± 2.8
+	–	130 ± 10	2.6 ± 0.2	1.9 ± 0.2	0.7 ± 0.1
+	+	N.C.	10.1 ± 0.9	1.0 ± 0.1	9.1 ± 0.8

plete μ chain mRNA (Fig. 2, C and E).

We confirmed the inhibitory effect of M-CSF on the differentiation by adding recombinant human M-CSF (200 ng/ml) (*15*). Addition of M-CSF during the first 5 days reduced the number of differentiated colonies to about one-third but had no influence on the formation of undifferentiated colonies (*10*). This indicates that M-CSF inhibits differentiation of ES cells into mesodermal cells. When M-CSF was added for the first 5 days of the 10-day culture, the number of day 10 hematopoieitc clusters was reduced to about 10%, and those of total cells and nonmacrophages to one-seventh irrespective of the presence of M-CSF during the second 5 days (Table 2). Thus, M-CSF inhibits not only differentiation of ES cells into mesodermal cells but also subsequent development to hematopoietic cells. When M-CSF was added during the second 5 days, hematopoietic cell clusters were hardly detectable, instead there was a tremendous proliferation of macrophages. Although five times more cells appeared, 90% of the cells were macrophages, and the number of nonmacrophages was reduced to about half. This increase of macrophages and decrease of nonmacrophages occurred regardless of the addition of M-CSF during the first 5 days. M-CSF unexpectedly inhibited the differentiation from ES cells to hematopoietic cells at more than one step. This explains why effective differentiation from ES cells to blood cells took place only on the M-CSF–deficient stromal cell line.

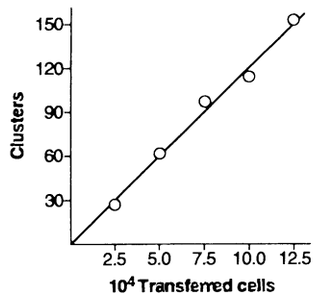

Fig. 3. Relation between numbers of transferred cells obtained by coculturing for 5 days on OP9 cells and those of hematopoietic clusters after culturing for another 5 days.

The other in vitro differentiation induction systems rely on the formation of embryoid bodies, suggesting the involvement of complex induction mechanisms for the development of blood cells (*1*, *2*). Differentiated mesodermal colonies in our system are different from embryoid bodies because (i) differentiated colonies grew flat and did not pile up until day 3 or 4, (ii) the colonies did not form complex structures such as cysts or lumina even after piling up, and (iii) erythroid and multipotential precursors appeared earlier (by day 3 and day 4, respectively) than in the embryoid body–forming systems (*2*, *10*). These observations suggest that direct interactions with an M-CSF–deficient stromal cell line may be sufficient to induce the development of lymphohematopoietic cells, although requirement for some interactions between the mesodermal cells during the first 5 days could not be excluded. Eighteen out of 30 randomly chosen G418-resistant ES cell clones differentiated into hematopoietic cells as efficiently as parental ES cells (*10*). This system will thus allow the study of hematopoietic cell development with in vitro genetic manipulation of ES cells (*16*) while avoiding the generation of lethal phenotypes that the targeted disruption of developmental genes often causes (*17*).

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8. The cell line OP9 was maintained in α-modified minimum essential media (α-MEM, Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Whittaker, Walkersville, MD). The same media was used for differentiation induction. The ES cells were maintained with standard procedures, on embryonic fibroblasts with recombinant leukemia inhibitory factor. For differentiation induction, ES cells were seeded onto confluent OP9 cell layers on six-well plates (Nunc, Roskilde, Denmark) at a density of 10^4 cells per well.
9. Both differentiated and undifferentiated cells were trypsinized with 0.25% trypsin for 10 min, suspended with α-MEM supplemented with 20% FCS, and pipetted vigorously until cells became single cells. Then the cells were seeded onto confluent OP9 cell layers on six-well plates at various cell densities.
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25. Preparation of high molecular weight DNA and Southern (DNA) blotting was carried out by standard procedures (*18*). DNA (10 μg) was digested with Eco RI, subjected to electrophoresis in 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled, 0.9-kb Hind III–Xba I JH4 probe (*19*).
26. RNA was prepared by using TRIzol (Life Technologies, Gaithersburg, MD). About 2 μg of total RNA was used for first strand synthesis by using random hexamer (*18*). The forward primer is VH1BACK, AGGT%G%CA%GCTGCAG%GAGTC%TG. Backward primers are CH1RC, AATGGGCACATGCAGATC-TC; and CH1HE, TCAGACAGGGGGCTCTCG (*20*). The complementary DNA was amplified with VH1BACK primer and CH1RC primer at first, and then the polymerase chain reaction (PCR) product was diluted 1000-fold and amplified with VH1BACK primer and internal primer CH1HE. Samples were amplified for 40 cycles under the following conditions: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min.
27. We cultured 10^4 D3 ES cells on OP9 cells for 5 days in the presence or absence of M-CSF as indicated. The numbers of differentiated and undifferentiated colonies were counted. Both differentiated and undifferentiated colonies were trypsinized, and 10^5 cells were transferred onto fresh OP9 cells and cultured for another 5 days in the presence or absence of M-CSF as indicated. Ten days after the initiation of the differentiation induction, the number of clusters were counted. After removal of OP9 cells the number of harvested cells were counted, and cytospin specimens stained with May-Grunwald Giemsa were examined to discriminate between macrophages and nonmacrophages. Data in Table 2 are shown as cumulative numbers yielded from 10^4 ES cells at the initiation of the induction.
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Delocalization of Vg1 mRNA from the Vegetal Cortex in *Xenopus* Oocytes After Destruction of Xlsirt RNA

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The Xlsirts are a family of transcribed repeat sequence genes that do not code for protein. Xlsirt RNAs become localized to the vegetal cortex of *Xenopus* oocytes early in oogenesis, before the localization of the messenger RNA Vg1, which encodes a transforming growth factor-β–like molecule involved in mesoderm formation, and coincident with the localization of Xcat2 transcripts, which encode a nanos-like molecule. Destruction of the localized Xlsirts by injection of antisense oligodeoxynucleotides into stage 4 oocytes resulted in the release of Vg1 transcripts but not Xcat2 transcripts from the vegetal cortex. Xlsirt RNAs, which may be a structural component of the vegetal cortex, are a crucial part of a genetic pathway necessary for the proper localization of Vg1 that leads to subsequent normal pattern formation.

Normal development of the vertebrate embryo is dependent on the proper spatial organization of maternally expressed macromolecules in the oocyte. In amphibian oocytes, mRNAs are localized at both the animal and vegetal regions (*1*). The vegetally localized mRNAs include Vg1 (*2*), which encodes a TGF-β–like molecule implicated in mesoderm formation, and Xcat2 (*3*), which encodes a nanos-like molecule. Based on the roles of their putative homologs in *Drosophila*, it is likely that these gene products are involved in axial patterning of the early amphibian embryo. Therefore, unraveling the genetic pathways involved in the localization of these transcripts would give greater insight into how the vertebrate body plan is established.

A group of nontranslatable interspersed repeat transcripts, Xlsirts, is also localized to the vegetal cortex of *Xenopus* oocytes during the early stages of oogenesis and may be a structural component of the cortex involved in the localization of other RNAs (*4*). To determine whether Xlsirts function to localize other RNAs at the vegetal cortex, we injected anti-sense oligodeoxynucleotides (AS ODNs) into stage 4 oocytes (*5*) to destroy the endogenous localized Xlsirt RNAs and analyzed the subsequent distribution of Vg1 and Xcat2 RNAs. Because Xlsirt RNAs do not code for protein, destruction of the RNA with AS ODNs should create a null mutant (*6*, *7*).

The Xlsirt AS ODNs consisted of a mixture of two different phosphothiolated 17-mers. We determined that the optimal dosage was 50 ng [that dose destroyed the Xlsirts without causing nonspecific toxic effects (*8*)]. Xlsirt RNA that was localized at the vegetal cortex was detected in oocytes injected with a control ODN for another maternally transcribed RNA, Xlcaax (formerly Xlgv 7) (*6*, *9*) (Fig. 1A). However, we did not detect any Xlsirt RNA localized at the vegetal cortex by in situ hybridization in oocytes that were injected with Xlsirt AS ODNs and cultured for 3 days (Fig. 1B). Xlcaax ODNs do not produce any nonspecific toxic effects when injected into oocytes (*6*–*8*).

Xlsirts are a heterogeneous population of RNAs consisting of transcripts from both strands of the genes (*4*). Those molecules found localized at the vegetal cortex consist of transcripts from one strand that are referred to as sense strand transcripts. Other Xlsirt RNAs are found throughout the cytoplasm and in the germinal vesicle (GV) and may exist as double-stranded molecules. Xlsirt RNAs appear as a smear on Northern (RNA) blots (*4*, *10*). On Northern blots of RNA isolated from oocytes injected with Xlsirt AS ODNs, we detected a small decrease in the amount of Xlsirt RNA but not the loss of any specific RNA species. This result made it difficult to determine the effi-

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