

Fc Receptors Initiate the Arthus Reaction: Redefining the Inflammatory Cascade



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Fig. 6. Reversion, in the presence of EGF and PD 153035 (0.3 μ M), of Swiss 3T3 cells that were transformed by overexpression of the human EGF receptor. (A) Control. (B) EGF (100 ng/ml). (C) EGF (100 ng/ml) and PD 153035 (0.3 μ M).

broblasts at 0.05 μ M but had no effect on early gene expression in response to the other growth factors at concentrations as high as 1 μ M (17). EGF-dependent expression of *c-jun* mRNA in the A-431 cells was inhibited at a concentration of 0.16 μ M (17). Finally, PD 153035 reverted the transformed morphology of fibroblasts transfected with human EGF receptor (20) in the presence of EGF (100 ng/ml). Exposure to 0.3 μ M PD 153035 for 24 hours caused the cells to flatten and resemble the non-transformed phenotype (Fig. 6).

These studies have disclosed an inhibitor of the EGF receptor tyrosine kinase of high potency and specificity. The properties of PD 153035 demonstrate that inhibition of a purified tyrosine kinase by molecules of low molecular weight is feasible at picomolar concentrations and that enough structural diversity exists among different tyrosine kinases to allow inhibitors to discriminate among closely related enzymes.

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- DNA coding for the soluble intracellular domains containing the active kinase of the PDGF receptor (full-length gene provided by L. T. Williams, Univ. of California, San Francisco), the CSF receptor (full-length gene provided by C. J. Sherr, St. Jude Hospital, Memphis, TN), and *erbB2* were cloned into the baculovirus transfer vector pETL. Baculoviruses containing the sequences for *v-src* and the intracellular domain of the insulin receptor were from R. Jove (Univ. of Michigan, Ann Arbor) and D. Lebowitz (Memorial Sloan-Kettering Cancer Center, New York), respectively. Enzyme assays contained 25 mM Hepes buffer (pH 7.4), 150 mM NaCl₂, 10 mM MnCl₂, 10 μ M [γ -³²P]ATP, a random polymer of glutamic acid and tyrosine (4:1; 250 μ g/ml), and 100 to 500 ng of enzyme. The incorporation of ³²P into the polypeptide was determined by precipitation with trichloroacetic acid. Values represent the mean of two experiments.
- Cells were grown to confluency in 24-well plates

containing a mixture of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F12 medium (Gibco), plus fetal bovine serum (10%), and then deprived of serum for 18 hours. The cells were then exposed to various concentrations of PD 153035 for 2 hours, after which different growth factors were added to each well. The cells were incubated for 24 hours at 37°C and then [³H]thymidine incorporation into DNA over the course of 2 hours was determined by precipitation with trichloroacetic acid. Values represent the mean \pm SEM of three separate determinations.

- Swiss 3T3 fibroblasts or A-431 human epidermoid carcinoma cells were grown to confluency in 100-mm plates, made serum-free for 18 hours, treated for 2 hours with various concentrations of PD 153035 or of solvent control, and stimulated with EGF, PDGF, or bFGF (20 ng/ml) or with serum (10%) for 45 min. Total RNA was isolated by means of the RNeasy B test (Tel Test, Friendswood, TX) and 20 μ g were electrophoresed on a 1% agarose gel. The RNA was transferred to hybrid N (Amersham) and blotted with a 40-mer probe to *c-jun* (Oncogene Science) that had previously been end-labeled with [³²P]ATP.
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Fc Receptors Initiate the Arthus Reaction: Redefining the Inflammatory Cascade

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Antibody-antigen complexes initiate the inflammatory response and are central to the pathogenesis of tissue injury. The classical model for this immunopathological cascade, the Arthus reaction, was reinvestigated with a murine strain deficient in Fc receptor expression. Despite normal inflammatory responses to other stimuli, the inflammatory response to immune complexes was markedly attenuated. These results suggest that immune complex-triggered inflammation is initiated by cell bound Fc receptors and is then amplified by cellular mediators and activated complement. These results redefine the inflammatory cascade and may offer other approaches for the study and treatment of immunological injury.

Immune complex deposition in such diseases as rheumatoid arthritis, systemic lupus erythematosus, and immune vasculitis is the pathogenic factor that triggers the inflammatory cascade, leading to tissue damage and the ensuing morbidity and mortality. The experimental model of immune complex-mediated pathogenesis described by Maurice Arthus in 1903 (1) was originally characterized by the edema, hemorrhage, and neutrophil infiltration resulting from intradermal injections of horse serum into sensitized rabbits. Because of its ease and reproducibility, the experimental vari-

ant most commonly used now is the reverse passive Arthus reaction: antibody is injected into the skin and antigen injected intravenously, resulting in the local formation of immune complexes and its sequelae (2). Experiments with this reaction support the currently accepted model of inflammation in which antibodies bind their antigen-forming immune complexes, which in turn bind and activate complement by means of the "classical pathway" (2, 3). This initiation phase of the response generates chemotactic peptides that cause neutrophil invasion and activation, with subsequent degranulation and release of inflammatory mediators. Depletion studies have confirmed the requirements for immune com-

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plexes, complement, and neutrophils in the Arthus reaction (4).

However, this model has not considered the potential role of cell surface receptors known to bind antibody-antigen complexes and activate effector cells. These receptors, known collectively as Fc receptors for their binding to the Fc portion of antibodies, can activate and trigger macrophage, neutrophil, natural killer, and mast cells *in vitro* (5). The contribution of Fc receptors to the inflammatory response *in vivo* has been difficult to dissect because of their genetic heterogeneity, promiscuity in ligand binding, and overlapping specificity with other Fc-binding molecules, such as complement.

We have evaluated the contribution of Fc receptors to the inflammatory cascade using the reverse passive Arthus reaction. We used a strain of mice that has a genetic deletion of the γ -subunit of the Fc receptor (FcR) complex and therefore lacks the low-affinity receptor for immunoglobulin G (IgG) immune complexes, Fc γ RIII, as well as the high-affinity receptors for IgG and IgE, Fc γ RI and Fc ϵ RI, respectively (6). This strain (-/-), along with heterozygous (+/-) or wild-type (+/+) littermates, was injected intravenously with 20 mg of chicken egg ovalbumin (OVA) per kilogram of body weight and then given intradermal injections of either rabbit IgG to OVA (anti-OVA), preimmune rabbit IgG, or buffer alone (7). The animals were killed 2, 4, 8, or 12 hours later, and the injected skin was removed and examined histologically for edema, hemorrhage, and neutrophil infiltration (Fig. 1). The -/- mouse showed a distinct reduction in these parameters relative to its +/+ counterpart; the reaction in +/- compared with +/+ mice was similar both qualitatively and quantitatively. Consistent with other studies (7), little or no detectable inflammatory reaction was observed in the absence of specific antibody. The residual response seen was probably secondary to nonspecific tissue trauma or direct complement activation, because it was independent of immune complex formation and observed in response to normal rabbit serum (8), and it was eliminated by depletion of complement with cobra venom factor (described below).

Blinded histologic scoring of at least 30 skin sections in each group showed a consistent and parallel reduction of all three parameters in the -/- compared with +/+ mice at all time points measured; edema peaked at 2 to 4 hours, whereas hemorrhage and neutrophil infiltration were maximal at 8 hours, consistent with reported results (7).

The edema was further quantitated at 2 hours visually with Evans blue or with intravenously injected 125 I-labeled human serum albumin (Fig. 2A) (9). An early and

distinct difference in edema formation between the -/- and the +/+ mice was evident. We quantitated hemorrhage macroscopically after 8 hours by measuring the size of the purpuric spot in inverted skin sections (Fig. 2B). There are differences in both the size and intensity of the hemorrhagic lesions; these differences were also present at 4 and 12 hours. Because myeloperoxidase (MPO) is abundant in neutrophils, it was colorimetrically measured to

quantitate infiltrating neutrophils (10). After verifying that -/- and +/+ neutrophils did not differ in MPO content, MPO was extracted from injected areas of skin and quantitated with purified MPO as a standard (Fig. 2C). There was a substantial difference between the -/- and +/+ mice after 8 hours, which was also present at similar, albeit somewhat reduced, values at 4 and 12 hours.

The lack of Arthus response in the -/-

Fig. 1. The Arthus reaction as seen in histologic sections of skin stained with hematoxylin and eosin at 8 hours in +/+ (top) compared with -/- (bottom) mice. Panels on the left (control) are from a mouse injected intradermally with either normal saline or preimmune rabbit IgG before intravenous injection of OVA (20 mg/kg). Panels on the right are from a mouse injected with 100 μ g of anti-OVA. Magnification of a small vessel with marginating neutrophils is shown in the inset. Results are representative of more than 20 mice. Original magnification, $\times 100$.

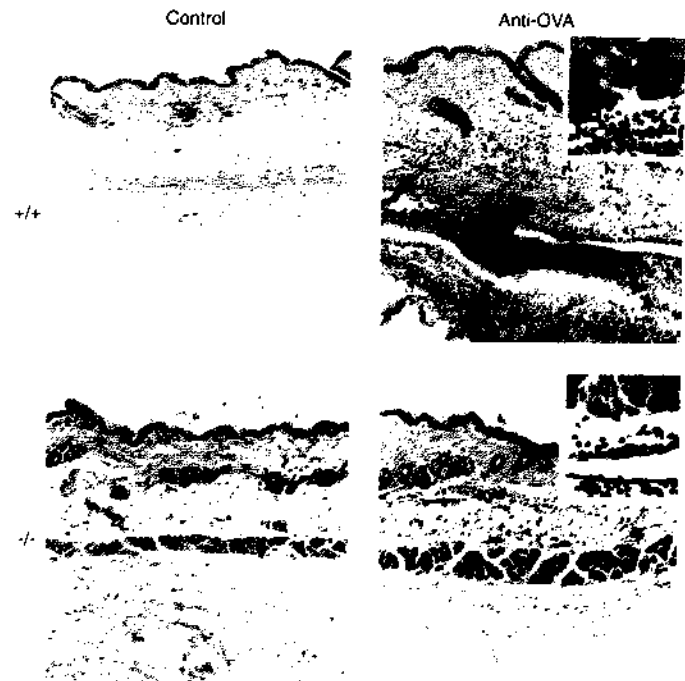
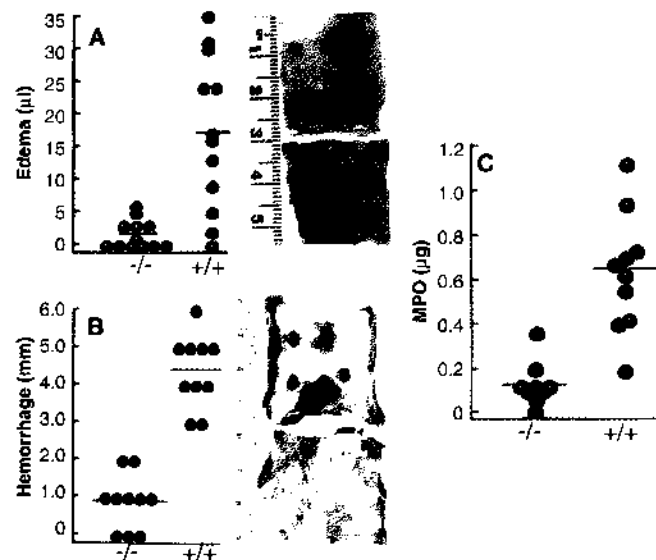


Fig. 2. (A) Edema from a 2-hour Arthus reaction. The dot plot compares the microliters of edema in +/+ and -/- mice in a typical 2-hour experiment. After intravenous injection of 10^6 cpm of 125 I-human serum albumin, we measured the counts per minute of 125 I in skin injected with rabbit anti-OVA (30 μ g) and subtracted out the negative control area; results are representative of at least 30 skin samples. The horizontal bar represents the mean value. The photograph shows Evans blue extravasation in +/+ (top) compared with -/- (bottom) mice after inclusion of 2% Evans blue in intravenous injectate. The upper



left quadrant in each skin section was injected with buffer alone; anti-OVA was injected in the other three quadrants. (B) Hemorrhage from 8-hour Arthus reaction. The dot plot gives the size (in millimeters) of the purpuric spots from a representative 8-hour experiment with 30 μ g of antibody; at right, the photograph shows inverted skin samples from +/+ (top) and -/- (bottom) mice. Negative control injections are in the upper right, and anti-OVA injections at the other three sites. (C) Micrograms of MPO from an 8-hour Arthus reaction as a measure of neutrophil infiltration. We colorimetrically quantitated MPO as previously described (10); the experiment is representative of results from more than 20 skin samples in each group.

mice was not due to a lag in kinetics, because minimal inflammation was seen at up to 24 hours. In addition, the differences seen were not dependent on the type of antibody used. Purified mouse monoclonal IgG2a to trinitrophenol (TNP), known to elicit an attenuated inflammatory response relative to heterologous antibody (2), nonetheless showed a detectable and consistent deficit in the $-/-$ mice (Figs. 3 and 4). In contrast, the IgG3 subclass of antibody, which does not interact with Fc γ RI, -II, or -III (2, 5), elicited the expected mild and indistinguishable response in the two mouse strains (Fig. 4).

To determine if the complement system was intact in the $-/-$ mice, total hemolytic complement concentrations were determined with the use of sheep red blood cells (SRBCs) coated with rabbit IgG to SRBC (anti-SRBC) (11). Both $+/+$ and $-/-$ animals had comparable and insignificantly different amounts of hemolytic complement, averaging 82 ± 30 and 187 ± 76 U/ml, respectively. The expression and function of the C3bi receptor CR3 was characterized on $-/-$ and $+/+$ macrophages (6, 12). Flow cytometry analysis of macrophages

derived from these two populations of mice demonstrated identical levels of CR3 expression. Similarly, SRBCs opsonized with C3bi were internalized normally by both $+/+$ and $-/-$ macrophages. Consistent with these observations, the complement cascade was able to function normally in vivo. Intradermal injection of zymosan, which activates complement independently of immune complexes through the "alternative pathway" (13), showed vigorous and indistinguishable inflammatory responses (Fig. 4). Therefore, the inflammatory deficit displayed by the $-/-$ mice can be attributed to the lack of Fc receptors in these animals.

The role of complement in the initiation of the Arthus reaction was assessed by depleting $+/+$ and $-/-$ mice of complement with cobra venom factor (Figs. 3 and 4) (14). The $+/+$ mice showed an attenuated inflammatory response, as described (2), whereas the minimal residual inflammatory response found in the $-/-$ mice was almost completely ablated.

Neutrophil chemotaxis and function were also qualitatively and quantitatively normal. We observed equivalently vigorous

neutrophil exudation into the peritoneum of both $+/+$ and $-/-$ mice 4 hours after inducing nonspecific inflammation with intraperitoneal thioglycollate. In response to this inflammatory challenge, the $+/+$ mice generated 4.87×10^6 cells, of which 64% were neutrophils, whereas the $-/-$ mice had 4.63×10^6 cells, of which 59% were neutrophils (15). Deposition of immune complexes was comparable in wild-type and FcR-deficient mice, as determined by the similar persistence of 125 I-labeled anti-OVA in these mice (16).

Deletion of the γ chain of the FcR complex results in the loss of surface expression of Fc γ RI, Fc γ RIII, and the IgE high-affinity receptor, Fc ϵ RI (6). To eliminate the possibility that the absent IgE receptor is responsible for the attenuated response, we assayed the reverse passive Arthus reaction in mice that lack the α subunit of this receptor yet have a normal expression of Fc γ Rs (17). In these Fc ϵ RI-deficient animals, a vigorous reverse passive Arthus reaction was elicited, comparable in magnitude with $+/+$ mice (18).

Our studies suggest a central role for the IgG Fc receptors in initiating the immune complex inflammatory response; the relevant Fc receptor-bearing cell types have yet to be determined. The mast cell, which expresses Fc γ RII and Fc γ RIII, is a likely candidate. Mast cells, which store and release many inflammatory mediators, are found in high density in the skin and are positioned around blood vessels; thus, they are easily accessible to circulating immune complexes (19). Moreover, mast cell-deficient mice exhibit an attenuated reverse passive Arthus reaction which is reconstituted by

Fig. 3. Arthus reaction with mouse IgG2a and in complement-depleted mice. Affinity-purified monoclonal mouse IgG2a against TNP (100 μ g) was injected intradermally, and dinitrophenol-coupled human serum albumin was injected intravenously. Animals were sacrificed at 8 hours. Skin samples were fixed in 10% buffered formalin and stained with hematoxylin and eosin. The complement-depleted mice were injected three times intraperitoneally with cobra venom factor (100 U/kg) at 8-hour intervals before the standard Arthus reaction was done. Representative sections were taken at 8 hours from $+/+$ (left) and $-/-$ (right) mice. Original magnification, $\times 100$.

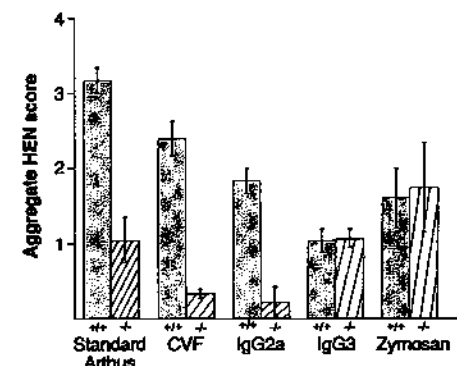
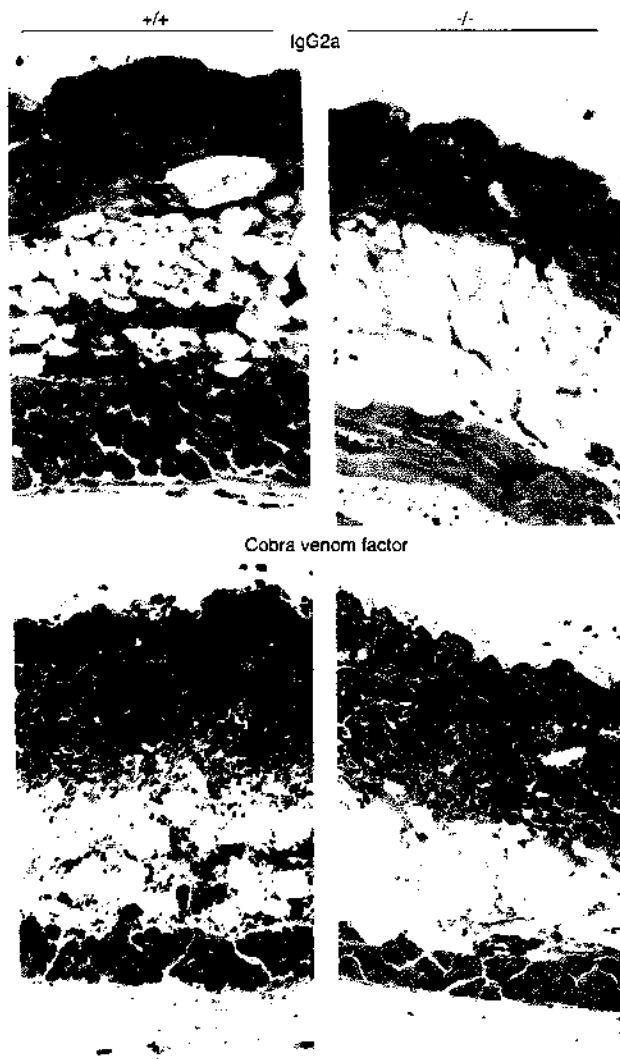


Fig. 4. Aggregate inflammation in $+/+$ compared with $-/-$ mice. The HEN score was derived by histologically scoring hemorrhage, edema, and neutrophil infiltration on a 1+ (minimal) to 4+ (vigorous) scale and averaging the three values for each section. This overall scoring process was found to be in good agreement with the quantitative values displayed in Fig. 2. Values for standard Arthus, cobra venom factor, IgG2a, and IgG3 were derived at 8 hours with 30 μ g of antibody and zymosan values for 100 μ g at 4 hours. Results are representative of more than four sections in each group.

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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Perussia, and T. Manser and others for discussion; J.-P. Kinet for providing FcεR1-deficient mice; D. Myers for assistance with histopathological interpretation; C. Rocco for slide preparation; N. Leibel for technical assistance; C. Chiang for figure designs; and C. Ritter for manuscript preparation. Supported by grant numbers AI34662 (J.V.R.) and AI01067 (D.S.) from the National Institute of Allergy and Infectious Diseases. All protocols used in the care and experimentation of mice were in accordance with NIH and institutional guidelines. We dedicate this study to the memory of Zarvil Cohn, an eminent scientist, teacher, and good friend whose contributions to our understanding of the inflammatory response were profound and whose insights we will sorely miss.

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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 RPO.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×C3H)F₂-*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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