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Short communication

A rapid rosetting method for separation of hemocyte sub-populations of *Drosophila melanogaster*

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Abstract

Hemocytes, cellular elements of the innate immune system in insects, play a crucial role in the cellular and humoral immune response. Although a significant amount of information has been collected on their differentiation and function, our understanding of hemocyte development is far from complete. Their characterisation is mostly based on morphological criteria. However, molecular markers were recently identified, defining functional subsets by the aid of monoclonal antibodies. Isolated subsets of hemocytes, in sufficient quantity and purity could help to analyse their development in vitro and also to further define their molecular characteristics. Here we describe an antibody-based rosetting technique for the physical separation of *Drosophila* hemocyte sub-populations. We have applied anti-hemocyte antibodies coupled to sheep red blood cells for separation. The method relies on the formation of rosettes between hemocytes and sheep erythrocytes, sensitised with discriminative anti-hemocyte monoclonal antibodies. Using this method the rosetting and non-rosetting hemocytes can be separated from each other by gradient centrifugation. Rosette-forming cells from the pellet and non-rosetting cells from the interface can be isolated in high recovery. The method can be used for functional and molecular characterisation of hemocyte sub-populations. The procedure is sensitive, reproducible and easy to perform.

Keywords: *Drosophila*; Hemocyte; Cell separation; Monoclonal antibody; Rosetting

1. Introduction

Hemocytes are immunoreactive cells of invertebrates, playing a central role in maintaining the host's integrity. They are involved in both humoral and cellular mechanisms such as synthesis of antimicrobial peptides, melanisation, phagocytosis, nodule formation and encapsulation. To date, the characterisation of different classes of hemocytes of

Abbreviations: CSSM3, Shields and Sang M3 insect medium containing stabilized glutamine and 5% fetal calf serum; FACS, Fluorescence Activated Cell Sorter; FCS, fetal calf serum; L1-SRBC, sheep red blood cells coated with the L1 antibody; mAb, monoclonal antibody; P1-SRBC, sheep red blood cells coated with the P1 antibody; PBS, phosphate buffered saline; PhHH3, Phospho-Histone H3; SRBC, sheep red blood cells.

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various invertebrate species is mainly based on morphological criteria [1,2]. More recently, the development of monoclonal antibodies helped to distinguish sub-populations of hemocytes of invertebrates [3–8]. Understanding the development and function of hemocyte subsets would be further facilitated by availability of methods for separation of subsets of hemocytes in sufficient amount, purity and viability.

Isolation of hemocyte sub-populations of invertebrates may be carried out by selective depletion of hemocytes [9–16] or by density gradient centrifugation through Ficoll [9] or Percoll [10–14]. However, our preliminary data show that none of these methods are suitable to obtain homogeneous sub-populations of hemocytes in *Drosophila* (data not shown). Our studies also revealed that the hemocytes can be separated neither with FACS since they are very sensitive for mechanical and osmotic stresses, nor with magnetic beads as they attach irreversibly to the magnetic beads. The aim of our work was to develop a highly reproducible method to isolate homogeneous sub-populations of functionally active hemocytes.

For the separation of *Drosophila* hemocytes we invented a rosetting procedure, using antibody-sensitized red blood cells, based on a technique originally developed for the separation of human lymphocytes [17]. Our method relies on the differential expression of cell surface molecules on functionally distinct subsets of *Drosophila* hemocytes. In *Drosophila*, the structural [18] and ultrastructural characteristics [19] of hemocytes are well known, but only a few cell surface molecules have been identified [20–23]. Recently we have developed a set of mAbs reacting with cell surface molecules of functionally distinct subsets of *Drosophila* hemocytes [22,23] and used them for separation of the corresponding subsets from a heterogeneous cell population. For this study, we used two discriminative antibodies. One of them is the P1 antibody, directed against plasmatocytes, a phagocytically active class of small round cells that normally comprise more than 90% of the blood cell population. The other one is the L1 antibody, which is specific for lamellocytes, a class of large flat cells that participate in the formation of a capsule around particles, which phagocytes cannot handle. This method may be applied with success in all those invertebrate species, where discriminative antibodies to cell surface antigens are available. No studies of this nature on invertebrate blood cells have appeared previously.

2. Materials and methods

2.1. Chemicals and reagents

A 0.15 M of NaCl solution was used for washing the Sheep red blood cells (SRBC, Phylmaster, Hungary) and Alsever's solution (24.6 g glucose, 9.6 g sodium citrate, 5.04 g sodium chloride dissolved in 1200 ml of distilled water and the pH adjusted to 6.1 with citric acid) was used for storing the SRBC. We used phosphate buffered-saline (PBS, 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) for washing both SRBC and hemocytes. The chromium-cloride ($CrCl_3 \times 6\ H_2O$, B.D.H. Chemicals Ltd, England) was used for coupling Rabbit anti-Mouse Immunoglobulin (DAKO, Copenhagen, Denmark dialyzed against 0.9% NaCl solution and adjusted to 1 mg ml^{-1}) to erythrocytes. Shields and Sang M3 insect medium (Sigma-Aldrich) containing stabilized glutamin (200 mM)(Pan System) and heat-inactivated fetal calf serum (FCS; Gibco) was used for isolation and washing of hemocytes. We used Ficoll-hypaque solution, $r = 1.077$ (Pharmacia) for separation of the rosetting, from the non-rosetting hemocytes. Distilled water was used for causing hypotonic shock, and sodium azide (Sigma), to prevent infection of the sensitized SRBC.

Anti-phospho-Histone H3 (PhHH3) antibody produced in rabbit (Upstate Biotechnology, Lake Placid) was used for detecting hemocytes showing mitotic activity. T2/48 antibody (0.5 mg ml^{-1}in 0.15 M NaCl solution) directed against the CD45 human leukocyte antigen [24] was used as a negative control. Monoclonal antibodies (mAbs) to hemocyte surface antigens were produced in mice [22,23]. The immunoglobulin fraction was separated from hybridoma supernatant by a Protein-G Sepharose column (Pharmacia) according to the manufacturer's instructions. These antibodies are designated as P1 and L1 reacting with plasmatocytes and lamellocytes, respectively [22,23], and stored as 0.5–0.7 mg ml^{-1} stock solutions in 0.15 M NaCl. Antibody H2 reacts with all circulating hemocytes of *Drosophila* larvae [22]. Goat anti-Mouse Immunoglobulin, FITC conjugate (DAKO, Copenhagen, Denmark), Goat

anti-Rabbit Immunoglobulin, FITC conjugate (DAKO, Copenhagen, Denmark) and Fluorolink Cy3 labelled streptavidine (Amersham Pharmacia) were used for visualise the staining pattern of the different markers. In all experiments the nuclei of hemocytes were stained with 4′,6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). A 1% (w/v) stock solution of $CrCl_3$ was prepared in 0.9% NaCl and the pH was immediately adjusted to pH 5.0 by 1 N NaOH [17]. The pH should not go above 6.0 as above this the $CrCl_3$ precipitates as insoluble chromate. The stock solution should be kept at room temperature and its pH should be readjusted to 5.0 pH four times, weekly. After this procedure, the 'mature' $CrCl_3$ stock solution is ready for use for coupling proteins to erythrocytes and is stable indefinitely at room temperature. For the coupling procedure a tenfold dilution of the stock solution is prepared in 0.9% NaCl immediately before use. This diluted solution may be used for coupling only on the day of the dilution.

2.2. Cells

Sheep red blood cells were stored in Alsever's solution at +4 °C for up to 2 months. *Drosophila* hemocytes were isolated from third instar larvae of homozygous *l(3)mbn-1* tumor suppressor mutant [25], by bleeding them directly into ice-cold Shields and Sang M3 insect medium containing stabilized glutamine and 5% fetal calf serum (CSSM3).

To prepare a stock suspension of anti-mouse Ig-sensitized SRBC a 300 μl aliquot of packed SRBC was washed four times with 0.9% NaCl (w/v) solution. After addition of 0.15 mg Rabbit anti-mouse Ig the sample was mixed and 150 μl of 0.1% $CrCl_3$ solution was added drop by drop while constant shaking. The suspension was gently layered under 2 ml of ice-cold 0.9% NaCl solution. After incubation overnight at 4 °C the reaction was stopped by 5.0 ml PBS. The cells were then washed four times with PBS and resuspended in 1.5 ml of PBS containing 10% FCS. For each coupling 0.1 mg of anti-hemocyte monoclonal antibody was added to 200 μl of SRBC suspension and incubated on ice for 1 h. Finally, the sensitized SRBCs were washed four times with PBS and resuspended in 400 μl PBS containing 10% FCS and 0.1% NaN_3. The sensitized SRBC are stable for 10 days at 4 °C.

3. Experimental design

The rosetting and separation were carried out at 4 °C in CSSM3 medium and pipetting was done with care to avoid fragmentation of the cells, in particular the lamellocytes. For each rosetting reaction, twenty third instar larvae were dissected, hemocytes collected in 200 μl CSSM3, washed once, resuspended in 200 μl of complete CSSM3 and counted. The sensitized SRBCs were washed tree times in CSSM3, counted and added at a hemocyte:erythrocyte ratio of 1:100. Samples were incubated in flat-bottomed, 96 well tissue culture plates (Greiner) on ice for 90 min. After incubation 2 ml of ice-cold CSSM3 was added, the cells were resuspended and layered on the top of 2 ml of Ficoll solution in a 10 ml glass test tube. The sample was spun with 500*g* for 30 min. The non-rosetting cells (negative population) were harvested from the interface, while the rosettes (the hemocytes covered by sensitized SRBC, the positive population) were collected from the bottom of the tube as the pellet. The volume of both samples was adjusted to 2 ml each, and centrifuged with 350*g* for 10 min. The pelleted cells of the rosetting fraction were exposed to a hypotonic shock of 200 μl of distilled water for one second and immediately 5 ml of complete CSSM3 was added to re-adjust ionic strength and osmolarity. Both the rosetting and the non-rosetting hemocytes were washed once with CSSM3 and used.

The immunological phenotype of the separated cells was determined by indirect immunofluorescence. *Drosophila* hemocytes were isolated from third instars by bleeding them directly into ice-cold complete Shields and Sang M3 insect medium (CSSM3). 25 μl cell suspension was placed on each spot of a multispot glass microscopic slide (C.A. Hendley Essex Ltd, GB), adhered to the glass surface by incubation at room temperature for 30 min, fixed with acetone for 6 min, saturated with PBS containing 0.1% BSA for 15 min and reacted with hybridoma supernatants at room temperature for 45 min. After three washes in PBS the hemocytes were incubated for 45 min with 1:100 dilution of FITC labelled anti mouse immunoglobulins, and 1:300 dilution of DAPI. After three washes in PBS the slides were mounted in 90% glycerol in PBS.

Phagocytosis of the FITC-labelled *E. coli* bacteria by hemocytes was recorded following a 15-minute incubation of hemocytes with heat-killed,

FITC-labelled bacteria [26]. Hemocytes taking up over five particles were regarded as positive. Hemocytes were adhered to microscope slides as mentioned above, fixed with acetone, saturated with PBS containing 0.1% BSA and reacted with hybridoma supernatants. After three washes with PBS, the cells were incubated for 45 min with 1:500 dilution of biotin labelled goat anti-mouse immunoglobulins as secondary antibody. Following three washes in PBS, cells were incubated for 45 min with 1: 3000 dilution of Cy3 labelled streptavidine, and 1:300 dilution of DAPI. After three washes in PBS the slides were mounted in 90% glycerol in PBS.

Mitotic cells were visualized by immunostaining for phospho-Histone H3 (PhHH3) [27], using 1:100 dilution of rabbit anti PhHH3 antibody, following three washes and 1:100 dilution of a FITC labelled anti rabbit immunoglobulin.

4. Results and discussion

Rosette formation. Hemocytes from *l(3)mbn-1* mutant larvae were incubated with the sensitized SRBC and the rosette-formation was monitored under a light-microscope. Any hemocyte binding more than four SRBC was defined as a rosette. The kinetics of rosette formation was monitored by using the lamellocyte-specific L1 antibody. Hemocytes from the l(3)mbn-1 mutant, which produces large numbers of hemocytes of all classes [25], were incubated with L1 antibody-sensitised erythrocytes and samples were taken for microscopic analysis. Single hemocytes and rosettes were scored every 30 min for 4 h. After the first 30 min of incubation 10% of the hemocytes were involved in rosette formation, after 60 min 17% rosette forming

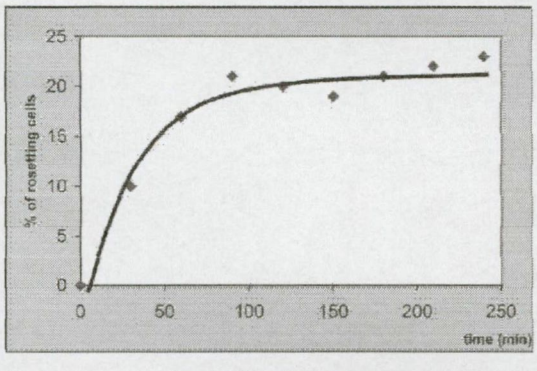

Fig. 1. The time course of rosetting. Hemocytes of *Drosophila melanogaster* were mixed with sheep red blood cells coupled with mAb specific for lamellocytes (L1) and the percentage of hemocytes forming rosettes was calculated every 30 min.

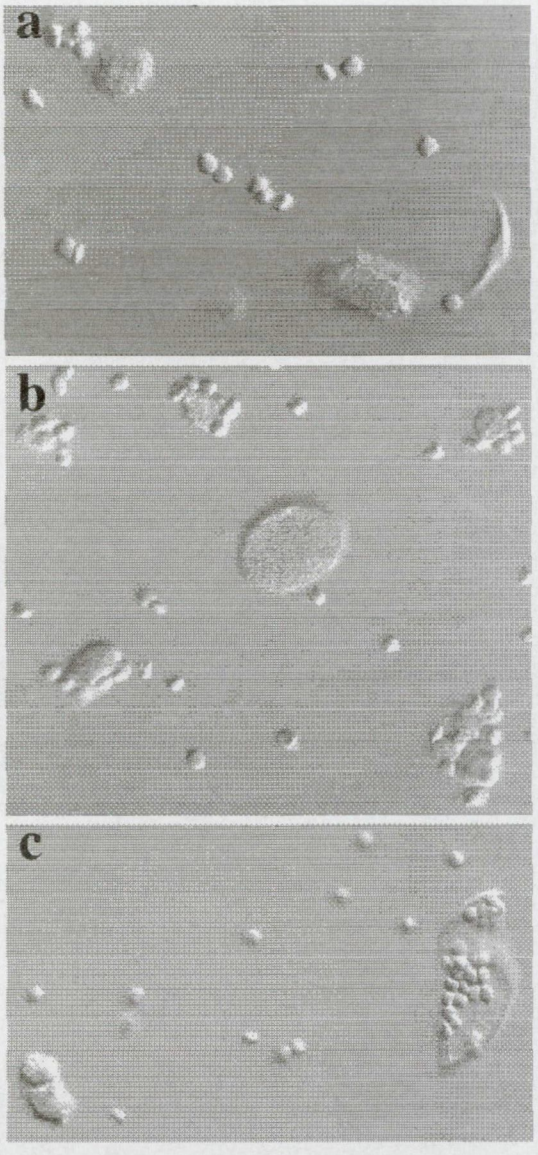

Fig. 2. The rosettes formed by erythrocytes coated with a negative control antibody (a), with a mAb binding to the plasmatocytes (b), and to the lamellocytes (c). 40 × magnification.

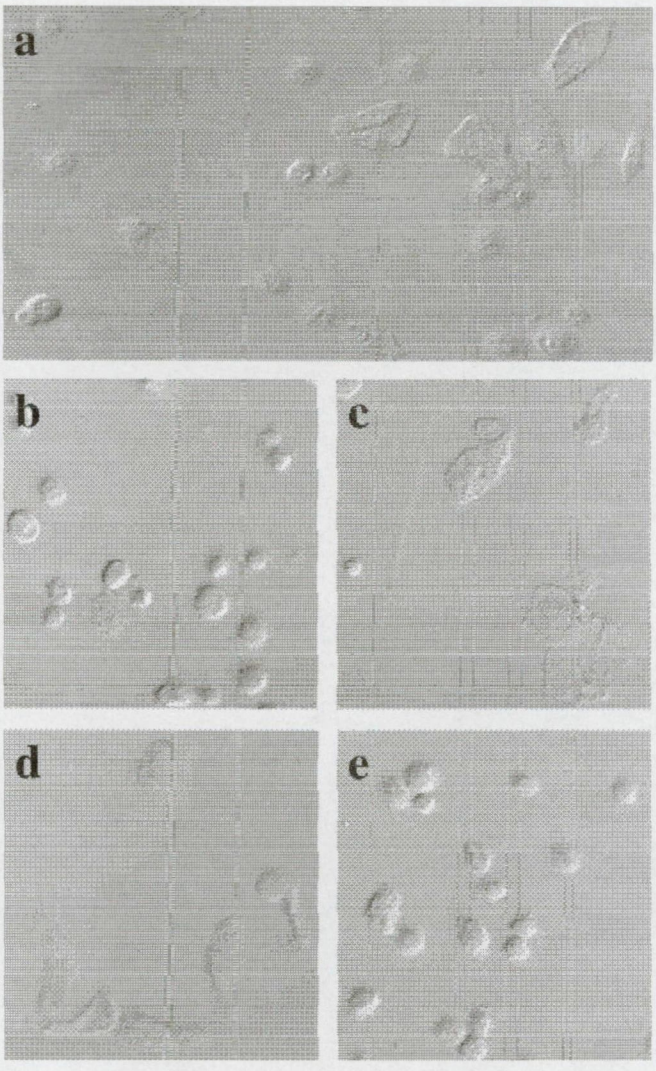

Fig. 3. Hemocytes separated with negative control antibody (a), and the sub-populations isolated with a mAbs binding to the plasmatocytes (b,c), and to the lamellocytes (d,e). 40 × magnification. (a) Using control antibody for separation the plasmatocytes, lamellocytes and cells containing crystalline inclusion in their cytoplasm were recovered from the interface. No hemocytes could be recovered from the pellet.; (b) sub-population of hemocytes recovered from the pellet when using P1-SRBC for separation; (c) non-rosetting hemocytes collected from the interface of the P1-SRBC separation; (d) L1 rosetting hemocytes recovered from the pellet of L1-SRBC separation; (e) hemocytes collected from the interface of the L1-SRBC separation.

hemocytes were observed; after 90 min the rosette formation was complete around 20% (Fig. 1). The rosettes were stable for at least 4 h. Erythrocytes coated with a control antibody (T2/48) did not form rosettes at all (Fig. 2a).

For the *separation of the two major hemocyte sub-populations*, the plasmatocytes and the lamellocytes, SRBC were coated with the plasmatocyte-specific P1 or the lamellocyte-specific L1 antibodies. Rosettes were formed around the small, round plasmatocytes

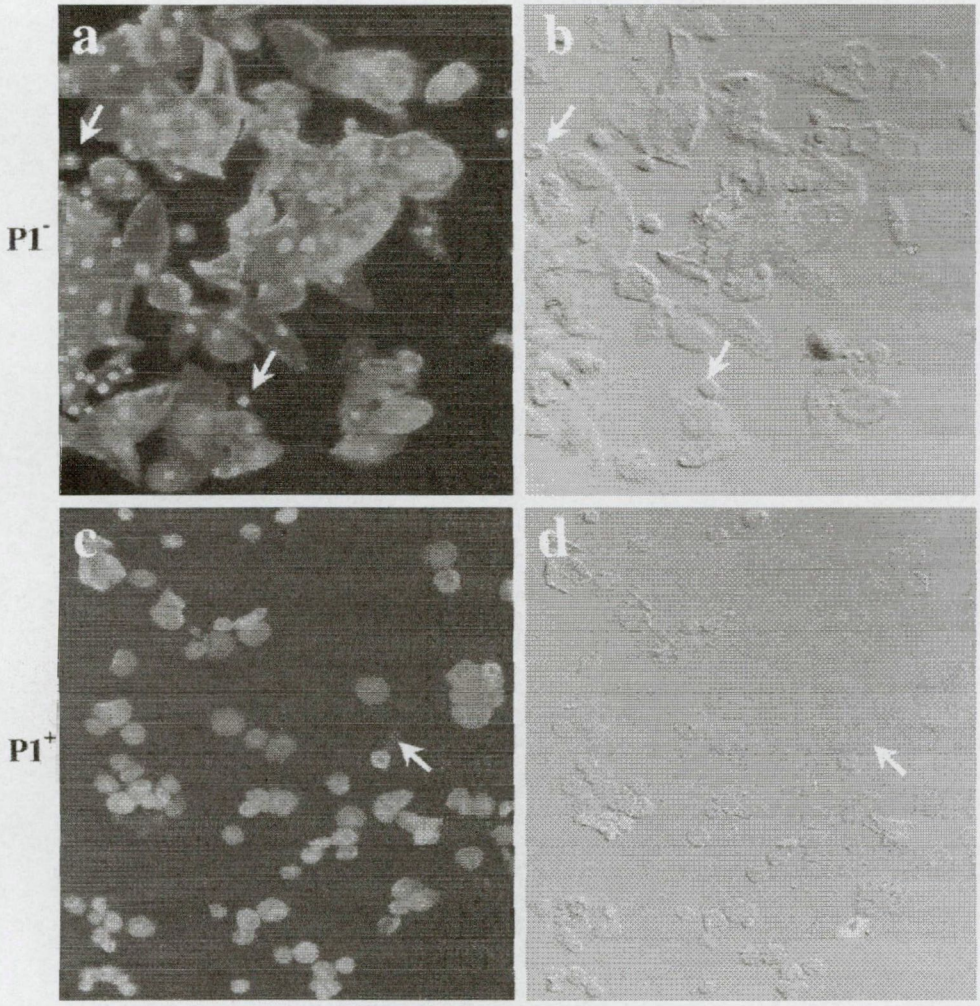

Fig. 4. Indirect immunfluorescense staining of the recovered hemocyte fractions after separation with the P1-SRBC. Magnification 20 ×. (a) Merged pictures of the non-rosetting fraction stained for the expression of lamellocyte-specific L1 marker visualized by FITC fluorochrome (green) and DAPI staining (blue); (b) Nomarski image of the same population. White arrows point to contaminating $L1^-$ hemocytes; (c) merged picture of the rosetting hemocytes which were analyzed for the expression of the plasmatocyte-specific P1 marker visualized by FITC fluorochrome (green) and DAPI staining (blue); (d) Nomarski image of the same population. White arrow points to a contaminating $P1^-$ cell.

by the P1-SRBC (Fig. 2b) and after gradient centrifugation the rosette-forming plasmatocytes were recovered from the pellet (Fig. 3b). The non-rosetting cells—the lamellocytes, small round cells having crystalline inclusions and small round cells without crystals—were recovered from the interface (Fig. 3c). The rosette formation of the L1-SRBC was restricted to the large flattened lamellocytes and a few small round cells (Fig. 2c) and these cells could be recovered from the pellet (Fig. 3d), while hemocytes with plasmatocyte morphology and cells with crystals in their cytoplasm were collected from the interface (Fig. 3e). Using the control T2/48 antibody for sensitization of the SRBC (Fig. 2a), the plasmatocytes, lamellocytes and cells containing crystalline inclusions were recovered from the non-rosetting fraction from the interface (Fig. 3a). There were no hemocytes in the pellet.

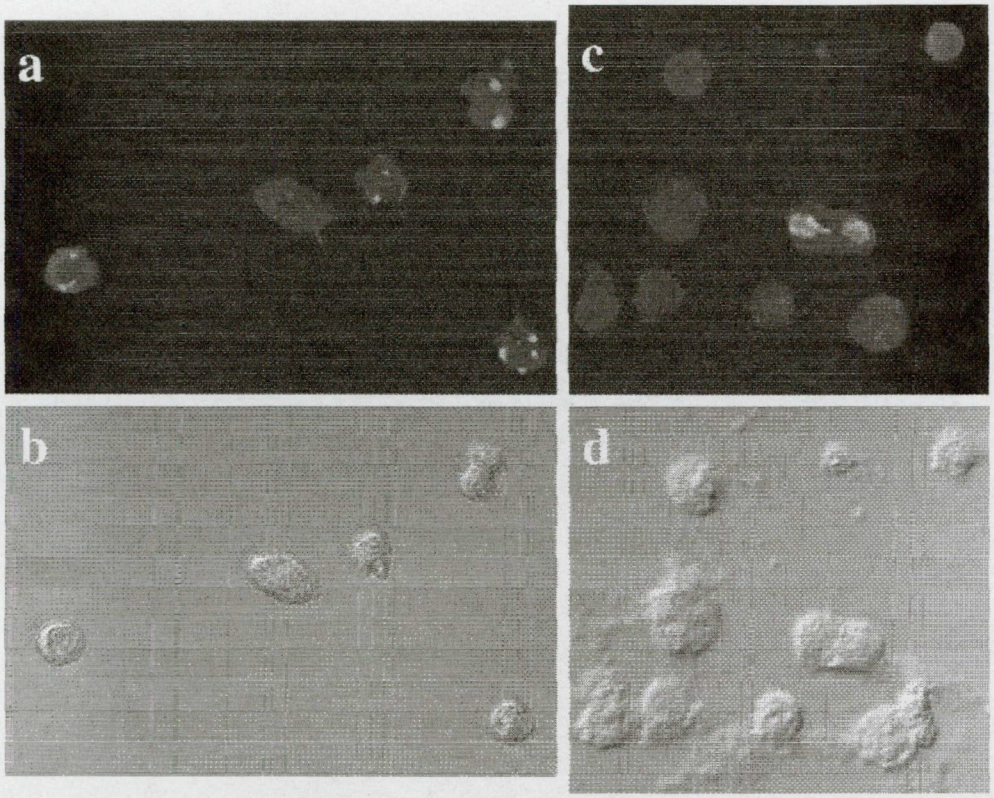

Fig. 5. Phagocytosis and mitotic activity of the separated hemocytes. (a,b) All hemocytes in the P1-positive fraction show phagocytic activity; (c,d) hemocyte in mitosis expressing the P1 antigen from the P1-positive fraction. 40 × magnification. (a) Hemocytes from the pellet of P1-SRBC separation express the P1 antigen (visualized with Cy3 fluorochrome) and they take up FITC labelled *E. coli*; (b) phase-contrast image of the hemocytes represented on (a); (c) Hemocytes from the pellet of P1-SRBC separation showing mitotic activity (detected with the aPhHH3 and visualized with FITC) express the P1 antigen; (d) Nomarski image of hemocytes represented on (c).

The *immunological phenotype* of hemocytes in each separated subset was determined by indirect immunofluorescence. All recovered cells, regardless of their origin, reacted with the pan-hemocyte H2 antibody [22] (data not shown). After separation with L1-SRBC 82.9 ± 9.0% of the rosette-forming cells were reacting with the L1 antibody [22]. This population comprised of cells with lamellocyte morphology and a minor population of small, round cells with plasmatocyte morphology; the former being the terminally differentiated large lamellocytes while the latter may be their precursors. In the L1-positive population few P1-positive small round cells were also detected (13.2 ± 5.8%). In the fraction of non-rosetting hemocytes P1-positive round cells (90.3 ± 6.4%) as well as hemocytes lacking the P1 marker were detected, the latter population being the crystal cells and supposedly an immature precursor cell population. After rosette formation with P1-SRBC the rosette forming hemocytes were small round cells with plasmatocyte morphology and 95.7 ± 3.6% of them stained with the P1 antibody (Fig. 4c and d). A small fraction (2.8 ± 1.6%) of L1 positive cells are also detected. In the non-rosetting fraction, hemocytes with lamellocyte, crystal cell and plasmatocyte morphology were found. The lamellocytes and a minor population of small round cells, the presumed lamellocyte precursors reacted with the L1 antibody 78.6 ± 8.1% (Fig. 4a and b). We also detected a minor subset, which expressed neither

the P1, nor the L1 marker; these cells represent the crystal cells and a yet unidentified sub-population, lacking both the P1 and the L1 antigens. These data show that the immunological phenotype of the cells separated with our mAbs corresponds to the phenotype of the main hemocyte subsets in *Drosophila*. The recovery exceeded 90% in each group. The viability of the recovered populations was 64–90% as determined by the trypan blue exclusion test (data not shown).

The *functional activity* of the separated hemocytes was tested in a phagocytosis assay. Fluorescein-labelled *E. coli* was taken up by the P1 rosetting cells (Fig. 5a and b), the L1 non-rosetting cells and by 1–3% of P1 non-rosetting hemocytes with plasmatocyte morphology (data not shown). The *mitotic activity* of the separated hemocytes was tested by an anti-phosphohistone H3 antibody. Cell proliferation was detected exclusively among of the P1 rosetting and the L1 and the P1 non-rosetting fractions suggesting that there is a mitotic pool in the $P1^+$ (Fig. 5c and d), $P1^-$ and in the $L1^-$ fractions (not shown).

We have been able to isolate functionally active subsets of *Drosophila* hemocytes by a rosetting method, using antibody-sensitized sheep erythrocytes. The high recovery, the excellent viability and the functional activity of the separated hemocytes make them suitable for molecular analysis and for 'in vitro' studies of hemocyte development. This method should be applicable also in other invertebrate species for high-recovery isolation of viable functional subsets of hemocytes, where monoclonal antibodies to cell surface antigens are available.

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Analysis of Ras-Induced Overproliferation in Drosophila Hemocytes

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ABSTRACT

We use the *Drosophila melanogaster* larval hematopoietic system as an *in vivo* model for the genetic and functional genomic analysis of oncogenic cell overproliferation. Ras regulates cell proliferation and differentiation in multicellular eukaryotes. To further elucidate the role of activated Ras in cell overproliferation, we generated a collagen promoter-Gal4 strain to overexpress Ras^{V12} in Drosophila hemocytes. Activated Ras causes a dramatic increase in the number of circulating larval hemocytes (blood cells), which is caused by cellular overproliferation. This phenotype is mediated by the Raf/MAPK pathway. The mutant hemocytes retain the ability to phagocytose bacteria as well as to differentiate into lamellocytes. Microarray analysis of hemocytes overexpressing Ras^{V12} *vs.* Ras^{+} identified 279 transcripts that are differentially expressed threefold or more in hemocytes expressing activated Ras. This work demonstrates that it will be feasible to combine genetic and functional genomic approaches in the Drosophila hematopoietic system to systematically identify oncogene-specific downstream targets.

THE ras genes encode highly conserved GTP-binding proteins that regulate cell growth, proliferation, and differentiation in almost all multicellular eukaryotes (reviewed in McCormick 1994). In addition, ras genes have been the subject of intensive research because they are mutated in almost 30% of human cancers (Bos 1989). These cancers include solid tumors and several types of leukemia, including chronic myelomonocytic leukemia, acute myelogenous leukemia, and acute lymphoblastic leukemia (Beaupre and Kurzrock 1999). Ras proteins function by switching between an active GTP-bound state and an inactive GDP-bound state. Activated Ras proteins bind to and activate several distinct downstream effector pathways, including Raf, Ral-GDS, and PI3-kinase. Many of the oncogenic mutations lock Ras in the activated state, leading to the constitutive activation of downstream effector pathways. The mechanisms by which Ras activation leads to increased cell proliferation and impaired differentiation, as is observed in many cancers, have still not been fully elucidated.

Much of our understanding of Ras-mediated signaling comes from a combination of biochemical experiments conducted in mammalian tissue culture cells and genetic screens conducted in Drosophila and *Caenorhabditis elegans* (reviewed in Greenwald and Rubin 1992 and McCormick 1994). In Drosophila, *Ras oncogene at 85D* (*Ras85D*, also known as *Ras1* and hereafter referred to as *Ras*) appears to be the ortholog of *H-ras*, *Ki-ras*, and *N-ras* found in mammals (Neuman-Silberberg *et al.* 1984). *Ras oncogene at 64B* (*Ras64B*, also known as *Ras2*) is the Drosophila counterpart of mammalian *R-ras*.

The most intensively studied Ras-mediated signaling pathway in Drosophila has been the one that leads to the specification of the R7 photoreceptor in the developing ommatidial clusters of the compound eye (Hafen 1991; Van Vactor *et al.* 1991; Greenwald and Rubin 1992). Since the R7 cell is specified from a group of postmitotic cells, the role of Ras in this pathway may differ from pathways where Ras promotes growth and cell proliferation. Indeed, it has been shown that the expression of activated Ras in Drosophila imaginal discs results in increased growth and cell proliferation (Karim and Rubin 1998; Prober and Edgar 2000). However, when clones of cells expressing activated Ras are induced in wing discs, they are eliminated from the intervein regions during differentiation later in development (Prober and Edgar 2000). Thus, in this aspect at least, their properties differ from mammalian tumor cells in which Ras mutations appear to sustain continued growth and proliferation. One reason for this difference may be that the rate of growth and proliferation decreases in the cells of the wing disc toward the end of larval development, and Ras activation may be toxic to the cells at that time. In contrast, Ras mutations detected in mammalian tumors often arise in populations of cells that are capable of sustained proliferation, such as the cells found in intestinal crypts (Stopera and Bird 1992; Losi *et al.* 1996).

As an alternative, the Drosophila hematopoietic system can be utilized to study the proliferative effects of Ras and other oncogenes. In contrast to imaginal disc cells, Drosophila hemocytes appear to be capable of

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sustained cell proliferation, as a number of mutations can lead to the increased numbers of circulating larval hemocytes (reviewed in DEAROLF 1998). Drosophila hemocytes serve several biological functions, including the secretion of extracellular matrix components and a role in the cellular immune response (DEAROLF 1998; LANOT *et al.* 2001; VASS and NAPPI 2001). Larval hemocytes apparently originate from the lymph glands and circulate freely in the hemolymph. There are three main subsets of larval hemocytes:

1. The plasmatocytes constitute ~95% or more of hemocytes and resemble cells of the vertebrate myeloid lineage. They contribute to host defense by engulfing microorganisms and are important during development for the phagocytosis of apoptotic cells.
2. Lamellocytes are larger cells that participate in encapsulating larger foreign invaders. They normally comprise ~1–5% of hemocytes, but increase in number when induced by parasites or by abnormally developing tissues in the body cavity.
3. Finally, the crystal cells are characterized by the presence of crystalline inclusions believed to contain prophenoloxidase (RIZKI *et al.* 1980, 1985). When activated and released, the phenol oxidase acitivity causes the melanization of the encapsulated targets.

Here we show that overexpression of activated Ras in Drosophila hemocytes results in sustained cell proliferation in a manner analogous to that observed in human leukemias. We also carry out a microarray analysis to identify changes in gene expression that result from increasing Ras activity in hemocytes. This work demonstrates the utility of the Drosophila hematopoietic system as a model for the genetic and functional genomic study of oncogenic cell overproliferation.

MATERIALS AND METHODS

Generation of transgenic flies: We generated flies that carry a 2.7-kb regulatory region located between the adjacent collagen type IV genes *Cg25C* and *viking* (YASOTHORNSRIKUL *et al.* 1997). We PCR cloned an *Eco*RI-*Bam*HI fragment, using the primers GAATTCGTTCGCCCGCAGACTC and GGATCCGA TGCCCTATGCACTTA, and fused it to the AUG-β-GAL vector (THUMMEL *et al.* 1988). A *Bam*HI-*Xba*I fragment encoding the *lacZ* gene was excised from this construct. This was replaced with a *Bam*HI-*Spe*I fragment encoding the *GAL4* gene excised from the pGATB vector (BRAND and PERRIMON 1993) to create the Cg-GAL4 construct. This construct was injected into w^{1118} embryos to generate transgenic flies carrying P[w, CgGAL4]. Three independent lines all mapping to the second chromosome were obtained. Two of the lines tested gave identical results and were used in this study.

Genetic crosses and phenotypic analyses: Flies were cultured on a standard medium containing corn meal, molasses, yeast, agar, and supplemented with Tegosept. When a low density of growth was desired, eggs (30–60) were collected from six pairs of parents for 4 hr in a bottle containing 25 ml of food and cultured at 25°. Under normal conditions at least 100 eggs were cultured in each bottle. To overexpress constructs in hemocytes, transgenic flies carrying the target genes under the control of GAL4 responsive elements (UAS; BRAND and PERRIMON 1993) were crossed to transgenic flies carrying the CgGAL4 construct. The hemocytes in the larval progeny that specifically express the target gene were then analyzed for any abnormal phenotype using phase-contrast microscopy. *e16E*, a GAL4 line expressed in the fat body but not in the hemocytes (HARRISON *et al.* 1995), was used as a negative control. The concentration of larval hemocytes from late third instar larvae was determined as described previously (ZINYK *et al.* 1993). The mutants used in this study include *Ras1* (85D), *phl* (*Draf*) (3A1), *rl* (41A), *hop* (10B5-6), and *l(3)mbn* (65A6) and are described in FLYBASE (1999).

Immunohistochemistry: The production and specificity of monoclonal antibodies to the different hemocyte subsets of Drosophila will be described elsewhere (E. KURUCZ, P. VILMOS, I. NAGY, Y. CARTON, I. OCSOVSZKI, D. HULTMARK, E. GATEFF and I. ANDO, unpublished results). H2, the pan-hemocyte antibody, recognizes all hemocytes. Antibodies P1 and L1 recognize plasmatocytes and lamellocytes, respectively. Requests for antibodies should be addressed to I. Ando. The rabbit antiphosphohistone H3 antibody was obtained from Upstate Biotechnology (Lake Placid, NY).

Wandering third instar larvae of the appropriate genotype were carefully pierced by a sharp tungsten wire and the hemolymph was collected in a drop of PBS on a glass slide. The hemocytes were incubated for 30 min in a humidified chamber to allow them to adhere to the glass slide. The buffer was removed and the cells fixed for 6 min in acetone. The cells were briefly air dried and incubated with 2% BSA in PBS for 30 min, followed by incubation overnight with the monoclonal antibody (1:10 dilution) or the phosH3 antibody (5 μg/ml). After three washes with PBS, the cells were incubated for 2 hr at room temperature with a 1:150 dilution of either Cy3-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) or FITC-conjugated goat anti-rabbit antibody (Sigma, St. Louis). After three washes in PBS, the slides were mounted in 90% glycerol in PBS. The experiment with the phosH3 staining was repeated more than three times and a total of 564 Ras-act hemocytes and 708 wild-type control hemocytes were counted to determine the number of phosH3-staining cells.

Functional assays: Hemocytes were tested for their ability to phagocytose in an *in vitro* assay. Hemocytes were seeded in 25 μl of Schneider medium on the spots of multiwell microscopic slides (SM-011, Hendley-Essex, England) and 2 μl of a 10% FITC-labeled *Escherichia coli* suspension were added immediately. Bacteria were added in saturating amounts, so that all the hemocytes capable of phagocytosis took up bacteria. The slides were incubated for 30 min in a humidified chamber at room temperature. After incubation, 2 μl PI solution (Sigma) was added to quench the fluorescence of nonphagocytosed bacteria and the slides were covered with a coverslip. The number of cells showing fluorescence as well as the number of phagocytosed bacteria per cell were counted. In each experiment 500 hemocytes were analyzed. In some experiments the phagocytic capacity of the cells was correlated with their immunological phenotype by using streptavidin-Cy3 (Amersham Pharmacia Biotech) in combination with biotinylated L1 and/or P1 antibodies.

We tested for increased lamellocyte differentiation induced by the parasitic wasp *Leptopilina boulardi*, strain G486 (RUSSO *et al.* 1996) in wild-type and mutant larvae. Approximately 40-sec instar larvae of each genotype were infested by exposure to four mature *L. boulardi* females for 4 hr at 25°. After 72 hr, we scored the total number of hemocytes and the number of $L1^+$ cells from these infested larvae. The larvae were bled and the hemolymph was collected on a glass slide in a drop of

Drosophila Ringer solution or Schneider medium. The cells were stained as described above.

Injection of hemocytes into adult flies: We collected the hemolymph from third instar larvae in ice-cold Drosophila Ringer solution and determined the cell concentration in a Bürker chamber. Wild-type, adult virgin females were anesthetized with diethyl-ether. Cell suspensions with the appropriate hemocyte concentrations or control buffer were injected into the abdomen of adult flies using a thin glass needle. The flies were transferred to fresh food, and the number of surviving flies was determined daily. For each genotype, the experiment was repeated at least three times and a total of 50–139 wild-type adult flies were injected with hemocytes. In each experiment, at least 50 adult flies were injected with the control buffer. To retrieve the hemocytes from the injected adults, the flies were anesthetized and perfused with ice-cold Drosophila Ringer medium. Cells were collected on a glass slide and stained as described above.

Analysis of mRNA expression using oligonucleotide microarrays: Drosophila larvae carrying either *UAS-Ras1^{V12}/CgGAL4* or *UAS-Ras1^{wt}/CgGAL4* were raised at 25°. The larval hemolymph from 80 larvae for those expressing *Ras^{V12}*(Ras-act) and from nearly 700 larvae for those expressing *Ras^{wt}*(Ras-wt) was carefully collected in PBS and the cells spun down and stored at −70°. Because wild-type larvae contain relatively small numbers of hemocytes, it was not possible to extract sufficient quantities of RNA for microarray analysis. Total RNA (15 μg) was extracted from the larval hemocytes using the Rneasy mini kit (QIAGEN, Chatsworth, CA). Gene expression analysis was performed using the Affymetrix Drosophila GeneChip, according to the laboratory methods described in the Genechip Expression Analysis Technical Manual (Affymetrix, 2001). Briefly, cDNA was synthesized from poly(A)$^+$ RNA and cRNA was labeled with biotin during its synthesis using the BioArray high-yield transcript-labeling kit (Enzo). Fifteen micrograms of labeled, fragmented cRNA was hybridized to each array. The arrays were washed and scanned according to the manufacturer's protocol. For each genotype, three independent RNA samples were used and hybridized to microarrays. The Ras-act microarrays were then compared to the Ras-wt microarrays, giving a total of nine comparisons.

GeneChip.DAT files were analyzed using the Affymetrix MAS 5.0 software that includes the statistical algorithms for GeneChip expression data analysis. The image files are scanned using the Affymetrix GeneChip software and then scaled to the same intensity value before they are compared. Each transcript is represented by a probe set on the Drosophila GeneChip. Each probe set has 14 pairs of perfect match (PM) and mismatch (MM) oligonucleotides. The expression level for each gene is determined by calculating the average of differences in hybridization intensities between the PM and MM oligos. The expression profile of genes in Ras-act hemocytes was compared to that of Ras-wt hemocytes, which was taken as the reference. Three comparisons out of nine were excluded from the analysis due to high noise, following the Affymetrix recommendations. Fold change for each transcript was calculated using the Affymetrix MAS 5.0 software and is represented by the average signal log ratio (SLR is the log to the base 2 value of the fold change). Only those genes that are expressed threefold (SLR = 1.6) or more in Ras-act compared to those in Ras-wt are included in Table 2. A more detailed analysis is presented in Table S1 of the supplementary data at http://www.genetics.org/supplemental/, which includes all genes that are increased in Ras-act compared to those in Ras-wt (SLR of 0.05 or more), along with the *P* values for "Detection" of each gene and "Change" of expression of each gene in the six comparisons. The genes that are decreased in Ras-act compared to those in Ras-wt are shown in Table S2 of the supplementary data (http://www.genetics.org/supplemental/) along with the corresponding *P* values. The average SLR values and the corresponding standard deviation values are also included in the supplementary data.

RESULTS

Identification of a hemocyte promoter: Our first goal was to identify a promoter that would enable us to preferentially express genes in the hemocytes of Drosophila. We previously showed that a DNA regulatory region located between two adjacent collagen type IV genes on the second chromosome, *Cg25C* and *viking*, can drive *lacZ* expression specifically in embryonic hemocytes (Yasothornsrikul *et al.* 1997). In the larva, *lacZ* expression is observed in the hemocytes and also in the fat body. We therefore generated *CgGAL4* transgenic lines, in which the yeast GAL4 transactivator is expressed under the control of this regulatory region. In the *CgGAL4* lines, GAL4 is expressed in the embryonic hemocytes from stage 13 until the end of embryonic development (data not shown). In the larva, GAL4 is expressed strongly in the circulating hemocytes (Figure 1, A and B), in the anterior-most pair of lobes of the lymph gland (Figure 1, C and D), and in the fat body (Figure 1, E and F). Expression of GAL4 is undetectable in lamellocytes (data not shown).

To verify the effectiveness of the *CgGal4* line, we used it to overexpress a cDNA encoding a hyperactive form of the Drosophila Jak kinase, *hopscotch* (*hop*). The gain-of-function mutation *hop^{Tumf}* has previously been shown to cause an overproliferation of larval hemocytes (Hanratty and Ryerse 1981; Harrison *et al.* 1995; Luo *et al.* 1995). At higher culture temperatures, *hop^{Tumf}* larvae have melanotic tumors and increased numbers of plasmatocytes and lamellocytes. Larvae carrying both the *CgGAL4* and the *UAS-hop^{Tumf}* transgenes have melanotic tumors and their hemolymph contains an increased number of plasmatocytes and lamellocytes (total number of hemocytes = $14 \times 10^3/\mu l$ at 25°; Figure 2B). In wild-type larvae grown at 25°, we observe $2–5 \times 10^3$ hemocytes/μl. These abnormalities are similar to those observed in *hop^{Tumf}* mutant flies. When *hop^{Tumf}* is expressed using the GAL4 driver *e16E*, which is expressed in the larval fat body but not in the hemocytes, the number and morphology of the hemocytes were similar to those of wild-type larvae (number of hemocytes = $2 \times 10^3/\mu l$ at 25°). These results indicate that the *CgGal4* lines provide sufficient expression in the hemocytes. They further suggest that the phenotypic abnormalities observed in *hop^{Tumf}* mutant larvae are most likely due to a cell-autonomous effect of *hop^{Tumf}* in hemocytes.

Activated Ras causes an overproliferation of hemocytes: Ras has been implicated in regulating cell proliferation and differentiation in many organisms. To examine the effects of increasing Ras activity in hemocytes, we expressed an activated form of Ras, Ras^{V12}, using the

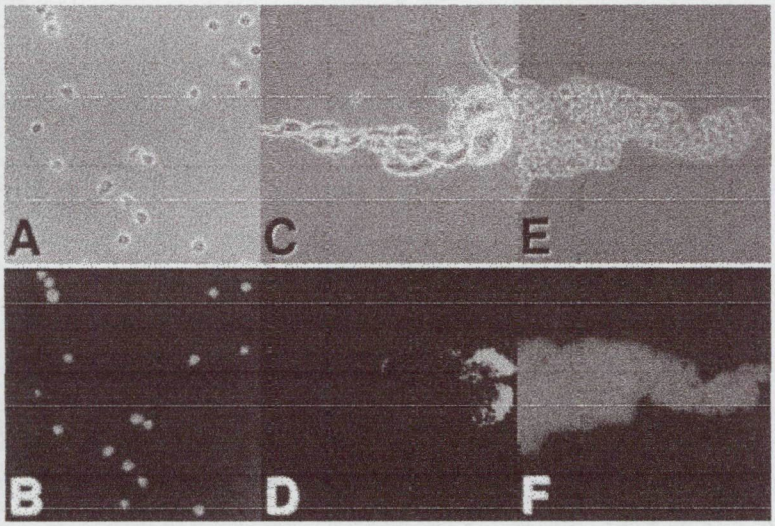

FIGURE 1.—The collagen promoter is expressed in the larval lymph glands and fat body. A, C, and E are phase-contrast images from third instar larvae carrying both the *UAS-GFP* and the *CgGAL4* transgenes and B, D, and E are the corresponding fluorescence images showing green fluorescent protein expression. (A and B) Circulating hemocytes. (C and D) The lymph gland showing strong collagen expression in the anteriormost pair of lobes. (E and F) Fat body.

CgGAL4 line. Late third instar larvae carrying both the *UAS-Ras*^{V12} and the *CgGAL4* transgenes (hereafter referred to as Ras-act) display a dramatic 40-fold increase in the number of hemocytes (Figure 2C and Figure 4). These cells express H2, an antigen that is expressed by all hemocytes (Figure 2, D and E). These cells morphologically resemble plasmatocytes, although they do not stain with the plasmatocyte-specific antigen P1 (data not shown). Under normal culture conditions at 25°, 99% of these animals die as early pupae. However, 33% of these larvae survive to adults when larvae are cultured at very low densities (see MATERIALS AND METHODS). Consistent with earlier data, (KARIM and RUBIN 1998), we found that the phenotypes produced by expressing *UAS-Ras*^{V12} are temperature sensitive. When raised at 18°, the Ras-act larvae display a 20-fold higher number of hemocytes than do wild-type larvae and develop into adults (data not shown). We do not observe an increased number of hemocytes in Ras-act embryos. Larvae carrying *UAS-Ras*^{wt} and *CgGAL4* (referred to as Ras-wt) display a milder increase in the number of hemocytes ($18 \times 10^3/\mu l$ at 25°) and develop into adults. In a control experiment, we used e16E as the GAL4 driver (HARRISON *et al.* 1995) to express *Ras*^{V12} in the fat body but not in the hemocytes (e16E also shows expression in the imaginal discs, gut, and cuticle). This leads to pupal lethality but has no effect on the number or morphology of circulating hemocytes (data not shown). Therefore the increased number of hemocytes in Ras-act larvae probably does not cause the observed lethality.

We next examined whether the large increase in Ras-act larval hemocyte numbers is due to an increase in

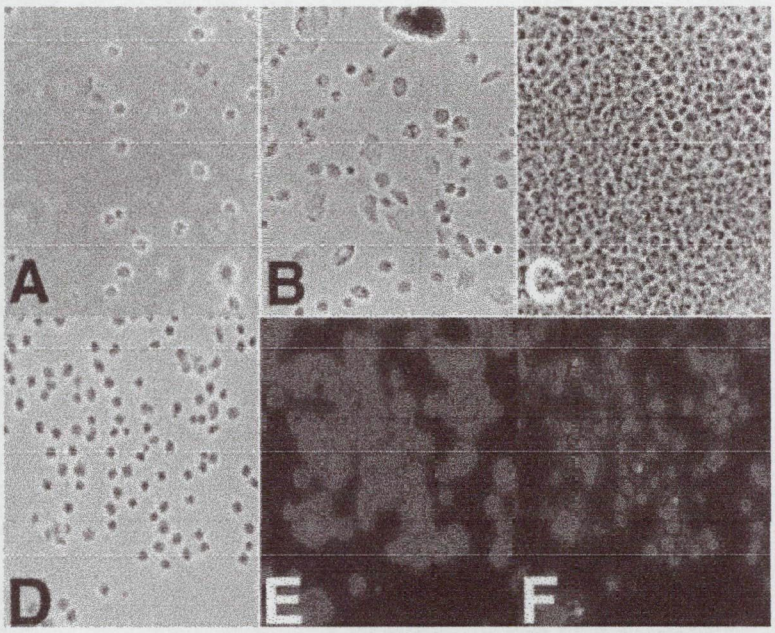

FIGURE 2.—Expression of activated *hop* or *Ras* causes abnormalities in the circulating larval hemocytes. (A–C) Phase-contrast images of unfixed hemocytes from third instar larvae of (A) wild type, (B) *CgGAL4/+ UAS-hop*^{Tuml}*/CgGAL4*, and (C) *UAS-Ras*^{V12}*/CgGAL4*. Overexpression of both *hop*^{Tuml} and *Ras*^{V12} causes increases in circulating hemocyte number compared to those in the control. (D) Phase-contrast image of fixed hemocytes from *UAS-Ras*^{V12}*/CgGAL4*. (E) Same field as in D, stained with H2, a marker expressed by all hemocytes and visualized by Cy3 fluorescence. (F) A merged image of hemocytes stained with phosH3 visualized by FITC (green) and H2 visualized by Cy3 (red).

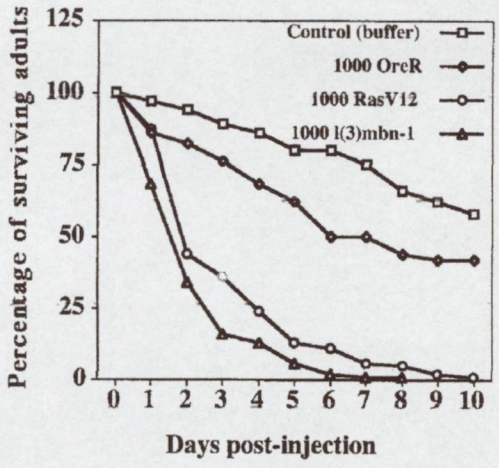

FIGURE 3.—Injection of Ras^{V12} hemocytes into wild-type adult females reduces survival. Survival curves of female flies injected with either control buffer or 1000 hemocytes each from OreR, Ras^{V12}, or *l(3)mbn* larvae. A total of 50, 131, or 139 wild-type female flies were injected with hemocytes from OreR, Ras^{V12}, or *l(3)mbn*, respectively. At least 200 wild-type flies were injected with the control buffer. Hemocytes from *l(3)mbn* or Ras^{V12} larvae reduce the survival of wild-type hosts compared to buffer or hemocytes from OreR larvae.

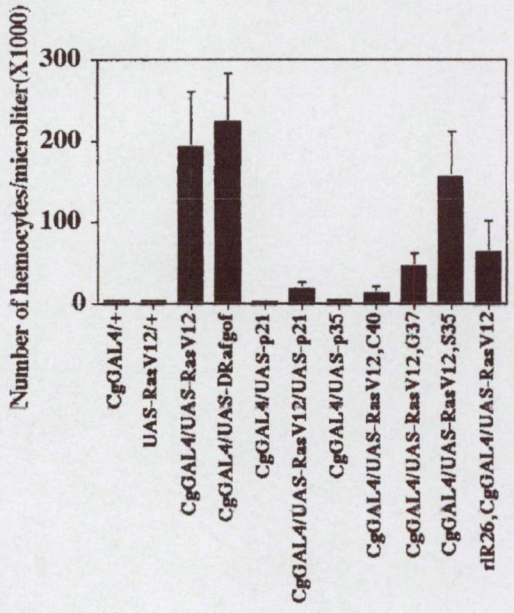

FIGURE 4.—Large increase in hemocyte number caused by hyperactivation of the Ras-Raf-MAPK pathway. The hemolymphs from at least eight different third instar larvae were examined for each genotype, and the average number of circulating hemocytes are presented with the standard deviation. The expression of Ras^{V12} or $DRaf^{gof}$ in hemocytes using CgGAL4 causes an ~40-fold increase in the number of circulating hemocytes. Co-expression of *p21*, the CDK inhibitor, with Ras^{V12} causes a significant decrease in hemocyte number compared with using Ras^{V12} alone. Expression of *p35*, which blocks caspase-mediated cell death, does not increase hemocyte cell number relative to controls. Ras double mutations that disrupt interactions with Raf, as well as reduction of *rl* MAPK gene dosage, suppress the Ras^{V12}-induced proliferation. Student *t*-tests show a significant difference in hemocyte numbers ($P < 0.01$) between *UAS-*Ras^{V12}*/CgGAL4* and all other genotypes shown except for *UAS-*$DRaf^{gof}$*/CgGAL4* and *UAS-*$Ras^{V12,S35}$*/CgGAL4*.

the proliferation of hemocytes or to a block in cell death. We obtained several lines of evidence to suggest that the mutant cells do indeed overproliferate. First, we cultured the Ras-act hemocytes in the abdomen of wild-type adult flies. Previous studies have shown that cells from tumorous lymph glands can be serially passaged in the abdomen of adult flies (HANRATTY and RYERSE 1981). Following injection with Ras-act hemocytes, the abdomen of the injected flies became enlarged, and 64% of the injected flies died within 3 days (Figure 3). These changes are associated with the overproliferation of cells within the host (HANRATTY and RYERSE 1981). A similar result was obtained when hemocytes from *lethal(3) malignant blood neoplasm-1* [*l(3)mbn-1*] mutant larvae were injected into the abdomen of adult flies (Figure 3). The *l(3)mbn* mutant is another example of a mutant that has increased numbers of circulating hemocytes (GATEFF 1978; KONRAD *et al.* 1994). In control animals injected with the same number of wild-type hemocytes, only 24% of the host animals died (Figure 3) and the abdomen of the injected flies showed no change in size. In control animals injected with the injection buffer, only 10% of the host animals died (Figure 3). The Ras-act hemocytes can be repeatedly passaged in the abdomen of adult flies (data not shown), and during this process they retain their original size and morphology and continue to express the pan-hemocyte marker H2. These results suggest that the mutant hemocytes proliferate in the adult abdomen and cause the death of the animal.

Second, we stained circulating larval hemocytes with anti-phosH3, a marker that stains chromosomes in cells undergoing mitosis. In a population of proliferating cells, only a small proportion of cells undergoes mitosis and stains with phosH3 at any given time (HENDZEL *et al.* 1997). We found that 0.32% (±0.49) of hemocytes stain with phosH3 in control larvae. QIU *et al.* (1998) obtained a comparable number of phosH3-staining hemocytes from wild-type larvae. In contrast, a significantly larger proportion ($P < 0.01$) of Ras-act hemocytes (3.93% ± 1.27) stained with this antibody (Figure 2, E and F), indicating that Ras-act hemocytes actively divide while in circulation.

Third, the cyclin-dependent kinase inhibitor, p21, has previously been shown to block the entry of cells into S-phase in Drosophila (DE NOOIJ and HARIHARAN 1995). If Ras^{V12} induced an overproliferation of hemocytes, expression of p21 would be expected to block their division. We found that the increase in Ras^{V12} hemocyte numbers produced is indeed largely suppressed by coexpression of p21 in hemocytes (Figure 4), suggesting that Ras^{V12} promotes cell proliferation.

TABLE 1

Induction of lamellocytes after parasitization with *L. boulardi*

Genotype	% of L1$^+$ cells
OreR control	0
OreR infested	24 (8)
Experiment 1	
GAL4/UASRas control	2.2 (0.4)
GAL4/UASRas infested	4.8 (0.6)
Experiment 2	
GAL4/UASRas control	11.0 (3.4)
GAL4/UASRas infested	26.8 (7.4)

In each group, at least eight larvae were dissected, and the mean number of L1$^+$ cells are presented with the standard deviation given within parentheses.

Fourth, the expression of the caspase inhibitor p35 in hemocytes does not cause an appreciable increase in hemocyte number. p35 has previously been shown to block apoptotic cell death in Drosophila (Hay *et al.* 1994). If the Ras^{V12} phenotype is caused primarily by a block in cell death, we would expect a suppression of cell death in wild-type larval hemocytes to cause an increase in their number. Our results suggest that the Ras^{V12} phenotype is not related to alterations in cell death.

Functional tests for Ras^{V12}-expressing hemocytes: We next determined whether the Ras-act hemocytes were capable of performing any of the functions associated with wild-type hemocytes, including the phagocytosis of bacteria and the accumulation of lamellocytes in response to an immune challenge. We performed an *in vitro* assay for phagocytosis, in which 500 hemocytes of each genotype were examined for their ability to engulf FITC-labeled *E. coli*. The control OreR plasmatocytes engulfed an average of 10 bacteria/cell, while the Ras-act counterparts engulfed an average of 5 bacteria/cell. Therefore, the Ras-act hemocytes are capable of phagocytosing bacteria, but are slightly less proficient than wild-type hemocytes.

We also observe cells that morphologically resemble lamellocytes in the Ras-act larvae. They range in number from 1 to 3.5% and express L1, the lamellocyte-specific marker (data not shown). If wild-type larvae are immuno-challenged by *L. boulardi*, a parasitic wasp that lays its eggs inside the Drosophila larvae, the lamellocytes increase in number and function in encapsulating the wasp eggs (Russo *et al.* 1996). To test whether the Ras-act larvae are capable of an increase in lamellocyte differentiation upon immune challenge, we allowed them to be parasitized by *L. boulardi*. In two independent experiments, the percentage of L1-positive cells increased in the wasp-infested Ras-act larvae compared to that in unchallenged Ras-act larvae (Table 1). This result indicates that lamellocyte differentiation can occur in Ras-act larvae.

The Ras^{V12} overproliferation phenotype is mediated by the Raf-MAPK pathway: The proliferative effect of Ras could be mediated via the Raf-mitogen-activated protein kinase (MAPK) pathway or could involve other effectors distinct from Raf. We therefore tested the effect of overexpressing a gain-of-function allele of Drosophila *Raf* ($DRaf^{gof}$) in the larval hemocytes. The expression of $DRaf^{gof}$ in hemocytes also results in a massive increase in hemocyte number (Figure 4), similar to that seen with the expression of Ras^{V12} (Figure 4). This indicates that Raf activation alone is sufficient to induce hemocyte proliferation.

We also looked at the effects of reducing the function of genes located downstream of Ras in this pathway. Ras-act larvae that were hemizygous for a *DRaf* loss-of-function allele ($DRaf^{l129}$) had fewer hemocytes than wild-type larvae did (data not shown). This is consistent with previous observations that a complete loss of *Raf* function causes an almost total loss of hemocytes, most likely due to a decrease in cell viability (Luo *et al.* 2002). Thus, expression of activated Ras cannot bypass this requirement for *Raf*. Ras-act larvae that were heterozygous for a loss-of-function mutation in the *rl* gene that encodes MAPK (Biggs *et al.* 1994) had a threefold decrease in hemocyte count compared to that of Ras-act (Figure 4). A complete lack of *rl* function (rl^{R26}/rl^{R26}) in Ras-act larvae results in very few hemocytes, similar to that seen in larvae homozygous for rl^{R26} or in larvae lacking *Raf* function (data not shown). These results indicate that MAPK function is essential for Ras-mediated signaling in cell proliferation.

Activated Ras has been shown to interact directly with other effectors in addition to Raf, including PI3-kinase and Ral.GDS (Katz and McCormick 1997). To test for the involvement of these effectors, we took advantage of three Ras effector loop mutants previously used in mammalian cell culture experiments (White *et al.* 1995; Joneson *et al.* 1996; Khosravi-Far *et al.* 1996) and in Drosophila wing development (Karim and Rubin 1998). In addition to the V12 mutation, these effector loop mutants carry a second amino acid substitution in the Ras effector domain. The mutant Ras^{V12C40} interacts with PI3-kinase but fails to interact with Raf or Ral.GDS. Ras^{V12C37} can bind to Ral.GDS but does not interact with Raf or PI3-kinase, and Ras^{V12S35} interacts with Raf but fails to bind Ral.GDS or PI3-kinase. The expression of the first two mutant forms of *Ras* (Ras^{V12C40} and Ras^{V12C37}) in hemocytes results in a significant reduction in their numbers ($P < 0.01$) compared with the massive hemocyte numbers seen with the expression of Ras^{V12} alone (Figure 4). These two mutants also partially suppress the lethality associated with the expression of Ras^{V12} in hemocytes. Since both these mutants fail to interact with Raf, these results indicate that Raf plays an important role in Ras1-mediated signaling. In contrast, the expression of Ras^{V12S35}, a mutant that interacts with Raf but fails to bind Ral.GDS or PI3-kinase, displays an overproli-

feration of hemocytes similar to that seen in Ras-act larvae (Figure 4). This result shows that an impaired ability of Ras to interact with PI3-kinase or Ral.GDS does not obviously affect Ras-induced proliferation. However, the number of hemocytes observed in the first two mutants (*i.e.*, those that do not activate Raf) is still increased compared to that in wild type. This result suggests that the effector loop mutants may retain some residual activity or that other effectors may also contribute in a minor way to the Ras-induced overproliferation.

Microarray analysis of Ras-expressing hemocytes: To identify those genes whose expression correlates with oncogenic Ras-induced cell overproliferation, we examined expression profiles of genes using oligonucleotide microarrays. There is a 10-fold increase in the number of Ras-act hemocytes ($192 \times 10^3/\mu l$) compared with that of Ras-wt hemocytes ($18 \times 10^3/\mu l$). Therefore, a comparison of the patterns of gene expression between these two populations of hemocytes is likely to identify those genes whose expression is altered by increasing Ras activity.

Using Affymetrix Drosophila oligonucleotide microarrays, we compared the expression profiles of >13,000 genes between the two populations of hemocytes. Expression of 6123 transcripts was detected as present in Ras-act and 4644 transcripts as present in Ras-wt hemocytes. In a comparative analysis of Ras-act to Ras-wt hemocytes (see MATERIALS AND METHODS) there is at least a threefold increase in the expression of 279 genes (Table 2 and supplementary information at http://www.genetics.org/supplemental/). The expression of 76 genes is decreased threefold or more in Ras-act compared with that in Ras-wt. These genes are not discussed further in this article, but are included as supplementary information at http://www.genetics.org/supplemental/.

Among the genes that are most strongly induced in Ras-act hemocytes are CG16731, a putative prophenoloxidase activator (128-fold), *string* (27-fold), and *Cks* (25-fold; Table 2). Crystal cells contain the enzymes and substrates required for melanization (RIZKI *et al.* 1985). Prophenoloxidase, the inactive precursor of phenol oxidase, is activated in the crystal cells by a series of serine proteases, the last of which is a prophenoloxidase activator. *string* encodes a phosphatase that activates the mitotic kinase, Cdk1 (Cdc2), and promotes the G_2/M transition in the cell cycle (EDGAR *et al.* 1994). The role of Ras in activating *string* transcription has not previously been documented. Cks encodes a protein that associates with cyclin/cdk complexes, but whose precise function is not known (SPRUCK *et al.* 2001).

Among the 279 genes that had a >3-fold increase in expression in Ras-act, 17 genes have functions related to cell cycle regulation and 16 genes function in DNA replication or mitosis (Table 2). The increased expression of some of these genes may be directly related to the increased proliferative capacity of Ras-act hemocytes.

TABLE 2

Gene expression induced by activated Ras in hemocytes

	Name	Average SLR
	Cell cycle	
CG1395	stg	4.77
CG3738	Cks	4.68
CG12306	polo	3.73
CG4274	fzy	2.79
CG3938	CycE	2.77
CG7838	Bub1	2.69
CG5363	cdc2	2.97
CG4488	wee	2.32
CG5814	CycB3	2.24
CG3000	rap/Fzr	2.15
CG1772	dap	2.08
CG11886	Slbp	1.95
CG10895	loki	1.88
CG17498		1.67
CG7581	Bub3	1.61
CG4711	grp	3.05
CG6235	tws	1.66
	Mitosis/DNA replication/repair	
CG10422	ham	4
CG5923	DNApol-alpha73	2.99
CG5371	RnrL	2.76
CG6920	mus309	2.71
CG5052	pim	2.67
CG4082	Mcm5	2.5
CG1616	dpa	2.35
CG11397	gluon	2.25
CG10726	barren	2.22
CG18608	prod	2.18
CG9633	Rpa-70	2.15
CG9193	mus209	2.11
CG4978	Mcm7	2.11
CG8975	RnrS	2
CG8142		1.63
CG13327	CapG	1.62
CG4208	XRCC1	1.62
CG3041	Orc2	2.35
CG9273		2.14
CG5553	DNAprim	3.04
	Ligand/receptor	
CG6127	Ser	3.97
CG6134	spz	1.93
CG5372	a PS5	3.94
CG3212		3.48
CG6536		2.45
CG16827	a PS4	2.13
CG6553		2.4
CG1762	betaIntnu	2.17
CG4823		1.99
CG5490	Tl	1.62
	Cytoskeleton/actin/microtubule	
CG12008	karst	3.78
CG18152	CalpA	2.93
CG18152	CalpA	2.2
CG7940		2.19

(*continued*)

TABLE 2

(Continued)

	Name	Average SLR
CG2092	scraps	2
CG14996	Chd64	2.4
CG2916	Sep	2.16
CG1768	dia	1.8
CG8705	pnut	1.72
CG7438	Myo31DF	1.72
CG10076	spir	2.44
CG6875	asp	3.51
CG9191	Klp61F	2.99
CG10923	Klp67A	2.96
CG7831	ndc	2.45
CG1258	pav	2.04
CG5981	stai	1.99
CG1451	Apc	1.74
CG6392	cmet	2.36
CG18436	Ppn	1.95
	Signal transduction	
CG1225		3.12
CG18511	Ggamma30A	2.67
CG13345	Rac GAP	2.32
CG10379	mbc	2.3
CG4276	aru	1.65
CG15015		2
CG5820	Gp150	3.15
CG5201	Dad	1.9
CG3048	Traf1	2.66
CG4394	Traf3	1.99
CG7207		2.73
CG7186	SAK	2.73
CG8173		2.33
CG10522		1.86
CG7719	Pk91C	1.84
CG14211		2.4
CG2096	flw	1.98
CG7643		1.88
CG18355	Btk29A	1.76
CG6656		1.67
CG4551	smi35A	1.63
CG3705	aay	1.6
CG18069	CaMKII	1.6
	Metabolism	
CG16731		7.05
CG5779	Bc	1.85
CG14527		2.02
CG16918		1.91
CG6639		1.62
CG4948	Tequila	1.99
CG17109		2.9
CG6687		2.61
CG17530		4.1
CG4381	pseudogene GstD	2.99
CG17523		2.13
CG5452	dnk	2.52
CG1411	CRMP	3.64
CG11811		1.69
CG10564	Ac78C	1.88

(*continued*)

TABLE 2

(Continued)

	Name	Average SLR
CG10214		2.27
CG3333	Nop60B	1.75
CG7487	RecQ4	2.4
CG7504		2.37
CG4152	1(2)35Df	1.71
CG4033	RpI135	1.27
CG5887	desat1	2.22
CG1633	Jafrac1	2.18
CG3635		1.84
CG10120	Men	1.98
CG3038		1.84
CG5999		2.04
CG5731		2.09
CG4829		1.92
CG4435	FucTB	2.15
CG10234	Hs2st	1.95
CG7098	dik	1.7
CG2674	M(2)21AB	2.19
CG4200	sl	1.97
CG12014		1.79
CG10242	Cyp6a23	3.82
CG10246	Cyp6a9	2.12
CG3523		2.23
CG7649		2.11
	Transporter/ligand binding/carrier	
CG1208		3.19
CG10960		3.15
CG1063	Itp-r83A	2.63
CG9023	Drip	1.69
CG3874	frc	1.94
CG5485		1.85
CG10997		1.96
	Transcription factor	
CG4059	ftz-f1	2.78
CG1689	lz	2.51
CG18376	lola	2.3
CG8933	exd	1.98
CG2670	Taf55	2.16
CG12223	Dspl	2.11
CG4029	jumu/Dom	2.02
CG8815	Sin3A	1.99
CG9207		1.88
CG5441	dei	1.85
CG7664	crp	1.82
CG11988	neur	1.6
CG6964	Gug	2.21
	Other	
CG10939	Sipl	4.11
CG5581	Ote	2.85
CG1847		1.63
CG1966	Acf1	1.78
CG4236	Caf1	1.68
CG12846	Tsp42Ed	2.38
CG14066	Larp	2.29
Cg4475	Idgf2	2.21

(*continued*)

TABLE 2

(Continued)

	Name	Average SLR
CG10939	plx	2.69
CG9772		1.66
CG13399	Chrac-14	1.66
CG3091		2.74
CG7565		2.59
CG10119	LamC	1.84
CG7421	Nopp140	1.63
CG14941	esc	2.75
CG2163	Pabp2	1.82
CG7741		1.68
CG1591	REG	1.93
CG3864	thioredoxin	1.8
CG5055	baz	1.83
CG5884	par-6	1.65
CG3525	eas	1.82
CG5354	pie	1.76
CG9695	Dab	1.72
CG9999	Sd	1.68
CG17252	BCL7-like	1.68
CG5303	mei-S332	1.6
CG7052	Tep2	2.48
CG5670	Atpalpha	2.36
	Unknown	
CG5807		1.99
CG5935	1(2)04154	1.93
CG2694		1.91
CG11207		3.9
CG10433		3.65
CG9188		3.31
CG14253		3.15
CG2213		2.81
CG6874		2.79
CG10364	msh1L	2.65
CG14610		2.52
CG18316		2.46
CG13283		2.42
CG15891		2.38
CG8902		1.88
CG7795		2.03
CG11451		2.98
CG17064		1.78
CG18253		1.74
CG6983		3.47
CG15740		3.27
CG7242		2.96
CG17269		2.72
CG9241		2.49
CG3278		2.33
CG7730		2.19
CG1558		2.1
CG7272		2.09
CG15713		2.09
CG8886	1(2)05714	2.09
CG8247		2.06
CG6321		2.03
CG18156		2.02

(continued)

TABLE 2

(Continued)

	Name	Average SLR
CG9328		2.02
CG14557		1.9
CG6264	best	1.88
CG6910		1.85
CG8436		1.84
CG9625		1.8
CG8317		1.77
CG5951		1.61
CG4239		1.61
CG12819		2.28
CG15211		1.63
CG11120		4
CG16873		1.9
CG15347		1.77
CG10722		3.02
CG17383		2.23
CG8486		3.54
CG9752		2
CG9917		1.8
CG16876		3.34
CG10927		1.85
CG6579		2.23
CG12592		1.88
CG15818		2.01
CG6014		1.81
CG7763		3.52
CG5100		1.74
CG6643		2.32
CG15707		2.23
CG6249		1.68
CG11399		1.66
CG11314		1.66
CG10191		2.76
CG15513		2.29
CG7845		1.89
CG10359		1.99
CG6954		1.95
CG12702		3.35
CG5690		2.04
CG3221		1.88
CG8924		2.55
CG2199		1.79
CG12260		2.02
CG12744		1.66
CG10631		1.62
CG10512		2.2
CG7120		2.05
CG18088		3.35
CG2065		2.05
CG8058		1.9
CG7593		1.64
CG18522		2.24
CG4735		2.85
CG7670		4.6
CG6477		4.44
CG6416		3.86

(continued)

TABLE 2

(Continued)

	Name	Average SLR
CG3533	*uzip*	1.89
CG18228		3.38
CG17219		2.7
CG13162		2.62
CG10420		1.78
CG3362		2.53
CG12065		1.75
CG9576		1.68
CG4880		1.61
CG3238		3.04
CG7139		1.88

Genes that are more than threefold induced in Ras-act hemocytes compared to those in Ras-wt hemocytes are presented here. SLR or signal/log ratio is a log to the base 2 value of the fold change; therefore a value of 1.6 is more than threefold.

Interestingly, the genes upregulated include both positive and negative regulators of cell cycle progression. Among the positive regulators that were induced are *string* (27-fold), *cyclin E* (6.8-fold), *cdc2* (7.8-fold), and *cyclin B3* (4.7-fold). Negative regulators of cell proliferation upregulated include *dacapo* (4-fold) and *wee1* (5-fold). Genes induced include those that regulate the G_1/S transition (*e.g., cyclin E, dacapo*) as well as those that regulate G_2/M (*string, wee1, cyclin B3*). Expression of *cyclin B* (2-fold), *cyclin A* (2.9-fold), *cdk2* (1.9-fold), and *cdk4/6* (2.4-fold) is also increased (supplementary information at http://www.genetics.org/supplemental/). Expression of *cyclin D*, a cyclin that has been shown to promote growth in some situations (DATAR *et al.* 2000), is not increased.

Among the genes known to regulate hemocyte fates in Drosophila, expression of *lozenge* (*lz*) is increased 5.7-fold in Ras-act hemocytes (Table 3). *lz* expression is necessary for crystal cell fate specification and *lz* mutants lack crystal cells (RIZKI *et al.* 1985; LEBESTKY *et al.* 2000). Although *lz* is induced in Ras-act hemocytes, we do not observe a significant increase in morphologically recognizable crystal cells in Ras-act larvae. However, these cells appear to express much higher levels of both a putative prophenoloxidase activator (128-fold) and *Black cells* (*Bc*; 3.6-fold), which encodes monophenol oxidase (Tables 2 and 3). Both these enzymes are believed to function in crystal cells (RIZKI *et al.* 1985). Thus, increased Ras activity may increase the expression of a subset of crystal-cell-specific genes. The transcription factor, *serpent* (*srp*), is essential for the development of all embryonic hemocytes (REHORN *et al.* 1996). *srp* expression is increased in Ras-act hemocytes by 2-fold (Table 3). The transcription factor, *gcm*, has been shown to be necessary for plasmatocyte fate specification (BERNARDONI *et al.* 1997). Expression of *gcm* is not detected in either Ras-act or Ras-wt hemocytes (Table 3). This may correlate with our finding that these cells do not express the plasmatocyte-specific marker, P1. However, *peroxidasin* (*Pxn*) and *croquemort* (*crq*), genes that are expressed in normal plasmatocytes (NELSON *et al.* 1994; FRANC *et al.* 1996), are expressed to similar levels in both Ras-wt and Ras-act hemocytes (Table 3). Thus, increasing Ras activity does not cause a general increase in the expression of genes normally associated with plasmatocytes.

TABLE 3

Changes in hemocyte gene expression

Hemocyte genes	Detection in Ras-wt	Detection in Ras-act	Average SLR	Fold change
srp	Present	Present	0.98	1.97
gcm	Absent	Absent	−1	1
lz	Absent	Present	2.5	5.7
Bc	Present	Present	1.85	3.6
crq	Present	Present	0.97	1.96
Pxn	Present	Present	−0.9	0.9
Cg	Present	Present	−0.72	0.72

DISCUSSION

Ras has been well studied as a mammalian oncogene. Mutations that activate Ras represent one of the steps in the formation of many types of human cancers (BOS 1989). Activation of Ras alone is rarely oncogenic. Thus the precise contribution of Ras to the multi-step transformation process and the way in which Ras interacts with other oncogenic events is still not well understood. We have shown that overexpression of wild-type Ras or activated Ras alone can induce hemocyte overproliferation in Drosophila. This one-step model of Ras-induced leukemia may thus represent a valuable system to characterize the link between increased Ras activity and uncontrolled cell proliferation.

Previous studies by others have shown that activated Ras can induce growth and cell proliferation in Drosophila (KARIM and RUBIN 1998; PROBER and EDGAR 2000). Expression of activated Ras in Drosophila imaginal discs results in disc overgrowth. Analysis of patterns of BrdU incorporation and apoptosis showed that some of the consequences of Ras overexpression were the result of nonautonomous mechanisms (KARIM and RUBIN 1998). These nonautonomous mechanisms have not yet been elucidated. Expression of activated Ras in clones of cells in the wing imaginal discs showed that increased Ras activity results in increased cell growth (mass accumulation). Increased Ras activity also results in cell death in the intervein regions of the wing when cells have to undergo terminal differentiation (PROBER and EDGAR 2000). In contrast, the hemocyte overproli-

feration that we describe generates a more uniform cell population, most likely resulting from a cell-autonomous effect of Ras in the hemocytes. Therefore, Drosophila hemocytes might represent a simpler system to elucidate the molecular mechanisms underlying Ras-induced neoplastic cell overproliferation.

Ras can induce an overproliferation of functional yet abnormal hemocytes: We have shown that the overexpression of activated Ras in Drosophila hemocytes results in a leukemia-like overproliferation. These circulating hemocytes appear to be normal by several functional criteria. The majority of the overproliferating hemocytes resemble wild-type plasmatocytes in their morphology and are capable of phagocytosis. Moreover, lamellocytes accumulate when Ras-act larvae are parasitized in the wasp assay. The Ras-act hemocytes also express several markers associated with normal plasmatocytes, including peroxidasin and croquemort.

At the same time, the mutant hemocytes differ from their wild-type counterparts in their expression patterns of known plasmatocyte markers. The mutant hemocytes do not express the epitope, recognized by the P1 monoclonal antibody that is found on all mature plasmatocytes (E. Kurucz, P. Vilmos, I. Nagy, Y. Carton, I. Ocsovszki, D. Hultmark, E. Gateff and I. Ando, unpublished results). Further, microarray analysis demonstrated that these cells do not express *gcm*, a transcription factor that is expressed in normal plasmatocytes (Bernardoni *et al.* 1997). These cells also appear to express several transcripts that are normally expressed in crystal cells, including the transcription factor *lozenge*, the CG16731 gene encoding a prophenoloxidase activator, and *Bc* encoding a monophenol monooxygenase. Although we did not observe an increase in morphologically recognizable crystal cells in Ras-act larvae, when these larvae are subject to a heat assay (Rizki *et al.* 1985; Lebestky *et al.* 2000), we find a considerable increase in the number of black cells (data not shown). These findings can be explained in several ways. The cells may represent multi-potential stem or precursor cells that are capable of giving rise to both plasmatocytes and crystal cells. Alternatively, these cells may not correspond to any population of cells found during normal hematopoiesis, as increasing Ras activity beyond physiological levels may result in the expression of inappropriate combinations of genes. The latter possibility is supported by the observation that wild-type plasmatocytes co-express *croquemort* and *gcm* (Bernardoni *et al.* 1997), unlike Ras-act hemocytes.

Our data indicate that activated Ras induces hemocyte overproliferation in Drosophila through a Raf/MAPK pathway. The Ras-induced overproliferation of hemocytes can be suppressed by mutations that reduce signaling via D-Raf or by reduced activity of the rl MAP kinase. Further, the overexpression of an activated Raf construct resulted in a large increase in the concentration of circulating hemocytes (Figure 4).

The Ras-induced overproliferation can also be suppressed by overexpression of the human cdk inhibitor p21. The Drosophila ortholog of p21, dacapo, has been shown to bind and inactivate cyclin E/cdk2 complexes (de Nooij *et al.* 1996; Lane *et al.* 1996). Thus the Ras-induced overproliferation appears to require active cyclin E/cdk2 complexes and cannot bypass their function. Moreover, the levels of cyclin E and *string* RNA, both of which have been shown to be induced by E2F (Neufeld *et al.* 1998), are elevated in Ras-act hemocytes. Thus Ras-induced hemocyte overproliferation may occur via increased E2F activity.

Changes in gene expression induced by Ras: By comparing the RNA samples from Ras-act and Ras-wt hemocytes, we have been able to assess the transcriptional changes over the entire predicted genome that result from an increase in Ras activity in a specific lineage. To our knowledge, this is the first description of a whole-genome, *in vivo* analysis of Ras-mediated changes in transcription. Other studies have examined the transcriptional changes resulting from an increase in Raf activity in a human breast epithelial cell line, although only a portion of the human genome was analyzed (Schulze *et al.* 2001).

One overall finding is that many of the genes that are upregulated in Ras-act cells include genes that function in cell cycle regulation and DNA replication. These genes include both positive and negative regulators of cell proliferation. The cyclin-dependent kinase inhibitor dacapo (which antagonizes the function of cyclin E/cdk2 complexes), as well as the wee1 kinase (which inactivates cdc2), are both induced. There is currently no known function for either gene in promoting cell cycle progression. Thus the induction of these genes may represent a negative feedback mechanism that attempts to reduce cell proliferation under conditions of excessive cell proliferation. Another possibility is that these two genes have roles in promoting cell cycle progression that are currently unknown. Our microarray data also show that regulators that promote all stages of cell cycle progression are induced, not only those that promote the G_1/S transition. Our data therefore suggest that both the G_1/S and G_2/M cell cycle transitions may be influenced by an increase in Ras activity.

A second finding is that many of the transcriptional targets known to be induced by Ras1 in other tissues are not induced in Ras-act hemocytes. Therefore, although the RTK/Ras pathway induces the expression of *phyllopod* (Chang *et al.* 1995; Dickson *et al.* 1995; Tang *et al.* 1997) in the eye disc, *mirror* in the ovary (Jordan *et al.* 2000), and *blistered* and *ribbon* in the tracheal cells (Nussbaumer *et al.* 2000; Bradley and Andrew 2001), none of these genes are obviously induced in Ras-act hemocytes. This is consistent with tissue-specific factors acting together with Ras to determine which target genes are expressed in each cell type. Another gene whose expression is modulated by Ras

activity is the pro-apoptotic gene, *hid* (also known as Wrinkled). It is believed that the anti-apoptotic effect of Ras in embryos is mediated in part by a reduction in *hid* transcription (KURADA and WHITE 1998). Our analysis demonstrates that the *hid* RNA level does not decrease in Ras-act cells, indicating that this mechanism may not be of importance in hemocytes. Ras may still inactivate *hid* in these cells via MAPK-mediated phosphorylation of the Hid protein. Other pro-apoptotic genes like *reaper* or *grim* are not expressed in either Ras-wt or Ras-act hemocytes.

Finally, our data indicate that the large overproliferation of hemocytes in response to activated Ras does not lead to a general activation of the immune response. Among the 134 Drosophila immune-regulated genes induced by septic injury and fungal infection (DE GREGORIO *et al.* 2001), only 6 genes are upregulated and 4 genes are downregulated by a factor of 3 or more in Ras-act hemocytes. The 6 upregulated genes in Ras-act are Tep2 (complement like), α-2M receptor like (complement binding), a trypsin-like serine protease (phenol oxidase cascade), a serpin (serine protease inhibitor), spz (antifungal response), and Tl (antifungal response). The 4 downregulated genes include Tep1 (complement like), Rel (transcription factor), Metchnikowin (antimicrobial response), and a lipase.

Concluding remarks: Activated versions of both Ras and the Hop Jak kinase induce leukemia-like phenotypes in Drosophila larvae. Further, it is possible to isolate sufficient quantities of larval hemocytes to conduct microarray expression studies. By comparing the expression profiles from different oncogene-induced leukemia cells, coupled with mutational analysis of the newly identified targets, it should be possible to systematically characterize the critical, oncogene-specific target genes. This approach could prove beneficial to the treatment of human cancers.

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ÖSSZEFOGLALÓ KÖZLEMÉNY

Ősi örökségünk: a veleszületett immunitás

A Drosophila sejtes immunitása

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A szerzők ismertetik az ecetmuslica sejtes immunitásának sejtes elemeiről eddig szerzett ismereteinket. Bemutatják az általuk kidolgozott Drosophila CD-rendszert, a CD antigének segítségével felismert differenciálódási vonalakat és a differenciálódás leírására kidolgozott kísérleti modellt.

veleszületett immunitás, Drosophila, vérsejtantigének

Our archaic heritage: the innate immunity. The cellular immunity of Drosophila

Authors describe the essentials of the cellular immunity of *Drosophila*. They describe the *Drosophila* CD system, the main blood cell lineages and a blood cell differentiation model based on the expression of the CD antigens.

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A soksejtű szervezetek számára a mikroorganizmusok állandó fenyegetést jelentenek. Ezért a fajok fennmaradásának és az evolúciónak alapvető feltétele a soksejtűek hatékony védekező rendszerének működése, ez elpusztítja és eltávolítja a szervezetből a mikroorganizmusokat és a parazitákat. E folyamatok első lépése a betolakodók felismerése a veleszületett immunitás elemei által, a mintázatfelismerő fehérjéken és -receptorokon keresztül. A felismerést fagocitózis, antimikrobiális peptidek termelése, vagy a testidegen részecskéknek a gazdaszervezeten belüli elhatárolása követi. A betolakodókat felismerő receptorok a mikroorganizmusok vagy paraziták egy-egy nagyobb rendszertani csoportjaira jellemző struktúrákat ismerik fel, ezek szerkezetükben alapvetően különböznek a gazdaszervezet saját antigénjeitől. A szerzett immunitás a törzsfejlődés során a porcos halak őseiben jelenik meg először, és a veleszületett immunitással ellentétben az egyedfejlődés során genetikailag átrendezett receptorokat használ a T- és a B-lymphocytákon. Ezek a receptorok végtelen számú, az adott mikroorganizmusra jellemző egyedi struktúrák felismerésére képesek, az egyedi struktúrák által módosított saját antigének felismerésén keresztül. Fontos megjegyezni, hogy a szerzett immunitással *rendelkező* szervezetek valamennyien *rendelkeznek* a veleszületett immunitással. Ezekben a szervezetekben a veleszületett immunitás elemei részt vesznek a szerzett immunitás (ellenanyag-termelés és T-sejt-válasz) működésében és az effektorfolyamatok irányának a megszabásában. Az elmúlt évszázadban a figyelem elsősorban a szerzett immunitás megismerésére összpontosult, és csak a századforduló felé kezdődött el a veleszületett immunitás részletesebb vizsgálata. Az érdeklődés először a nagytestű ízeltlábúak *(Cecropia, Manduca)* felé fordult, de a *Drosophila* (ecetmuslica) genetikai rendszerének megismerése, majd genomjának szekvenálása és a kódolórégiók meghatározása ezt a fajt helyezte a kísérletek középpontjába. A mole-

kuláris genetika eszköztára a *Drosophila* genom megismerésével kombinálva egyedi lehetőségeket kínál; nemcsak in vitro, hanem in vivo is végezhető a veleszületett immunitás komplex genetikai, genomikai analízise. Mindez akkor válik lehetővé, ha azonosítjuk a veleszületett immunitás sejtes elemeit, a differenciálódási vonalakat a prekurzoroktól a terminálisan differenciálódott sejtekig, jellemezzük az effektorfunkciókat és ezen keresztül egy-egy sejtpopulációhoz vagy effektorfunkcióhoz kötjük a génexpressziós mintázatot.

A rovarok immunvédekezése

A fajok számát és változatosságát tekintve a rovarok az élővilág legsikeresebb tagjai. Becslések szerint a jelenleg élő fajok 90%-át alkotják; mind a szárazföldön, mind a vizekben széleskörűen elterjedtek. Élőhelyüket potenciális ellenségeikkel, mikroorganizmusokkal és soksejtű parazitákkal osztják meg, amelyekkel állandó küzdelemben állnak. A múlt század elején már nyilvánvalóvá vált, hogy a rovarok humorális és sejtes elemekből álló, hatékony immunrendszerrel védekeznek a betolakodókkal szemben.

A humorális immunválasz, a fertőzések által kiváltott antimikrobiális peptidek termelésének folyamata, jól ismert. Tudjuk, hogy a fertőzés a rovarok zsírtestjét, a gerincesek májának funkcionális homológját és egyes vérsejteket indukál, ennek eredményeként antimikrobiális peptidek termelődnek. A peptidek a mikroorganizmusokkal történt kölcsönhatást követően beépülnek a sejtmembránba és az ionháztartás felborításával okozzák a mikroorganizmusok pusztulását. Ez a humorális válasz nem rendelkezik az ellenanyagokhoz hasonló specifitással, memóriával, viszont nagyfokú hasonlóságot mutat a gerincesek akutfázis-válaszával.

A sejtes immunválaszt elsősorban morfológiai szempontból sikerült jellemezni; nagyon keveset tudunk azokról a sejtekhez kapcsolt folyamatokról, amelyek során a támadó mikroorganizmusok és paraziták felismerését követően a mikroorganizmusokat bekebelezi, vagy a parazitákat elhatárolja a gazdaszervezet.

A sejtes és a humorális immunválaszt megelőzően, illetve azzal egy időben proteolitikus kaszkádok aktiválódnak; ezek a testnedvek koagulációját, valamint melanizációs folyamatokat indítanak el és végső soron a sérülés helyének reparálásához vezetnek, vagy a betolakodó pusztulását eredményezik.

Az egyedfejlődés különböző stádiumaiban különböző védelmi stratégiák szükségesek. Az embriókat a mikroorganizmusok számára áthatolhatatlan burok veszi körül. A lárvákat a mikroorganizmusok behatolásától lágy kitinkutikula védi, de ezen egyes parazitáták, valamint a fürkészdarazsak tojócsöve könnyedén áthatolnak. A testnyílások – a száj, a végbélnyílás és a légzőnyílások – a mikroorganizmusok számára nyithatnak kaput. A kifejlett rovarok kemény kutikulája a mechanikai ártalmakkal szemben hatékony védelmet biztosít, azonban a légzőnyílások és az emésztőcső nyílása ugyanúgy bemeneti kapuként szolgálhat a mikroorganizmusok számára, mint a lárvákban. Az immunvédekezés elemei is ennek megfelelően változnak az egyedfejlődés során. Az embrióban az úgynevezett embrionális macrophagok elsősorban az apoptotikus sejteket távolítják el, és az embrionális élet során az immunvédekezésben feltehetően nem játszanak szerepet. Az immunvédekezési folyamatok és az immunszervek a lárvában jelennek meg. A tápanyaggal az emésztőcsőbe jutó mikroorganizmusokat a helyben termelődő lizozim, a tracheanyílásokon behatolni készülő baktériumokat és gombákat pedig a nyílásoknál elhelyezkedő hámsejtek által termelt antimikrobiális peptidek támadják meg. A testüregbe bejutott mikroorganizmusokat a testnedvekben keringő vérsejtek egy csoportja, a plasmatocyták kebelezik be, illetve a zsírtest által termelt antimikrobiális peptidek pusztítják el. Az elpusztított mikroorganizmusokat ugyancsak a vérsejtek takarítják el környezetükből.

A lárvák számára jelentős veszélyt jelentenek a paraziták, köztük is a leggyakrabban előforduló fürkészdarazsak. Petéiket a lárva testüregébe rakják és a testüregben fejlődő darázslárvák számára a *Drosophila*-lárva szolgál táplálékul. A parazitafertőzést követően az esetek egy részében a *Drosophila* lárvája a paraziták felnevelése során elpusztul. Ha a parazita inváziója minden esetben sikeres lenne, előbb-utóbb a gazdaszervezet és ezzel együtt maga a parazita is kihalna. Egy populációszinten finoman szabályozott immunreakció megléte vagy hiánya azonban biztosítja mind a gazdaszervezet, mind pedig a parazita túlélését. Néhány esetben a gazdaszervezet hatékony immunreakciót indít a betolakodóval szemben, ennek során nagyméretű, lapos vérsejtek – lamellocyták – differenciálódnak. A lamellocyták több rétegben burkolják be a plasmatocyták által nem fagocitálható nagyméretű parazitapetéket, majd az így képződött tok, a petével együtt, egy proteolitikus láncreakció aktiválódásának eredményeként melanizálódik. A melanizációs folyamatban kitüntetett szerepet játszanak a kristályszerű zárványokat – feltételezések szerint profenoloxidázt – tartalmazó vérsejtek, az úgynevezett kristálysejtek.

A kifejlett rovarra a mikroorganizmusok jelentik a legnagyobb veszélyt. Az emésztő- vagy a légzőszerven keresztül behatoló mikroszervezeteket a lárvához hasonlóan az antimikrobiális peptidek és a fagocitáló sejtek távolítják el. További veszélyforrást jelentenek a

Drosophila számára a szövetburjánzások, tumorok. Ezek kifejlődése szinte minden esetben a szervezet pusztulásához vezet. Az immunrendszerben képződő szövetburjánzások és tumoros sejtek invázióját követően emelkedik a vérsejtek száma, ezt a legtöbb esetben a burjánzó szövetekkel szemben kialakuló lamellocytadifferenciálódás kíséri. Érdekes, hogy erősen proliferáló vérsejtek kizárólag az embrióban és a lárvában fordulnak elő. A kifejlett rovarban nincsenek osztódó sejtek. A lamellocyták eredete, az egyes vérsejt-differenciálódási vonalak fejlődése és a különböző differenciálódási vonalak egymáshoz való viszonya alig ismert. Ugyancsak nem világos, hogy az immunválasz kialakulása során milyen típusú sejt-sejt kölcsönhatások zajlanak. Miután a *Drosophila* veleszületett immunitásának számos eleme megtalálható a gerinces szervezetekben, ahol a veleszületett immunitás alapvetően szükséges a szerzett immunitás elemeinek aktiválódásához, valószínű, hogy a *Drosophilában* található sejtes és humorális immunválasz az evolúciónak mintegy alapfeltételeként egyetlen nagy, sikeres egységként maradt fent a törzsfejlődés során. Így a veleszületett immunitás sejtes és molekuláris szintű folyamatainak vizsgálata hozzájárulhat a gerinces szervezetek homológ immunfolyamatainak megismeréséhez.

A Drosophila immunválasza

Humorális immunitás

A mikroorganizmusok, a Gram-pozitív baktériumok és a gombák, valamint a Gram-negatív baktériumok egymástól eltérő antimikrobiális peptidek termelését indukálják, sőt, a peptidek termelését is eltérően szabályozzák *(1. ábra)*[1]. Az előbbi csoportba tartozó mikrobák elsősorban a Toll-szerű transzmembránreceptor által közvetített jelátviteli utat, míg az utóbbiak az *imd* (immune deficiency) utat indukálják. A *Drosophilában* a Toll-szerű transzmembránreceptort először a dorsoventralis polaritást szabályozó génként ismertük meg, majd kiderült, hogy az immunválasz szabályozásában is kulcsszerepet játszik. A Toll aktivációs út szabályozásában központi szerepet játszanak a Rel családba tartozó transzkripciós faktorok, közülük is a DIF és a Dorsal; ezek transzkripciója drozomicin termeléséhez vezet. Az *imd* génről elnevezett aktivációs út ugyancsak Rel faktorokon – a Relishen – keresztül fejti ki hatását; ennek aktiválódása több, antimikrobiális peptidet kódoló gén átíródásához és diptericinek, cekropinok, drozicinek és attacinok termeléséhez vezet. Mindkét jelátviteli útra proteolitikus láncreakció jellemző, elemei megtalálhatók a gerinces szervezetekben, sőt, egyes alkotórészei a növényekben is. A humorális immunválasz effektormolekulái az antimikrobiális peptidek: a mikrobák sejtfalába épülve az ionháztartás felborításával azok azonnali pusztulásához vezetnek. A humorális immunválasz szabályozásában szerepet játszó Rel faktorok közvetlenül csak az antimikrobiális peptidek termelésének a szabályozásában vesznek részt, a celluláris immunválaszt nem érintik[2-3].

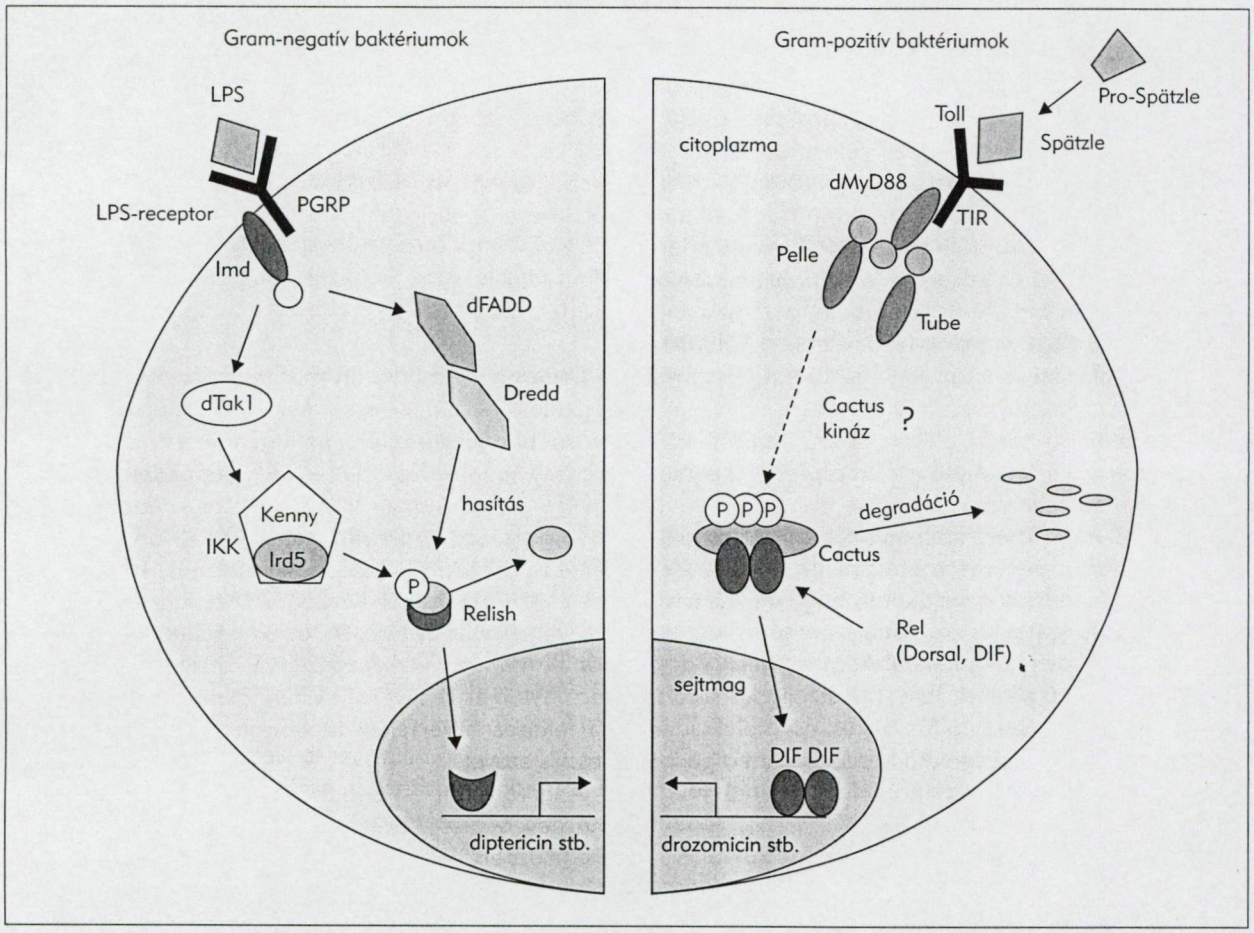
Sejtes immunitás

A *Drosophila*-embrióban már az embrionális élet korai stádiumában megjelennek a mesodermából származó phagocytasejtek, az úgynevezett embrionális macrophagok. Később – a peterakást követően 14–16 órával – ugyancsak a mesodermából alakul ki a fő nyirokszerv, a nyirokmirigy; ez a lárvában a fő vérsejt-populációt alkotó plasmatocyták, a lamellocyták és a kristálysejtek képződési helyéül szolgál majd. Az embrionális élet végén, az embrióburkot elvető lárvának már van vérképző szerve. Testnedveiben plasmatocyták és kristálysejtek keringenek; testüregének belső falához is vérsejtek tapadnak, ez az úgynevezett sessilis szövet.

A lárva – mielőtt bebábozódna – kétszer vedlik. A második vedlést követően, közvetlenül a bábozódást megelőzően, lamellocyták figyelhetők meg a keringésben; eredetük és funkciójuk ebben a stádiumban nem ismert. A parazitafertőzést követően nagyfokú lamellocytadifferenciálódás figyelhető meg; eredetük azonban nem ismert, egyes feltételezések szerint a nyirokmirigyből származnak.

A bábban az immunrendszer alapvető morfológiai és funkcionális átalakuláson megy keresztül. A nyirokmirigy szerkezete felbomlik, a bábban azonban megfigyelhető néhány fagocitáló sejt. A kifejlett rovar testnedveiben ugyancsak megtalálhatók a vérsejtek, ezek eredete szintén nem tisztázott, és az sem világos, hogy esetleg mely – eddig ismertetett – vérsejtpopulációhoz tartoznak. A *Drosophilában* folyó vérsejt-differenciálódás tehát komplex folyamat; ennek megismerése nemcsak a rovarok immunrendszerét érintő érdekes kérdésekre adhat választ, hanem segítheti egy, a filogenezis során mindmáig fennmaradt és a gerinces szervezetekben is igen hatékony funkcionális egység működésének megértését.

Az embrionális macrophagok szabadon vándorolnak a fejlődő szövetek közötti virtuális résekben. Elsősorban az embrionális élet során zajló szöveti átrendeződést kísérő sejtpusztulás termékeit fagocitálják. Az apoptotikus sejteket a Croquemort (Crq) transzmembránfehérje ismeri fel; ez az emberi CD36 szerkezeti homológja. A vérsejtek újabb csoportjai a lárva testnedveiben keringő vérsejtek, a haemocyták; a fő vérképző szerv az embrióban indul fejlődésnek. Eb-



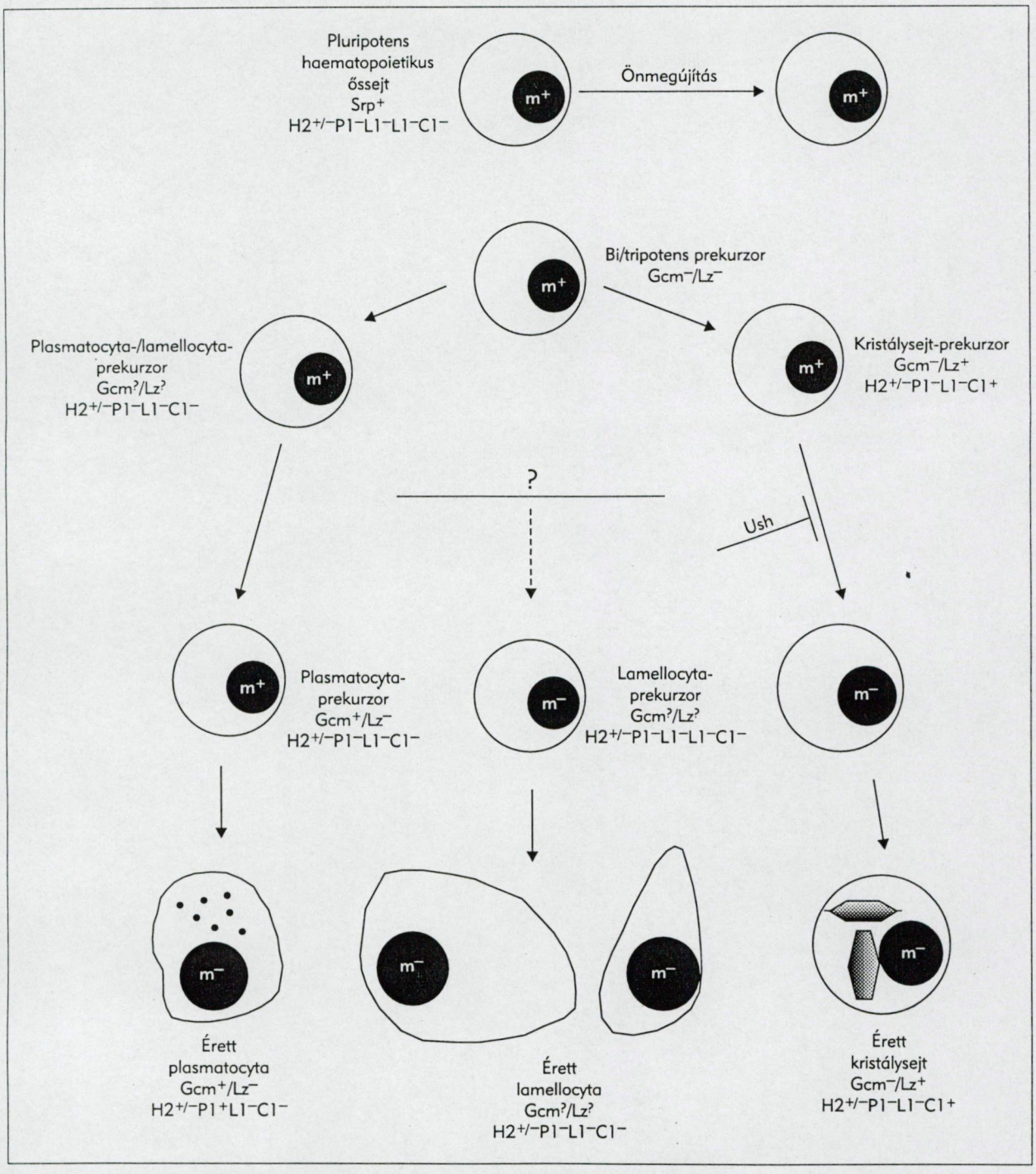
1. ábra. Az antimikrobiális peptidek termelését szabályozó fő jelátviteli utak *Drosophilában* (Hoffmann JA, Reichhart J-M után, módosítva[1])

ben a stádiumban vérsejtek differenciálódnak egy, a mesodermából kialakuló sejtcsomóban; ezek a sejtek – a törzsfejlődés során konzervált egyes transzkripciós faktorok expresszióját tekintve – heterogenitást mutatnak. A transzkripciós faktorok a vérsejtek differenciálódásának szabályozásában vesznek részt. Az őssejtek differenciálódását a Serpent (Srp) irányítja *(2. ábra)*. Ezekből a sejtekből az embrióban két differenciálódási vonal származik. A Lozenge – egy Runx proteinhomológ – a kristálysejtek, míg a Gcm1 és a Gcm2 – Glial cell missing – a plasmatocyták differenciálódását szabályozzák[4]. Ezzel szemben az Ush – U-shaped, „Friend of GATA" faktor – a kristálysejtek differenciálódását gátolja. Az Srp, az Lz és az U-shaped különböző kombinációkban – egymással kölcsönhatásban – hajtják végre a vérsejt-differenciálódási programot[5–6].

A lamellocyták differenciálódásának szabályozása még kevésbé ismert; egyes eredmények arra utalnak, hogy a Toll aktivációs út egyes elemei részt vesznek benne[7].

A Drosophila CD-rendszerének alapjai

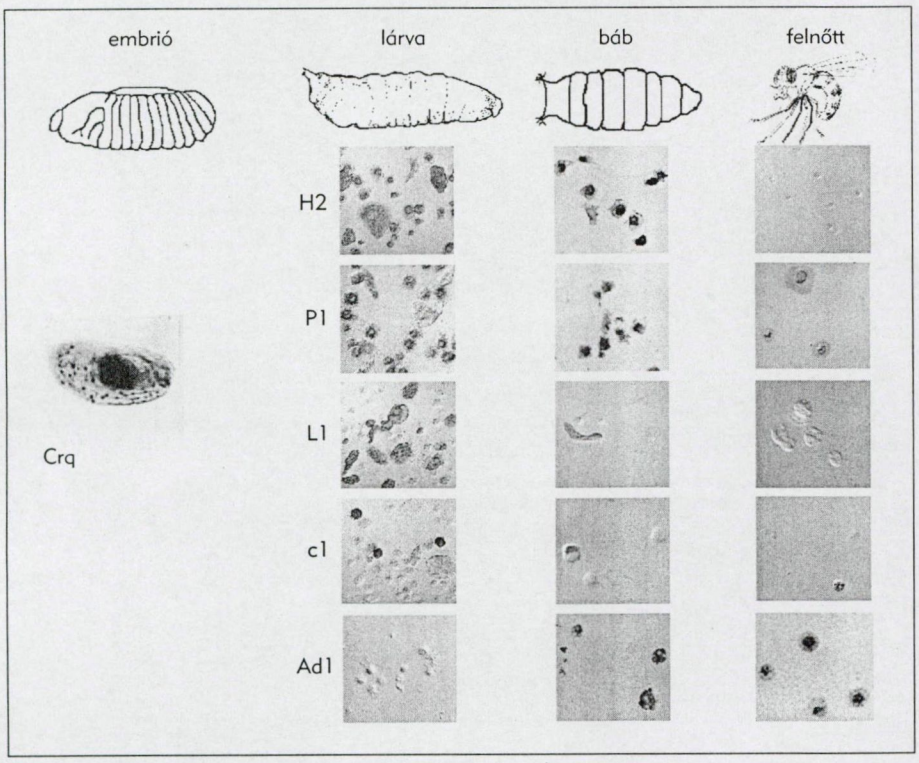
A *Drosophila* szervezete alapvető változásokon megy keresztül az embrió-lárva-báb-kifejlett rovar átmenet során. A különböző fejlődési stádiumokban különböző vérsejt-populációk differenciálódnak, ennek megfelelően az egyes fejlődési stádiumokban más-más sejtpopuláció dominál *(3. ábra)*. A differenciálódás szabályozásának vizsgálatában alapvető fontosságú a megfelelő – egyes alpopulációkra, differenciálódási vonalakra jellemző – molekuláris markerek használata. Bár az egyes, terminálisan differenciálódott sejtpopulációk morfológiai sajátságaik alapján elkülöníthetők egymástól, a közvetlen előalakok és az őssejtek morfológiai bélyegek alapján nem ismerhetők fel, nem jellemezhetők. Az immunológiai markerek használata komoly sikereket hozott a gerincesek immunrendszerének megismerésében; ezek nemcsak egyes vérsejt-alpopulációkat, differenciálódási vonalakat jel-



2. ábra. A vérsejt-differenciálódás feltételezett fő útjai *Drosophilában*.

lemezhetnek, hanem lehetőséget adnak az egyes populációk szeparálására is. Miután expressziójuk egy-egy adott sejttípusra jellemző, gyakran azonosíthatók jelátviteli utak részeként is. Megismerésük segítheti a veleszületett immunitás általános folyamatainak a megértését is. Laboratóriumunkban a *Drosophila* vérsejtjeit jellemző immunológiai markereket azonosítunk és a vérsejt-differenciálódás, a veleszületett immunitás folyamatainak a megértéséhez használjuk.

A markerek azonosítása során alkalmazott tesztrendszer biztosította azt, hogy a markerek meghatározott köre sejtfelszíni vagy intracelluláris antigén legyen. Az antigének csoportokba rendezése, cluster-



3. ábra. A Drosophila CD-antigének mintázata az egyedfejlődés során.

analízise során azonosítottuk az egyes vérsejt-alpopulációkra jellemző markereket, és kialakítottuk a *Drosophila* CD-ket [CD (cluster of differentiation): differenciálódási marker cluster], amelyek – az egér és az emberi CD-hez hasonlóan – alkalmasak a haemocyták azonosítására és funkcionális jellemzésére. Az azonosított clustereket az *1. táblázatban* foglaltuk össze. Az antigének expressziómintázata alapján megállapítottuk, hogy a lárvában

– a fagocitáló és az antimikrobiális peptideket termelő sejtek a H és a P antigének expressziójával, az L és a C antigének hiányával,

– a tokképzésben részt vevő sejtek a H és az L antigének expressziójával, a P és a C antigének hiányával,

– a kristályokat tartalmazó, a melanizációban részt vevő sejtek a H és a C antigének expressziója alapján, valamint a P és az L antigének hiányával,

– a vérsejtelőalakok a H antigének expressziójával, a P, az L és a C antigének hiányával jellemezhetők *(2. ábra)*.

A vérsejtek antigénmintázata változik az egyedfejlődés során; tehát úgy tűnik, hogy az egyedfejlődés során a vérsejtek sajátos érési folyamaton mennek keresztül *(3. ábra)*. Az embrionális macrophagokon egyik vérsejtantigén sem található meg, de valamennyin jelen van a Crq antigén. A lárva vérsejtjein a H antigének, valamint egymást kizáró módon a P, az L és a C antigének vannak jelen. A kifejlett egyedek vérsejtjeinek túlnyomó többségén a P antigének, egy alpopuláción a C antigének expresszálódnak. Valamennyi vérsejten megnyilvánul az Ad1 antigén, ez kizárólag a felnőtt rovar haemocytáin fejeződik ki. Ezek az eredmények azt sejtetik, hogy a *Drosophila* fejlődése során a vérsejtek az egyedfejlődést követően három fő – fejlődéstanilag egymástól különböző és egymáshoz eddig tisztázatlan módon kapcsolódó – kompartmentet alkotnak. Az egyik fő kompartment az embrionális macrophagok, a másik kompartment a larvalis vérsejtek, a harmadik a kifejlett egyed vérsejtjei.

A vérsejtek differenciálódását a lárvában vizsgáltuk a legrészletesebben. Megállapítottuk, hogy a plasmatocyták, a lamellocyták és a kristálysejtek egymástól különböző differenciálódási vonalat képeznek *(2. ábra)*. Az osztódó pool a $H^{+/-}P^{-}L^{-}C^{-}$, a $H^{+}P^{-}L^{-}C^{-}$, a $H^{+}P^{+}L^{-}C^{-}$ és a $H^{+}P^{+}L^{-}C^{+}$ populációban található. A paraziták és egyéb, eddig nem definiált ingerek a lamellocytákon és előalakjaikon az L antigének expresszióját indukálják *(1. táblázat)*. Az L1 és az L2 antigén először kis, plasmatocytaszerű sejteken jelenik meg, majd ezek az $L1^{+}$, $L2^{+}$ sejtek nagy, lemezszerű lamellocytává differenciálódnak. Az L1, L2 antigének megjelenését követő terminális differenciálódás során a sejtek nem osztódnak. (A terminális differenciálódás, a kis kerek sejtekből a testidegen részecskéket beborítani képes nagy lemezes sejtekké történő átalakulás komplex folyamata az antigénmintázat változásaival jellemezhető. A kis kerek sejteken megjelenik az L4 antigén, majd a terminálisan differenciálódott sejteken az L6 antigén. A folyamat a sejtváz átrendeződésével is jár, ebben szerepet játszik a filaminnak (aktinkötő fehérje) a haemocytákban L5 antigénként definiált új, magas izoformájú változata. A lamellocyták differenciálódása során az antigének szekvenciálisan jelennek meg. Szabályozásuk tanulmányozására lehetőséget ad a sejtek szeparálása és ezzel kapcsolt komplex genomikai és proteom szintű vizsgálata[8–9].

Markereink expresszióját az embrióban sikeresen használt transzkripciós faktorok expressziójával kapcsolva megerősítve láttuk azt, hogy az eddig azonosí-

1. táblázat. Sejttípus-specifikus antigének clusterezése haemocytákon, adott reakciómintázatuk és biokémiai sajátságaik alapján.

Fő cluster (CD)	Sejttípus	Molekula-tömeg[+]	Klón
H1	minden haemocyta és embrionális macrophag	135–160	H11
H2	minden keringő haemocyta	30–60	1.2
H3	minden keringő haemocyta	16	4A12
P1a	plasmatocyta	100–110	N1
P1b	plasmatocyta	100–110	N47
P3	plasmatocyta-alpopuláció	*	8B1
L1	lamellocyta	16	H10
L2	lamellocyta	44	31A4
L4	lamellocyta-alpopuláció	82–86	1F12
L5	lamellocyta-alpopuláció	85–100, 240	4B8
L6	lamellocyta-alpopuláció	96	H3
C1	kristálysejtek és kristálysejt-prekurzorok	84	12F6
C2	kristálysejtek és kristálysejt-prekurzorok	*	21D3
C3	kristálysejtek és kristálysejt-prekurzorok	*	10D2
C4	érett kristálysejtek	100	9C8
C5	érett kristálysejtek	66,135	1.19
Ad1	felnőtt haemocytái	10	7C8

[+] kDa-ban, nem redukált körülmények között
* konformációfüggő epitóp

tott transzkripciós faktorok feltehetően részt vesznek a vérsejt-differenciálódás szabályozásában *(2. ábra)*. Larvalis vérsejtekben a szabályozás a faktorok egymás közti komplex kölcsönhatása révén valósul meg; ez lényegesen különbözik a faktoroknak az embrionális macrophagokban betöltött kizárólagos szabályozó szerepétől[4–6].

Mivel az antigének kizárólag vérsejteken vagy alpopulációkon találhatók, megismerésük segíthet a veleszületett immunitás működésének a megértésében. Az antigének biokémiai jellemzése és a gének klónozása eddig sok érdekes információval szolgált. A H2 antigén, a Hemese[10] a lamellocyták differenciálódását az aktiválódáson keresztül szabályozó transzmembránfehérje; a glikoforinok családjának új tagja. A P1 antigén EGF-doméneket hordozó transzmembránfehérje; közvetlen környezetében tíz, vele nagyfokú szerkezeti homológiát mutató gént azonosítottunk. Meglepő lenne, ha ez a géncsalád nem játszana alapvető szerepet a vérsejt-differenciálódás szabályozásában. Az L1 antigén GPI-kapcsolt transzmembránfehérje; több szerkezeti homológja található az élővilágban, köztük gerincesekben. Legközelebbi eddig azonosított gerinces homológja a leukocytákban végbemenő jelátviteli folyamatokat szabályozza.

A *Drosophila* CD antigének, a gerincesekben azonosított funkcionális homológok megismerésén túlmenően nemcsak az antigének in vivo vizsgálatát teszik lehetővé, hanem a vérsejt-differenciálódás komplex genomikai elemzését is. A gének szabályozórégióinak izolálása és felhasználása a genetikai rendszerekben elősegíti a veleszületett immunitásban részt vevő jelátviteli utak térképezését. A szabályozórégiók segítségével a szövet- és sejttípus-specifikusan expresszált markermolekulák lehetővé teszik az immunválasz „in vivo" nyomon követését.

Köszönetnyilvánítás

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