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Genetics

A recessive mutation, immune deficiency (*imd*), defines two distinct control pathways in the *Drosophila* host defense

(antibacterial peptides/antifungal peptides/insect immunity)

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ABSTRACT **In this paper we report a recessive mutation, immune deficiency (*imd*), that impairs the inducibility of all genes encoding antibacterial peptides during the immune response of *Drosophila*. When challenged with bacteria, flies carrying this mutation show a lower survival rate than wild-type flies. We also report that, in contrast to the antibacterial peptides, the antifungal peptide drosomycin remains inducible in a homozygous *imd* mutant background. These results point to the existence of two different pathways leading to the expression of two types of target genes, encoding either the antibacterial peptides or the antifungal peptide drosomycin.**

The powerful innate defense of higher insects involves proteolytic cascades (coagulation and phenoloxidase cascades), phagocytosis and encapsulation of invading microorganisms, and the synthesis by the fat body of a battery of potent antimicrobial peptides (reviewed in refs. 1 and 2). In *Drosophila*, several genes encoding inducible antibacterial peptides [cecropins (3, 4); diptericin (5); defensin (6); drosocin (7); M. Charlet, personal communication] and one inducible antifungal peptide [drosomycin (8); L.M. and J.-M.R., unpublished results] have been cloned. Understanding the mechanism of the coordinate control of their expression after immune challenge (e.g., septic injury) is a major goal in the field. Significant similarities exist between the control of antimicrobial peptide gene expression in insects and that of acute phase response genes in mammals (reviewed in refs. 1 and 2). This is illustrated by the involvement of common cis-regulatory motifs in the promoters of most of the insect and mammalian immune genes [e.g., NF-κB and NF-IL6 response elements, interferon consensus regulatory sequences (9–11)]. Furthermore, members of the Rel/NF-κB family play a crucial role in the transactivation of mammalian acute phase response genes; similarly, Rel proteins have been recently implicated in the control of the immune genes in *Drosophila* (12, 13) as the genes encoding the two Rel proteins dorsal (dl) and Dif (dorsal-related immune factor) are up-regulated in the fat body upon immune challenge and both proteins are translocated in the nucleus (refs. 12 and 13; B.L. and E.N., unpublished results). The precise roles of these proteins in the immune response of *Drosophila* are not yet established (discussed in refs. 14 and 15).

While analyzing the expression of antibacterial genes in a mutant of the phenoloxidase cascade, we have found, by serendipity, a recessive mutation, immune deficiency (*imd*), that impairs the inducibility of the antibacterial peptides described so far in *Drosophila*. When challenged with bacteria, flies carrying this mutation show a lower survival rate than wild-type flies. We also report that, in contrast to the antibacterial peptides, the antifungal peptide drosomycin remains inducible in a homozygous *imd* mutant background. These results point to the existence of two different pathways leading either to the expression of the genes encoding antibacterial peptides or to the expression of the drosomycin gene.

MATERIALS AND METHODS

***Drosophila* Stocks and Culture Medium.** Oregon^R flies were used as a standard wild-type strain. The transgenic strain, Dipt2.2-*lacZ*:1, is a ry^{506} C.S. line carrying a diptericin reporter gene on the X chromosome (5). The fusion gene contains 2.2 kb of diptericin upstream sequences fused to the bacterial *lacZ* coding region and was inserted into the Carnegie 20 vector (16). The developmental and the inducible expression of the Dipt2.2-*lacZ* transgene has been described (5, 17); it is similar to that of the resident diptericin gene at the end of the third larval instar.

1046Bc is a line homozygous for the *Bc* (Black cells) mutation (18, 19) obtained from the Bloomington Stock Center (Bloomington, IN). Deficiency lines are described in ref. 20.

Stocks and crosses were maintained on a standard corn meal medium. All experiments were performed at 25°C unless otherwise stated. For complete descriptions of the marker genes and balancer chromosomes, see ref. 20.

Bacterial Challenge. Bacterial challenge was performed by pricking third instar larvae, pupae, or adults with a thin needle previously dipped into a concentrated bacterial culture of *Escherichia coli* and *Micrococcus luteus*.

β-Galactosidase Localization. The fat bodies were dissected in phosphate-buffered saline and treated as described in ref. 21.

RNA Preparation and Analysis. Crosses were performed at 25°C and wandering third instar larvae, 24- to 48-h pupae, or 2- to 4-day-old adult flies were collected. Total RNA extraction and Northern blotting experiments were performed as in ref. 15. The following probes were used: diptericin cDNA (22), drosocin cDNA (7), drosomycin cDNA (8), rp49 cDNA [a PCR fragment of ≈400 bp generated between two oligonucleotides designed after the rp49 coding sequence (23)], a 21-mer oligonucleotide (5′-GATTCCCAGTCCCTGGATTGT-3′) complementary to part of the coding sequence of cecropin A1, which is identical for cecropin A2 (3). Poly(A) RNA extraction was prepared as in ref. 15 except that extraction of total RNA was performed with the RNA Trizol (GIBCO/BRL) method.

Preparation of Extracts and Electrophoretic Mobility Shift Assay. The method was that described in ref. 10. In short, 10

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μg of protein extracts from control and bacteria-challenged adult males were incubated with 20,000 cpm (2 fmol) of a labeled 16-bp double-stranded probe containing a κB-related motif (5′-ATCGGGGATTCCTTTT-3′) for 15 min at room temperature, and were analyzed in a standard gel-shift assay.

RESULTS

Characterization of immune deficiency (*imd*), a Mutation That Alters the Inducible Expression of the Genes Encoding Antibacterial Peptides. The initial part of this study was based on a fly line, 1046Bc, obtained from the Bloomington Stock Center, that probably corresponds to the original *Bc* (Black cells) line isolated by Grell after ethyl methanesulfonate mutagenesis in 1969 (ref. 18; K. Matthews, personal communication). *Bc* is a second chromosome mutation that is homozygous viable. The most striking phenotype of homozygous and heterozygous *Bc* mutants is the presence of circulating melanized crystal cells in larvae (19). Phenoloxidase activity is undetectable in the cell-free hemolymph of *Bc/Bc* larvae (19). We have also observed that the 1046Bc line exhibits a thermosensitive tumor phenotype; larvae raised above 21°C have a high frequency of melanotic tumors and a higher than normal hemocyte count (data not shown).

When challenging homozygous 1046Bc flies with bacteria, we observed that the expression of the antibacterial peptide genes was severely impaired. In fact, as will be demonstrated below, this impairment was independent from the *Bc* mutation but was due to a closely related, as yet undescribed, mutation, which we call immune deficiency (*imd*). We will refer to the fly line presenting the *Bc* and the immune deficiency phenotypes as *Bc,imd* hereafter.

We first studied by a transgenic approach the expression of the diptericin gene, which encodes an antibacterial peptide. We have performed histochemical staining of the fat body in larvae carrying a transgene in which 2.2 kb of upstream sequences of the diptericin gene are fused to the *lacZ* reporter gene (Dipt2.2-*lacZ*:1; see ref. 5). We constructed a fly line carrying the reporter transgene and the *Bc,imd* mutations by standard crosses. Fig. 1 illustrates the β-galactosidase activity (5-bromo-4-chloro-3-indolyl β-D-galactoside staining reaction) in the fat body of challenged wild-type larvae and homozygous *Bc,imd* mutant larvae; a deep blue coloration is apparent in all fat body cells of the wild-type larvae, whereas only a reduced number of cells shows a weak expression of the transgene in the mutant fat body. In challenged mutant adults, in contrast to wild type, only a faint level of expression was observed in the fat body (data not shown).

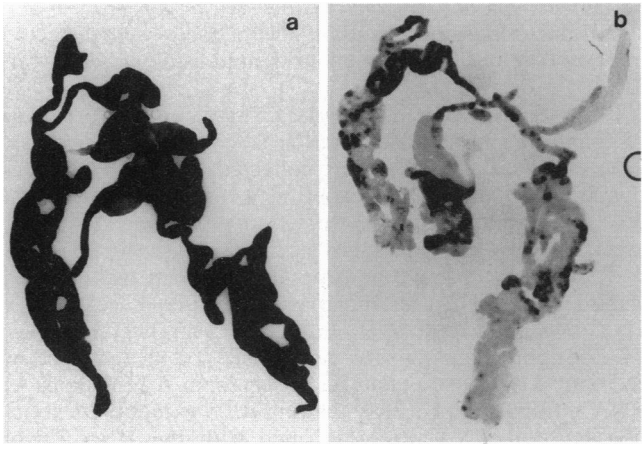

FIG. 1. Induction of a diptericin-*lacZ* reporter gene in challenged wild-type and homozygous *Bc,imd* mutant larvae. Histochemical staining of β-galactosidase activity is shown in the fat body of a 4-h bacteria-challenged diptericin-*lacZ* transgenic larva (*a*) and of a 4-h challenged transgenic larva carrying the homozygous *Bc,imd* mutation (*b*). Both stainings were performed in the same run of experiments. (×20.)

We have corroborated these results by an analysis of the expression of all previously identified *Drosophila* genes encoding antibacterial peptides. Total RNAs from larvae, pupae, and adults of control and challenged wild-type and *Bc,imd* mutants were probed with cDNAs corresponding to diptericin, drosocin, and cecropin A (encoded by the *CecA1* and the *CecA2* genes; see ref. 3), rp49 serving as a control. In wild-type animals, the four genes were strongly induced at all stages. In striking contrast, the induction was severely impaired for all four genes in *Bc,imd* mutant larvae, pupae, and adults (Fig. 2 *a* and *b*). However, the inducibility was not totally abolished, as a low hybridization signal remained detectable in challenged mutants, particularly for cecropin A transcripts. Radioactivity measurements indicated that the overall reduction in the level of inducibility was ≈10-fold with some variability among different genes. Heterozygous *Bc,imd/+* larvae and adults retained a full inducibility of the diptericin gene after challenge (Fig. 2*c*; data not shown).

In *Drosophila*, the gene encoding insect defensin is induced at a significantly lower level than that of the other antibacterial genes. We asked whether its induction was also affected in the *Bc,imd* background. Poly(A)-enriched RNA from challenged and unchallenged wild-type and *Bc,imd* larvae and adults was prepared and probed with defensin cDNA. Fig. 3 shows that the inducibility of the defensin gene was also abolished in mutant larvae and adults.

We next studied in *Bc,imd* mutants the expression of the drosomycin gene, which encodes an antifungal peptide. Northern blots were probed with drosomycin cDNA and, as shown in Fig. 2 *a* and *b*, the inducibility of this gene by bacterial challenge was unaffected in *Bc,imd* background, indicating that at least two distinct control pathways exist for induction of antimicrobial (antibacterial/antifungal) peptides.

The fact that the impaired induction of the antibacterial genes was not related to the *Bc* mutation but to a closely linked mutation was established by the analysis of deficiencies and, finally, by recombination of the two mutations. Deficiency *Df(2R)PC4*, which deletes the 55A–55F interval, uncovers the *Bc* mutation (mapped at 55A; ref. 20) and *imd*. However, two more limited deficiencies, *Df(2R)Pcl-7B* and *Df(2R)Pcl-11B* (deleting 54E8-F1;55B9-C1 and 54F6-55A1;55C1-3, respectively), which also uncover the *Bc* mutation (20), did not uncover the *imd* mutation, which alters the immune response. *imd* was therefore mapped to the 55C–55F interval. Finally, a series of recombination experiments has enabled us to map the *imd* mutation at 3.5% from the *Bc* mutation (based on seven recombinants).

We have repeated the most significant experiments on the impairment of the inducible expression of antibacterial peptides with recombinant homozygous *imd* flies, lacking the *Bc* mutation. The results were identical (data not shown), in Northern blot analyses and β-galactosidase staining, to those presented above. Homozygous *imd* flies did not generate a melanotic tumor phenotype nor any other striking morphological defects. Conversely, homozygous recombinant *Bc* mutants (devoid of the *imd* mutation) showed a wild-type inducibility of antibacterial peptide genes and also exhibited the thermosensitive melanotic tumor phenotype previously observed (see above). We have not yet clarified whether this phenotype is directly related to the *Bc* mutation or to a mutation proximal to *Bc*.

Protein Extracts from *imd* Homozygous Mutants Bind Poorly to κB-Related Nucleotide Sequence Motifs. The genes encoding inducible antibacterial peptides that have been characterized so far in *Drosophila* all contain κB-related sequence motifs in their promoters. These sequences have been shown to confer bacteria inducibility to the diptericin and cecropin

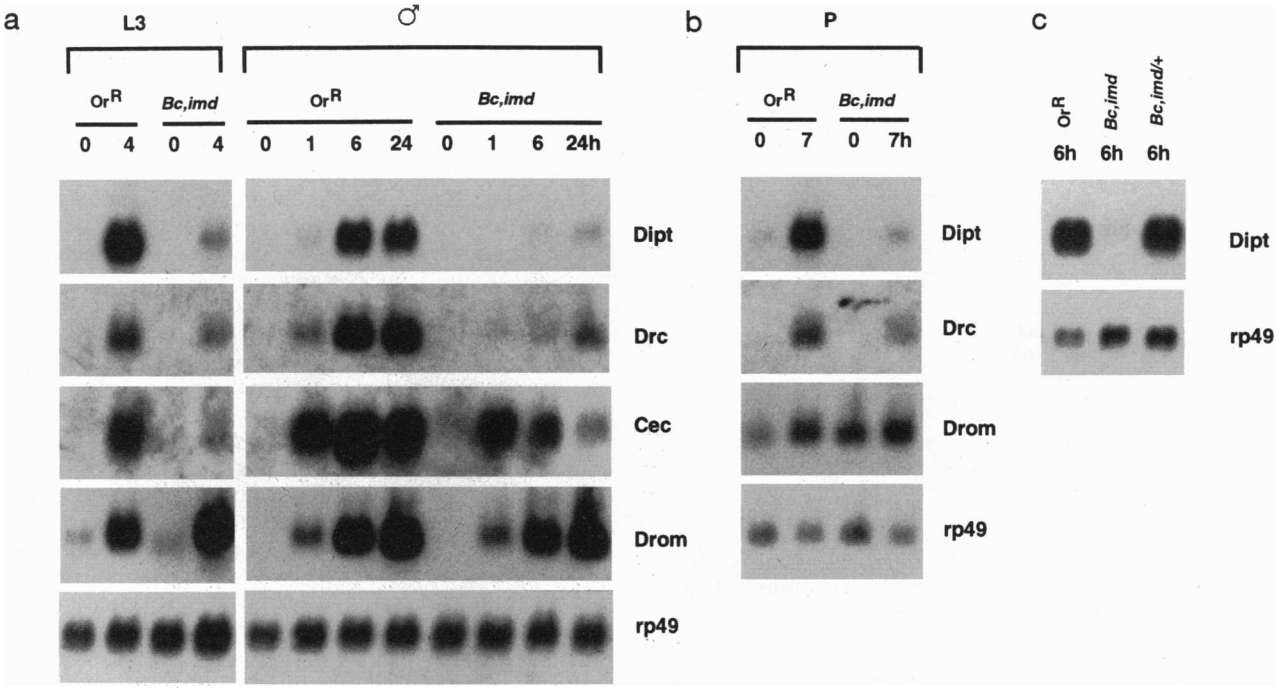

FIG. 2. Transcriptional profiles of diptericin, drosocin, cecropin A, and drosomycin in wild-type and *Bc,imd* mutant *Drosophila*. Total RNA was extracted at different time intervals (as indicated) after septic injury from wild-type Oregon^R (Or^R), *Bc,imd* homozygous, and *Bc,imd*/+ heterozygous mutants. Twenty-microgram samples were fractionated by denaturing 1% agarose/formaldehyde gel electrophoresis, transferred onto a nylon membrane, and successively hybridized with nick-translated cDNA probes (Dipt, diptericin; Drc, drosocin; Drom, drosomycin; rp49) and an oligonucleotide probe (Cec, cecropin A). L3, wandering stage third instar larvae; P, 24- to 48-h-old pupae; 48- to 72-h adults. Adults were used in *c. a, b*, and *c* were obtained separately.

genes by experiments based on transgenic fly lines and transfection of an immune-responsive blood cell line (9, 10). Protein extracts from challenged but not from naive insects form a major complex with the κB-related sequences of the promoters of both genes in electrophoretic mobility shift assays. We were curious to determine if the alteration of the antibacterial response in *imd* mutants was correlated to a defect in the formation of this protein–DNA complex. We used a labeled oligonucleotide containing a diptericin κB-related motif in electrophoretic mobility shift assays with nuclear protein extracts from control and bacteria-challenged wild-type and *imd* male adults. As previously reported (10), bacterial challenge induces a strong DNA-binding activity in extracts of wild-type *Drosophila*, but only a weak binding activity was detectable in our experimental conditions with extracts from *imd* flies (Fig. 4).

Susceptibility of *imd* Homozygous Mutants to Septic Injury. To our knowledge, *imd* is the first mutation known to alter the induction of genes encoding antibacterial peptides. We were

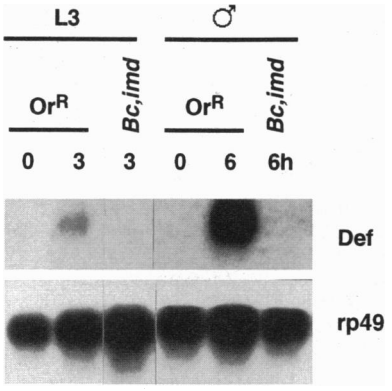

FIG. 3. Transcriptional profiles of defensin in wild-type and *Bc,imd* mutant *Drosophila*. Poly(A) RNA was extracted at different time intervals (as indicated) after septic injury from wild-type Oregon^R (Or^R) or *Bc,imd* homozygous mutants. Five-microgram samples were fractionated by denaturing 1% agarose/formaldehyde gel electrophoresis, transferred onto a nylon membrane, and successively hybridized with nick-translated defensin (Def) and rp49 cDNA probes.

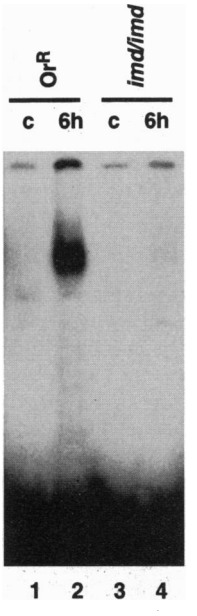

FIG. 4. DNA-protein binding activity in nuclear extracts of wild-type and homozygous *imd* mutant *Drosophila*. Control (c) or 6-h bacteria-challenged Oregon^R (Or^R) or mutant (*imd*/*imd*) male adults were sacrificed and the corresponding nuclear protein extracts were incubated in the presence of a κB-related 16-bp radioactive oligonucleotide probe (10). The specificity of the binding activity was ascertained by competition assays with excess of native or mutated probe (data not shown).

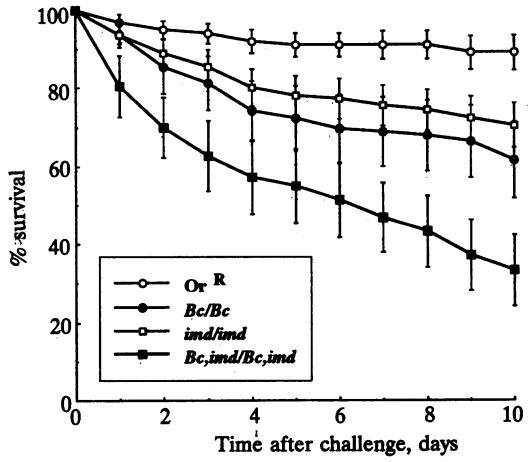

FIG. 5. Survival of wild-type and homozygous *imd* mutant male adults after immune challenge. The survival levels after bacterial challenge of *imd/imd*, *Bc/Bc*, and *Bc,imd/Bc,imd* mutants and of wild-type (Or^R) flies are displayed with their confidence intervals ($P < 0.05$). Groups of 25–30 adults, aged 2–4 days, were challenged and transferred to a fresh vial every 4 days. When tested in the same conditions, the survival levels of unchallenged *imd/imd*, *Bc/Bc*, and *Bc,imd/Bc,imd* mutants were identical to that observed with wild-type line (>95% after 10 days). At least 20 replicates were used for the determination of the survival levels.

interested to determine if *imd* homozygous flies were more sensitive to bacterial infection than wild-type flies. Groups of 20–25 mutant and wild-type adults were subjected to bacterial challenge and their survival was monitored over a 10-day period. As shown in Fig. 5, challenged *imd/imd* mutants exhibited a slight but significantly lower level of survival after septic injury. Ten days after treatment, around 70% of the *imd/imd* males survived compared to >90% of wild-type males. This lower survival rate does not reflect a generally decreased level of viability since the survival is not affected in unchallenged *imd* adults over a 10-day period (see legend to Fig. 5). Heterozygote adults presented a survival rate after challenge that was similar to that of wild-type flies (data not shown). Interestingly, homozygous *Bc,imd* flies showed a very low level of survival. Four days after treatment, <40% of the *Bc,imd/Bc,imd* males survived. This low level probably reflects the contribution of both mutations, since adults carrying only the *Bc* mutation show also a lower than wild-type level of survival, comparable to that of *imd* mutants (Fig. 5).

DISCUSSION

In this paper we report a recessive mutation that impairs the inducibility of the genes encoding antibacterial peptides during the immune response of *Drosophila*. This mutation, *imd* for immune deficiency, was found by serendipity, linked to the *Bc* mutation, in the Bloomington *Bc* stock. We did not observe an additional phenotype in *imd* mutants.

To date, the genes encoding cecropins, diptericin, drosocin (all predominantly active against Gram-negative bacteria), and insect defensin (active against Gram-positive bacteria), have been characterized in *Drosophila* (reviewed in refs. 1, 2, and 24). These genes are expressed with acute phase kinetics following bacterial challenge, although their expression patterns show some variability. It is significant that the four genes are affected in homozygous *imd* flies and that they all show an inducibility which is reduced roughly 10-fold. This result indicates that the coordinate expression of these genes shares a common pathway in which the imd^+ product is a crucial element. It is of interest to note in this context that the promoters of these genes all contain a certain number of common cis-regulatory sequences, among which κB-related motifs appear to play a pivotal role in the inducibility, as has been demonstrated in the case of the diptericin and cecropin genes (9, 10). As oligonucleotides containing a κB-related sequence fail to form a detectable complex with nuclear extracts from immune-challenged *imd* mutant flies, in contrast to wild-type challenged flies, the imd^+ product either is a component of this complex or is required for the formation of the complex. The cloning of the *imd* gene is an obvious goal that will presumably clarify this problem.

Our data on the survival rate of *imd* mutants lend credit to the relevance of the inducible antibacterial peptides in the host defense of *Drosophila*. Clearly, survival of the bacteria-challenged insects is reduced when the induction of the antibacterial peptide genes is impaired. However, the involvement of other defense mechanisms, such as phagocytosis or the low level of antibacterial peptides still present in mutant flies, may account for the survival of a high proportion of challenged *imd* mutants. Interestingly, our data also support the long-standing idea that the prophenol oxidase cascade plays a role in the host defense, as judged from the decreased survival rates of *Bc* mutants. As the lines used were not isogenic, we cannot exclude that the survival rate could also have been affected by other unrelated mutations. Isolation of new *imd* alleles by mutagenesis should clarify this point and reveal whether the *imd* gene's product is involved in additional biological processes.

A surprising result was the observation that in *imd* mutants, the drosomycin gene, in contrast to the antibacterial peptide genes, retains its full inducibility. This gene encodes a peptide that has a marked sequence homology to plant antifungal peptides and indeed exhibits a high *in vitro* activity against fungi (8). This result indicates that two distinct regulatory cascades lead to the expression of the antimicrobial genes. Although in this study, we were limited to the only inducible gene encoding an antifungal peptide characterized so far in *Drosophila*, we propose that the two regulatory pathways correspond indeed to two distinct functional defense responses directed against either bacteria or fungi.

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