INSTITUT DE BIOLOGIE MOLÉCULAIRE ET CELLULAIRE

Strasbourg, le 21 Juin 1996.

**Professor Peter RIGBY**
**CELL / European Editor**
*Division of Eukaryotic Molecular Genetics*
MRC National Institute for Medical Research
The Ridgeway, Mill Hill
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Dear Professor Rigby,

Enclosed we are sending the revised manuscript **"The dorsoventral regulatory gene cassette *spaetzle/Toll/cactus* controls the potent antifungal response in *Drosophila* adults"** (Ref. X0314E). In the revision, we have carefully taken into account the comments of the referees as explained below in our General Comments and, subsequently, in a precise point-to-point analysis.

**GENERAL COMMENTS**

Two major requests for additional experimental data were raised by the reviewers :

• **(1)** the demonstration that the genes of the cassette *spaetzle/Toll/cactus* are expressed in adult insects, in which our studies were performed. We have now completed these experiments and the results are clearcut : the genes are actually all expressed in adults and their expression is significantly upregulated by bacterial challenge. These results are indeed important for this study and we have integrated them into the text and added a figure (Figure 7). We also provide circumstantial evidence that the expression of these genes occurs predominantly in the fat body, which is the major source of antimicrobial peptides;

• **(2)** a stronger evidence that within the Toll pathway the genes other than Toll play a significant role. We have undertaken a comprehensive study of the survival rates of mutants for all these genes and the results considerably strengthen our inference as they show that survival is dramatically affected in *spz, tub, pll* mutants. Interestingly, survival is not affected in *ea* and *dl* deficiencies, which is in keeping with all the other data of this study. We have included a Table (Table 1) and a short text of comment.

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Given these additions which were requested by the reviewers and which are actually highly relevant for our conclusions, and in order to follow your recommandation that we remain within the length limits layed out by Cell, we have deleted the results (one Figure, several sentences) concerning the larval immune response. We have selected to delete this aspect because the paper focusses on adults (as stated in the title) and that all our conclusions are based on the studies obtained with adults. This deletion is in keeping with the suggestion of reviewer 3, who noted that he was unclear about the justification that components of the DV pathway are also functional in larvae. However, to indicate that results with larvae are available, we have introduced into the Discussion a short paragraph mentioning the salient results and their conclusion.

SPECIFIC COMMENTS

• *Reviewer 1*

*Dif nuclear translocation.*

Nuclear translocation of Dif by immune challenge could be visualised only in one third of the polyploid fat body cells of larvae by Ip et al. (1993) but has not been visualized so far in adults. If better antibodies became available, the demonstration of nuclear translocation of Dif after immune challenge would not be a final demonstration for the role of Dif in the control of expression of the antimicrobial peptide genes : indeed dorsal is present in the fat body and it can be assumed that its nuclear translocation is controlled by the Toll pathway. Nevertheless the data with *dorsal* deficient mutants show that this protein is not involved in the control of antimicrobial gene expression. Clearly, only dif mutants, when they become available, will clarify this point. In our manuscript, we only propose Dif as a candidate for the Rel-X protein and the identity of this Rel-X protein is not the scope of this paper, although we agree that it is an important issue.

*The drosomycin promoter.*

We have just cloned and sequenced the drosomycin gene and noted that it contains several κB-related binding sites. This information has been included into the revised version.

*Induction of the antimicrobial peptides.*

This again is an important issue and we fully understand the point made by the reviewer. To facilitate comparison between the various experiments in this study, we have preferred to use one single system of immune challenge (bacterial challenge), which had been shown earlier to induce all the antimicrobial peptides, including drosomycin (which actually was isolated from *bacteria*-challenged *Drosophila* by Fehlbaum et al., 1994). We have also verified that fungal infection induces the transcription of all antibacterial genes and of drosomycin, and this is now stated in the revised manuscript. The underlying problem here pertains to the recognition mechanisms of microorganisms which are not yet adequately understood. We have made this clearer by adding several sentences in the text.

*The hypersensitivity of spz, tub and pll mutants to fungal infection.*

This important issue has now been addressed and the results are included (see General Comments).

*Toll dominant and cact mutants.*

These mutants have a lower level of viability in the absence of challenge and we could not establish survival curves under meaningful conditions.

The "minor points" raised by this reviewer have all been corrected as requested.

• *Reviewer 2*

We understand the two major points made by the reviewer and believe that they are now adequately clarified by the introduction of the new data. In particular the results showing that the dorsoventral genes are expressed in adult insects and, most importantly, that they are strongly upregulated upon immune challenge are very strongly in favour of a direct implication of these genes in the immune response. Is it difficult to imagine that these genes would somehow act indirectly on a developmental process which would then affect the antifungal but not the antibacterial response. If the impaired inducibility of drosomycin would simply reflect global physiological abnormalities, there would be no reason to explain that immune challenge upregulates the expression of the dorsoventral genes in the fat body. The suggestion of using temperature sensitive mutants is interesting but we think the new data which we have now included are strong enough to eliminate an indirect effect of the Toll signaling pathway. We have nevertheless clarified in which way we have used the temperature sensitive mutants namely that the mutants were raised at the permissive temperature of 18° C and were shifted to 29° C at the adult stage when the survival rates or the gene expressions were determined.

The second point raised by this reviewer is addressed by the new data which are included in the revised manuscript (see General Comments). In particular, the role of spätzle is strengthened by the dramatic effect on survival of *spz* deficiency (whereas *ea* deficiencies, for instance, does not affect the survival rates) and by the fact that the spz gene is upregulated by immune challenge.

Overall the text has been made clearer by the introduction of these data and the corresponding comments.

• *Reviewer 3*

We agree that it would be interesting to study the drosomycin gene expression in $Tl^{-}$;cact mutants. Unfortunately these flies are difficult to contruct due to the lethality of cact and we have been unsuccessful to date.

The important second point raised by the reviewer on testing sensitivity to infections in at least one further member of the pathway has been addressed, as given in our General Comments. Indeed, all the members have been successfully tested.

We agree also with the third point and have clarified our position in the Discussion. As stated in the General Comments, we have now omitted the data on larvae in this text and we just mention that in larvae with a constitutively active Toll signaling pathway, drosomycin is expressed in the absence of immune challenge. There are clearly additional pathways active in larvae, which will be for future research to unravel.

*Minor points.*

All minor points have been modified as requested, except for the suggestion that the data in Figure 2 would be easier to understand if presented as points or lines with error bars, with all genotypes pooled. We believe that our representation is both easily readable and most informative as it shows the variability between the various mutant genotypes.

CONCLUSION

We believe that we have been able within the two months of reception of the review of our manuscript, to meet all major criticisms made by the reviewers. The revised manuscript which includes new important data requested by the reviewers, is admittedly clearer and strengthened in its conclusions.

We thank the reviewers for their comments and hope that the manuscript is now acceptable for publication in Cell.

With many thanks and best wishes,

Yours sincerely,

Jules A. Hoffmann