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Evaluation of Synergism among *Bacillus thuringiensis* Toxins†

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A simple test for synergism among toxins is described and applied to previously reported data on independent and joint toxicities of insecticidal proteins from *Bacillus thuringiensis*. The analysis shows synergism between a 27-kDa (CytA) toxin and 130- or 65-kDa (CryIV) toxins from *B. thuringiensis* subsp. *israelensis* against *Aedes aegypti* larvae. No positive synergism between 130- and 65-kDa toxins or among three CryIA toxins tested against seven species of Lepidoptera occurred. Comparisons with the original interpretations of these data show one case in which synergism occurred but was reported previously as absent and two cases that were not synergistic but were reported previously as suggestive of synergism. These results show that lack of an appropriate test for synergism can produce misleading conclusions. The methods described here can be used to test for synergistic effects of any poisons.

Many strains of *Bacillus thuringiensis* produce several proteins with insecticidal activity (8). The strain used most widely to control lepidopteran pests, *Bacillus thuringiensis* subsp. *kurstaki* HD-1, contains genes that code for at least five insecticidal crystal proteins: CryIA(a), CryIA(b), CryIA(c), CryIIA, and CryIIB (8). The relative toxicities of individual CryIA proteins from HD-1, which have been reported for at least 18 different species of Lepidoptera, vary widely among species (8, 11, 14, 15, 22). These differences in specificity, which occur despite 82 to 90% amino acid identity between pairs of CryIA proteins (8), indicate functional differences among toxins. *Bacillus thuringiensis* subsp. *israelensis*, which is active against dipterans, contains a heterogeneous and functionally diverse group of insecticidal proteins, including CryIVA, CryIVB, CryIVC, CryIVD, and CytA (8).

It has been suggested that mixtures of functionally diverse toxins might be more effective than single toxins and might also delay evolution of resistance in target insects (6, 20, 23). Little is known, however, about interactive effects of the toxins. In particular, hardly any data to test the hypothesis that interactions among toxins are synergistic are available.

If synergism occurs, the toxicity of a mixture cannot be estimated from the effectiveness of the individual ingredients (2). When a mixture is more toxic than expected, the interaction among ingredients is called synergistic. Antagonistic interactions among components cause the potency of a mixture to be less than expected (5). Although these concepts are simple, some statistical procedures for evaluating synergism are complex (5, 7, 17) and rarely used by biologists. When an appropriate test for synergism is not used, however, one may draw misleading conclusions about interactions of toxins. In particular, reanalysis of previously published data on interactions of *B. thuringiensis* toxins shows evidence for synergism in one case in which absence of synergism was reported (3). Conversely, two other cases previously described as suggestive of synergistic interactions (22) show no evidence of synergism.

The primary objective of this paper is to describe a simple test for synergism and illustrate its use with previously reported data on the individual and joint effectiveness of toxins from *B. thuringiensis* subsp. *israelensis* (3) and *B. thuringiensis* subsp. *kurstaki* (22). Reevaluation of these data provided here shows evidence for synergism between CytA and CryIV toxins from *B. thuringiensis* subsp. *israelensis* but not between two CryIV toxins or among CryIA toxins from *B. thuringiensis* subsp. *kurstaki*. A secondary objective of this paper is to briefly discuss two other approaches for assessing synergism. Although the focus of this paper is joint effects of *B. thuringiensis* toxins on insects, the methods described here can be used to test for synergism of any poisons (e.g., aflatoxins).

MATERIALS AND METHODS

A test for simple similar action. To test for synergism, one must first propose a model without interactive effects. If such a model enables prediction of the toxicity of a mixture directly from the toxicities of the constituents and their relative proportions in the mixture, synergism is absent. Rejection of such a null model provides evidence for synergism.

When different components of a mixture have similar effects, so that one component can be substituted as a constant proportion of another, the null model is called simple similar action or similar joint action (2, 5). This model, which assumes that dose-response regression lines for different components of a mixture are parallel, is most appropriate for testing synergism of chemically similar poisons such as *B. thuringiensis* toxins.

I first illustrate this approach with a hypothetical example. Assume that the dose causing 50% mortality (LD_{50}) is 5 ng for toxin *a* and 500 ng for toxin *b*. If no synergism occurs, what is the expected LD_{50} of a mixture containing toxins *a* and *b* in a ratio of 0.25:0.75? To determine the expected LD_{50} of a mixture under the assumptions of the simple similar effects model, one first expresses the effectiveness of the components of the mixture in terms of their potency relative to one of the components of the mixture. In this hypothetical example, the potency of toxin *b* relative to that of toxin *a* (p_b) is estimated as

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$$p_b = \frac{LD_{50(a)}}{LD_{50(b)}} = \frac{5\text{ ng}}{500\text{ ng}} = 0.01 \quad (1)$$

The next step is to estimate the potency of the mixture (p_m) relative to that of toxin *a* on the basis of the relative proportions of *a* (r_a) and *b* (r_b) in the mixture:

$$p_m = r_a + (r_b \times p_b) = 0.25 + (0.75 \times 0.01) = 0.2575 \quad (2)$$

The expected LD_{50} of the mixture ($LD_{50(m)}$) relative to the LD_{50} for toxin *a* can be estimated:

$$LD_{50(m)} = \frac{LD_{50(a)}}{p_m} = \frac{5\text{ ng}}{0.2575} = 19.4\text{ ng} \quad (3)$$

Even though toxin *b* makes up 75% of the mixture, its potency is lower than that of toxin *a*, and thus *b* contributes little to the effectiveness of the mixture. Twenty nanograms of the mixture of *a* and *b* contains 5 ng of *a* (0.25 × 20 ng = 5 ng), which by itself is sufficient to kill 50% of the target population. Note that the expected LD_{50} of the mixture (19.4 ng) is only slightly less than the LD_{50} expected if toxin *a* was 25% of a mixture with 75% inert ingredients (20 ng).

Substituting the definition for potency of the mixture (equation 2) into equation 3 yields

$$LD_{50(m)} = LD_{50(a)}/[r_a + (r_b \times p_b)] \quad (4)$$

which is equivalent to equation 11.8 of Finney (5). Substitution of the definition of potency of *b* (equation 1) into equation 4 and algebraic simplification yield

$$LD_{50(m)} = \left[\frac{r_a}{LD_{50(a)}} + \frac{r_b}{LD_{50(b)}}\right]^{-1} \quad (5)$$

Equation 5 shows that the expected LD_{50} is the harmonic mean (19) of the LD_{50}s of the components of the mixture weighted by their proportions in the mixture. In other words, the potency (which is inversely related to LD_{50}) of the mixture is the weighted arithmetic mean of the potencies of the components of the mixture.

Equation 5 can be extended to a mixture with any number of components. For example, the LD_{50} of a three-component mixture could be estimated as

$$LD_{50(m)} = \left[\frac{r_a}{LD_{50(a)}} + \frac{r_b}{LD_{50(b)}} + \frac{r_c}{LD_{50(c)}}\right]^{-1} \quad (6)$$

Sources of data. I used equations 5 and 6 to calculate the expected effectiveness of mixtures of toxins (assuming no synergism) from *B. thuringiensis* subsp. *israelensis*, studied by Chilcott and Ellar (3), and from *B. thuringiensis* subsp. *kurstaki*, studied by van Frankenhuyzen et al. (22).

Chilcott and Ellar (3) determined the individual and joint toxicities to *Aedes aegypti* larvae of proteins of 130, 65, and 27 kDa purified from *B. thuringiensis* subsp. *israelensis*. These proteins apparently correspond to CryIVA or CryIVB, CryIVC or CryIVD, and CytA, respectively (8). Each individual protein or protein mixture was assayed four times. Mortality was estimated at 24 h, and 50% lethal concentrations (LC_{50}s) were calculated by using interpolation.

van Frankenhuyzen et al. (22) examined the toxicities of HD-1 proteins against seven species of defoliating forest Lepidoptera. CryIA(a), CryIA(b), and CryIA(c) were obtained from cloned prototoxin genes expressed in *Escherichia coli*, activated with gut juice from the Chinese silkworm, *Bombyx mori* L., and force-fed to larvae. The dose of protein (nanograms per larva) that caused failure of frass production in 50% of larvae from each species (termed FFD_{50}, analogous to LD_{50}) was estimated with probit analysis (5). The black army cutworm, *Actebia fennica* (Tauscher), was not sensitive to HD-1 toxins (FFD_{50} > 3,500 ng per larva). To test for synergistic effects of CryIA proteins in the other six species, the FFD_{50} observed for native HD-1 toxin containing all three CryIA proteins was compared with the FFD_{50} expected on the basis of results from independent tests of the three individual proteins. In both the original analysis reported by van Frankenhuyzen et al. (22) and the reanalysis reported here, the proportions of CryIA toxins in the HD-1 crystal were assumed to be 0.136 for CryIA(a) (r_a), 0.542 for CryIA(b) (r_b), and 0.322 for CryIA(c) (r_c), as determined by Masson et al. (12).

TABLE 1. Observed toxicity to *A. aegypti* larvae of mixtures of toxins from *B. thuringiensis* subsp. *israelensis* versus toxicity expected assuming no synergism

Toxin(s) (proportions)[a]	LC_{50} (ng of toxin protein/ml)			
	Observed[b]			Expected[c]
	Mean	Lower	Upper	
130	32.0	25.6	38.4	
65	4.0	2.4	5.6	
27	115.0	95.1	134.9	
130-27 (0.20:0.80)	7.5	4.6	10.4	75.7
65-27 (0.43:0.57)	2.0	1.0	3.0	8.9
130-65 (0.25:0.75)	15.0	11.7	18.3	5.1
130-65-27 (0.125: 0.375:0.5)	5.5	3.8	7.2	9.8

[a] Listed according to size of protein (in kilodaltons). 130, CryIVA or CryIVB; 65, CryIVC or CryIVD; 27, CytA (8).
[b] 95% confidence limits. Data from Tables 1 and 2 of Chilcott and Ellar, reprinted with permission (3).
[c] Calculated from toxicities of individual proteins as described in text.

RESULTS

***B. thuringiensis* subsp. *israelensis*.** Analysis of the data of Chilcott and Ellar (3) by the method described here shows that the 27-kDa (CytA) protein interacted synergistically with both the 130-kDa and the 65-kDa (CryIV) proteins (Table 1). For each of these mixtures, the LC_{50} expected without synergism was greater than the upper limit of the 95% confidence interval for the observed LC_{50}. For the mixture of the 27- and 130-kDa proteins, the observed LC_{50} was less than 1/10 of the LC_{50} expected in the absence of synergism. In other words, synergism caused a 10-fold increase in the potency of the mixture. The data show about fourfold positive synergism between the 27- and 65-kDa proteins.

In contrast, the observed LC_{50} for the combination of the 130- and 65-kDa proteins was about triple the LC_{50} expected in the absence of synergism. These data show no positive synergistic effect between the 130- and 65-kDa proteins and suggest weak antagonism between these toxins. The results for the mixture of all three toxins show a slight synergistic effect, which is the net result of weak antagonism between the 65- and 130-kDa proteins and synergism between these two proteins and the 27-kDa protein.

***B. thuringiensis* subsp. *kurstaki*.** Analysis of the data of van Frankenhuyzen et al. (22) shows no evidence for positive synergistic interaction among CryIA toxins (Table 2). For

TABLE 2. Observed toxicity of HD-1 versus toxicity of HD-1 expected on the basis of toxicities of individual CryIA proteins in HD-1

Insect species	FFD_{50} (ng of toxin protein/larva)			
	Observed[a]	95% confidence limit of observed value[a]		Expected[b]
		Lower	Upper	
Choristoneura fumiferana	16.8	12.5	22.2	15.8
Choristoneura occidentalis	18.1	13.4	27.0	14.8
Choristoneura pinus	13.1	8.5	19.2	18.3
Lymantria dispar	28.6	19.2	40.0	36.8
Malacosoma disstria	24.0	15.9	35.0	27.0
Orygia leucostigma	93.8	44.3	162.9	40.3

[a] Data from Table 2 of van Frankenhuyzen et al., reprinted with permission (22).
[b] Calculated from toxicities of individual proteins as described in text.

five of six species, the expected FFD_{50} of the mixture was within the 95% confidence interval for the FFD_{50} observed for HD-1, which contains a mixture of the three toxins. The results suggest that for these five species, a simple additive model adequately explains the effects of the mixture; i.e., no synergism occurred.

For the sixth species, the white-marked tussock moth, *Orygia leucostigma* (J. E. Smith), the expected FFD_{50} (40.3 ng) was less than the lower limit of the 95% confidence interval (44.3 ng) of the observed FFD_{50} (93.8 ng). Although these data suggest that the mixture was less effective than expected on the basis of the potencies of its components, the evidence for antagonism between toxins is weak. Because the expected value was outside the 95% confidence interval in only one of six comparisons and the discrepancy was relatively small, additional experimental evidence would be needed to support an interpretation of antagonism among toxins for *O. leucostigma*.

DISCUSSION

The results reported here clarify interactions among toxins from *B. thuringiensis* and resolve some apparent inconsistencies previously noted in the literature. In bioassays with *A. aegypti* larvae, Wu and Chang (24) found that mixtures of the 27- and 65-kDa proteins from *B. thuringiensis* subsp. *israelensis* were more toxic than expected on the basis of their individual toxicities, but Chilcott and Ellar (3) concluded from their own data that no synergism between these two proteins occurred. Analysis of the data of Chilcott and Ellar (3) reported here shows a synergistic interaction between the 27- and 65-kDa proteins. With this new interpretation, both studies (3, 24) support the same conclusions: positive synergism between the 27-kDa protein (CytA) and either of the CryIV proteins (65 and 130 kDa) and no such synergism between CryIV proteins (65 and 130 kDa).

Methods for evaluating synergism rely on accurate identification, isolation, and determination of potency of the individual components of a mixture. Thus, these methods cannot readily resolve confusion about toxicity of individual proteins from *B. thuringiensis* subsp. *israelensis* caused by contamination or other methodological problems (references 4, 6, 9, and 16 and references therein). Studies of cloned gene products should help to overcome difficulties of purifying closely related proteins (2a, 4).

On the basis of a study of individual proteins obtained from cloned prototoxin genes and mixtures of proteins obtained from native HD-1, van Frankenhuyzen et al. (22) suggested a possible synergistic effect of CryIA toxins against the gypsy moth, *Lymantria dispar* L., and the white-marked tussock moth, *O. leucostigma*. The method of calculating the expected FFD_{50} used by van Frankenhuyzen et al. (22), a weighted arithmetic mean of the FFD_{50}s of the individual toxins, tends to produce expected values that are biased upwards. Thus, the expected values for the FFD_{50} of the mixture tend to be greater than the observed FFD_{50}, causing one to conclude incorrectly that synergism occurs. This tendency increases as the difference in toxicity among components in the mixture increases. Thus, the low levels of toxicity of CryIA(c) to *O. leucostigma* (FFD_{50} = 560 ng per larva) and *L. dispar* (FFD_{50} = 2,484 ng per larva) relative to the toxicities of CryIA(a) and CryIA(b) (range in FFD_{50}, 22 to 56) led to inflated values for expected FFD_{50} (200 ng for *O. leucostigma* and 819 ng for *L. dispar*) and the misleading suggestion of synergism.

Reevaluation of their data provided here shows no evidence for synergism among toxins in the two aforementioned species or in the other five species examined in the study. A simple additive model adequately explains the results for all species except *O. leucostigma*, in which weak antagonism among toxins may occur. Moar et al. (15) also found no evidence for synergism of CryIA toxins from the HD-1 or NRD-12 strain of *B. thuringiensis* subsp. *kurstaki* against the beet armyworm, *Spodoptera exigua* (Hübner). In light of the analysis described here, a consistent overall pattern emerges: no positive synergism among CryIA toxins has been observed for larvae of eight species of Lepidoptera examined so far. The analysis reported here confirms the conclusion of van Frankenhuyzen et al. (22) that CryII toxins either were absent from their native HD-1 toxin preparations or were present but did not increase toxicity.

Definitive treatment of models of joint toxicity can be found elsewhere (2, 5, 17). It is worthwhile to mention here two additional approaches for evaluating synergism: independent joint action and a simple empirical approach. When the components of a mixture act independently and have different modes of action, the appropriate null model is called independent joint action (5). For example, this approach can be used to assess synergism between synthetic insecticides and *B. thuringiensis* (18) or between bacterial and viral pathogens (13).

In perhaps the simplest approach to evaluating synergism of two toxins, one first determines the dose-response relationship for each toxin separately. Next, one tests a mixture containing enough of toxin *a* to kill 10 to 50% of the target population and a sublethal amount of toxin *b*. The same dose of toxin *a* that is included in the mixture is tested by itself in a simultaneous experiment with subjects randomly assigned to the mixture treatment or toxin *a* alone. In this case, assuming no synergism, toxin *b* is not expected to increase the effectiveness of the mixture. Thus, significantly greater mortality caused by the mixture than by toxin *a* alone is evidence for synergism. This approach has been used to examine synergism among *B. thuringiensis* toxins (1, 4, 24, 25) and between *B. thuringiensis* toxins and protease inhibitors (10, 21).

In conclusion, the simple similar action model is useful for evaluating synergism among toxins that are alike, such as various *B. thuringiensis* toxins. The independent joint action model is most appropriate for evaluating synergism between toxins that differ in their mode of action. The simple empirical approach described above can be used to examine

synergism between similar or dissimilar toxins. Designing and conducting experiments that test appropriate models will facilitate progress toward understanding the interactive effects of toxins.

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