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Analysis of Synergism in Hepatocarcinogenesis Based on Preneoplastic Foci Induction by 10 Heterocyclic Amines in the Rat

Ryohei Hasegawa,[1] Isao Yoshimura,[3] Katsumi Imaida,[1] Nobuyuki Ito[2] and Tomoyuki Shirai[1]

[1]First Department of Pathology, Nagoya City University Medical School and [2]Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467 and [3]Department of Industrial Management and Engineering, Science University of Tokyo, 1-3 Kagurazaka, Shinjuku-ku, Tokyo 162

The effects of simultaneous treatment with 5 or 10 heterocyclic amines at low dose levels on hepatocarcinogenesis in rats were investigated using a medium-term liver bioassay protocol based on the two-stage carcinogenesis hypothesis with diethylnitrosamine initiation (200 mg/kg, i.p.). Five carcinogenic heterocyclic amines in experiment 1 (Trp-P-1, Glu-P-2, IQ, MeIQ, MeIQx) and experiment 2 (Trp-P-2, Glu-P-1, MeAαC, AαC, PhIP) were administered together or individually in the diet at levels of 1/1, 1/5, or 1/25 carcinogenic doses, and all 10 chemicals were given at 1/10 or 1/100 levels in experiment 3. Induction of preneoplastic glutathione *S*-transferase placental form (GST-P)-positive foci in the liver was generally increased in the combination groups over the sums of the 5 or 10 individual effects. Thus, based on the heteroadditive concept, synergism was observed for each combination, being most obvious in the group given all 10 chemicals at the 1/10 dose levels. However, the values for the combined groups were generally close to the averages of the 5 or 10 data gained for the heterocyclic amines alone at the corresponding higher doses, indicating the possibility of isoadditivity. Based on these findings, we propose here a new statistical method for analysis of combined effects of multiple chemicals, and, using this, we demonstrated (true) synergism with some heterocyclic amine combinations. The importance of dose-response curves for evaluation of combination effects is discussed.

Key words: Synergism — Statistical analysis — Hepatocarcinogenesis — Heterocyclic amine — GST-P-positive foci — Rat

Our environment contains a great variety of carcinogenic factors including naturally occurring and synthetic chemicals, radiation and viruses[1, 2] and humans may be concurrently or sequentially exposed to these environmental factors at only very low individual doses over their lifetime. Therefore, as an adjunct to detection and exclusion of hazardous agents from our environment, examination of their low dose combination effects is an important area for research aimed at evaluation of human cancer risk.[3, 4]

It is well known that carcinogenic substances such as heterocyclic amines are produced in our foods under normal cooking conditions.[2, 5–7] For 10 of those heterocyclic amines, carcinogenicity has been demonstrated in animal experiments[8]: 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Except for PhIP,[9, 10] all these compounds are carcinogenic to the liver in either rats or mice.

Therefore, we have examined the combined effects of dietary administration of 5 or 10 heterocyclic amines on the development of rat liver preneoplastic lesions using our medium-term liver bioassay for prediction of hepatocarcinogenicity. Detailed results have already been published.[11–13] In the present paper, the theoretical basis underlying the concept of synergism is reviewed and a new mathematical method for analysis of synergism in carcinogenesis induced with multiple chemicals is proposed based on our data.

The animal model applied is a so-called medium-term liver bioassay for rapid detection of carcinogenic agents, which was developed to overcome various disadvantages of both short-term screening and conventional long-term carcinogenicity testings.[14, 15] Since the model requires only about 15 rats per group for reliable statistical analysis, has an experimental duration of only 8 weeks and features simple performance and easy quantitative analysis, we consider that the protocol is eminently suitable for precise evaluation of the combined effects of multiple carcinogenic agents.[16]

METHODS AND FOCI DATA

Fig. 1 shows the protocol which is now employed in our laboratory as a rat liver medium-term bioassay for rapid detection of carcinogenic agents. Male F344 rats are initially injected with diethylnitrosamine (DEN, 200 mg/kg, i.p.) to initiate hepatocarcinogenesis and after a 2-week recovery period, receive test compound(s) (group 1). Groups 2 and 3 serve as DEN alone and test compound(s) alone controls, respectively. All animals are subjected to two-thirds partial hepatectomy at week 3 and killed at week 8. Development of the immunohistochemically demonstrated glutathione *S*-transferase placental form (GST-P)-positive focus, recognized as one of the most reliable marker lesions for rat liver carcinogenesis, is quantitatively evaluated. Heterocyclic amines used in the present series of experiments with their full doses in parentheses were Trp-P-1 (0.015%), Glu-P-2 (0.05%), IQ (0.03%), MeIQ (0.03%), MeIQx (0.04%), Trp-P-2 (0.05%), Glu-P-1 (0.05%), MeAαC (0.08%), AαC (0.08%) and PhIP (0.04%). These doses were carcinogenic in previously conducted long-term studies. The combinations and dose levels of chemicals use here are summarized in Table I along with the major target organs for carcinogenesis in the rat. All chemicals were incorporated into the powdered diet along with corn oil at a concentration of 2% to moisten the diet.

In the first two experiments (combinations 1 and 2), five of the above-listed heterocyclic amines were administered simultaneously at 1/5 and 1/25 of the full doses. Other groups were given individual chemicals at the full doses or at 1/5 or 1/25 of these. In the subsequent experiment (combination 3), groups receiving all 10 chemicals added to the diet at dose levels of 1/10 or 1/100 were compared with groups given individual chemicals at the 1/10 dose level. The numbers of GST-P-positive foci larger than 0.1 mm in diameter per cm^2 of liver section are summarized in Table II. The data are also illustrated in Figs. 2–4.

Dose-response curves for the groups given each chemical alone and chemical mixtures were generally nonlinear, as shown in Fig. 2. Levels of GST-P-positive foci development in the full dose groups generally paralleled hepatocarcinogenic potential. MeIQ, Trp-P-2 and AαC were positive in the present system, although clear liver carcinogenicity has not been reported in the rat, suggesting that they are in fact weak hepatocarcinogens. PhIP was negative in this study, in line with the results of

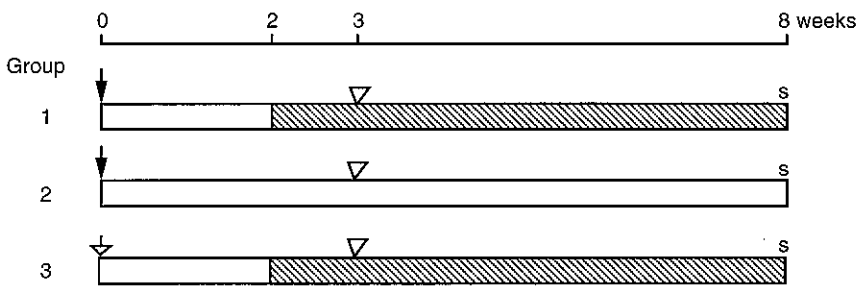

Fig. 1. Experimental protocol of the medium-term liver bioassay. Animals: F-344 male rats (6 weeks old), ↓: DEN, 200 mg/kg i.p., ▽: Saline, i.p., ▽: Two-thirds partial hepatectomy, ▨: Test chemical(s), □: No treatment, S: GST-P immunohistochemistry on the liver.

Table I. Combinations and Doses of Heterocyclic Amines

Experiment	Chemical	Main targets (rat)	Full dose (%)	Concentration in the diet[a]	
				Independently	Mixtures
(1)	Trp-P-1	Liver	0.015	1/1, 1/5, 1/25	
	Glu-P-2	Liver, Colon, Small intestine	0.05	1/1, 1/5, 1/25	
	IQ	Liver, Colon, Small intestine	0.03	1/1, 1/5, 1/25	1/5, 1/25
	MeIQ	Colon, Oral cavity	0.03	1/1, 1/5, 1/25	
	MeIQx	Liver	0.04	1/1, 1/5, 1/25	
(2)	Trp-P-2	Urinary bladder	0.05	1/1, 1/5, 1/25	
	Glu-P-1	Liver, Colon, Small intestine	0.05	1/1, 1/5, 1/25	
	MeAαC	Liver, Pancreas	0.08	1/1, 1/5, 1/25	1/5, 1/25
	AαC	—	0.08	1/1, 1/5, 1/25	
	PhIP	Colon, Mammary gland	0.04	1/1, 1/5, 1/25	
(3)	All 10 chemicals			1/10	1/10, 1/100

a) Ratio to the full dose.

Table II. Numbers of GST-P-positive Foci in the Livers of Rats Treated with Heterocyclic Amines

Heterocyclic amine	Dose levels									
	1/1		1/5		1/25		1/10		1/100	
	Value	Effect	Value	Effect	Value	Effect	Value	Effect	Value	Effect
Trp-P-1	69.55	49.17	28.38	8.00	21.20	0.82	15.59	−0.83		
Glu-P-2	29.78	9.40	22.24	1.86	20.08	−0.30	11.69	−4.73		
IQ	85.70	65.37	28.27	7.89	21.07	0.69	17.29	0.87		
MeIQ	37.82	17.44	34.68	14.30	19.49	−0.89	14.86	−1.56		
MeIQx	48.74	28.36	21.55	1.33	20.02	−0.36	14.65	−1.77		
Mean	54.32	33.95	27.02	6.64	20.37	−0.01				
Sum of effects	—	—	—	33.22	—	−0.04				
Mixture	—	—	56.85	36.47	26.80	6.42				
Control	20.38		20.38		20.38					
Trp-P-2	26.53	5.97	25.53	4.97	23.43	2.87	14.05	−2.37		
Glu-P-1	76.04	55.48	35.89	15.33	26.82	6.26	21.69	5.27		
MeAαC	38.49	17.93	25.25	4.69	23.32	2.76	16.80	0.38		
AαC	29.98	9.42	23.02	2.46	24.03	3.47	12.37	−4.05		
PhIP	18.25	−2.31	21.99	1.43	21.01	0.45	15.32	−1.10		
Mean	37.86	17.30	26.34	5.78	23.72	3.16	15.43	−0.99		
Sum of effects	—	—	—	28.88	—	15.81	—	−9.89		
Mixture	—	—	62.95	42.39	28.12	7.56	58.74	42.32	14.60	−1.82
Control	20.56		20.56		20.56		16.42		16.42	
Mean of 10	46.09	25.62	26.68	6.23	22.05	1.58				

Data are group means (No./cm^2). Values: actual data. Effects: values obtained by subtraction of the control value from the actual data.

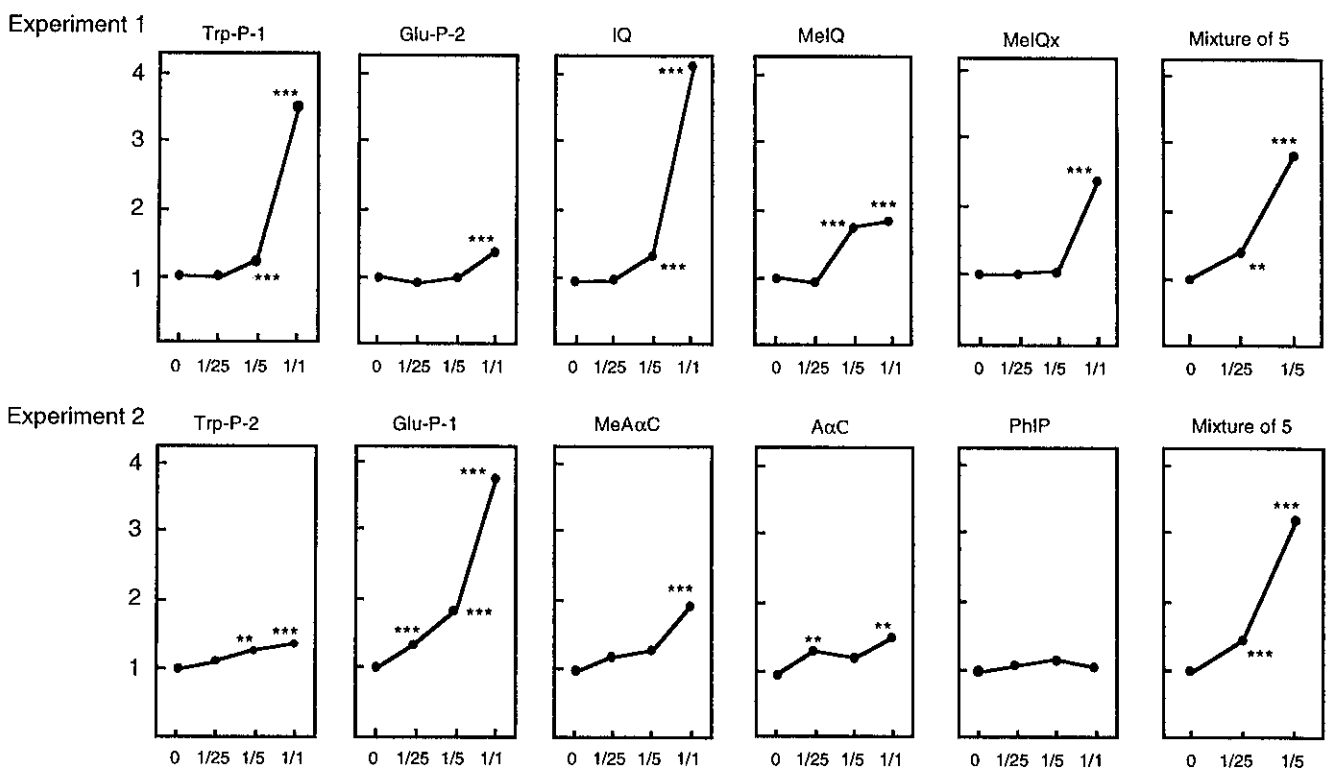

Fig. 2. Dose-response curves for numbers (ratio to the control value) of GST-P-positive liver cell foci in experiments 1 and 2. Dose is expressed on a logarithmic scale. Generally, the heterocyclic amine dose response was nonlinear, including the cases where chemical mixtures were given. ** $P<0.01$, *** $P<0.001$.

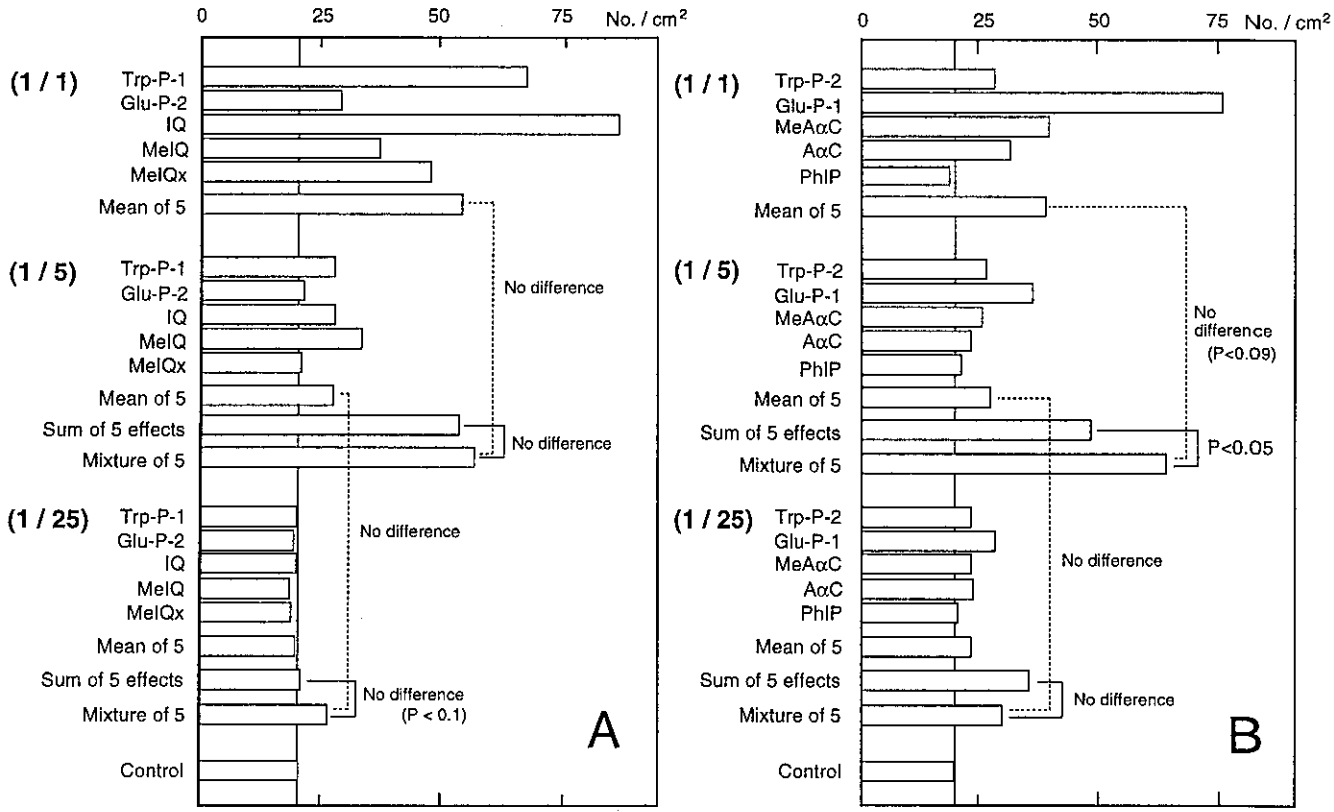

Fig. 3. Results for numbers of GST-P-positive foci in experiments 1 (A) and 2 (B). Levels with the highest doses were mostly parallel to liver carcinogenicity in the rat. The sum of effects means simple addition of all values, in each case minus the control level (background). With the statistical analysis, pairs compared for heteroadditivity are indicated with solid lines and pairs assessed for isoadditivity by our simple method are indicated with dotted lines. A significant difference ($P<0.05$) was only observed for heteroadditivity in experiment 1 at the 1/5 dose levels (B).

long-term studies.[9, 10] For most chemicals, the maximum no-effect dose levels were around 1/10 of the full dose, except for Glu-P-1, which showed an effect even at the 1/25 level. Similar results were observed for areas of foci (data not shown).

Induction of preneoplastic GST-P-positive foci was increased in some combination groups over the sums of the effects in the groups treated separately at the same dose levels, and the pairs for comparison are indicated with solid lines in Figs. 3 and 4. The effect was most obvious in experiment 3 (Fig. 4). When 10 heterocyclic amines were mixed in the diet at the 1/10 full dose levels, induction of GST-P-positive foci was very high, while no clear effects were evident for any of the chemicals given separately at the 1/10 dose levels. A similar tendency was also seen with the 1/5 mixture in combination 2 (Fig. 3B). Thus, enhancement of foci development by the combined treatments differed depending on the combination.

In most published studies, synergism is discussed simply on the basis of findings such as those presented above. However, to obtain a mathematically sound appreciation of whether synergism has actually occurred in combination treatment groups requires more rigorous evaluation. This subject has previously been discussed in Japanese.[17]

STATISTICAL ANALYSIS

Adequate data on dose-response curves for individual chemicals are essential to permit a scientifically based conclusion of synergism.[18] However, most studies lack such data and a simple additive model is most often applied. There are at least two approaches for analysis of synergism using heteroadditive and isoadditive models.

Heteroadditive model A simple definition is that synergism occurs when the effect of two or more substances acting together exceeds the sum of their effects when acting separately. This idea is based on the heteroadditive concept of synergism. "Effect" can be obtained by subtracting the background (control) level from actual

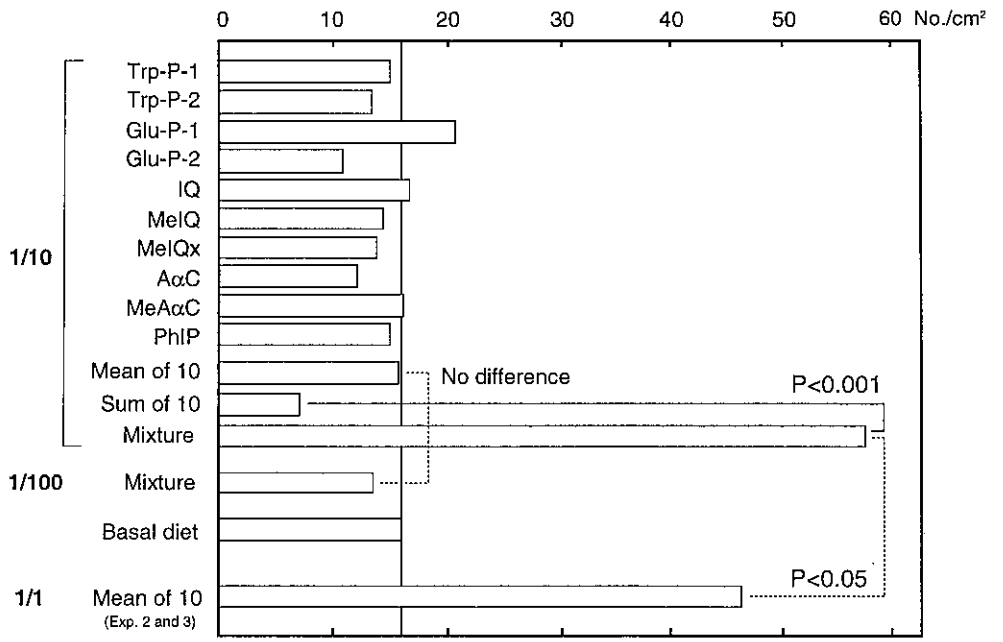

Fig. 4. Results for numbers of GST-P-positive foci in experiment 3. The sum of effects means simple addition of all values, in each case minus the control level (background). With the statistical analysis, pairs compared for heteroadditivity are indicated with solid lines and pairs assessed for isoadditivity by our simple method are indicated with dotted lines. The most pronounced synergism was found for the mixture of 10 chemicals at the 1/10 dose levels, and this was revealed to be a true (strict) synergism.

values. With the above definition, synergism occurs between carcinogens A and B when

$$T_{ab}-T_0>(T_a-T_0)+(T_b-T_0), \qquad [1]$$

where T_{ab} is the value for the group exposed to both A and B, T_a and T_b are those for the groups exposed to A and B alone, and T_0 is that for the group exposed to neither A nor B. This can be expressed as

$$T_{mix}-T_0>\Sigma\,(T_i-T_0), \qquad [1a]$$

where T_{mix} is the mixture group value, T_i is that for the groups treated with each chemical alone, and T_0 is that for the control group.

In this model, dose-response curves for carcinogens acting separately are not a prerequisite, and a positive result obtained when equation 1 or 1a is tested for statistical significance signifies that the chemicals have acted with apparent synergism. The addition of the term "apparent" here indicates that the conclusion of synergism might be invalidated if dose-response data were available for more rigorous testing using the isoadditive concept, as will be discussed later.

Based on this heteroadditive model for synergism, we propose here an equation which is applicable to the cases of multiple chemicals, as in this report. The t test using test statistic t and the degrees of freedom, df, defined below was carried out on quantitative data on GST-P-positive foci as follows;

$$t=\frac{Y_{mix}+(m-1)Y_0-\Sigma Y_i}{\sqrt{Ve(1/n_{mix}+(m-1)^2/n_0+\Sigma(1/n_i))}} \qquad [2]$$

$$df=n_{mix}+n_0+\Sigma n_i-(m+2)$$

where Y represent a mean focus value, m is the number of chemicals mixed, n_{mix}, n_0 and n_i are the effective numbers of rats in the combined, control and single chemical treated groups, respectively, and Ve is the mean square corresponding to error.

The significance for numbers of foci (number/cm^2) is indicated in Figs. 3 and 4 with solid lines. In experiment 1 (combination 1), the value for the mixture at 1/25 dose levels (26.80) was greater than the sum of the 5 effects ($20.34=20.38-0.04$) and the P value was 0.097 (not significant). In experiment 2 (combination 2), the value for the mixture at 1/5 dose levels (62.95) was significantly ($P<0.05$) higher than the sum of the 5 effects ($49.44=20.56+28.88$). The most pronounced effect was observed in experiment 3 (combination 3). When all 10 heterocyclic amines were mixed in the diet at the 1/10 dose levels, the resultant value (58.74) was markedly

higher ($P<0.001$) than the sum of the 10 individual effects at the 1/10 dose level (6.53=16.42−9.89). Thus, apparent synergism was indicated for heterocyclic amines at the 1/5 dose level with combination 2 and at the 1/10 level with combination 3, based on the heteroadditive model.

Isoadditive model It is noteworthy, however, that the numbers of foci in the combination treatment groups were always similar to the averages (means) of those with the 5 or 10 chemicals individually given at 5 or 10 times higher dose levels. With regard to the numbers of foci (number/cm^2), the average of the 5 full dose data in experiment 1 (Fig. 3A) was 54.32 as compared to 56.85 when the same compounds were given together at the 1/5 doses. Similarly, the average of the five 1/5 dose level data was 27.02 and the 1/25 dose level combination gave a value of 26.80. For experiment 2 (Fig. 3B), the same close relation was found for the lower doses (26.34 and 28.12). Similarly, the mean of the 10 data at the 1/10 doses (15.43) was very close to the result for the group given all 10 heterocyclic amines at the 1/100 levels (14.60) (Fig. 4). The effects might thus be explained in terms of isoadditivity, as stressed by Reif.[18] Exceptions were observed in the 1/5 dose mixture of experiment 2 and the 1/10 dose mixture of experiment 3. The combination value at the 1/5 doses (62.95) was considerable greater than the average for the 5 full dose values (37.86) in experiment 2 and, whereas the average of all 10 heterocyclic amines from the two separate experiments was 46.09 (=((20.38+33.95)+(20.56+17.3))/2), the value for the combination treatment of 10 chemicals at the 1/10 dose level was 58.74 (Fig. 4).

Consideration of the dose-response curves is necessary here. Since these are usually convex (lower half of an S-shape) (Fig. 2), the effect of applying a dose a of carcinogen A twice (dose $2a$) is to produce a tumor incidence (T_{2a}) that is higher than twice that obtained with the single dose ($T_a \times 2$) as shown in Fig. 5. In this

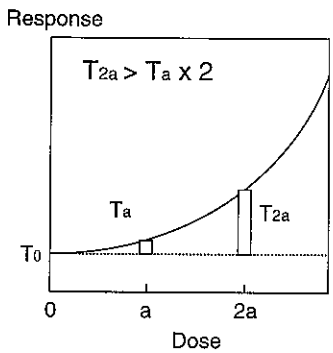

Fig. 5. Autosynergism. When a dose response curve has an S-shape (nonlinear), the effect at dose $2a$ of carcinogen A is greater than twice the single dose response ($T_a \times 2$).

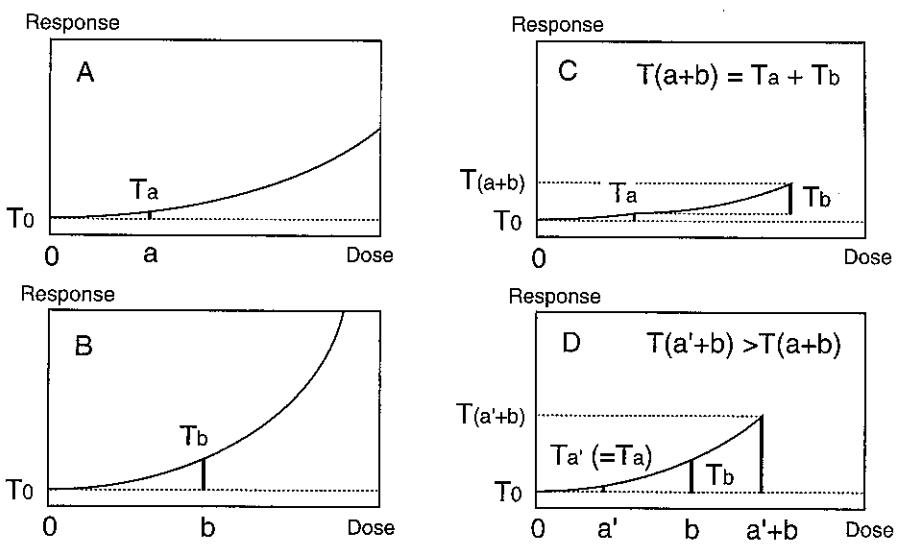

Fig. 6. Analysis of combined effects using dose-response curves. A, Dose-response curve for chemical A. B, Dose-response curve for chemical B. C, Heteroaddition defined by equation 1. The expected effect is T_a+T_b. D, Example of isoaddition. The effect for the group given both chemical A at dose a and chemical B at dose b is expected to be $T_{(a'+b)}$. Dose a' is determined as the level for chemical B at which the same effect as with chemical A at dose a is produced. When the dose-response curve is nonlinear, $T_{(a'+b)}$ may be greater than the simple sum of T_a and T_b: $T_{(a'+b)}>T_a+T_b$.

situation, carcinogen A is synergistic with itself based on the definition of synergism expressed in equation 1 or equation 1a. This may be termed as "autosynergism," but since a conclusion of synergistic action would clearly be inappropriate, the effect is fundamentally additive. This is the basis of the isoaddition concept and it is reasonable that the combined effects of chemicals, especially those with biologically similar characteristics, should be evaluated on the same basis.

Fig. 6 shows an example of combined effects based on the isoadditive concept: combined effects of chemical A at dose a and chemical B at dose b estimated by using the dose-response curve for chemical B. The effect for the subgroup given both chemical A (T_a) and chemical B (T_b) is expected to be $T_{(a'+b)}$ (Fig. 6D), obtained as the sum of the effects of dose a' and dose b of chemical B. Dose a' is determined as the level with which the same effect as that of chemical A at dose a is produced with chemical B. When the combined effect significantly exceeds an estimated value using a model based on dose-response curves, such as the isoaddition model, it can be termed true or strict synergism. In Fig. 6C, heteroaddition defined by equation 1 is also illustrated as an aid to understanding. When a dose-response curve is not linear (curves upwards with increasing dose), $T_{(a'+b)}$ in Fig. 6D is greater than the simple sum of T_a and T_b in Fig. 6C (T_a+T_b).

Proposal of model With mixtures of 5 or 10 chemicals, however, it is quite difficult to apply the above procedure to the data. Therefore, a simple method of analysis which is fundamentally based on the idea of isoaddition, and which can be easily applied, is proposed here as follows: synergism occurs when

$$T_{mix} > \Sigma(T_i)/m, \qquad [3]$$

where m is the number of chemicals mixed, T_{mix} is the value for the subgroup treated with all m chemicals together, and T_i is the value for each individual chemical at the m-times higher dose level (Y_i). Based on this model, the t test using test statistic t and the degree of freedom, df, defined below was carried out on quantitative data for GST-P-positive foci as follows:

$$t = \frac{Y_{mix} - \Sigma(Y_i/m)}{\sqrt{Ve(\Sigma(1/m^2 n_i) + 1/n_{mix})}} \qquad [4]$$

$$df = n_{mix} + \Sigma n_i - (m+1)$$

where Y represents a mean focus value, m is the number of chemicals mixed, and n_{mix} and n_i are the effective numbers of rats in the combined and single chemical treated groups, respectively. Ve is the mean square corresponding to the error, obtained as follows:

$$Ve = (n_{mix} V_{mix} + \Sigma n_i V_i)/df$$

The significances reached for numbers of foci (number/cm^2) are illustrated in Figs. 3 and 4 for comparison with dotted lines. In experiment 1, the value for the mixture at 1/5 dose levels (56.85) was similar to the average of the 5 individual results at the full dose (54.32) and the value for the mixture at 1/25 dose levels (26.80) was similar to the average of 5 individual results at the 1/5 dose levels (27.02). In experiment 2, the value for the mixture at the 1/5 dose levels (62.95) was greater than the average of the 5 results at the full doses (37.86), and the value for the mixture at the 1/25 dose levels (28.12) was similar to the average of the 5 individual results at the 1/5 dose levels (26.34). The most pronounced effects were again observed in experiment 3. When all 10 heterocyclic amines were mixed in the diet at the 1/10 dose levels, the resultant value (58.74) was higher than the average of the 10 individual results at the full dose levels (46.09). The difference was significant ($P<0.05$) based on our mathematical model for synergism (equation 4). The result for the mixture at the 1/100 dose levels (14.60) was, however, again similar to the average of the 10 individual results for the 1/10 doses (15.43).

Thus, whereas isoadditivity was commonly observed with the mixtures, true synergism was only apparent for heterocyclic amines at the 1/10 level in combination 3, based on the isoadditive model, as shown with dotted lines (Fig. 4). The 5 chemicals at the 1/5 dose levels in experiment 2 demonstrated a non-significant ($P<0.09$) tendency for true synergism, but, without statistical validation, the effect must be concluded to be only apparently synergistic.

DISCUSSION

It is obvious from the present investigation that the conclusion of the presence or absence of synergism may differ depending on the mathematical model used for the evaluation. The two approaches assessed here are not of comparable validity, since isoaddition is based on dose-response relationships while heteroaddition is not. Many authors have emphasized that analysis of synergism without appropriate data for dose-response relationships provides only limited information.[18, 19] A knowledge of the dose-dependence of agents acting separately is therefore a prerequisite for accuracy, and where appropriate data are not available, statistically evaluated positive results based on heteroadditivity can only be concluded to demonstrate apparent (seeming) synergism.[18] Due to a lack of appropriate data, unfortunately, precise analysis is not possible in many of the reported cases.

As mentioned above, our data are too complicated to allow analysis by mathematical methods, such as the model in Fig. 6 and the isobolic diagram, recommended by Reif[18] and others,[19] and we propose here an alterna-

tive mathematical model theoretically based on the concept of the isoadditive model (equation 3), and a precise procedure (equation 4) based on this idea.

Berenbaum[20] reported in 1981 the following index to detect and characterize interactions for m compounds:

$$\Sigma(x_i/X_{iE}) \quad [5]$$

where a value of <1 stands for synergy, $=1$ for additivity, and >1 for antagonism and in which x_i is the concentration of the i-th compound in the combination that yields response E, and X_{iE} is the concentration of the i-th compound that yields response E when given alone ($i=1, \ldots, m$). The idea is based on the isobolic diagram, while ours is similar to the isoadditive concept.

In the present analysis, although highly significant synergistic effects were observed for heterocyclic amines when the heteroadditive model was adopted, the significance was reduced or no longer observed when the mathematical model based on the isoadditive concept was applied. From our data, we concluded that synergism with the heteroadditive model may be more clearly demonstrated when the number of chemicals in mixtures is increased.

In line with "autosynergism," combination effects evaluated based on dose-response curves provide information on whether the effect is true (or strict) synergism or not. The relationships between heteroaddition, isoaddition, and the terms *apparent* and *true* synergism are illustrated in Fig. 7.

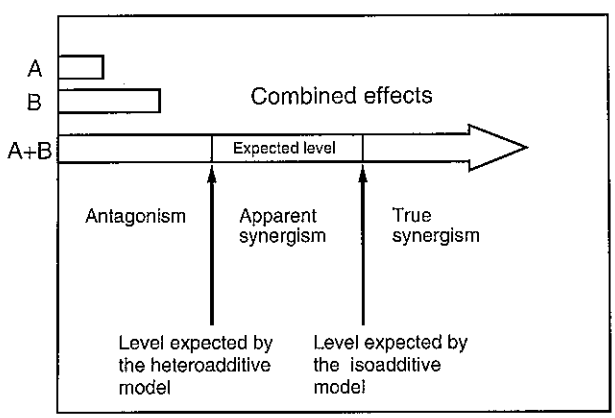

Fig. 7. Apparent synergism and true synergism. Generally, the expected level with the isoadditive model is greater than that with the heteroadditive model, since dose-response curves are usually nonlinear. For any combination, the effect in a mixture group is expected to result in levels between those for heteroaddition and isoaddition. The more the biological properties of chemicals are similar and inter-dependent, the closer the result for the mixture will approach the isoadditive level. Apparent synergism means that the effect is fundamentally additive.

Although there are some exceptions, the present data thus indirectly indicate that the 10 heterocyclic amines biologically act in a similar manner and that their interaction in combination is fundamentally additive rather than synergistic. Actually, all these heterocyclic amines are reported to be activated through similar basic metabolic pathways mediated by cytochrome P450s, the principle responsible isozyme being cytochrome P450IA2, and specific cytochrome P450 isozymes are inducible by various chemicals, including heterocyclic amines themselves.[21, 22]

However, it might be concluded that true synergism occurred with combinations including PhIP, MeIQ, Trp-P-2 and AαC as seen in experiments 2 and 3. This observation might be explained by the fact that these heterocyclic amines are not hepatocarcinogenic in the rat, although they can induce the key metabolic enzyme (CYP1A2) in the rat liver.[21, 22] Its induction by each chemical alone or in combination has been demonstrated for each chemical used in experiment 2.[23] On the other hand, we have shown that DNA adduct formation in the livers of rats given 5 heterocyclic amines in the diet for 6 weeks is basically heteroadditive (simple additive).[24]

Finally, it should be borne in mind that dose levels are very important for evaluation of synergism and additivity. The response obtained depends upon which portions of the dose-response curves are involved and whether the response levels are similar for each individual chemical. Analysis has no meaning in dose ranges where the effects for mixtures reach a maximum response and the dose-response curves become flat (plateau), in any mathematical model.

The present analysis allows the following conclusion. Although a single environmental agent might alone present only a low risk, individuals are usually exposed to a variety of such influences and in concert they may be capable of inducing tumors. In the case of the compounds investigated here, this is very important, since mixtures of heterocyclic amines may be generated in certain cooked foods.[6, 7] However, since a clear dose-response relationship exists for complex mixtures, as with each individual chemical, risk should decrease with decrease in the dose so that measures taken to reduce chemical concentrations offer an effective approach to control. Furthermore, since appropriate dose-response data are necessary for adequate evaluation of combined effects of chemicals, synergism validated only by heteroadditivity (apparent synergism) may be of only limited significance and does not deserve strong emphasis.

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