

# Synergy, additivism and antagonism in immunosuppression

## A CRITICAL REVIEW

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### INTRODUCTION

In spite of the successes of immunosuppressive therapy, the agents in use are not as effective as desired, partly because their dosage is to a large extent limited by their toxicity. Methods to increase their effectiveness are therefore of practical importance. One of the approaches to this end is the search for synergistic combinations of agents and, over the past 25 years, there has been a steady stream of publications in this field. Synergy, however, is a topic on which confusion reigns. The relevant pharmacological literature is often obscure (some papers, indeed, are models of incomprehensibility) and is profusely littered with technical terms that are not always clearly defined. Several different terms are used to describe the same phenomenon and the same term means different things to different authors. Goldin & Mantel (1957) listed seven different definitions of synergy and implied that, in this chaotic state of affairs, the choice of definition was a matter of personal preference. So far as most published work is concerned, there appears to have been little progress since that time. Reference to pharmacological text-books does not help; they generally deal with the topic cursorily and/or incorrectly, or avoid it altogether.

It is not surprising, therefore, that this confusion is reflected in investigations on synergy in immunosuppression. Experiments are commonly designed in such a way that they could not detect synergy if it were present, results are interpreted as showing synergy when there is no evidence for it, as showing additivism where there is clear antagonism, and so on.

The basic difficulty is that most investigators use fallacious criteria for determining the nature of drug interactions—they compare the effect of the agents used in combination with the sum of their effects when used alone. This comparison is experimentally straight-forward but, as it is based on assumptions that are wrong, it leads to endless confusion, and conclusions based on it are generally valueless. The correct method for analysing drug interactions is, in most cases, more laborious and involved, but conclusions based on it may be relied on.

It is worth pointing out that procedures for analysing interactions between various agents have a wide application in immunology and are not restricted to the study of immunosuppressive agents. Topics eminently suitable for the application of such methods are the study of cell interactions and the treatment of neoplasms by combined immunization and chemotherapy. These fields, especially the latter, have suffered from the effects of confused ideas about synergy and they would benefit from the more rigorous approach to be described here.

### THE FALLACY IN ADDING DRUG EFFECTS

A combination of agents that is more effective than is expected from the effectiveness of its constituents is said to show synergy (other terms loosely used in the immunological literature are augmentation, potentiation, super-additivism and sometimes, quite wrongly, additivism). One less effective than expected is said to show antagonism (or depotentiation, negative interaction, negative synergy, etc.), and one no more and no less effective than expected is said to show additivism. Synergy and antagonism imply that the different constituents affect each other's actions, i.e., they interact pharmacologically;

additivism implies that they do not. Of course, the key question is what is to be 'expected' from a combination of agents. Most workers simply add the effects of the constituents used separately, and synergy, antagonism and additivism are deemed to be present if the effect of the combination is respectively more than, less than or equal to this sum.

For example, Bareham, Griswold & Calabresi (1974), in a study of the effect of methotrexate (MTX) and 5-fluorouracil (FU) on the antibody response of mice to sheep red cells, found that 1 mg/kg of MTX suppressed the response by 35.9% and 50 mg/kg FU suppressed it by 35.4%. They reasoned that both drugs together should suppress the response by  $35.9 + 35.4\%$ , or 71.3%. When, however, both were given (the MTX 30 min before FU), the response was suppressed by 93.3%, and they concluded that this was a case of synergy. Similar reasoning has been followed by the great majority of workers in this field.

The fallacy in this approach may be illustrated as follows. Suppose that two drugs, A and B, each suppress a response by 10% when given at a dosage of 1 mg/kg but that, when both are given together at this dose, the suppression is not 20 but 90%. One might conclude that this is a case of marked synergy but, as the dose-effect curves of these drugs show (Fig. 1), a 2 mg dose of either drug alone also produces

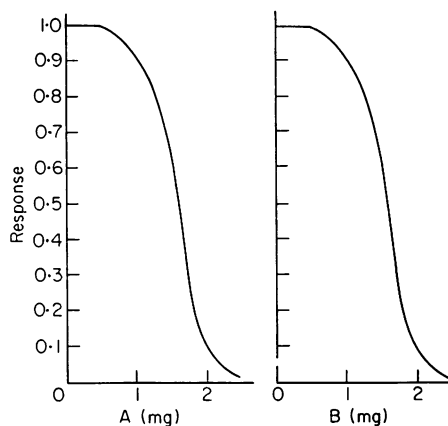


FIG. 1. Dose-effect curves of agents A and B, to show that the nature of a drug interaction cannot be determined by adding drug effects. 1 mg of A or of B each suppress the response by 10% and it is known that both together (i.e., 2 mg of a mixture of A and B) suppress it by 90%. This does not indicate synergy, for the dose-effect curves show that 2 mg of either drug alone also suppresses the response by 90%.

a 90% suppression. As 2 mg of the combination produces exactly the same effect as 2 mg of either drug by itself, the drugs are clearly not more effective when used in combination than when used singly. It is equally wrong to conclude that a drug combination is synergistic merely because the doses chosen produce no effect on their own but are effective when given together, for dose-effect curves may have thresholds. In Fig. 1, for example, doses up to 0.5 mg have no evident effect and, if the effect of a combination were the sum of effects of its constituents, we would have to conclude that any number of doses of 0.5 mg given together should also have no effect, which would be absurd.

This approach would be correct only if the effects of drugs were simply proportional to dose, when the effect of a dose of one drug would be the sum of the effects of its constituent quanta. If two or more such drugs given together did not interact pharmacologically, the effect of the combination should similarly be the sum of effects of its constituent quanta. However, because of the nature of drug-receptor interactions, dose-effect curves for biologically active agents are rarely if ever linear (Ariëns, 1964). The dose-effect curves for immunosuppressive agents so far investigated have all been found to be markedly non-linear (Berenbaum, 1969) and, considering the complexities of the responses they affect, the likelihood of any not yet investigated being linear appears to be remote, so that this condition is unlikely to be met.

There is only one common and relevant situation in which the effect of an agent is proportional to

dose, and that is when ionizing radiation is used and the duration, not the intensity, of the effect is measured. Proportionality to dose is seen, for instance, in the time taken by irradiated animals to recover immunity to tetanus (Silverman & Chin, 1956), to form serum haemolysins after injection of sheep red cells (Taliaferro & Taliaferro, 1964) and to reject skin allografts (Brent & Medawar, 1966). Ionizing radiation impairs cell reproductive integrity exponentially with dose, and the proportionality could be explained by assuming that (1) depletion of cells by radiation followed a simple exponential curve with no shoulder, so that log surviving fraction of cells was inversely proportional to dose and (2) recovery of the response was due to these survivors proliferating exponentially without a lag and at a rate independent of the degree of depletion. Thus, if two or more agents behaving like radiation were used together and did not interact pharmacologically, the duration of the effect of the combination could reasonably be expected to be the sum of the durations of the effects of its constituents. However, no other agent has so far been found to resemble radiation in showing simple proportionality between dose and duration of immunosuppression.

It appears then that the ideas underlying a good deal of work on drug interactions, and certainly most work on drug interactions in immunology, are wrong. Except in special circumstances, where the effect measured is known to be simply proportional to dose, the effect of a combination of drugs can rarely if ever be expected to be the sum of the effects of its constituents and, except in these circumstances, the assumption that it should be leads to absurd conclusions.

However, it is easy to avoid the snare represented by this approach. The proper way to compare different agents having the same effect and non-linear dose-effect curves is to find what amount or concentration of each produces the same quantitative effect, i.e., to titrate them, a procedure long familiar to immunologists. Titration of different agents to the same, easily identifiable end-point is performed as readily with combinations of agents as with single ones and it can therefore be used to compare the effectiveness of combinations of drugs with the effectiveness of their constituents. This approach avoids the pitfall of non-linearity of dose-effect relations and, as will now be shown, it enables the formulation of unequivocal definitions of synergy, additiveness and antagonism which can be described in simple mathematical terms.

## METHODS FOR DETERMINING THE NATURE OF DRUG INTERACTIONS

*Algebraic method.* It is self-evident that combinations of various doses of one and the same drug cannot produce effects greater or less than those expected from its dose-effect curve, i.e., such combinations can only show additive effects; they cannot be synergistic or antagonistic.

Consider, for example, a drug that doubles the survival time of skin allografts when given in a dose of 10 mg. We put this drug into two containers, labelled A and B, and conduct an experiment to find out what combinations of A and B will produce a given quantitative effect (in this case, doubling of graft-survival time). Of course, we find that this specified effect is produced by 10 mg of sample A, or 10 mg of sample B, or 5 mg A + 5 mg B, or 1 mg A + 9 mg B, and so on. Expressing this algebraically, let the doses of A and B producing the same quantitative effect (the equi-effective doses) be  $A_e$  and  $B_e$  respectively (in the example we are considering,  $A_e = B_e = 10$  mg). Then, if the relation between A and B is additive, our example shows that this specified effect will also be produced by any combination of A and B such that:

$$\frac{\text{dose of A}}{A_e} + \frac{\text{dose of B}}{B_e} = 1. \quad (\text{Equation 1})$$

Suppose now that sample B is mixed with equal parts of inert material which has no effect itself and does not influence the effect of the drug. Again, there can be neither synergy nor antagonism between samples A and B, but only additive effects. Now,  $A_e$  is 10 mg and  $B_e$  20 mg, and a doubling of graft survival time is now produced by 10 mg of A, 20 mg of B, or by such combinations as 5 mg A + 10 mg B, 7 mg A + 6 mg B, 1 mg A + 18 mg B, and so on. Equation (1) evidently still holds, so that the fact that the two samples now have different equi-effective doses does not alter the algebraic description of their

additivism. Additive relations between pairs of drugs can therefore be expressed by equation (1), whether their equi-effective doses are the same or not.

Now, suppose that A and B are not samples of one and the same drug but of two different drugs, which may show additivism, synergy or antagonism, as the case may be. If the relation between A and B is additive, it will satisfy equation (1). If it is synergistic, the drugs will be more effective in combination than separately; therefore, smaller than expected fractions of  $A_e$  and  $B_e$  given together will produce the same effect as either of them given alone, so that the sum of the fractions in equation (1) is less than 1. If the relation is antagonistic, greater than expected fractions of  $A_e$  and  $B_e$  are needed to produce this effect when they are given together than when either is given separately, so that the sum of the fractions exceeds 1.

The method may be illustrated by the data in Table 1 for suppression of antibody production in mice, taken from Bieber *et al.* (1962). We first find some effect that is produced by a combination and by each drug alone in the dose-range covered. For thioguanine and thiodeoxyuridine (Table 1a) we shall use depression of the 'antibody index' to 0.37 of control levels, an effect produced by a combination of 0.3 mg/kg thioguanine and 50 mg/kg thiodeoxyuridine. The equi-effective doses for this effect, found by interpolation in the respective dose-effect curves, are 0.9 mg/kg thioguanine and 275 mg/kg thiodeoxyuridine. Substituting in equation (1), we have, for this specified effect:

$$\frac{0.3}{0.9} + \frac{50}{275} = 0.52.$$

As the sum of these fractions is  $<1$ , this dose-combination is synergistic.

Similarly, by interpolation, Table 1b suggests that the doses of azathioprine and bromodeoxyuridine that depress the response to 0.58 of controls are about 37 mg/kg and 9 mg/kg respectively. In combination, this same effect is produced by 20 mg/kg azathioprine with 10 mg/kg bromodeoxyuridine. The respective fractions of the equi-effective doses here are 0.54 and 1.11. This combination therefore shows antagonism, for the sum of these fractions is greater than 1.

It will be obvious that the sum of fractions calculated in this way allows one to quantitate the degree

TABLE 1. (a) Synergy of 6-thioguanine and 4-thiodeoxyuridine and (b) antagonism of azathioprine and 5-bromodeoxyuridine in inhibiting haemagglutinin response of mice to sheep red cells. Values are 'antibody indices' (Bieber *et al.*, 1962)

(a) Dose of thiodeoxyuridine (mg/kg)	Dose of thioguanine (mg/kg)			
	0	0.3	1.0	3.0
0	1.00	0.77	0.33	0.19
50	0.72	0.37	0.16	
150	0.52	0.31	0.13	
450	0.31			
(b) Dose of bromodeoxyuridine (mg/kg)	Dose of azathioprine (mg/kg)			
	0	7	20	60
0	1.00	0.92	0.72	0.48
3	0.93	0.72	0.79	0.48
10	0.56	0.69	0.58	0.42
30	0.31	0.56	0.46	0.30

of synergy or antagonism shown by a drug combination and thus provides a useful yardstick for comparing different combinations.

These examples illustrate some of the problems in determining the nature of a drug interaction from examination of tabulated results. Generally, the equi-effective doses must be found by interpolation, and it is almost impossible from a table to envisage the nature of the drug interaction over the whole dose range investigated, especially when many dose-combinations are studied.

*Geometric method. Isoholes.* The algebraic method described above has an elegant geometric counterpart. If combinations of drugs A and B are represented by points in a Cartesian plane, the two axes of which represent doses of A and B, then equation (1), describing the additive relationship, is the equation of the straight line joining  $A_e$  and  $B_e$  (for example, the straight line in Fig. 2). Therefore, whether any particular dose-combination is additive, synergistic or antagonistic is shown immediately by whether

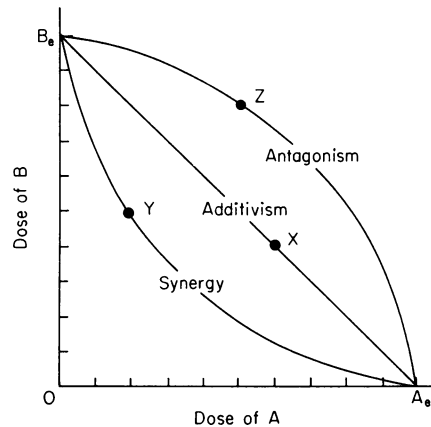


FIG. 2. Isoholes of additivism, synergy and antagonism.  $A_e$  and  $B_e$  are doses of drugs A and B that produce an equal effect, and the three isoboles shown are lines joining points representing dose-combinations of equal effect. For all combinations of A and B represented by points on the additivism isobole, which is the straight line joining  $A_e$  and  $B_e$ , the sum of the fractions of  $A_e$  and  $B_e$  constituting the combination equals 1 (e.g., combination X consists of  $0.6 A_e + 0.4 B_e$ , and so on). For all combinations on the synergy isobole, which is concave, the sum of these fractions is  $< 1$  (e.g., combination Y consists of  $0.2 A_e + 0.5 B_e$ ). For combinations on the antagonism isobole, which is convex, the sum of fractions is  $> 1$  (e.g. combination Z consists of  $0.5 A_e + 0.8 B_e$ ).

the point representing that combination lies on, below or above the straight line joining the doses  $A_e$  and  $B_e$  of the two drugs that, when given alone, produce the same effect as that combination. When a dose combination is synergistic the sum of the fractions in equation (1) is  $< 1$ , and therefore the point representing the combination lies below the line (e.g., combination Y in Fig. 2); when it is antagonistic, the sum of these fractions is  $> 1$  and the point representing it lies above the line (e.g., combination Z in Fig. 2). The line joining all doses and dose combinations producing any given quantitative effect is termed the isobole for that effect. When all the dose combinations producing that effect are synergistic, the isobole is concave; when they are antagonistic, the isobole is convex (Fig. 2).

The term 'isobole' was coined by Loewe & Muischnek (1926), who described the characteristic isoboles for synergy, additivism and antagonism, and their application has been discussed in detail by Loewe (1928; 1953; 1957) and de Jongh (1961). However, the method is a good deal older than this, for Fraser (1870-1; 1872) was the first to realize the convenience and expository power of representing drug interactions in this way, and who first illustrated an isobole showing drug antagonism.

The data in Table 1 may be used again to illustrate the graphic method. With combinations of thio-deoxyuridine and thioguanine, it is evident that the isoboles for antibody responses 0.2, 0.3 and 0.5 of normal are concave, showing that these two drugs act synergistically. With combinations of azathioprine and bromodeoxyuridine, the isoboles for responses 0.5 and 0.6 of controls are markedly convex, showing antagonism (Fig. 3).

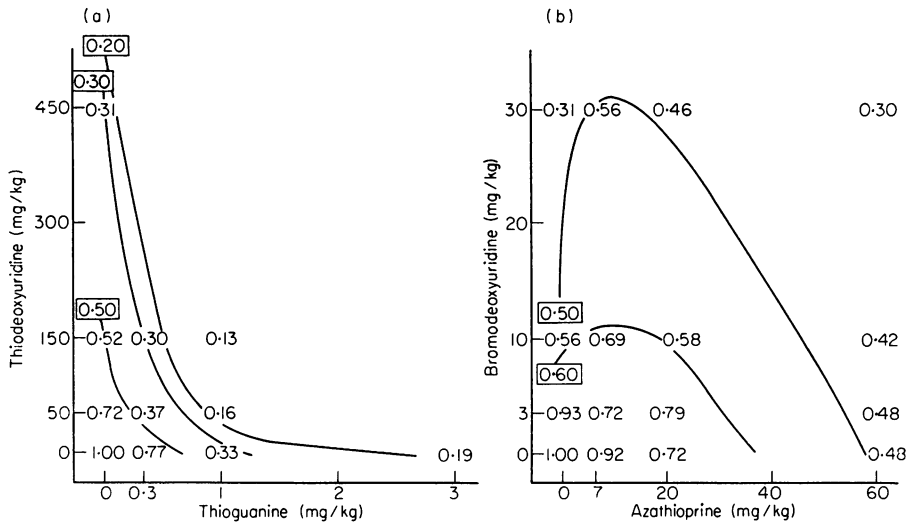


FIG. 3. Isoboles showing (a) synergy of thioguanine and thiooxyuridine and (b) antagonism of azathioprine and bromodeoxyuridine in inhibiting antibody production in mice. Data of Bieber *et al.*, (1962) as in Table 1. Isoboles have been fitted by eye. Boxed figures at the ends of isoboles indicate the effects produced by dose-combinations lying on the respective isoboles.

The advantage of the graphic over the algebraic method is that it makes it possible to depict in readily comprehensible form the interactions of two agents in several dose-combinations. In fact, the more combinations used, the more confidence can be attached to the isoboles and the more informative they are. Plotting isoboles makes it easy to identify combinations of maximum synergistic or antagonistic effect, to show which parts of the dose-combination map are worth more detailed exploration and to locate combinations with anomalous effects. Its disadvantage is that it is readily used only with combinations of two agents: application to combinations of three agents is cumbersome, for the graph is in three dimensions (see below), and it is not applicable to combinations of four or more agents. The algebraic method has no such limitations.

*Combinations of three or more agents.* The argument used earlier for two samples of the same drug, one of which was mixed with inert material, can be extended to any number of samples mixed with different amounts of inert material. So, equation (1) can be generalized for use with any number of agents:

$$\frac{\text{dose of A}}{A_e} + \frac{\text{dose of B}}{B_e} + \frac{\text{dose of C}}{C_e} + \dots + \frac{\text{dose of X}}{X_e} = \begin{cases} < 1 & \text{for synergy} \\ 1 & \text{for additivism (Equation 2)} \\ > 1 & \text{for antagonism.} \end{cases}$$

For combinations of three agents, equation (2) becomes the equation of the plane passing through  $A_e$ ,  $B_e$  and  $C_e$  when doses of A, B and C respectively are represented by three coordinate axes. It follows that, if the interaction between drugs A, B and C is additive, and their doses are represented by points on three axes, then the points representing all doses and dose combinations with the same effect as  $A_e$ ,  $B_e$  or  $C_e$  will lie on the plane connecting these three points. (The two-drug combinations A-B, B-C and C-A are represented by the three lines bounding the plane) (Fig. 4). Synergistic combinations producing the same effect as  $A_e$ ,  $B_e$  or  $C_e$  are represented by points below the plane; when all dose combinations producing this effect show synergy, they make up a concave isobolar surface. Antagonistic combinations producing this effect are represented by points above the plane and, when they all show antagonism, the isobolar plane is convex.

*Logarithmic scale isoboles.* A problem arises in plotting isoboles when the drugs studied have very shallow dose-effect curves, for the doses used may extend over a wide range, sometimes several orders of magnitude. It is then impracticable to use linear scales for drug doses and logarithmic scales are

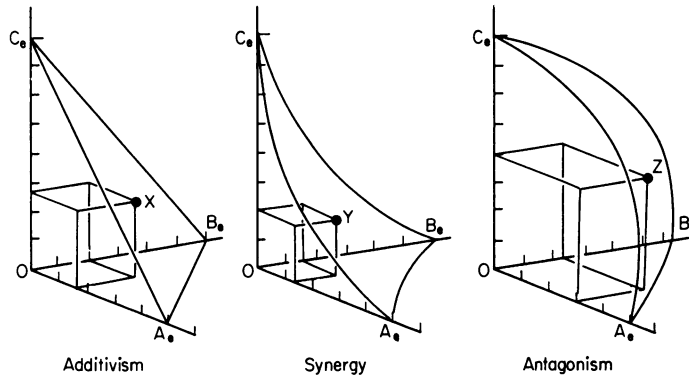


FIG. 4. Isobolar surfaces for additivism, synergy and antagonism with combinations of three agents, A, B and C.  $A_e$ ,  $B_e$  and  $C_e$  are doses producing an equal effect. The isobolar surface is flat when all combinations of A, B and C producing that effect are additive, and the sum of fractions of  $A_e$ ,  $B_e$  and  $C_e$  constituting any combination on this surface equals 1 (e.g., combination X consists of  $0.33 A_e + 0.33 B_e + 0.33 C_e$ ). The isobolar surface for synergy is concave and the sum of these fractions is  $< 1$  (e.g., combination Y consists of  $0.25 A_e + 0.25 B_e + 0.25 C_e$ ). The isobolar surface for antagonism is convex and the sum of fractions is  $> 1$  (e.g., combination Z consists of  $0.6 A_e + 0.4 B_e + 0.5 C_e$ ).

preferred. When isoboles are plotted on such scales, they are greatly distorted. Figure 5 illustrates this effect, and shows that the additive isobole becomes markedly convex on a log-log plot and that any less marked convexity, or a straight line, indicates synergy. Examples of such isoboles are given elsewhere (Berenbaum, Cope & Bundick, 1977). It is in any case possible to demonstrate the nature of the drug interaction involved by transferring such isoboles back to the less convenient linear scales, or by measuring the sums of the fractions of the equi-effective doses which together form equi-effective combinations as described above.

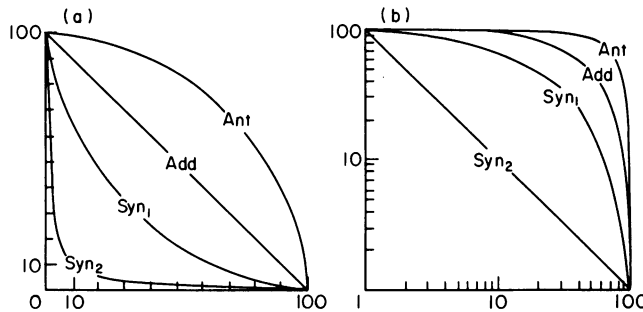


FIG. 5. Isoboles of additivism (Add), antagonism (Ant) and synergy, moderate ( $Syn_1$ ) or marked ( $Syn_2$ ) plotted on (a) linear and (b) logarithmic scales. When the logarithmic scale covers two or more decades, isoboles with anything less than a marked convexity indicate synergy.

### HETERERGIC COMBINATIONS

The interactions considered above are between drugs each of which can independently produce the effect being measured. Effects that all the constituents of a combination have in common are termed homergic (Loewe, 1957). It is clear that such agents may act either synergistically (Fig. 3a), additively or antagonistically (Fig. 3b) with respect to the effect they have in common.

We have now to consider interactions in which one agent increases or decreases the effect of another without itself being able to produce that effect. Effects produced by some but not all the constituents of a drug combination are termed heterergic (Loewe, 1957). One agent may, for example, delay or accelerate the activation, degradation or excretion of another agent, or sensitize cells to its action, without itself having the effect specific to that agent, or it may have an effect itself on the target cells which is the

opposite of the effect under consideration. For instance, the immunosuppressive effects of 6-mercaptopurine are increased by administration of the xanthine-oxidase inhibitor HPP (Elion *et al.*, 1963) or of duazomycin A (Rosenberg & Calabresi, 1963; Vogel & Calabresi, 1969), and the effect of 6-azauracil is said to be increased by chloramphenicol (Fischer, Cassidy & Welch, 1966), but it has been claimed that HPP, duazomycin A and chloramphenicol are not in themselves immunosuppressive. On the other hand, folinic acid antagonizes the immunosuppressive effects of methotrexate (Berenbaum & Brown, 1965), the microsomal enzyme inhibitor SKF-525A reduces the immunosuppressive effects of cyclophosphamide (in rats) (Berenbaum, Cope & Double, 1973) and cysteine reduces the immunosuppressive effects of nitrogen mustard (Addison & Berenbaum, 1971).

Claims that an effect is heterergic rather than homergic require to be made with caution for they depend on showing that the agent lacking the specific effect under consideration does not do so merely because of inadequate dosage. No claim so far is entirely satisfactory in this respect. In the work of Elion *et al.* (1963) and Fischer *et al.* (1966), both HPP and chloramphenicol showed weak immunosuppressive activity in the single experiments in which they were tested alone and, in the work of Rosenberg & Calabresi (1963) and Vogel & Calabresi (1969), duazomycin A was tested only in low and non-toxic dosage. Folinic acid, SKF-525A and cysteine have not so far been adequately investigated for possible immunosuppressive effects but this is perhaps unimportant in the present context, for the mechanisms by which they antagonize the effects of methotrexate, cyclophosphamide and nitrogen mustard are well understood.

In the pharmacological literature, heterergic effects have acquired their own special terminology (Loewe, 1957; de Jongh, 1961). Ignoring this, as it complicates matters unnecessarily, we may say that heterergic combinations, like homergic, may show synergy (when the immunologically ineffective agent increases the effect of the immunosuppressive agent), antagonism (when it reduces its effect) or additivism (when it has no modifying effect), and there is no difficulty in using equation (2) to analyse such cases. The fraction corresponding to the immunologically ineffective agent in equation (2) is zero as its equi-effective dose is theoretically infinite, and isoboles for such combinations do not intersect the dose-axis of the ineffective agent.

## EXPERIMENTAL DESIGN

### *Minimum requirements for demonstrating synergy or additivism*

Equation (2) shows that dose combinations claimed to show synergy must be made up of fractions of equi-effective doses of the agents used alone such that the sum of these fractions is less than 1. For dose combinations claimed to be additive, the sum of the fractions must be 1. It follows that, in experiments designed to investigate possible synergy or additivism, the dose of each agent in the combination(s) in question must be less than at least one of its doses used alone, otherwise the sum of the fractions can never be shown to be  $< 1$ . Therefore, each agent must be tested in at least the following three dose levels: (a) one level of the agent used alone; (b) one level, less than (a), used in a combination; (c) a nil-dose level (as a corollary of (a), if each agent is tested alone, each of its partners must be tested at a nil-dose level).

Similarly, to show that an isobole runs a concave or straight course between its two ends, we require at least three points, i.e., its ends (each representing a dose of each agent used alone with a nil dose of the other) and at least one intermediate point (representing a combination in which each agent is given in a dosage less than its dose used alone).

Experiments in which any of the agents is tested at less than these three levels can therefore never demonstrate synergy or additivism. This simple consideration alone is enough to invalidate the great majority of published claims for synergy in immunosuppression, for most of these are based on experiments in which each agent was tested in only two doses, including a nil dose, forming a  $2 \times 2$  array when plotted graphically (or a  $2 \times 2 \times 2$  array when three agents were used).

Consider, for example, the data of Bareham *et al.* (1974) referred to at the outset of this paper, in which 1 mg/kg MTX and 50 mg/kg FU together (the former given 30 min before the latter) sup-



pressed the response by 92.3%, and each given alone caused 35.9 and 35.4% suppression respectively. Substituting in equation (2) for 92.3% suppression we have:

$$\frac{1}{>1} + \frac{50}{>50} < 2.$$

This result does not permit us to draw any conclusions as to the nature of the interaction involved—it is consistent equally with synergy, additiveness and antagonism and, when the data are plotted graphically, it is evident that the shape of the isobole for the effect of the combination is quite indeterminate (Fig. 6).

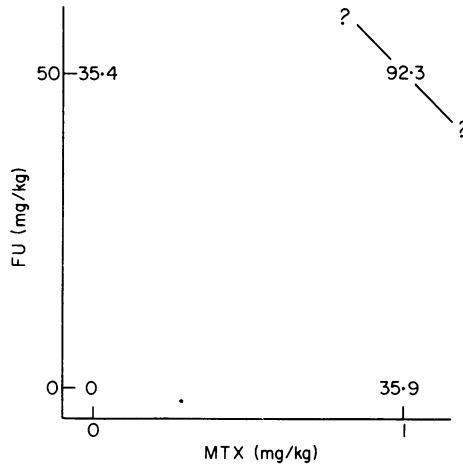


FIG. 6. Impossibility of demonstrating synergy with a  $2 \times 2$  array of dose-levels. Data of Bareham *et al.*, (1974) for suppression of plaque-forming cell response of mice by methotrexate and fluorouracil, the former being given 30 min before the latter. The shape of the isobole for 92.3% reduction in the response cannot be determined from the data.

The same consideration applies to all investigations in which one of the agents is tested at two levels only (including the nil-dose level), irrespective of the number of dose levels of the other(s). The work of Hansen *et al.* (1964) will illustrate this point. These authors claimed that azathioprine and azaserine acted synergistically in suppressing antibody production in the rat. Azaserine was tested at five dose levels but azathioprine at two only, 0 and 10 mg/kg. The impossibility of demonstrating synergy or additivism with this sort of experimental design is illustrated by Fig. 7. The ten groups form a  $2 \times 5$  rectangular array and therefore cannot provide the information required for drawing a concave or straight isobole, whatever the results.

#### Requirements for demonstrating antagonism

Antagonism is obviously present when a drug combination is less effective than one or more of its constituents (for instance, in Fig. 3b, the combinations of 7 mg/kg of azathioprine with 10 or 30 mg/kg bromodeoxyuridine have less effect than these doses of bromodeoxyuridine on their own). Positive claims to have demonstrated antagonism in immunosuppression usually refer to antagonism of this marked degree, which needs no elaborate procedure for its detection. In such cases, the isobole is so convex that it forms an angle of more than  $90^\circ$  with one or more of the dose-axes (Fig. 3b, Fig. 10).

The situation is not so simple when antagonism is less marked and the combination is more effective than any of its constituents used alone. Antagonism is then detectable only by making measurements sufficient to show that the sum of fractions in equation (2) is  $> 1$  or that the isobole for the effect of the combination is convex. This sort of situation causes great confusion when incorrect criteria are used for

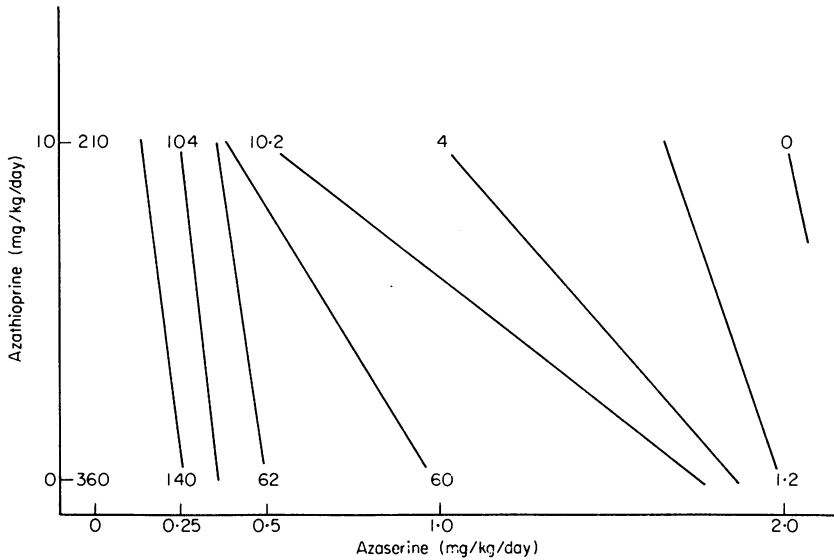


FIG. 7. Impossibility of demonstrating synergy with a  $2 \times n$  array of dose-levels. Data of Hansen *et al.* (1964) for suppression of production of antibody to a mouse tumour in the rat by combinations of azathioprine and azaserine. Values are titres  $\times 10^{-6}$ . The approximate directions of the lower parts of the isoboles can be guessed (they are drawn here as straight lines although there is no evidence from the data that they must be so), but the ends of these isoboles on the azathioprine dose-axis, and therefore their overall shapes, are quite indeterminate. Although as many as ten groups of animals were used, the experimental design (a  $2 \times 5$  array) has made it impossible to demonstrate synergy.

analysing drug interactions. For example, Roth, Friedman & Syverton (1957) found that *Candida*-infected mice given cortisone and radiation together had a shorter survival time and higher mortality than mice given either agent alone, and they concluded from this that the combination was synergistic. Their results are shown in Fig. 8a and b, and it is clear from the convexity of the isoboles that the combination was, in fact, antagonistic, a conclusion that may readily be confirmed by appropriate use of equation (2). It should be emphasized further that a combination may be antagonistic even when its effect exceeds the sum of the effects of its constituents, as shown in Fig. 8c. This shows again, if any further demonstration were needed, that drug interactions cannot usefully be examined by comparing the effect of a combination with the sum of the effects of its constituents.

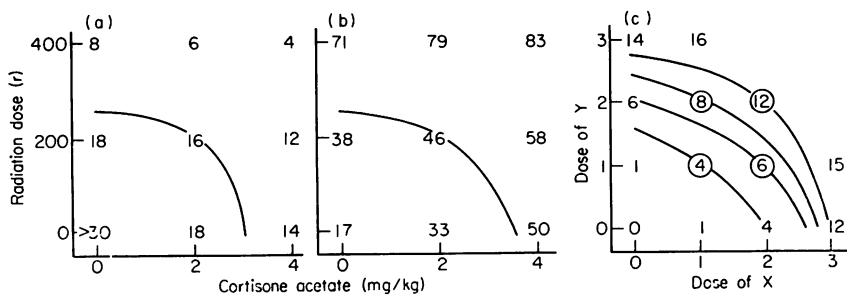


FIG. 8. Antagonism may be present when the effect of a combination exceeds that of any of its constituents or even the sum of their effects. (a) and (b) show the effect of cortisone acetate and radiation on mean survival time in days (a) and per cent mortality (b) in mice infected with *Candida albicans* (Roth *et al.*, 1957). The authors concluded that the combinations were synergistic because their effects exceeded those of their constituents, but the convex isoboles show that they were, in fact, antagonistic. (c) The four hypothetical combinations represented by the ringed points are all antagonistic, as shown by convex isoboles, but the effect of each exceeds the sum of the effects of its constituents.

*Determination from incomplete data*

Ideally, experiments on drug interactions should be designed to allow determination of equi-effective doses for all the agents used, which means constructing a sufficiently detailed dose-effect curve for each agent. However, it is often possible to demonstrate synergy or antagonism without going to these lengths, as all we require to know is whether the sum of the fractions in equation (2) is less or more than 1. We may be sure that this is the case if the minimum or maximum limits for doses of the agents used alone that are equi-effective with the combination examined are sufficiently high or low, even if we do not know their values more precisely.

A case in point is provided by the data of Friedman, Gelfand & Bernheimer (1971), who measured

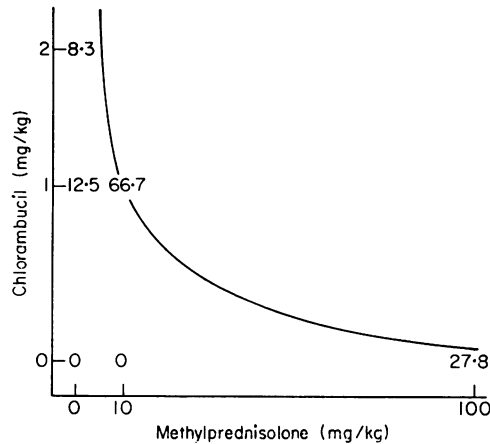


FIG. 9. Demonstration of synergy with incomplete data. Effect of chlorambucil and methylprednisolone on tetanus antitoxin production in mice. Selected data of Friedman *et al.* (1971). Values are percentages of animals in which drugs prevented the protective effect of immunization. The isobole for 66.7% prevention must be concave, indicating synergy, although the doses of the two drugs that produce this effect on their own were not determined.

the effect of chlorambucil and methylprednisolone on tetanus antitoxin production in mice (assayed by protection against toxin). The data are illustrated in Fig. 9. Abolition of protection in 66.7% of animals is obtained with a combination of 1 mg/kg chlorambucil and 10 mg/kg methylprednisolone but, if these drugs are used alone, it would require considerably more than 2 mg/kg chlorambucil or 100 mg/kg methylprednisolone to produce the same effect. Substituting in equation (2) we have:

$$\frac{1}{>2} + \frac{10}{>100} = <0.6.$$

As this sum is less than 1 (and the isobole for 66.7% protection is correspondingly concave), this combination is undoubtedly synergistic, although these results do not enable us to say what doses of the two agents alone would produce this effect nor, indeed, do they enable us to determine the equi-effective doses for any effect.

An even simpler experimental design may suffice to prove the existence of antagonism. Consider, for example, the data of Bareham *et al.* (1974) for the effect of 5-fluorouracil and methotrexate on the antibody response of mice to sheep red cells, illustrated in Fig. 10. A 12.4% suppression of the response is produced by 1 mg/kg of methotrexate with 50 mg/kg fluorouracil provided the former is given 30 min after the latter. We do not know what doses of these drugs separately have this effect, only that they are respectively, less than 1 mg/kg methotrexate and less than 50 mg/kg fluorouracil. Substituting in equation (2) we have:

$$\frac{50}{<50} + \frac{1}{<1} > 2.$$

As this sum exceeds 1, the combination is antagonistic, as shown also by the marked convexity of the isobole for this effect.

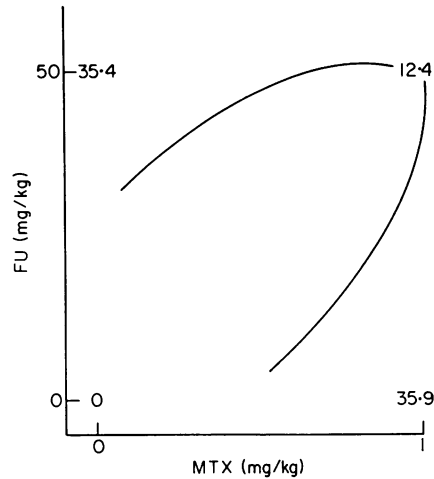


FIG. 10. Demonstration of antagonism with a  $2 \times 2$  array of dose-levels. Data of Bareham *et al.* (1974) for suppression of plaque-forming cell response of mice by methotrexate and fluorouracil, the former being given 30 min after the latter. The isobole for 12.4% reduction in the response must be markedly convex, showing antagonism, although the doses of the two agents that produce this effect on their own were not determined.

However, in most published work on drug interactions in immunosuppression, the results do not allow us to obtain a value for the sum of the fractions that is unequivocally less or greater than 1.

#### *Efficiency and economy of experimental design. Diagonal arrays*

Inspection of Fig. 2 shows that, for combinations of two agents, departure from linearity of the isobole for any specified effect would most efficiently be detected by testing the combination at the mid-point of the straight line joining the equi-effective doses, i.e., that made up of half the equi-effective dose of each agent. For combinations of three agents, Fig. 4 shows that concavity or convexity of the isobolar surface is detected most easily by testing the combination at the mid-point of the flat plane joining equi-effective doses i.e., that made up of one-third of the equi-effective dose of each agent. In either case, the degree of synergy or antagonism is measured by titrating along the line joining the point in question to the origin, the arrangement thus constituting what will be termed a diagonal array. In general, therefore, the most efficient and economical procedure for  $n$  agents is as follows:

(a) Test each agent alone at several dose levels. Construct dose-effect curves and so find doses of the different agents that have equal specified effects (e.g., reduction of antibody levels to 0.5, 0.1 and 0.01 of controls, prolongation of graft survival by 5, 10 and 20 days, and so on). Usually these equi-effective doses must be found by interpolation in the dose-effect curve, and the confidence to be attached to them depends on the closeness of the dose levels tested, the errors of the measurements and the regularity of the curve.

(b) Test each agent again (1) at the above-determined equi-effective doses for one (or more) of the specified effects and (2) in combinations made up of  $1/n$  of the equi-effective dose of each agent, and of serially decreasing or increasing doses in the ratio of this combination (represented by the line passing through this combination and the origin) so as to find a combination that produces the specified effect. Determine the nature of the drug interaction by use of equation (2) or by plotting isoboles.

(c) Explore in more detail doses and dose-combinations found to be of interest in step (b).

## VALID CLAIMS FOR SYNERGY IN IMMUNOSUPPRESSION

Some fifty-six papers dealing with synergy or additiveness in immunosuppression have been examined, appearing principally in the main immunological journals over the past 25 years. These may be taken as representative of published work in this field and probably constitute the bulk of it. These papers incorporate 104 claims that particular combinations of immunosuppressive agents are synergistic and seventeen claims to have shown additivism. The great majority of these claims can be dismissed without further consideration because the experimental design used (a  $2 \times 2$  array or other  $2 \times n$  array) could not have demonstrated the existence of synergy or additivism even if they had been present. The most consistent offenders are workers in transplantation; these account for seventy-seven of the claims, only one of which is valid (Floersheim, 1969), and even this was based on the incorrect grounds that the effect of the combination exceeded that 'which would have been obtained by simple addition of the individual prolongations'.

In fifteen papers, the use of  $3 \times 3$  or higher order assays is reported, and the validity of the claims has been tested by using the results to calculate in each case the sum of fractions in equation (2). In three of the papers (Roth *et al.*, 1957; McKneally *et al.*, 1964; Fischer *et al.*, 1966), none of the combinations tested was synergistic according to the criteria used here. The remaining papers are summarized in Table 2. Fourteen of the claims for synergy are valid. In another seven cases, equi-effective doses for use in equation (2) had to be found by interpolation between widely spaced dose levels. They are therefore approximate only and the sum of fractions is accordingly uncertain but, where it is probably  $< 1$ , the claim for synergy has been deemed to be probably valid.

Perhaps the most important conclusion to be drawn from Table 2 (and the papers excluded from it) is that the paucity of valid claims for synergy in immunosuppression may be due mainly to the widespread use of inappropriate experimental designs. When appropriate designs are employed, commonly used immunosuppressive agents quite often show synergy. It should be emphasized, however, that many of the claims deemed to be valid or probably valid were based on single experiments and/or single dose-combinations in experimental systems of notorious inherent variability, and they therefore require confirmation.

There is little point in detailing here the invalid claims that constitute the vast majority. However, it is worth noting that, in the papers reviewed, only one of the groups of workers investigating the potentially promising manoeuvre of treating transplant recipients with combinations of donor antigen and immunosuppressive agents, especially ALG, adopted an experimental design capable of demonstrating synergy. Yet their claims to have shown that such combinations are synergistic underlie the belief that 'this is probably the next major application of antilymphoid globulin' (Monaco & Codish, 1976). (The sole exception was that of McKneally *et al.* (1964), and their claim that azathioprine and radiation acted synergistically with donor antigen was invalid.) It should also be pointed out that at least one current hypothesis about the mechanism of immunological enhancement (Davies, 1976) is said to be supported by the finding that enhancing antisera and ALG act synergistically (Russell, 1971; Batchelor, Fabre & Morris, 1972). However, these claims were based on experiments with  $2 \times 2$  arrays of dose levels which, as shown above, cannot detect synergy.

Of course, these criticisms of the bulk of published work do not imply that combinations claimed to be synergistic were not so, but that the experimental designs used were incapable of demonstrating the fact.

As explained earlier, the detection of marked antagonism presents little difficulty; claims to have demonstrated this are generally correct and will not be examined further here. It should be noted, however, that the confusion in this field is so pervasive that it leads some authors to overlook (Cerilli & Hattan, 1972) or positively to deny (Koene *et al.*, 1975, Table 3) the existence of antagonism shown in their experiments, and even (Roth *et al.*, 1957) to claim that clearly antagonistic combinations are synergistic (Fig. 8).

TABLE 2. Valid claims for synergy in immunosuppression

Reference	Experimental system	Agents	Experimental design*	Combinations tested	Relevant combinations†	Sum of fractions‡
<i>Antibody production</i>						
Bieber <i>et al.</i> (1962)	CD mouse, SRC agglutinins	6MP, BUdR	4×4	9	4	0.75, <0.67, 0.75, <0.43
Table 1	CD mouse, SRC agglutinins	6TG, ThioUdR	4×4	4	4	<0.60, <0.67, 0.52, <0.44
Table 2	C57BL mouse, SRC agglutinins	6CP, HPP	4×3	6	2	<0.46, <0.68
Elion <i>et al.</i> (1963)	Swiss mouse, antitetanus immunity	MePr, Chlor	6×6	1	1	<0.25
Friedman <i>et al.</i> (1971)	Swiss mouse, antitetanus immunity	MePr, AzaT	6×6	1	1	<0.22
Tables 1, 2	Swiss mouse, antitetanus immunity	MePr, Rad	6×4	1	1	<0.46
Tables 1, 3	Swiss mouse, antitetanus immunity	AzaT, Rad	6×4	1	1	<0.58
Tables 1, 3	Swiss mouse, antitetanus immunity	MePr, AzaT, Chlor	6×6×6	1	1	<0.34
Tables 1, 2	Swiss mouse, antitetanus immunity	MePr, AzaT, Rad	6×6×4	1	1	<0.51
Tables 1, 3, 4	Swiss mouse, antitetanus immunity	AzaT, Chlor, Rad	6×6×4	1	1	<0.58
Tables 1, 3, 4	Swiss mouse, antitetanus immunity	MePr, AzaT, Chlor, Rad	6×6×6×4	1	1	<0.60
Hyman <i>et al.</i> (1975)	NZB/W mouse, antibody to poly I. poly C.	Cyclo, MTX	7×7	5	5	2.9, 0.89, 0.46, 0.81, 2.13

<i>Graft rejection</i>							
Floersheim (1969) Table 3	BALB/c skin to CBA	ALS, Ro-4/6824	4 × 3	6	2	< 0.79, < 1.07	
<i>Graft-vs-host disease</i>							
Glucksberg & Fefer (1973) Tables 1, 2	C57BL cells to BALB/c	Cyclo, Procarb.	5 × 5	1	1	< 0.58	
<i>Autoimmune disease</i>							
Gelfand & Steinberg (1972) Tables 1, 2	NZB/W mouse nephritis	AzaT, MePr, Cyclo	3 × 3 × 3	1	1	< 1.0	
Gelfand <i>et al.</i> (1972) Table 1							
<i>Resistance to infection</i>							
Syverton <i>et al.</i> (1952) Fig. 2	Swiss mouse, Coxsackie virus, 30-day survival	Rad, Cort Ac	6 × 6	4	4	0.37, 0.42, 0.52, 0.65	
<i>In vitro systems</i>							
Mendelsohn <i>et al.</i> (1973) Table 1	Human lymphocytes, PHA response	Cortisol, DBcAMP	3 × 3	4	1	0.32	
Table 1	Human lymphocytes, unstimulated, thymidine uptake	Cortisol, DBcAMP	3 × 3	4	1	0.19	
Schrek & Stefani (1976) Table 3	Human lymphocytes, unstimulated, 5-day survival	CArab, Rad	3 × 3	4	1	< 0.66	
Berenbaum <i>et al.</i> (1977) Fig. 1	Human lymphocytes, PHA response	Cortisol, PGE <sub>2</sub>	4 × 6	15	8	90% of values < 1; 50% < 0.25	

Where more than one relevant combination was tested, fractions are given in the order of the combinations in an isobologram, from top left to bottom right, the dose-scale of the first named agent along the abscissa. Fractions in bold type show valid claims for synergy, i.e., doses of the individual agents equi-effective with the combinations tested were found by interpolation between reasonably close dose-levels or their lower limits could be determined. Fractions < 1 not in bold type show claims deemed probably valid, i.e., lower limits for the equi-effective doses could not be determined or dose levels were too widely spaced for confident interpolation. AzaT = azathioprine; BUdR = 5 bromodeoxyuridine; CArab = cytosine arabinoside; Chlor = chlorambucil; 6CP = 6-chloropurine; Cyclo = cyclophosphamide; Cort Ac = cortisone acetate; DBcAMP = dibutyl cyclic AMP; HPP = 4-hydroxypyrazolo (3,4-d) pyrimidine; MePr = methylprednisolone; 6MP = 6-mercaptopurine; MTX = methotrexate; Procarb = procarbazine; Rad = radiation; ThioUdR = 4-thiodeoxyuridine; 6TG = 6-thioguanine; SRC = sheep red cells.

\* Number of dose-levels of each agent, including nil dose, in same order as in preceding column.

† Combinations that include the maximum dose level of any agent are irrelevant for determination of synergy (see text).

‡ Calculated by equation (2).

## CLINICAL IMPLICATIONS

The demonstration that a particular combination of drugs shows synergy in the effect being measured does not guarantee that it will show synergy with regard to other effects. Indeed, if all the biological effects of the combination showed equal degrees of synergy or antagonism, there could be no therapeutic gain or loss. Synergy in the pharmacological sense would be disadvantageous and antagonism advantageous if they were more marked for the toxic than the therapeutic effects of the drugs used. It might seem therefore that, in practice, we need not be concerned unduly with the detailed analysis of drug interactions and that what is important is simply the relation between the therapeutic and toxic effects of various dose-combinations, which may be determined without elaborately measuring equi-effective doses, plotting isoboles, and so on. Certainly, information of great practical use may be obtained in this way, but this is not an argument for restricting investigations to this simple level, but for extending them. If a dose combination of two or more agents, chosen more or less at random from the innumerable possible combinations of those agents, turns out to be therapeutically better than some other combination, is it not highly probable that other combinations, not tested, will be better still? Indeed, the laws of chance, and the fact that isoboles for qualitatively different effects such as immunosuppression and

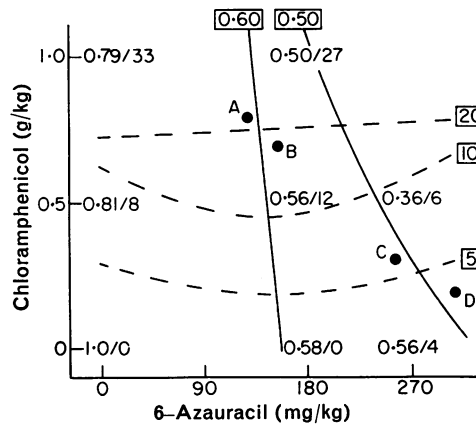


FIG. 11. Immunosuppressive and toxic effects of chloramphenicol and 6-azauracil in the mouse. (Data of Fischer *et al.*, 1966.) In each pair of values, the first indicates the 'antibody index' (Bieber *et al.*, 1962) for the response to sheep red cells, the second the percent mortality. Isoboles for antibody response (—), mortality (---). Boxed figures indicate the effect produced by dose-combinations on the isobole.

toxicity are not parallel, make this a virtual certainty. Consider, for instance, the experiments of Fischer *et al.* (1966), who measured both the immunosuppressive and toxic effects of combinations of chloramphenicol and 6-azauracil in mice. Their results are illustrated in Fig. 11, which shows isoboles for the degree of immunosuppression and for mortality. It is immediately apparent, from the positions and directions of these isoboles, that combinations A, B, C and D for example, in that order, show not only increasing immunosuppressive effects but decreasing toxicity and therefore that, in searching for combinations of these two drugs of optimal therapeutic effectiveness, the region most profitable to explore is the bottom right-hand corner of the graph and the area to the right of this. It is unlikely that this conclusion would easily have been reached as a result simply of comparing one or two randomly selected combinations. Optimum therapy therefore requires the exploration of a range of dose combinations sufficient to provide the information for constructing a map of isoboles for both therapeutic and toxic effects. Further, without constructing such maps, it may be difficult to detect, or even suspect, the existence of markedly anomalous effects produced in particular ranges of dose-combinations. This consideration is especially important when it is proposed to explore in man therapeutic regimens suggested by work in laboratory animals.



Many substances found *in vivo* have immunosuppressive effects, for instance, corticosteroids, prostaglandins, some tumour products (such as  $\alpha$ -foeto-protein), certain  $\alpha$ -globulins, fibrin-degradation products, interferon and probably other, as yet, unidentified materials. In view of the frequency of immunological impairment found in malignant disease, in some chronic non-malignant diseases, after trauma, during pregnancy and so on, the possibility that some of these substances act synergistically to cause clinically significant immunosuppression requires examination (Berenbaum, Cope & Bundick, 1977). The relation between immunosuppressive and toxic effects is irrelevant here and, in any case, most of these substances are not significantly toxic at levels occurring *in vivo*. Investigation of the possibility that depressed immunity *in vivo* is due to synergistic interactions of a number of naturally occurring substances requires exploration of the whole range of concentrations found *in vivo*.

Lastly, it is not only for scientific reasons that the study of synergy is important. Cost and shortage of supply are potent factors (although not the only ones) in limiting the dosage in man of at least one agent, ALG. Indeed, this limitation may be partly responsible for the doubtful therapeutic status of this agent in man, for there is no doubt of its effectiveness in laboratory animals which are given proportionately much higher doses. ALG of greater selectivity and less toxicity than at present available, and therefore potentially capable of being given in larger doses, will doubtless be even more expensive and limited in supply, and the same may be true of sera with special selectivities, e.g., for particular sub-populations of T cells or B cells, that may eventually be required clinically. There is thus a direct economic motive in searching for synergistic combinations of ALG and other antisera with other agents, so as to permit the use of smaller amounts of serum, even where toxicity is not a limiting factor.

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#### REFERENCES

- ADDISON, I.E. & BERENBAUM, M.C. (1971) The effect of cysteine on the immunosuppressive activity of busulphan, cyclophosphamide and nitrogen mustard. *Brit. J. Cancer* **25**, 172.
- ARIÈNS, E.J. (ed.) (1964) *Molecular Pharmacology. The Mode of Action of Biologically Active Compounds, volume 1* Academic Press, New York.
- BAREHAM, C.R., GRISWOLD, D.E. & CALABRESI, P. (1974) Synergism of methotrexate with Imuran and with 5-fluorouracil and their effects on hemolysin plaque-forming cell production in the mouse. *Cancer Res.* **34**, 571.
- BATCHELOR, J.R., FABRE, J. & MORRIS P.J. (1972) Passive enhancement of kidney allografts. Potentiation with anti-thymocyte serum. *Transplantation*, **13**, 610.
- BERENBAUM, M.C. (1969) Dose-response curves for agents that impair cell reproductive integrity. A fundamental difference between dose-response curves for antimetabolites and those for radiation and alkylating agents. *Brit. J. Cancer*, **23**, 426.
- BERENBAUM, M.C. & BROWN, I.N. (1965) The effect of delayed administration of folic acid on immunological inhibition by methotrexate. *Immunology*, **8**, 251.
- BERENBAUM, M.C., COPE, W.A. & BUNDICK, R.V. (1977) Synergistic effect of cortisol and prostaglandin E<sub>2</sub> on the PHA response. Relation to immunosuppression induced by trauma. *Clin. exp. Immunol.* (In Press.)
- BERENBAUM, M.C., COPE, W.A. & DOUBLE, J.A. (1973) The effect of microsomal enzyme inhibition on the immunosuppressive and toxic effects of cyclophosphamide. *Clin. exp. Immunol.* **14**, 257.
- BIEBER, S., ELION G.B., HITCHINGS, G.H., HOOPER, D.C. & NATHAN, H.C. (1962) Suppression of the immune response by drugs in combination. *Proc. Soc. exp. Biol. (N.Y.)*, **111**, 334.
- BRANT, L. & MEDAWAR, P. (1966) Quantitative studies on tissue transplantation immunity. VIII. The effects of irradiation. *Proc. roy. Soc. B.* **165**, 413.
- CERILLI, J. & HATTAN, D. (1972) The effect of combined immunosuppressive agents and cell-free antigen on xenograft survival. *Transplantation*, **14**, 207.
- DAVIES, D.A.L. (1976) The true mechanism of immunologic enhancement, deduced from studies of heart transplants in rats. *Transplant. Proc.* **8**, 37.
- ELION, G.B., CALLAHAN, S., NATHAN, M., BIEBER, S., RUNDLES, R.W. & HITCHINGS, G.H. (1963) Potentiation by inhibition of drug degradation: 6-substituted purines and xanthine oxidase. *Biochem. Pharmacol.* **12**, 85.
- FISCHER, D.S., CASSIDY, E.P. & WELCH, A.D. (1966) Immunosuppression by pyrimidine nucleoside analogs. *Biochem. Pharmacol.* **15**, 1013.
- FLOERSHEIM, G.L. (1969) A study of combined treatment with chemical immunosuppressants and antilymphocytic serum to prolong skin allograft survival. *Transplantation*, **8**, 392.
- FRASER, T.R. (1870-1) An experimental research on the antagonism between the actions of physostigma and atropia. *Proc. roy. Soc. Edinb.* **7**, 506.
- FRASER, T.R. (1872) The antagonism between the actions of active substances. *Brit. med. J.* **ii**, 485.
- FRIEDMAN, E.A., GELFAND, M. & BERNHEIMER, H.P. (1971) Synergism in immunosuppression. I. Effect of methylprednisolone, azathioprine, chlorambucil, and radiation on tetanus antitoxin production. *Transplantation*, **11**, 479.
- GELFAND, M.C. & STEINBERG, A.D. (1972) Therapeutic studies in NZB/W mice. II. Relative efficacy of azathioprine, cyclophosphamide and methylprednisolone. *Arthr. and Rheum.* **15**, 247.
- GELFAND, M.C., STEINBERG, A.D., NAGLE, R. & KNEP-SHIELD, J.H. (1972) Therapeutic studies in NZB/W mice. 1. Synergy of azathioprine, cyclophosphamide and methyl-

- prednisolone in combination. *Arthr. and Rheum.* 15, 239.
- GLUCKSBERG, H. & FEFER, A. (1973) Combination chemotherapy for clinically established graft-versus-host disease in mice. *Cancer Res.* 33, 859.
- GOLDIN, A. & MANTEL, N. (1957) The employment of combinations of drugs in the chemotherapy of neoplasia: a review. *Cancer Res.* 17, 635.
- HANSEN, H.J., VANDEVOORDE J.P., BENNETT K.J., GILES, W.G. & NADLER, S.B. (1964) Azaserine and thiopurines. 1. Inhibition of S-180 mouse tumor and antibody synthesis. *J. Lab. clin. Med.* 63, 801.
- HYMAN, L.R., KOVACS, K. & STEINBERG, A.D. (1975) Drug-induced tolerance: selective induction with immunosuppressive drugs and their synergistic interaction. *Int. Arch. Allergy*, 48, 248.
- DE JONGH, S.E. (1961) Isoboles. *Quantitative Methods in Pharmacology* (ed. by H. de Jonge), p. 318. North-Holland Publishing Co., Amsterdam.
- KOENE, R.A.P., GERLAG, P.G.G., MCKENZIE, I.F.C., HAGEMANN, J.F.H.M. & WIJDEVELD, P.G.A.B. (1975) Enhancement of skin grafts in the mouse. The combined use of specific and non-specific immunosuppression. *Transplantation* 20, 241.
- LOEWE, S. (1928) Die quantitativen Probleme der Pharmakologie. *Ergebn. Physiol.* 27, 47.
- LOEWE, S. (1953) The problem of synergism and antagonism of combined drugs. *Arzneimittelforsch.* 3, 285.
- LOEWE S. (1957) Antagonisms and antagonists. *Pharmacol. Rev.* 9, 237.
- LOEWE, S. & MUISCHNEK H. (1926) Über Kombinationswirkungen. 1. Mitteilung: Hilfsmittel der Fragestellung. *Arch. f. exper. Path. u. Pharm.* 114, 313.
- MCKNEALLY, M.F. KELLY, W.D., RESNICK, J. & MARTINEZ, C. (1964) Induction of tolerance with disrupted antigen, Imuran, and sublethal irradiation. *Surg. Forum* 15, 131.
- MENDELSON, J., MULTER, M.M. & BOONE R.F. (1973) Enhanced effects of prostaglandin E<sub>1</sub> and dibutyryl cyclic AMP upon human lymphocytes in the presence of cortisol. *J. clin. Invest.* 52, 2129.
- MONACO, A.P. & CODISH, S.D. (1976) Survey of the current status of the clinical uses of antilymphocyte serum. *Surg. Gyn. Obstet.* 142, 417.
- ROSENBERG, S. & CALABRESI, P. (1963) Enhanced suppression of the secondary immune response by combination of 6-mercaptopurine 'Duazomycin A'. *Nature (Lond.)*, 199, 1101.
- ROTH, F.J., FRIEDMAN, J. & SYVERTON, J.T. (1957) Effects of roentgen radiation and cortisone on susceptibility of mice to *Candida albicans*. *J. Immunol.* 78, 122.
- RUSSELL, P.S. (1971) Immunological enhancement. *Transplant. Proc.* 3, 960.
- SCHREK, R. & STEFANI, S.S. (1976) Cytarabine: cytotoxic effect on normal and leukemic lymphocytes. Synergism with X-rays and comparison with mechlorethamine. *Exper. mol. Path.* 24, 84.
- SILVERMAN, M.S. & CHIN, P.H. (1956) The effect of whole body X-irradiation of mice on immunity to tetanus toxoid. II. The delayed immune response to injections of tetanus toxoid. *J. Immunol.* 77, 266.
- SYVERTON, J.T., WERDER, A.A., FRIEDMAN, J., ROTH, F.J. JR., GRAHAM, A.B. & MIRA, O.J. (1952) Cortisone and roentgen radiation as synergistic agents for production of lethal infections. *Proc. Soc. exp. Biol. (N.Y.)*, 80, 123.
- TALIAFERRO, W.H. & TALIAFERRO, L.G. (1964) The relation of radiation dosage to enhancement, depression, and recovery of the initial Forssman hemolysin response in rabbits. *J. infect. Dis.* 114, 285.
- VOGEL, C.L. & CALABRESI, P. (1969) Enhanced suppression of experimental allergic encephalomyelitis by combination therapy with duazomycin-A and 6-mercaptopurine. *Proc. Soc. exp. Biol. (N.Y.)*, 131, 251.