Synergistic Interactions of Antibodies in Rate of Virus Neutralization

Pietro Paolo Sanna, Fernando Ramiro-Ibañez, and Alessandro De Logu

*Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037; and †Sezione di Microbiologia e Virologia, Dipartimento di Scienze Chirurgiche e Trapianti d’Organo Universita’ di Cagliari, Cagliari, Italy

Received November 29, 1999; returned to author for revision February 17, 2000; accepted February 24, 2000

Antibodies and antibody combinations are often evaluated only by their potency in inactivating a known quantity of virus in dose–effect assays. However, a crucial additional parameter is the rate at which neutralization takes place, or kinetics. Synergism of certain antibody combinations in dose–effect assays has been previously demonstrated. In the present report, using a battery of murine monoclonal antibodies to herpes simplex virus (HSV), we investigated whether antiviral antibodies can also synergize in neutralization kinetics. To determine whether synergism in dose–effect assays can predict synergism in neutralization rate, the ability of neutralizing antibodies to synergize in neutralization rate (kinetics) was compared to their ability to synergize in dose–effect assays (potency) in cell-free assays. Although certain antibody combinations synergized in both neutralization rate and potency, combinations that did not clearly synergize in potency could still significantly synergize in neutralization rate. Weak neutralizing antibodies could also greatly increase the neutralization rate of more potent antibodies. These results suggest that evaluating antibody combinations in dose–effect assays but not in neutralization kinetics provides a partial picture of neutralizing antibody dynamic interactions and may prevent the identification of certain favorable antibody combinations. These findings also support the importance of establishing defined antibody cocktails for prophylactic and therapeutic purposes. A simple strategy to evaluate antibody interactions in neutralization kinetics is proposed in which a quantitative prediction of additivity is made on the basis of the neutralization rate constants of the individual antibodies in the combination.

INTRODUCTION

Advances in antibody technology have greatly increased our ability to isolate human recombinant monoclonal antibodies and to humanize murine antibodies of therapeutic value (Barbas and Burton, 1996; Williamson et al., 1993). This has cleared the way for the potential use of selected monoclonal antibodies, or cocktails of monoclonal antibodies, in the prophylaxis and treatment of several viral human maladies. The potential therapeutic exploitation of passive immunization with human or humanized monoclonal antibodies of defined specificities makes the study of the dynamic interactions of antibodies of great significance, especially if implications relevant to their potential clinical use can be derived.

While a general role for antibodies in the prevention of viral infection, reinfection, and recovery from infection is widely accepted, the exact mechanisms by which antibodies confer protection in vivo remain a matter of controversy. This is at least in part due to the difficulty of teasing apart the roles of different antibody-mediated mechanisms such as cell-free virus neutralization, lytic and nonlytic mechanisms in different settings, and virus–host systems. In addition, different antibodies to the same virus can act through different mechanisms (see, for instance, Eis Hubinger et al., 1991; Lefrancois, 1984; Mester et al., 1991). However, great attention is usually given to the ability of antibodies to neutralize cell-free virus, and it appears reasonable that, at least in the prophylactic setting, this is likely to be a major protective mechanism (Moore and Burton, 1999).

Neutralization can be described in terms of potency, efficacy, and the rate of neutralization, or kinetics. Potency and efficacy are best represented by dose–effect (or log dose–effect) curves, in which the magnitudes of neutralization at different antibody concentrations are plotted against the different concentrations of the antibody or the log₁₀ of such concentrations, respectively. Pharmacologically, potency refers to the location of the dose–effect curve on the dose axis or, in other words, the concentration required to produce a defined effect (e.g., 50% of the maximal effect of a drug or other biological response modifier) (Goodman Gilman et al., 1985). For neutralizing antibodies, potency is usually defined as the concentration required to produce 50 or 80% reduction in infectivity. Efficacy—which is independent of potency—can be defined as the maximal response obtained on the y or response axis (Goodman Gilman et al., 1985). The ability to produce a maximal response (in the case of neutralization, total loss of infectivity) can therefore be
defined as 100% efficacy, while the presence of a non-neutralized or persistent fraction despite excess of antibody present indicates a less than 100% efficacy.

The kinetics (or the time course) of the neutralization reaction is described by plots in which the log10 of the residual infectivities at predetermined time points after the addition of a fixed concentration of antibody are plotted against time (Dulbecco et al., 1956). This plot, which also allows determination of the neutralization rate constant, has been utilized for several purposes, from serotyping of related viruses (Figueroa and Rawls, 1969; McBride, 1959), to gaining insight into the mechanisms of neutralization itself (Della Porta and Westaway, 1978; Dimmock, 1993; Dulbecco et al., 1956).

In the present study, using a battery of murine monoclonal antibodies to herpes simplex virus (HSV), we investigated whether antiviral antibodies can synergize in neutralization kinetics and whether synergism in dose–effect assays can predict synergism in neutralization kinetics. The ability of certain combinations of antibodies to display increased neutralizing potency (Lussenhop et al., 1988) or reduced persistent fractions and enhanced neutralization rate has been noted before (Howard et al., 1985; Kingsford, 1984). We have observed that certain antibody combinations synergized both in neutralization rate and in potency, while other combinations that did not clearly synergize in potency could still significantly synergize in neutralization rate. Finally, weak neutralizing antibodies could also greatly increase the neutralization rate of more potent antibodies. Representative situations encountered are reported. Examples of significant synergism in dose–effect neutralization assays were also demonstrated in some recent reports (Li et al., 1997, 1998; Mascola et al., 1997; Vijh-Warrier et al., 1996). However, the in vivo significance of neutralizing antibody synergism remains to be elucidated.

A simple strategy for evaluating quantitatively the dynamic interactions of antibodies in neutralization kinetics was devised. The neutralization rate constant observed for the antibody combinations (Kd) was compared to the predicted neutralization rate for additivity (Kp), and the isobologram analysis of Chou and Talalay, (1984) was then adapted to the neutralization rate to validate the conclusions reached. HSV was used as a model system of general relevance because of the large number of specific mAbs available against this agent (Eisenberg and Cohen, 1986; Muggeridge et al., 1990) and because of the potential use of antibodies of exceptional protective properties in the prophylaxis and, possibly, therapy of HSV infections (Co et al., 1991; Sanna et al., 1995, 1996). Combinations of two neutralizers displayed synergism or additivity/indifference in their rate of neutralization. Interestingly, significant increases in neutralization rate could sometimes be seen even when two neutralizers that did not clearly synergize in terms of potency in dose–effect neutralization experiments were combined. Last, certain weak neutralizers could substantially increase the neutralization rate of potent neutralizing antibody even at concentrations well below those needed to display significant neutralization when used individually.

These results suggest that evaluating antibody interactions by potency alone and not in neutralization kinetics may prevent the identification of certain favorable antibody combinations. This conclusion is of great relevance to the design of cocktails of monoclonal antibodies for the prophylaxis and therapy of viral disease in which synergism in neutralization rate could be a major benefit.

RESULTS

Quantitative prediction of residual infectivity using the neutralization kinetic constant

In neutralization kinetics, the neutralization kinetic constant relationship [Eq. (1); see Materials and Methods] allows for quantitative predictions of the residual infectivities (V/V0) at different antibody concentrations as described in the methods. As commonly seen, the kinetics obtained showed a leveling off at later time points. They were nevertheless interpreted as pseudo-first order since their initial linear segment extrapolated through the origin (Della Porta and Westaway, 1978; McLain and Dimmock, 1994). The kinetics constants were calculated for the initial linear segments (see Materials and Methods) as done by others (McLain and Dimmock, 1994). This is demonstrated in Fig. 1 with a monoclonal antibody (mAb) preparation. As expected, when the antibody was used alone at 10 μg/ml, the same kinetic constant was obtained as when it was used at 5 μg/ml in the presence of 5 μg/ml normal purified mouse antibody of the same subclass (IgG 2A) (Fig. 1). Consequently, as outlined under Materials and Methods, using the residual infectivity data obtained at 10 μg/ml an accurate prediction could be made of the residual infectivity obtainable at 5 μg/ml and vice versa (Fig. 1). Similarly, predictions of additivity for antibody combinations could also be carried out on the basis of the neutralization rate constants of the individual antibodies in the combination. To this aim, the product of the predicted residual infectivities of each antibody in a mixture at their concentration in the same mixture was used as a prediction of the residual infectivity of the mixture at additivity (see Materials and Methods and below).

Synergism of antibody combinations in both potency and rate of neutralization

H170 is an antibody directed to the group VII antigenic determinant of gD, while H128 is a mAb to the group Ib antigenic determinant of the same glycoprotein. The initial neutralization rates of these two antibodies on HSV-1
Synergism of antibody combinations in rate of neutralization but not in potency

H170 and H128 neutralized HSV-2 with higher neutralization rates and potencies on HSV-2 than on HSV-1 (Figs. 3A and 3D and Table 1). The efficacy of H170 was substantially higher on HSV-2 than on HSV-1, approaching 90% (Fig. 3C). As with HSV-1, a mixture containing 5 μg/ml of H128 and 5 μg/ml of H170 displayed a much greater rate of neutralization on HSV-2 than 10 μg/ml of each of the antibodies alone (Fig. 3A). The \( K_o \) was significantly higher \((P < 0.001)\) than the \( K_o \) (Table 1), as were the differences between \( K_a \) and the kinetics constant of either antibody alone \((P < 0.001)\). The \( K_o/K_a \) ratio of the combination was 0.56 ± 0.04, signifying a rate of neutralization almost double of that predicted by additivity \((P < 0.001)\). A CI of 0.58 ± 0.06 was obtained for this combination, consistent with synergism in rate of neutralization. Conversely, in dose–effect neutralization experiments (Figs. 3C and 3D and Table 1), the two antibodies only displayed additivity at the 50% neutralization end point \((P < 0.001)\).

These results demonstrate that antibodies that do not clearly synergize in potency can substantially synergize in rate of neutralization. Therefore, evaluating antibody interactions in dose–effect assays alone may prevent the identification of such potentially favorable antibody interactions.

Weak neutralizing antibodies can increase the neutralization rate of potent neutralizing antibodies

Whether weak neutralizing antibodies could synergize with more potent neutralizers was then addressed. For this purpose, several antibody combinations including mAb H170 with H222, a weak neutralizer specific for HSV-2 gE, were tested. In neutralization kinetics, the neutralization rate of potent neutralizing antibodies only displayed additivity at the 50% neutralization end point \((P = 0.25)\) and vice versa. The \( K_o \) of the mixture of H170 + H222 was significantly higher \((P < 0.001)\) than the \( K_o \) and the \( K_o/K_a \) ratio was 0.61 ± 0.05 \((P = 0.25)\).
FIG. 2. The combination of H170 and H128 synergized both in neutralization rate and in potency in HSV-1 neutralization. (A) As in Fig. 1, the kinetics (or the time course) of the neutralization reaction is described by plots in which the log_{10} of the residual infectivities (V/V_{0}) at predetermined time points after the addition of a fixed concentration of antibody are plotted against time. In neutralization kinetics of HSV-1, the combination of 5 μg/ml of H170 and 5 μg/ml of H128 was clearly synergistic. The neutralization rate of the mixture was approximately double of the predicted one as indicated by a K_{c}/K_{o} ratio of 0.52 ± 0.03. The difference between K_{c} and K_{o} was highly significant (P < 0.001); see also Table 1. A point-by-point prediction of additivity is also shown (dashed line, open squares). (B) When the neutralization rates observed for the combination and the antibodies alone were compared by isobologram analysis using an adaptation of the Chou and Talalay equation (see Materials and Methods) to the analysis of the neutralization rate, the interaction was also clearly synergistic. The predicted concentration for the combination required to obtain the same neutralization rate observed if the antibodies were additive, D_{c} (dashed line, open square), was significantly higher (P < 0.001) than the concentration of the antibodies in the mixture, D (solid line, black circle). A combination index (CI) of 0.52 ± 0.03 was obtained. (C) To evaluate the potency of neutralization of antibodies or antibody combinations, the number of plaques obtained at each antibody concentration in 1 h was plotted against the log_{10} of the antibody concentrations (log_{10} dose–effect curves) and expressed as percentage of virus neutralized. The combination of H170 and H128 showed clear synergism in potency, its profile laying to the left of both those of H128 and H170 alone. The combination also displayed 100% efficacy, as observed with HSV-2 (Fig. 3C). (D) Isobologram representation of the interaction of mAbs H128 and H170 at 50% neutralization of HSV-1. Error bars in the graphs represent SE.

Consistently, a CI of 0.66 ± 0.06 was calculated for the neutralization rate (Fig. 4B, Table 1). Therefore this antibody combination was deemed synergistic in neutralization rate. In dose-effect neutralization experiments (Fig. 4B), the combination was judged to be indifferent/additive, despite a nonsignificant trend toward synergism. In

### TABLE 1

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Virus serotype</th>
<th>K antibody alone</th>
<th>K combination observed (Ko)</th>
<th>K additivity predicted (Ko)</th>
<th>Ko vs. Ka difference</th>
<th>Ka/Ko ratio</th>
<th>Interaction (Kinetics)</th>
<th>CI potency (50%)</th>
<th>Interaction (Potency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H170</td>
<td>HSV-2</td>
<td>6.7 x 10^{4} ± 6.3 x 10^{3}</td>
<td>9.1 x 10^{4} ± 1.2 x 10^{3}</td>
<td>5.1 x 10^{4} ± 4.7 x 10^{3}</td>
<td>P &lt; 0.001</td>
<td>0.56 (±0.04)</td>
<td>0.58 (±0.06)</td>
<td>synergistic</td>
<td>0.94 (±0.09)</td>
</tr>
<tr>
<td>H128</td>
<td>HSV-2</td>
<td>4.5 x 10^{4} ± 3.7 x 10^{3}</td>
<td>2.4 x 10^{4} ± 1.3 x 10^{3}</td>
<td>2.7 x 10^{4} ± 3.6 x 10^{3}</td>
<td>P &lt; 0.001</td>
<td>0.52 (±0.03)</td>
<td>0.52 (±0.03)</td>
<td>synergistic</td>
<td>0.26 (±0.03)</td>
</tr>
<tr>
<td>H170</td>
<td>HSV-1</td>
<td>3.1 x 10^{4} ± 3.0 x 10^{3}</td>
<td>5.2 x 10^{4} ± 3.6 x 10^{3}</td>
<td>2.7 x 10^{4} ± 1.9 x 10^{3}</td>
<td>P &lt; 0.001</td>
<td>0.52 (±0.03)</td>
<td>0.52 (±0.03)</td>
<td>synergistic</td>
<td>N.A.</td>
</tr>
<tr>
<td>H128</td>
<td>HSV-1</td>
<td>2.4 x 10^{4} ± 0.9 x 10^{3}</td>
<td>3.9 x 10^{4} ± 3.2 x 10^{3}</td>
<td>2.4 x 10^{4} ± 2.2 x 10^{3}</td>
<td>P &lt; 0.001</td>
<td>0.61 (±0.05)</td>
<td>0.66 (±0.06)</td>
<td>synergistic</td>
<td>N.A.</td>
</tr>
<tr>
<td>H170</td>
<td>HSV-2</td>
<td>1109</td>
<td>4.5 x 10^{4} ± 4.6 x 10^{3}</td>
<td>2.4 x 10^{4} ± 0.9 x 10^{3}</td>
<td>3.9 x 10^{4} ± 3.2 x 10^{3}</td>
<td>2.4 x 10^{4} ± 2.2 x 10^{3}</td>
<td>P &lt; 0.001</td>
<td>0.61 (±0.05)</td>
<td>0.66 (±0.06)</td>
</tr>
<tr>
<td>H128</td>
<td>HSV-1</td>
<td>1122</td>
<td>3.0 x 10^{4} ± 3.9 x 10^{3}</td>
<td>2.1 x 10^{4} ± 2.4 x 10^{3}</td>
<td>1.8 x 10^{4} ± 1.7 x 10^{3}</td>
<td>2.4 x 10^{4} ± 2.2 x 10^{3}</td>
<td>P &gt; 0.05</td>
<td>0.86 (±0.04)</td>
<td>0.86 (±0.16)</td>
</tr>
</tbody>
</table>

* The 50% neutralization end point was considered to determine the nature of the interaction in potency since the 80% end point could reflect the ability of the mixture to neutralize the non-neutralized or persistent fraction rather than a true increase in potency (see Figs. 2 and 3).

* This combination was interpreted as indifferent in neutralization kinetics because the Ko was not significantly higher than Ka and the neutralization rate of the combination did not statistically differ from the neutralization rate of the better antibody in the mixture used alone, suggestive of indifference. In dose/effect neutralization the interaction of this combination also appeared to be of indifference, although a non-statistically significant trend toward antagonism was present.

* A meaningful end point, such as 50% neutralization, was not achieved even at the highest concentration tested. Therefore isobologram analysis was not conducted.

* The combination did not statistically differ from the antibody alone at the concentration in the mixture or when used alone at double the concentration (see text). The precise interaction could not be determined.
fact, while a 1:1 combination of 5 mg/ml each of H170 and H222 had a potency similar to that of 10 mg/ml of H170 used alone, the combination also did not significantly differ from the prediction of indifference (i.e., the predicted potency of the more potent antibody in the mixture used alone at its concentration in the mixture). An isobologram evaluation of the potency could not be carried out because mAb H222 did not reach the 50% end point (Fig. 4C). The results with this antibody combination demonstrate that a weak neutralizer can substantially increase the neutralization rate of a potent neutralizing antibody even at concentrations well below those needed to display a neutralizing effect of its own. In addition, the weak neutralizer used in this experiment, mAb H222, also synergized in neutralization rate with mAb H128, another potent neutralizer with a $K_a/K_o = 0.52 \pm 0.05$ (not shown).

However, not all weak neutralizing antibodies could increase the neutralization rate of more potent antibodies. When mAb H128 was tested on HSV-1 in combination with a weak type-1-specific anti-gB neutralizing antibody, H126, in neutralization kinetics experiments (Fig. 5A), the combination of the two antibodies resulted in a $K_o$ not significantly different from the predicted $K_o$ ($P = 0.44$, Table 1). The interaction of antibody H126 with the potent neutralizer H170 in neutralization kinetics was also one of indifference/additivity (not shown).

**DISCUSSION**

Antimicrobial combinations are desirable because of reduced likelihood of selecting resistant strains; poten-
tial synergistic interactions, which may allow for reduced dosage and lower toxicity (i.e., higher therapeutic indices); and, sometimes, expanded antimicrobial spectrum. In the case of neutralizing antibodies, limiting the risk of neutralization-escape mutants and exploiting favorable pharmacodynamic interactions are reasons for the establishment of cocktails of therapeutically relevant antibodies.

Dynamic interactions of antibacterial agents have been more intensely investigated than interactions of antiviral agents and neutralizing antibodies. With antibacterial combinations, two types of assays have gained widespread acceptance, checkerboard/isobologram methods and time-kill methods (Krogstad and Moeller-Ing, 1987). Discordance between drug interactions investigated with the two methods are sometimes observed, suggesting that the two kinds of assays measure different antimicrobial properties. In fact, in bacteriology, checkerboard/isobologram assays are usually interpreted as measures of the bacteriostatic activity, while time-kill assays tend to be interpreted as tests of bactericidal activity (Krogstad and Moeller-Ing, 1987). Checkerboard/isobologram methods are based on dose–effect experiments. Time-kill assays are time-course experiments and are therefore somewhat similar to neutralization kinetics. One substantial difference that should be kept in mind is, however, that bacteria multiply during these assays, unlike virus inocula being neutralized in a cell-free medium. Checkerboard/isobologram methods are very practical and allow for easy determination of the

![FIG. 3. Antibodies, which do not display clear synergistic interactions in terms of potency, can substantially synergize in neutralization rate.](image-url)
nature of the dynamic interaction. Conversely, time-kill assays are usually interpreted with arbitrary guidelines (Krogstad and Moellering, 1987).

Using variations of isobologram type analyses, neutralizing antibody combinations have been investigated in dose-effect experiments in recently published studies (Li et al., 1998; Mascola et al., 1997; Vijh-Warrier et al., 1996). In these studies, increases in potency of antibody combinations over additivity were demonstrated. The rate of virus neutralization is instead not usually considered in evaluating neutralizing antibodies interactions, despite its great potential importance in antibody neutralization (Della Porta and Westaway, 1978; Dimmock, 1993; Dulbecco et al., 1956; McLain and Dimmock, 1994). In fact, as discussed by Dimmock and McLain, the action of a neutralizing antibody can be seen as "a race between the neutralization and the escape of the virus particle by entry into a host cell" (McLain and Dimmock, 1994). Thus desirable kinetic qualities in neutralizing antibodies are the ability to induce rapid virus inactivation (high neutralization rate constant) and the ability to neutralize by an apparent first-order reaction (i.e., virus inactivation proceeding linearly without an initial lag).

In the present study, the interactions of murine monoclonal antibodies to herpes simplex virus (HSV) in rate of neutralization in comparison to their interactions in potency were investigated. An unambiguous prediction of additivity was necessary to draw meaningful conclusions from the analysis of the effect of antibody combinations in neutralization kinetics. In the absence of established methods to quantify and compare antibody interactions, it was postulated that for a mixture to be additive the relative infectivity (V/V₀) of the mixture should be equal to the product of the relative infectivities of the two antibodies used alone at the same concentrations at which they are present in the mixture, V₁/V₀ and V₂/V₀ (see Materials and Methods). These can be precisely derived using the kinetics constant relationship [Eq. (1)], which correlates relative infectivity, time, and antibody concentration. In this way, the predicted kinetics constant of the mixture at additivity (Kᵢ) can be mathematically determined, allowing for its statistical comparison with the kinetics constant experimentally observed in the actual antibody combination (Kᵢ). Such comparison allowed us to interpret the nature of the dynamic interactions of the combinations studied. This approach is practical and allows for the evaluation of antibody combinations in neutralization kinetics without the use of arbitrary end points as is often done in time-kill assays in bacteriology. Similarly designed analyses could be devised for multiple antibody comparisons. To validate our conclusions, an isobologram type analysis was also

FIG. 4. A weak neutralizer can substantially increase the neutralization rate of a potent neutralizing antibody at concentrations below those needed for it to display a neutralizing effect of its own. (A) Neutralization kinetics on HSV-2 of mAbs H170 and of a weak neutralizer, H222, individually and in combination (open circle). The mixture of 5 μg/ml of H170 and 5 μg/ml of H222 displayed a neutralization rate not significantly different from that of 10 μg/ml of H170 alone (P = 0.25), while 10 μg/ml of H222 alone produced only a marginal reduction in viral infectivity. The neutralization rate constant observed (Kᵢ) for the mixture of H170 and H222 was significantly better (P < 0.001) than the estimated additivity constant (Kᵢ). The Kᵢ/Kᵢ ratio was 0.66 ± 0.06. (B) The interaction of these two antibodies was also clearly synergistic when the kinetics constants were compared in an isobologram format: Dᵢ was significantly higher (P = 0.0213) than D, the mAb concentration in the combination (solid line, black circle). A similar result was obtained if, for the weaker of the two antibodies (H222), the constant observed at the concentration used in (A) was replaced with the neutralization constant obtained at a higher concentration, 50 μg/ml (black square) because at the concentration used in (A), the reduction of infectivity was minimal and the determination of the constant less reliable. (C) Dose–effect neutralization of HSV-2 by mAbs H170, H222 and H170 + H222. mAb H222 displayed a neutralization rate not significantly different from that of 10 μg/ml of H222 (gray square) because at the concentration used in (A), the reduction of infectivity was minimal and the determination of the constant less reliable. (D) A 1:1 combination of H170 and H222 had potency similar to that of H170 used alone at double the concentration of H170 in the mixture, but at the 50% end point it also did not significantly differ from the predicted behavior of H170 used by itself at its concentration in the mixture (not shown), suggesting additivity or indifference. Error bars in the graphs represent SE.
adapted to the evaluation of the neutralization rate of antibody combinations (see Materials and Methods). A very good agreement was found between the two approaches (Table 1).

All the antibodies tested displayed an apparent first-order neutralization reaction. With regard to the rate of neutralization, antibody combinations often displayed significantly increased neutralization rates when compared to predicted additivity even in the absence of clear improvements in potency. Increases in neutralization rates are highly desirable and should be considered when designing antibody cocktails for clinical applications, irrespective of whether such combinations are also synergistic in potency. In this regard, it is also important to notice that neutralization potency, efficacy, and rate are independent variables. For instance, antibody H128 had higher efficacy than antibody H170 against both HSV-1 and -2 (Figs. 2 and 3). However, antibody H170 was more potent than H128 on HSV-2 and was at least as potent on HSV-1 if potency is defined as the concentration required to produce 50% of maximal effect (half-maximal effect or EC50) (Goodman Gilman et al., 1985). The initial rates at which these antibodies reduced viral infectivity in neutralization kinetics were instead very similar on both HSV serotypes, despite the fact that on type 1 H170 barely reached a 50% efficacy in our hands (Fig. 2C). Actually, H170 consistently displayed a higher (although nonsignificant) initial rate of neutralization than H128 in the direct comparison experiments (Figs. 2 and 3 and Table 1). Also supporting the independence of neutralization potency and rate, increased rate of neutralization did not always correlate with increased potency of the antibody mixture (Fig. 3 and Table 1).

Mechanisms of virus neutralization are quite diverse and multiple factors may contribute to antibody synergy. Antibodies directed against different epitopes of the same antigen can bind synergistically (Gomez and Retegui, 1994; Klonisch et al., 1996). Synergistic binding of cocktails of antiviral antibodies directed to the same virus glycoprotein has been proposed to be responsible for increased neutralization (Li et al., 1998; Lussenhop et al., 1988; Vijh-Warrier et al., 1996), for the neutralization properties of cocktails of nonneutralizing antibodies (Lussenhop et al., 1988), and for the broadening of the spectrum of virus strains neutralized (Vijh-Warrier et al., 1996). However, strict proportionalities between binding affinity and either neutralization potency (Bachmann et al., 1997; Brown et al., 1990; Dimmock, 1993) or neutralization rate are often not demonstrable (Schofield et al., 1997). Possible exceptions are viruses like HIV that appear to be neutralized primarily by preventing attachment to host cells (Parren et al., 1998). In other cases, including HSV, which are neutralized primarily by postattachment mechanisms, epitope specificity appears to be important, given that different glycoproteins and specific domains within them may be crucial in virus penetration (Fuller et al., 1989; Fuller and Spear, 1985, 1987). In such a scenario, simultaneous inhibition of two crucial determinants for HSV interaction with receptor interaction could account for the synergistic properties of a cocktail of neutralizing antibodies. In the present study, a combi-
nation of two type-common antibodies to glycoprotein gD, H170 and H128, directed to two highly neutralizing determinants involved in binding to HSV entry mediator A (HveA) (formerly HVEM) (Nicola et al., 1998) was tested against HSV-1 and -2. Consistent with the role in infectivity of the epitope recognized by these antibodies, the cocktail was synergistic in rate of neutralization against both serotypes. Unexpectedly, however, the same antibody cocktail displayed a significantly higher potency over predicted additivity only against HSV-1. In the process of HSV penetration, different glycoproteins are believed to be sequentially recruited in a scenario which would appear prone to produce synergistic interactions between neutralizing antibodies directed to epitopes on different glycoproteins participating in the infection processes. The role of gB in virus infection is well established. Interestingly, an antibody directed to this glycoprotein failed to synergize with antibodies to gD, while an antibody to gE did. These observations suggest a complex picture behind synergistic interactions of neutralizing antibodies, which defies simple interpretations. Future studies will be needed to dissect the mechanisms determining the nature of the dynamic interaction in each specific example.

In the present study, antibody interactions in neutralization kinetics and in dose-effect neutralization experiments were compared. Synergism in both types of assays was observed in some cases, as were examples of discordance between the two types of assays. Indifference and additivity were also seen. The neutralization kinetics of antibody combinations were analyzed using quantitative predictions of additivity based on the neutralization rate constants of the individual antibodies in the mixture. The results obtained with this method agreed with isobologram comparisons of the neutralization rate constants carried out with an adaptation of the method of Chou and Talalay, which defies simple interpretations. Future studies will be needed to dissect the mechanisms determining the nature of the dynamic interaction in each specific example.

In the present study, antibody interactions in neutralization kinetics and in dose-effect neutralization experiments were compared. Synergism in both types of assays was observed in some cases, as were examples of discordance between the two types of assays. Indifference and additivity were also seen. The neutralization kinetics of antibody combinations were analyzed using quantitative predictions of additivity based on the neutralization rate constants of the individual antibodies in the mixture. The results obtained with this method agreed with isobologram comparisons of the neutralization rate constants carried out with an adaptation of the method of Chou and Talalay, which defies simple interpretations. Future studies will be needed to dissect the mechanisms determining the nature of the dynamic interaction in each specific example.

**Materials and Methods**

Antibodies, cells, and viruses

HSV-1 (F) and HSV-2 (G) were obtained from Professor Bernard Roizman of the University of Chicago. Vero cells were obtained from ATCC. All the antibodies used for the study were obtained from the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, FL) as concentrated (~1 mg/ml) purified IgG. The following antibodies were used: H107 (Catalog No. 1103), a group VII type-common to gD; H128 (Catalog No. 1110–2), a group Ib type-common to gD; H126, a type-common antibody to gB (Catalog No. 1122); and H222 (Catalog No. 1109), an antibody to gE-2 (Cohen et al., 1984; Pereira, 1982; Pereira et al., 1980; Sanchez-Pescador et al., 1992). Additional antibodies from this or different sources were tested in preliminary experiments, but only the most representative antibody combinations were investigated in detail and are presented here. Normal purified mouse IgG 2A was obtained from Jackson Laboratories (West Grove, PA).

**Neutralization kinetics**

Neutralization kinetics were performed as follows: 5000 plaque forming units (pfu)/ml of HSV-1(F) or HSV-2(G) were incubated with the indicated concentrations of the different monoclonal antibodies or antibody combinations in a total volume of 1 ml. Incubations were carried out at 20°C because at this temperature the initial rate can be monitored more reliably and reproducibly than at higher temperatures. Fifty-microliter aliquots were removed from the virus–antibody mixtures at the different time points. Antibody–antigen reactions were terminated by immediately diluting such aliquots 100-fold with 5 ml of serum-free medium prechilled at 4°C. Such 5-ml suspensions were then adsorbed on confluent Vero monolayers in 100-mm plates for 1 h at 37°C with intermittent rotary shaking. After removal of the inoculum, a nutrient overlay based on DMEM and containing 0.5% agarose and 2% heat-inactivated FCS (final concentrations) was added to the plates. After the appearance of plaques, the plates were fixed with 10% Formalin in PBS, rinsed, and stained with crystal violet (10% w/v in 70% methanol). The neutralization rate constant, K (hereby referred to simply as neutralization rate constant), was calculated according to the equation (McBride, 1959)

\[
K = (D/t) \cdot 2.3 \log\left(\frac{V_o}{V}\right),
\]

where \(V_o\) and \(V\) are infectious virus at time 0 and time \(t\), respectively; and \(D\) is the reciprocal of the molar concentration of the antibody solution (1/C). The neutralization rate constant was usually calculated at three time points (180, 360, and 600 s) since these points tend to be more linear (see figures). The log\(_{10}\) of the residual infectivities (\(V/V_o\)) at the different time points were then plotted against time (Dimmock, 1993; Dulbecco et al., 1956). For kinetic analyses, all antibodies were tested at 10 \(\mu\)g/ml alone and 5+5 \(\mu\)g/ml in combination (additional concentrations of 5 \(\mu\)g/ml for antibodies alone, 10+10 \(\mu\)g/ml for combinations, etc., were also tested as warranted, see Results).

**Dose–effect neutralization experiments**

Potency and efficacy were determined as follows: serial dilutions of antibodies or antibody combinations were incubated for 1 h at 37°C with ~100 pfu of virus; they were then adsorbed on confluent monolayers of Vero cells in six-well plates for one more hour with intermittent shaking; after removal of the inoculum, a
nutrient overlay was applied to wells as described above. Monolayers were fixed and stained as above. The number of plaques obtained at each antibody concentration was plotted against the log_{10} of the antibody concentrations (log_{10} dose–effect curves). To compare the interaction of antibodies in dose–effect experiments, dilution series of each antibody alone and dilution series of a 1:1 mixture of the two antibodies being tested were tested, similarly to the neutralization kinetics experiments.

Definitions of the interactions

Standard definitions were employed for antibody interactions. The interaction of two antibodies was defined as additive if their combined effect was equal to the predicted sum of their individual effects, synergistic if they produced an effect statistically significantly greater than the one predicted by simple additivity, antagonistic if their combined effect was smaller than the one predicted by simple additivity, and indifferent if their combined effect was not statistically different from that of the most potent antibody used alone at the same concentration at which it was present in the mixture. Indifference and additivity may sometimes be indistinguishable (indifference/additivity).

Interpretation of the results and data analysis

Given that all of the antibodies in question neutralized by an apparent first-order reaction (Dimmock, 1993; Dulbecco et al., 1956), the neutralization rate constants of the antibodies and their combinations could be directly compared. The neutralization rate constants for all the antibodies and antibody combinations were calculated with Eq. (1). A prediction of additivity for the kinetics experiments was then made as follows on the basis of their individual neutralization rate constants. The predicted residual infectivity (V/V_o) expected to be effected by each antibody in the mixture at its concentration in the mixture was derived from Eq. (1) knowing their neutralization rate constants,

\[ V/V_o = 10^{-\left(K_o/2.3D\right)}, (2) \]

where \( D \) is the concentration of each antibody in the mixture. The reciprocal of the product of the reductions of infectivity expected to be effected by the two antibodies (antibody 1 or 2) in the mixture individually at their concentration in the same mixture (V/V_o/V_1/V_2) was then used in Eq. (1) to predict the neutralization rate constant (K_o) of the mixture of the two antibodies if their interaction was one of simple additivity (prediction of additivity):

\[ K_o = (D/t) \cdot 2.3\log(V/V_o \cdot V_1/V_2). (3) \]

If the kinetics constant experimentally observed for the combination (K_o) was statistically significantly greater than K_o, the combination was considered synergistic. The ratio of K_o over K_o was also determined. A K_o/K_o < 1 was interpreted as synergistic, K_o/K_o = 1 as additive, and K_o/K_o > 1 as antagonistic. In this fashion, it was also possible to make point by point V/V_o additivity predictions (see figures).

The data obtained in the dose–effect experiments allowed for isobologram comparisons at the 50% neutralization end point, as well as the determination of the combination index (CI) (Chou and Talalay, 1984; Elion et al., 1954; Li et al., 1997). With this commonly used method to evaluate the effects of drug or antibody combinations, the concentrations of one of the two drugs tested in multiples and fractions of the concentration needed by the drug to achieve the chosen end point (50% neutralization in the present case) are tabulated in arithmetic scale on the x axis, the same data for the other drug are tabulated on the y axis, while combinations of different concentrations of the two drugs are plotted (Chou and Talalay, 1984; Elion et al., 1954; Li et al., 1997). The diagram connecting the experimental points that achieve the designated end point can produce a straight line (additivity, CI = 1), a concave profile (synergism, CI <1), or a convex profile (antagonism, CI >1). The isobologram equation of Chou and Talalay was used for this analysis (Chou and Talalay, 1984),

\[ CI = D_1/D_{x1} + D_2/D_{x2}. (4) \]

where \( D_{x1} \) and \( D_{x2} \) are the concentrations required by the two antibodies alone to achieve the chosen end point (50% neutralization), and \( D_1 \) and \( D_2 \) are the concentrations of antibody 1 or 2, respectively, required to achieve the chosen end point in combination. Interactions that produce CI <1 are interpreted as synergistic, CI = 1 as additive, and CI >1 as antagonistic. Given that 1:1 mixtures were employed in the study (i.e., \( D_1 = D_2 \) or simply, D), the antibody concentration that would result in additivity, \( D_o \), can be calculated as follows from Eq. (2) providing, by definition of additivity, that CI = 1:

\[ D_o = 1/(1/D_{x1} + 1/D_{x2}). (5) \]

Combinations that included antibodies that by themselves had lower effectiveness than 50% could not be evaluated in this fashion.
While this isobologram analysis is usually employed for dose–effect experiments, we also adapted it to kinetics data to validate the conclusions obtained by comparing $K_1$ and $K_2$ as outlined above. To this aim, the following modifications of Eq. (4) were introduced: $D_{x_1}$ and $D_{x_2}$ were the concentrations required by the two antibodies alone to achieve the neutralization rate observed in the combination mix and $D_1$ and $D_2$ were the concentrations of antibody 1 or 2 in the combination mix and were therefore known. Again, since 1:1 mixtures were employed, $D_1 = D_2 = D$. Given that the neutralization rate constants for each antibody had been determined, $D_{x_1}$ and $D_{x_2}$ could be determined from Eq. (1). $D_x$ values were considered to statistically differ from $D$ if they differed by more than two standard deviations.

Calculations were carried out with the spreadsheet program Excel (Microsoft, Seattle, WA) with specifically designed macros. Statistical analyses were carried out by ANOVA followed by post hoc analyses as warranted using the StatView statistical package (Abacus Concepts, Berkeley, CA). Interpolations to determine 50% neutralization endpoints in dose–effect experiments were carried out by linear regression analysis with the same statistical package. The data presented are compiled from three to five independent replications.

ACKNOWLEDGMENTS

We thank Drs. Dennis Burton, Lindsay Whitton, Michael Buchmeier, and John Polich of TSLI for critically reviewing the manuscript and Dr. Lenore Pereira of the University of California, San Francisco, for advice and discussion. This work was partially supported by Public Health Service Grant AI-273582 to (P.P.S.).

REFERENCES


