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On the Regular Lines of Antigen-Antibody Interactions, Incorrect Hypotheses, and Misconceptions

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Abstract

This analytical review of antigen-antibody interactions is intended as a guide for researchers and university teachers until the regular reaction lines of antigen-antibody interactions have been widely understood and recognized by the immunological community.

All significant relationships characterizing in vitro antigen-antibody interactions are described in detail in Section 2 and further recapitulated and listed coherently in Section 2.3. The standard formula for antigen-antibody interactions, with aggregation absent/eliminated, is $k_{st} = \frac{[Ab][Ag]^q}{T}$ *This multivariable formula shows the influence of each variable, one of which is exponential, and indicates the lines to follow to produce antibody-antigen assays of acceptable sensitivity.*

The following false hypotheses or misleading or incomplete concepts in antigen-antibody interactions, including a special antibody-mediated condition that does not seem to have been widely recognized, were found relevant to highlight:

- *The equilibrium theory of virus neutralization, or virus binding, by antibodies, including the occupancy theory*.

  These theories arose from the lack of awareness of the complexity of antigen-antibody interactions. Investigations conducted in 1978 by the author clarified the bifactorial nature of the response in conventional neutralization tests not influenced by complement. One reaction was the rapid, short-lasting "over-neutralization" (*direct virus aggregation*), and the second was the *fundamental, monovalent,* and *enduring first-order* reaction that documented irreversible binding between the virus and neutralizing antibodies under physiological conditions, thereby invalidating these two unsubstantiated theories.

- *The Percentage Law, comprising two claimed aspects: 1) the rate of neutralization is independent of the concentration of the virus antigen, and 2) a percentage of the antigen is regularly resistant to neutralization. This second aspect had led to the use of a specific term: a persistent fraction.* The 1978 analyses revealed that both aspects of the Percentage Law are incorrect. The response in neutralization rate to a change in virus concentration *is significant but disproportionately low*. This particular low response relates to the extraordinary virus-binding capability of antibodies, but unfortunately led to the perception that the antigen's (virus) influence was insignificant. The term "neutralization rate constant" used to indicate the neutralizing potency of an antibody medium is incorrect, and a "regular persistent fraction" of the virus does not exist. Use of the term "the Percentage Law" shall be abandoned.

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However, the original observation of irregular neutralization under certain experimental conditions clearly called for further study. It is related to the formation over time of a physical barrier created by non-neutralizing antibodies under extreme experimental conditions, not to the existence of a "persistent fraction of virus", and shall not be explained by "steric hindrance".

- *The theory of various weak chemical forces binding antibodies with antigens, and the related term* "*antibody avidity*".

  The theory of weak binding forces, accepted in chemistry, has long been widely recognized: non-covalent interactions, i.e., electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions, mediate antigen-antibody binding. However, this hypothesis 1) has never been substantiated in investigations and 2) does not explain the specificity of antigen-antibody binding. The forces binding 1) antigens with antibodies and 2) the C1q component of complement with the Fc region of sensitized antibodies are, therefore, *so far unknown.* The definition of "antibody avidity" *is incorrect.* (The strength of antibody binding will be related to the function of the factor q in the formula above.)

- *Broadly neutralizing antibodies.*

  This expression seems to originate from a limited understanding of the regular lines of viral inactivation. *In vitro*, both non-neutralizing and neutralizing antibodies bind to their antigenic determinants within a specific range where they are influenced by their attractive binding force. This opens the way for two different virus-inactivating aggregation reactions: 1) the antibodies react either directly, in a *synergistic* action by di- or polyvalent antibodies, or 2) indirectly by interfering with the C1q aggregator of antigen-antibody complexes. Non-neutralizing antibodies are the most potent *in vitro* virus inactivators because of their high concentrations and *rapid, aggregative inactivation of the virus*. Antigen-antibody interactions *in vivo* shall be evaluated against regular and even irregular *in vitro* reactions. The term "broadly neutralizing antibodies", not related to specified regular reactions, appears both imprecise and misleading and shall be avoided.

- *The secondary, inflammatory antibody-mediated, complement-activated disease complex in special tissues or organs* (CADC).

  This item is included here because secondary disease symptoms associated with antigen-antibody interactions are not widely understood or accepted. Severe inflammatory lung disease has occurred associated with SARS-CoV-2 infection and has been described as a condition included in the *acute respiratory distress syndrome*, *ARDS*, the cause of which does not seem to have been explained when associated with infectious diseases. The secondary, antibody-mediated, complement-activated disease complex described by the author appears suddenly, approximately 7-9 days after infection, triggered by the attainment of a threshold level of newly produced IgM and/or IgG antibodies. The background for ARDS associated with SARS-CoV-2 infection is logically explained by the virus variant's pronounced lung tissue tropism and the sudden onset of the inflammatory, complement-activated disease. So, ample logical reasons exist to include ARDS associated with SARS-CoV-2 infection into the *secondary, antibody-mediated, complement-activated inflammatory disease complex*. See *Section 3.5* for further information on CADC.

The most critical reasons for false hypotheses, misleading designations, and difficulties encountered in attempts to publish innovative research on this topic appear to be its extreme complexity, the establishment's lack of impartiality or open-mindedness, and a reluctance to accept results that diverge from widely accepted but possibly erroneous relationships.

Reflective, unbiased mathematical thinking; systematically planned and performed analyses; logical deduction; and respect for science, justice, and other researchers may be the required capabilities and attitudes for researchers to promote open-mindedness and prevent future inadequate conclusions and hypotheses.

Adequate lines for publishing scientific research are discussed.

In vitro antigen-antibody interactions are essential for understanding both regular and irregular *in vivo* reactions. Investigations into the functional mechanisms of the two distinct attractive forces involved, including their potential control, are urgently needed.

1. Introduction

Antigen-antibody interactions are a complex key topic in immunobiology. The development of suitable antigen-antibody assays, demonstrating either antigen or antibody, is usually based on documented *in vitro* antigen-antibody interactions. Furthermore, observations on reactions *in vivo* should be evaluated against both the regular lines of

virus-antibody interactions *in vitro* and irregular *in vitro* observations. This underlines the significance of *in vitro* investigations and analyses.

The enigmatic nature of antigen-antibody interactions, due to the complexity of the reactions and the difficulties they pose for understanding, has led to false hypotheses, misinterpretations, and misleading or incomplete designations, which, once published and widely acknowledged, have been difficult to correct later. Both inadequate publications, even by reputable publishers, and difficulties in publishing innovative results that diverge from recognized but erroneous relationships have contributed to the severity of the problems.

The various modes of *in vitro* virus inactivation by antibodies, especially their reaction lines, as revealed by the author's investigations and analyses, are described in a detailed overview (*Section 2*), against which false or partially false hypotheses, insufficiently substantiated conclusions, and imprecise or misleading designations are analyzed.

False hypotheses are frequently encountered when relations are complex and problem-solving consequently requires systematic analyses. It is therefore not surprising that studies of antigen-antibody interactions have led to many theories that were later found invalid. False or unsubstantiated theories published and widely accepted must be countered and effectively refuted. Otherwise, misunderstandings will be ongoing.

Investigations into antigen-antibody interactions have been conducted almost exclusively using viral antigens. However, the antibody is the essential reactant and is likely to bind immutably to the antigen, regardless of the antigen's character, so this should not be considered a limitation.

This overview highlights and discusses the most serious mistakes in antigen-antibody interactions. The *in vitro* interactions found in investigations up to 1970, and by this author in later analyses, are described in *Section 2*, while relevant false or partly false hypotheses, including misinterpretations and misleading designations, are accentuated in *Section 3* and further discussed in *Section 4* with respect to their causes and future standard requirements and attitudes that might prevent the observed unfortunate evolution.

All relationships characterizing *in vitro* antigen-antibody interactions are recapitulated and listed coherently in Section 2.3.

Definitions

Neutralizing and *non-neutralizing antibodies*: Neutralizing antibodies inactivate viruses simply by binding to their antigenic determinants on the virion. In contrast, non-neutralizing antibodies cannot inactivate the virus simply by binding to their specific antigenic determinants.

Measurements of antigens or antibodies can be performed *in screening or titration assays*. A *37°C/24h* test modification employs reaction at 37 °C for 24 hours.

The reacting antibodies at the highest concentration determine the titer recorded in an antibody titration test. This titer also reflects the *test sensitivity*.

A gold-standard assay is a test modification with a specified activity and sensitivity, recognized as the benchmark for comparison when evaluating other tests.

A reference standard assay is a test modification recommended by authorities for practical use or to indicate a required sensitivity level.

Antigen and antibody assays demonstrate antigens and antibodies, respectively.

Aggregation of infectious agents: direct aggregation is the simple aggregation by di- or polyvalent antibodies, while *indirect aggregation* is the aggregation of antigen-antibody complexes by the complement component C1q.

Keywords: Antigen-antibody interactions; Di- or polyvalency of antibodies; False hypotheses; Equilibrium theory of neutralization; Occupancy theory of neutralization; Percentage law; Persistent fraction; Steric hindrance; Broadly neutralizing antibodies; Complement activation; Antibody-mediated; Complement-activated; Disease complex; CADC; ARDS.

2. The Fundamental Antigen-Antibody Interactions *in vitro*

2.1. The early investigations

The earliest *in vitro* studies of virus neutralization used bacteriophages or animal viruses with *artificial* hyperimmune sera. Samples were taken at intervals from a mixture of virus and antibodies to measure the progress of neutralization and the influence of variables.

The neutralization rate in a virus-antibody mixture was found to be semi-logarithmically linear with the reaction time [1], proportional to the antibody concentration [2], temperature-dependent [3], and, erroneously, independent of the virus concentration [1].

However, when the reaction in titration neutralization tests was investigated, it proved inconsistent. No substantial progression of neutralization was concluded to occur after reaction at 37 °C for approximately 1 hour or at 4 °C for 1 day, which led to the perception that after a particular temperature-dependent reaction time, the reaction would reach a state of equilibrium in compliance with the *Law of Mass Action*, also called the *Law of Chemical Equilibrium*. Actually, it wasn't realized that a neutralization test reaction might be bi- or even multifactorial, implying that not a single mode of virus inactivation, but several, should be considered.

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2.2. Personal investigations and analyses

In 1969, an acute BoHV-1 genital/respiratory infection was diagnosed at an artificial insemination bull center in Denmark. The possibility of this infection spreading with semen from bull centers to numerous herds was unacceptable. Early examinations had indicated that the neutralization reaction in a neutralization test: 1) was dependent on the virus concentration (virus dose), 2) was highly dependent on the reaction time, and 3) allowed testing of serum undiluted. A 37°C/24h test version was therefore relatively soon introduced as the reference standard antibody assay for control of quarantined animals before admittance to the centers.

After the pioneering decision in 1970 to eradicate the BoHV-1 infection in bull centers, all animals were controlled annually, and no BoHV-1-infected animals were ever found to have passed access control.

To gain further insights into antigen-antibody interactions, systematic analyses of the reactions were performed, not in virus-antibody mixtures as earlier done, but primarily in *titration neutralization tests*; first in *conventional neutralization tests* with *natural antibody samples* [4], and then in *complement-enriched neutralization tests* [5].

2.2.1. Reactions in conventional titration neutralization tests

All investigations [4] were planned and performed systematically to clarify the influence of the four independent variables: the concentrations of the reacting antibody and virus, and the reaction time and temperature.

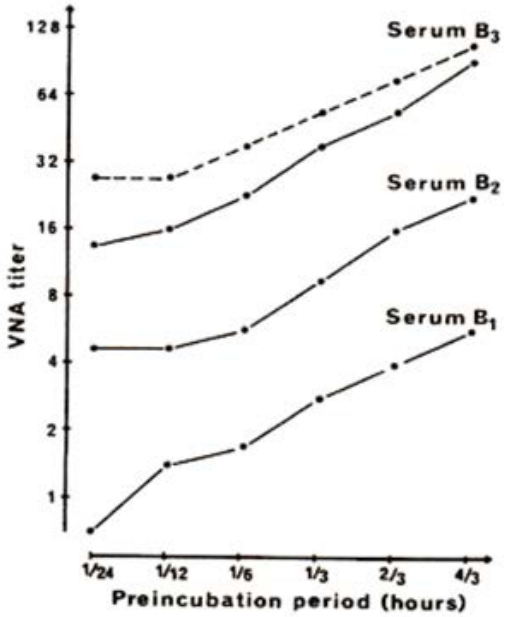

Figure 1: The progression of virus neutralization in titration neutralization tests at 37 °C with short reaction times. (From Bitsch, 1978).

VNA: virus-neutralizing antibody. Preincubation period: reaction time. Three sera with different late-infection antibody levels (IgG) were investigated. The reaction time was planned and plotted on a logarithmic scale. It should be noted that if the reaction time had been shown non-logarithmically, the neutralization lines would have been seen to "flatten out", which, by many, would be interpreted as an indication of almost no improvement of the test sensitivity by increasing reaction time.

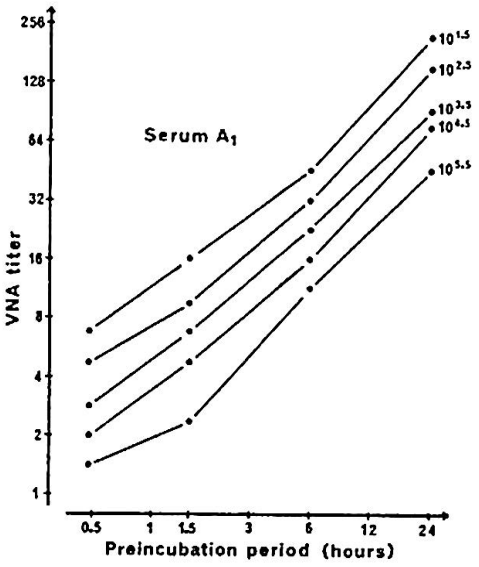

Figure 2: The progression of virus neutralization in titration neutralization tests at 37 °C with extended reaction times, and with virus concentrations (virus doses) varying from 101.5 to 105.5 TCID50. (From Bitsch, 1978).

The neutralization lines can be considered the continuation of those shown in Figure 1. These log-log reaction lines approach a straight line with a slope of 1. After reaction onset, they can be considered linear from 2-3 hours onwards, indicating a first-order relationship between antibody titer and reaction time (see text). Please note that 1) if for a fixed virus concentration with extended reactions the reaction time is doubled, the antibody titer, or antibody test sensitivity, will also be doubled, and 2) if for a fixed antibody concentration, the reaction time is doubled, or for a fixed reaction time, the antibody concentration is doubled, the number of virus particles bound or neutralized will be increased by a factor of approximately 100, demonstrating an immense virus-neutralizing capability of antibodies, see text.

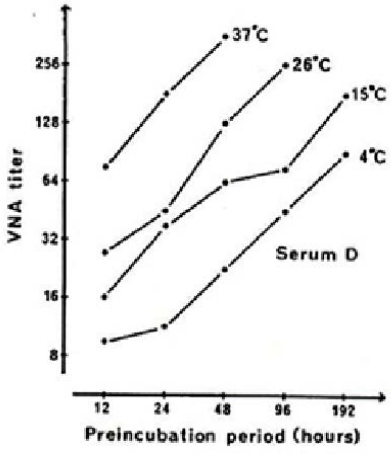

Figure 3: Progression of virus neutralization in titration neutralization

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tests with excessive reaction times from 12 hours to 8 days, and at reaction temperatures of 4, 15, 26, and 37 °C. (From Bitsch, 1978).

The log-log neutralization lines are linear with a slope of 1, indicating a first-order antibody reaction. It is worth noting that the sensitivity (titer) of an antibody test with a reaction at 37 °C for 24 hours is not achieved at room temperature or 4 °C until after reactions of 4 and 16 days, respectively, i.e., another logarithmic relationship. The virus stock was prepared to ensure that the active virus was only a few hours old at harvest and storage, and controls were included in all experiments, demonstrating that no time-dependent virus inactivation occurred other than antibody-mediated neutralization.

Figures 1, 2, and 3 illustrate the relationships between antibody titers and 1) short, 2) extended, and 3) excessive reaction times, respectively. Additionally, the influence of virus concentration is included in Figure 2, and the effect of reaction temperature is in Figure 3.

As reaction time increases, the log-log neutralization lines in these figures approach a straight line with a slope of 1. At 37 °C, the reaction in the virus-neutralization test is strictly first-order from 2-3 hours onwards. This first-order antibody reaction will be understood from the following. In the simple first order equation, y = ax + b, the y intercept b shows where, in a coordinate system, the straight line will cross the y axis; the equation y = ax will, logarithmically transformed, be log y = log x + log a, which documents that a straight line with a log-log slope of 1 identifies a first order relationship for the variables y and x. For further details of the regular virus-antibody interactions in vitro, see Bitsch, 1978, 2017, 2024a, 2024b, 2025 [4,6,7,8,9] and Bitsch &Eskildsen, 1982 [5].

The rapid neutralization with short reaction times, which exceeds what would be expected for a first-order antibody reaction, was termed *over-neutralization* and concluded to be a regular phenomenon [4]. This reaction, which is highly dependent on a sufficient antibody concentration, was not observed in the investigations by Andrewes and Elford because their experimental antibody preparations were appropriately diluted.

Brioen et al. [10] demonstrated in 1983 that a virus was inactivated by being aggregated by a monoclonal non-neutralizing antibody, which logically explained that over-neutralization [4] was caused by this second mode of virus inactivation, i.e., simple *direct aggregation of the virus* by antibodies (here: divalent IgG antibodies) without interference from complement [6,8,9].

The neutralization reaction in a conventional titration neutralization test, where the antibody medium has been heated to inactivate complement, is therefore bifactorial, consisting of: 1) *the prompt and short-lasting over-neutralization*, i.e., virus inactivation via aggregation by predominantly non-neutralizing antibodies, which are, or will very soon become, within the range from their antigenic determinant, where they are influenced by their magnetism-like, attractive force also binding the reactants [8], and 2) *the enduring, but very slowly progressing, monovalent, first-order reaction by specifically neutralizing antibodies* observable separately only with extended reaction times [4].

It should be recalled that 1) all the various antibodies react *synergistically* in the early direct aggregation, and 2) the reason for the *monovalent* characteristic of the enduring neutralization process is that the reaction with increasing reaction time is determined exclusively by new *hits* arising from molecular movements of the reactants. A *hit* occurs when related binding sites come close enough to attract one another and bind.

The standard formula for the antigen-antibody interactions, not influenced by aggregation reactions, presented in 1978 is $k_{st} = \frac{[Ab][Ag]^q}{T}$ [4,6,9]. In this multivariable equation, k_{st} is the *standard reaction rate factor*, Ab and Ag are *titer of the reacting antibody and antigen* (or test sensitivity), respectively, T is the *reaction time*, and the factor q, a new co-determiner of the reaction rate unexpectedly observed, is a particular *log-log antibody/antigen binding ratio* independent of the reacting antibody and antigen concentrations but varying with the reaction temperature. In this way, *q indicates the strength of the attractive force that binds the reacting antibody to its antigenic determinant*. The reaction temperature, the fourth independent variable, is indirectly indicated in the equation by the factor q, which varies with it. Theoretically, q ranges between 0 and 1: the lower q, the stronger the attractive force. In the 1978 neutralization study with *a herpesvirus* and *IgG antibodies*, q was reported to be approximately 0.15 at 37 °C but 0.24 at 4 °C. More vivid molecular movements at higher temperatures may *explain* or *contribute to* the temperature dependence of the factor q.

Detailed knowledge of the binding mechanisms will be needed to better understand and, if possible, control/improve/repair these reactions**.**

From the formula, the neutralization rate factor k_{st} is seen to be a reaction velocity (the denominator T is the reaction time; compare the fraction in the formula with the expression *kilometers per hour*) that depends on several variables, one of which is further exponential. Nevertheless, understanding the reactions will be easy for readers remembering basic mathematics and exponential equations, because the functions of these variables are known and/or controllable. Based on the antigen-antibody interactions described in this formula, assays for the detection of antigens and antibodies with previously unknown high sensitivity have been developed and used in veterinary medicine for many years [7,8,9]. With extended reaction times and complement inactivated, the sensitivity, or antibody titer, of an *antibody assay* will be proportional to the reaction time, yielding a considerable response. In contrast, the sensitivity, or antigen titer, of an

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antigen assay increases exponentially with reaction time, to a degree depending on the factor q, a measure of the binding force. The value of q was approximately 0.15 at 37 °C, but 0.24 at 4 °C for the IgG-herpesvirus reaction.

It should be realized: 1) that the reaction in an *antibody titration test* is the progress of reaction in a series of virus-antibody mixtures, all with one and the same virus concentration and a regularly decreasing antibody concentration, and 2) that the reaction in an *antigen titration assay* is the progress of reaction in a series of mixtures with one and the same antibody concentration and a regularly decreasing antigen concentration. *Both relationships are expressed in the formula*.

The results in *Figure 2*, with extended IgG antibody reactions with a herpesvirus (without aggregations), illustrate:

- For an *antibody assay*:

  Figure 2: A changed reaction time results in a directly proportional change in antibody titer or test sensitivity. In the formula, the neutralizing potency of the reacting antibodies will be expressed by the value of *Ab* (reacting antibody titer or test sensitivity). The influence of $[Ag]^q$ can be considered negligible because the virus dose is kept constant, and k_{st} will remain unchanged. As *T* changes, the now dependent variable *Ab* will change proportionally, and the reaction will be first-order. However, as mentioned earlier, the early reaction in *Figure 2* is not entirely first-order. Because of low residual over-neutralization (i.e., simple direct aggregation) recorded after 1 hour of reaction, the increase in reaction time from 1 to 24 hours does not increase *Ab* or sensitivity by a factor of 24, but by a factor of 16-18. Still, the reaction is fully first-order after 2-3 hours, and the achieved improvement demonstrates the high sensitivity of the recommended 37°C/24h gold-standard titration neutralization test for specifically neutralizing IgG antibodies [4,7]. It is worth noting that aggregation reactions are not possible in conventional antibody ELISAs, which opens the way for sensitive, short-reacting antibody hybrid ELISAs by incorporating controlled aggregation reactions.

- For an *antigen assay*:

  Figure 2: If the reaction time is doubled, or alternatively the antibody concentration is doubled, the number of virus particles neutralized or bound by the reacting antibodies will increase at 37 °C by approximately a factor of 100, illustrating the immense neutralizing or antigen-binding potency of antibodies. These relations will appear from the formula when q is substituted with 0.15, its value here at 37 °C. A doubling of *T* implies that the exponential variable $[Ag]^{0.15}$ will double when other variables are unchanged. This means that the value of *Ag* (antigen titer or sensitivity) will increase exponentially by approximately a factor of 100. (Example: if in the exponential variable $[Ag]^{0.15}$, the value of *Ag* is 10, its value in the doubled exponential expression $2[Ag]^{0.15}$ will be 1000: $2\times10^{0.15} = 1000^{0.15}$ (= 2.82)). Similarly, doubling *Ab* (i.e., inserting the factor 2 in the numerator of the fraction of the formula, but keeping all variables except $[Ag]^{0.15}$ unchanged) implies that *Ag* in the exponential variable $[Ag]^{0.15}$ will change as before. This illustrates the hardly imaginable, overwhelming sensitivity of a conventional 37°C/24h antigen ELISA, which would be an ideal gold-standard antigen assay [7,9]. If the reaction is performed at 4 °C (q = 0.24), the improvement in sensitivity for the antigen, corresponding to either a doubling of the antibody concentration or a doubling of the reaction time, will be reduced from 100 to approximately 18 times. It should be noted that antigen aggregation is not possible in a conventional antigen ELISA.

The formula above expresses regular antigen-antibody interactions under conditions that exclude aggregation. These relations are both fundamental and essential. Therefore, the formula can be used for two purposes: 1) to evaluate and compare sensitivities of antibody or antigen assays and decide the one wanted, and 2) for the indication of the reaction characteristics in mixtures of antigen and antibody.

2.2.2. Reactions in the complement-enriched neutralization test

The investigations [5] were undertaken to understand the regular inactivation of the virus by non-neutralizing antibodies in combination with the C1q component of complement. Again, a complement-enriched titration neutralization test was anticipated to be better suited to investigation than individual antigen-antibody mixtures, and, most importantly, investigations with short reaction times were omitted to avoid the influence of *over-neutralization*, i.e., early direct aggregation.

The results of the investigations have been published in detail in various articles [5,7,8,9]. The C1q component is hexavalent and binds to the Fc region of antibodies sensitized for this binding by the action of being bound to its antigenic determinant on the antigen. This means that C1q will aggregate, not antigens directly as the di- or polyvalent antibodies do, but antigen-antibody complexes. The concentration of C1q can be adjusted to ensure immediate reaction with such virus-antibody complexes under *in vitro* conditions by using an appropriately low dilution of a stored complement source, e.g., “fresh” guinea pig serum.

Figure 4 illustrates the complement-mediated supplementary inactivation of the virus by non-neutralizing antibodies. Only extended reaction times were used. First, immediately after addition, the complement reacts readily

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with all complexes of virus and non-neutralizing antibodies already formed, inactivating them by aggregation. Thereafter, the inactivation elicited by complement is first-order with increasing reaction time, following the first-order binding of the highest-concentration IgM antibodies to their antigenic determinants. It is also evident from reactions 1-1, 2-2, 3-3, and 4, all at extended reaction times, that, regardless of when the complement is added, binding of the non-neutralizing antibodies is strictly first-order, *indicating monovalent, firm binding*. If a considerable part of the bindings had been reversible, a decelerating binding rate would have been seen.

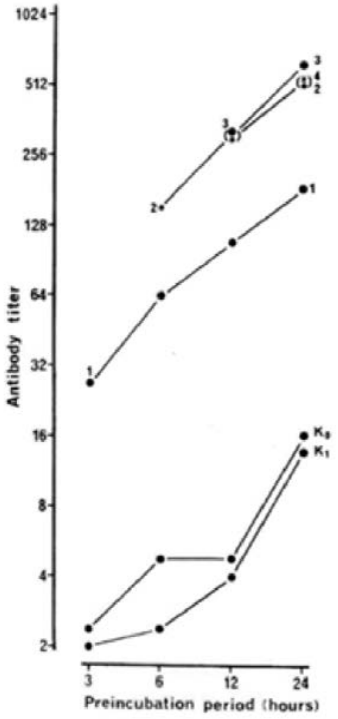

Figure 4: The effect of complement in neutralization tests at extended reactions on the progress of reaction with an early convalescent-phase serum with IgM and IgG antibodies after nasal herpesvirus infection. (From Bitsch and Eskildsen, 1982).

The SuHV-1 antigen and titration neutralization tests with extended reaction times at 37 °C were used. Preincubation period: reaction time. K_0 and K_1: no complement (K_0) or heat-inactivated complement (K_1) was added at the start of virus-serum incubation. For the neutralization test results 1-1, 2-2, 3-3, and 4, the complement was added at the start of the reaction or after 5, 11, and 23 hours, respectively. The deviating 1-1 neutralization line illustrates that complement should be added late in a complement-enriched neutralization test. Due to the relatively high concentration of the reacting IgM antibodies, only IgM reactions will be shown in titrations.

Figure 5 shows the consecutive development of both non-neutralizing and neutralizing IgM and non-neutralizing and neutralizing IgG antibodies after experimental nasal infection with SuHV-1 virus, as demonstrated in 37°C/24h conventional and complement-enriched neutralization tests.

A *significant* virus-inactivating effect of non-neutralizing IgM antibodies is demonstrated by the complement-enriched neutralization test in serum collected 4 days after experimental nasal infection and in serum diluted 1:10.000 after 8-15 days. The binding of a single non-neutralizing antibody molecule to the virus results in immediate inactivation by inclusion of this infectious virus-antibody complex into a non-infectious aggregate due to the immediate action of C1q [5,6,8,9].

The effect and significance of the simple, direct virus-inactivating aggregation by di- and polyvalent antibodies are described in detail above in *Section 2.2.1*. It was emphasized that it was practically immediate but highly dependent on antibody concentration, because the synergistic effect of different antibodies would be drastically reduced at lower concentrations. Furthermore, an antigen at low concentration will require a relatively high antibody concentration to ensure

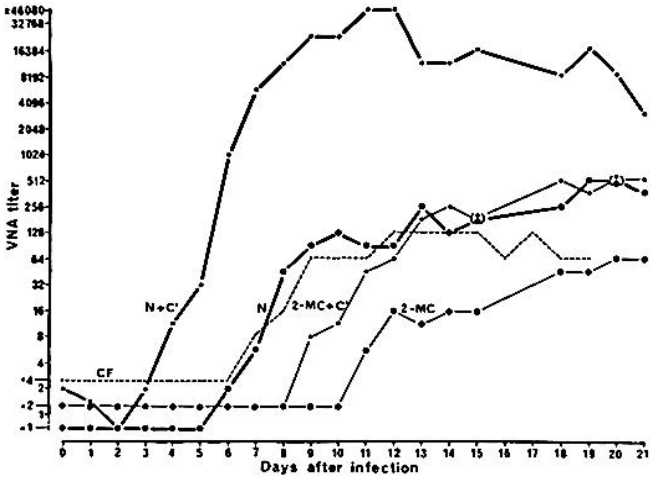

Figure 5: The consecutive appearance of non-neutralizing and neutralizing IgM and IgG antibodies during the first 21 days after experimental nasal herpesvirus infection, as shown by conventional and complement-enriched 37°C/24h titration neutralization tests. (From Bitsch and Eskildsen, 1982).

Virus: SuHV-1. The sera were complement-inactivated at 56 °C for 30 min. and tested, either untreated (N) or treated with 2-mercaptoethanol (2-MC), which will inhibit the neutralizing effect of IgM antibodies, but leave IgG antibodies unchanged. In both cases, they were tested with and without the addition of complement (C´). The virus-serum mixtures were incubated at 37 °C for 24 hours, and where used, complement was added after 23 hours of reaction. Results from a complement fixation test are also shown (CF). The CF titers follow the titers measured for the dominant non-neutralizing IgM antibodies, albeit at much lower levels. Titers of non-neutralizing IgG antibodies were approximately 8 times (3 log base 2 units) higher than those of the neutralizing ones, as found earlier for other herpesvirus IgG antibodies. Titers measured for the antibodies are indicated by the symbols as follows:

Symbols:	N+C':	non-neutralizing IgM antibody titers
	N:	neutralizing IgM antibody titers
	2MC+C':	non-neutralizing IgG antibody titers
	2MC:	neutralizing IgG antibody titers
	CF:	titers in a conventional complement-fixation test

rapid aggregation and inactivation by antibody *hits*. In these situations, the IgM antibodies would seem simply perfect: 1) they are highly aggregative due to their pronounced polyvalency and a relatively wide span between binding sites, and 2) their concentrations during the acute infection period are extreme, promoting rapid inactivation by aggregation.

IgM antibodies are typically present only during the acute infection and shortly thereafter, and there is no reason to doubt that *their primary function is to combat infections*. The *in vitro* investigations shown in *Figure 5* even indicate that the non-neutralizing antibodies, present at high concentrations to a degree that may be largely proportional to the number of related antigenic determinants on the antigen, will have by far the highest virus-inactivating potency compared to neutralizing antibodies [5,8].

The titers of the reacting non-neutralizing IgG antibodies at the highest concentrations are approximately 8 times those of neutralizing antibodies (3 base-2 log units in *Figure 5*), a feature also observed for BoHV-1 herpesvirus IgG antibodies [5,9]. For IgM antibodies, the corresponding difference varied from approximately 100 to 10, decreasing over the observed infection period (*Figure 5*).

In conclusion, in addition to neutralization of the virus by neutralizing antibodies, there are two rapid aggregation modes of virus inactivation *in vitro*: 1) the simple, direct aggregation of virions by di- or polyvalent antibodies, involving predominantly non-neutralizing but to some extent also neutralizing antibodies and 2) the C1q-dependent aggregation of virus-antibody complexes, again involving both groups but predominantly non-neutralizing antibodies. The number of neutralizing antibody types against a virus is unknown, but it is presumably 1 or a few, while the number of non-neutralizing antibody types is typically high. The total concentration of non-neutralizing antibodies will therefore be much higher than that of neutralizing antibodies, being probably largely proportional to the number of related antigenic determinants. A synergistic aggregation reaction will be highly dependent on the total antibody concentration and will decrease dramatically at lower concentrations. A single non-neutralizing antibody bound to its antigenic determinant will, under *in vitro* conditions in the presence of C1q, result in immediate inactivation of the virus. These characteristics, which imply that non-neutralizing antibodies are the most significant virus inactivators *in vitro*, suggest that the *in vivo* virus-inactivating effect in the extracellular space and, possibly, on mucous membranes, may be extraordinary [8].

Three separate virus-inactivation modes by antibodies can thus be identified, and their reaction lines are described above. Although separate reactions, their functions may be integrated if circumstances allow.

It appears crucially important that the *in vitro* antigen-antibody interactions described above, including their significance for sensitive and safe assays of antigens or antibodies, as well as the extraordinary importance of non-neutralizing antibodies, are acknowledged by the immunological community to enable their full exploitation and to counter and dispel erroneous concepts and related misunderstandings. Until then, this analytical review article, which presents the fundamental characteristics of antigen-antibody interactions *in vitro*, along with the most serious mistakes and misinterpretations, will be valuable to researchers and university teachers.

2.3. The characteristics of *in vitro* antigen-antibody interactions recapitulated

Antigen-antibody interactions *in vivo* are extremely complex and should be evaluated based on conclusions drawn from *in vitro* analyses.

Investigations of antigen-antibody interactions have primarily been conducted using viruses. However, antibodies are likely to bind immutably to various antigens, indicating that interactions with viruses may account to a wide extent for other antigens.

The characteristics of the *in vitro* interactions are described and listed coherently below:

1. Antibodies are regularly bound firmly and irreversibly to their antigenic determinants under physiological conditions.
2. The binding force is *so far unknown*, as is also the force binding the hexavalent complement component C1q to the Fc fragment of antibodies sensitized for that binding by being bound to their antigenic determinant (see below). The force binding antigens and antibodies is highly *specific*.
3. The function of the two distinct binding forces is magnetism-like, meaning that, within a certain range, related binding sites are attracted to each other and subsequently bound firmly.
4. Antibodies are flexible molecules, and their attractive binding forces will draw and bend a reacting arm towards its related binding site.
5. Detailed knowledge of the involved binding mechanisms will be needed to better understand and possibly even control or influence them.
6. Virus-neutralizing antibodies constitute a minor part of all specific antibodies to antigenic determinants on the virion. It is unclear whether individual viruses have one or a few types of neutralizing antibodies. The number of different antigenic determinants on a virus that elicits non-neutralizing antibodies is also generally unknown.

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7. The structure of antibody molecules, either in a divalent form (e.g., IgG with 2 binding sites) or a polymerized, polyvalent form (e.g., IgM with 10 binding sites), illustrates their capability of aggregating infectious agents. Polyvalent versions have the advantage of a wider span between their binding sites. It is evident that the di- or polyvalent structure of antibodies serves one specific function: aggregating agents, thereby facilitating inactivation in various ways.

8. IgG antibodies persist regularly for life. For herpesviruses, the concentration of the non-neutralizing IgG antibodies at the highest concentration is approximately 8 times that of neutralizing antibodies, whereas for IgM antibodies, the difference is greater, ranging from approximately 100 to 10, decreasing over their reaction period.

9. For IgM antibodies, a *significant* virus-inactivating effect of the *non-neutralizing antibodies* was demonstrated in a complement-enriched neutralization test (see *Point 15* below) in serum collected 4 days after experimental nasal infection and in serum diluted 1:10.000 after 8-15 days. IgM antibodies, of which a minor part is neutralizing: 1) are *typically* present from a very few days after infection, 2) are found in extremely high concentrations during the acute infection phase, and 3) disappear after some weeks, indicating that production stops as soon as stimulation by the presence of antigen has faded out. Their ability to aggregate is immense, especially due to their extremely high concentration, which greatly enhances their rapid synergistic effect during direct aggregation (see below). This justifies classifying IgM antibodies as *those to defeat acute infections*.

10. Viruses are inactivated by antibodies *in vitro* in three ways: 1) via attachment to neutralizing antibodies, 2) via direct virus aggregation by the di- or polyvalent antibodies, or 3) via aggregation of virus-antibody complexes by C1q, the hexavalent aggregator of antigen-antibody complexes. The effect of C1q has so far been shown for IgG and IgM antibodies. Although these reactions can be identified as separate, they will be integrated if circumstances allow.

11. The reaction in a conventional titration neutralization test, where complement in the antibody medium has been inactivated, is bifactorial. The initial prompt and short-lasting virus-inactivating reaction, originally called *over-neutralization*, is almost exclusively direct virus aggregation, *synergistically* mediated by the di- and/or polyvalent antibodies. The second reaction is the *very slowly progressing, enduring, and first-order monovalent* neutralization reaction induced by neutralizing antibodies, which, for herpesvirus IgG antibodies, can be observed as a separate first-order reaction with reaction times exceeding 2-3 hours at 37 °C.

12. The temperature-dependency of the neutralization reaction implies that the herpesvirus antibody titer, or the test sensitivity, at 37 °C for 24 hours in a titration neutralization test, is not achieved at room temperature or 4 °C until after 4 and 16 days, respectively. Antigen-antibody tests should therefore not be performed at temperatures below 37 °C.

13. The reason for the *monovalent*, *one-hit* characteristic of the *enduring* neutralization process is that the reaction with increasing reaction time is determined exclusively by new *hits* arising from molecular movements of the reactants. A *hit* occurs when related binding sites come close enough to attract and bind. The reaction remains *enduring* in a titration neutralization assay because the dilution of the antibody medium is compensated for by the increased reaction time.

14. The *direct virus aggregation* by di- or polyvalent antibodies will primarily be *prompt* (the antibody concentration may be too low for this synergistic reaction, even with a measurable first-order neutralization), because all related binding sites within a certain range will react immediately. The subsequent aggregation reaction will depend on new hits arising from reactant movements, but will progress very slowly and be short-lasting. The antibodies react synergistically. The total effect is highly dependent on the antibody concentration and will be dramatically reduced at lower general antibody concentrations. It is readily diluted away in a titration neutralization test.

15. The *in vitro* aggregation of virus-antibody complexes by C1q is also *prompt* for the same reason. In a complement-enriched titration neutralization test with extended reaction times, the reaction immediately after complement addition is rapid (the C1q concentration can be adjusted to ensure immediate binding to the formed complexes). The inactivation by C1q will thereafter be first-order, reflecting the first-order *hits* from reactant molecular movements as the reaction time increases. An infectious complex formed by a single non-neutralizing antibody in the presence of C1q is promptly inactivated by its inclusion into an aggregate.

16. The antigen-antibody interactions, *where aggregation is absent or eliminated*, e.g., by extended reaction and inactivation of complement, are indicated in *the standard, multivariable, antigen-antibody interaction formula*: $k_{st} = \frac{[Ab][Ag]^q}{T}$.
This formula can be used to evaluate the influence of the individual variables, to compare the sensitivity of antigen-antibody assays, and to determine an appropriate sensitivity for practical use. See text for detailed information about *symbols* and *relations*.

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17. In *antibody assays*, the sensitivity, or antibody titer, will be determined by the reacting antibodies present in the highest concentration and will be proportional to the reaction time. It is worth noting that aggregation is not possible in conventional antibody ELISAs; therefore, the formula will account for the test reactions.

18. In *antigen assays*, the sensitivity will be raised exponentially with both increased reaction time and increased antibody concentration, illustrating the immense antigen-binding capability of antibodies. In the formula, this effect is influenced by the temperature-dependent factor q, which represents the actual strength of the attraction between the antibody and the antigenic determinant. The factor q will have values between 0 and 1: the lower q's value, the stronger the force. In the herpesvirus-IgG study, q was approximately 0.15 at 37 °C but 0.24 at 4 °C. More vivid molecular movements at higher temperatures may *explain* or *contribute to* the temperature dependence of the factor q. It is worth noting that aggregations are eliminated in conventional antigen ELISAs.

19. On the basis of the demonstrated antigen-antibody reaction lines, 1) the ideal gold-standard assay for specifically virus-neutralizing IgG antibodies, and 2) the ideal gold-standard assay for reacting non-neutralizing IgG antibodies in the highest concentration, are 37°C/24h modifications of 1) a conventional neutralization test and 2) a conventional antibody ELISA, respectively. Similarly, the ideal gold-standard assay for demonstrating antigens can be concluded to be a conventional 37°C/24h antigen ELISA.

20. Additionally, *reference standard assays* or *adequately sensitive tests for practical use* can be elaborated from the reaction lines of the formula.

21. The reaction recorded in an antibody blocking ELISA reflects the reactions in antigen-antibody mixtures indirectly. This reaction is essentially log-log linear in time, but with a slope that differs from 1, actually indicating it is decelerating.

3. Incorrect Hypotheses, Misconceptions, or Misleading Designations in Antigen-Antibody Interactions

A considerable number of false hypotheses about virus-antibody interactions appear in the literature, obviously because this topic is very complex, difficult to overview, and therefore enigmatic.

In the following, the perceptions of greatest importance found invalid by the author are highlighted together with a few selected unfortunate or misleading designations. Also included is a particular, important but apparently non-acknowledged *antibody-mediated* disease complex.

The following subsections will address: 1) the *equilibrium theory of neutralization,* including the occupancy theory and the Law of Mass Action, 2) the *Percentage Law*, claimed to comprise both independence of the neutralization rate on virus concentration and a regular existence of a so-called "fraction of virus resistant to neutralization, a persistent fraction*",* 3) *the binding forces in antigen-antibody interactions*, 4) *broadly neutralizing antibodies*, and 5) *the secondary, inflammatory antibody-mediated*, *complement-activated disease complex* in special tissues or organs which seems to be unknown but relevant in this context because there is ample evidence that it is triggered by antibodies.

3.1. The equilibrium theory of neutralization, including the occupancy theory, and the Law of Mass Action

The 1978 study documented that the reaction in titration neutralization tests was bifactorial, consisting of: 1) an *initial prompt and short-lasting* "*over-neutralization"* and 2) an *enduring but very slowly progressing first-order neutralization*, becoming visible as a separate reaction from 2-3 hours of reaction onwards at 37 °C [4]. Highly sensitive *reference-standard antibody assays* and *antigen ELISAs* with reaction at 37 °C for close to 24 hours, based on the lines of the standard antigen-antibody interaction formula, were introduced and used to diagnose, control, and even eradicate widespread infectious animal diseases in Denmark.

An *equilibrium theory of neutralization* in antigen-antibody media, based on the perception that the reaction would follow the *Law of Mass Action*, had been widely accepted, cf. *Svehag* (1964) [11], and newer review articles [12,13]. The antigen-antibody interactions should, accordingly, follow the simple equilibrium equation known from chemistry, $Ag + Ab \leftrightarrow AgAb$, and not the more complex standard multivariable formula above in *Section 2.2*, which is based on the substantiated lines of firm and irreversible bindings, not influenced by aggregation reactions [4,5,6.8,9].

The 1978 study [4,6] documented that the neutralization reaction, in the absence of complement interference, was bifactorial, and subsequent investigations clarified the significance and reaction lines of complement-mediated virus inactivation [5,6]. The firm and irreversible binding implies that test sensitivity can be adjusted and improved, consistent with the substantiated interaction lines described in *Section 2* and expressed without aggregations in the standard formula [4,5,6,7,8,9].

These investigations were decisive for understanding basic *in vitro* neutralization reactions. Yet they went unnoticed by the immunological community, and misunderstandings and misperceptions about relationships of extreme significance persisted. The difficulty of publishing innovative research is part of the problem posed by the persistence of non-

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substantiated perceptions regarding antigen-antibody interactions.

Discussions up to around 2000 were mainly about whether neutralization might result from a *one-hit* reaction, as suggested by the earliest findings [1], or would require *multiple hits*, because observed reactions did not seem consistent with any perception of regular antigen-antibody interactions. Unfortunately, the fundamental 1978 and 1982 studies [4,5] were not taken into account. In 2001, a group of researchers argued for a more specified multi-hit model, later referred to as the occupancy theory, cf. *Burton et al.* [13]. The overall implication of that hypothesis was that virus neutralization depended mainly on a sufficient number of antibodies binding to the virion's antigenic determinants, suggesting that the effects of specifically virus-neutralizing antibodies and other potential virus-inactivation mechanisms would be of minor relevance.

Conclusions recapitulated

It will be understood from the documentation presented in *Section 2.2* that neither the equilibrium theory, including the occupancy theory, nor the Law of Mass Action applies to antibody-mediated virus neutralization. Virus inactivation by antibodies *in vitro* occurs in three ways, each following separate reaction lines, cf. *Section 2*. Use of the term *Law of Mass Action* shall be abandoned.

3.2. The Percentage Law

Andrewes and Elford [1] published on a so-called *Percentage Law* that should imply: 1) that the rate of virus neutralization in a virus-antibody mixture is independent of the virus concentration, and 2) that a fraction of the virus resistant to neutralization, *a persistent fraction*, is a regular feature, constituting a certain percentage of the amount of virus.

These two aspects are addressed separately.

Influence of the virus concentration on the rate of neutralization

The authors used artificial immune sera [1]. The progress of neutralization in a virus-antibody mixture was found to be linear on a semi-logarithmic scale.

Furthermore, they concluded that neutralization was independent of the virus concentration. Although their investigation was limited, this led to the general acknowledgment that the *neutralizing potency* of an antibody medium could be expressed by its *neutralization rate constant*, rather than by a neutralization rate or a neutralization rate factor that would vary with antigen concentration.

However, the neutralization rate *does depend* on the virus concentration and follows the standard formula for antigen-antibody interactions *in vitro*, as shown in Section *2.2* [4,6,9].

If the virus concentration increases, the neutralization rate will not increase proportionately because the factor q is not 1 but much smaller, closer to 0. In other words, the influence of a change in antigen concentration on the reaction rate, although significant, is disproportionately small. It should be understood that this particular effect is closely associated with the immense neutralizing or antigen-binding capacity of antibodies, as described above in *Section 2.2.1*. The disproportionately small change in the reaction rate with changes in antigen concentration plausibly explains why the authors reached their erroneous conclusion.

A fraction of the virus resistant to neutralization

A certain percentage of the virus was found not to be neutralized by an experimental antibody medium in the investigations by Andrewes and Elford, and they concluded that this was a regular condition.

The problem of irregular neutralization illustrated in *Figure 6* from the 1978 study was unexpected.

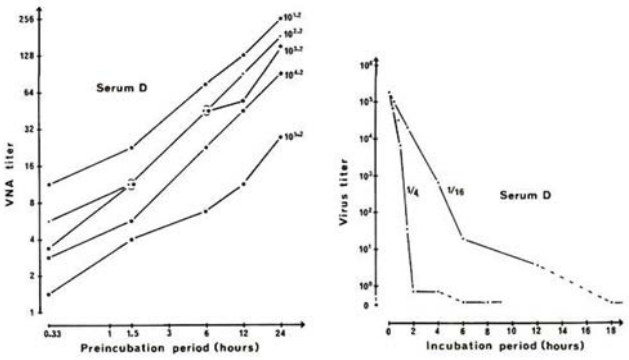

Figure 6: Irregular progression of neutralization for a particular serum sample in a titration neutralization test, with low to exceptionally high virus doses and extended reaction periods. From Bitsch (1978).

To the left, virus-neutralizing antibody titers; to the right, the course of neutralization for the highest virus dose with two dilutions of the antibody medium.

The neutralization line for the highest virus dose with long reaction times to the left is irregular, and to the right is seen that the neutralization of this high virus dose by serum diluted 1:4 proceeds semi-logarithmically linear within 2 hours, while neutralization with serum diluted 1:16 requires a longer reaction time: *a residual part* is neutralized at a reduced rate under the present extreme conditions.

The deviating results for the highest virus dose with the longest reaction times indicate a reduced neutralization rate under extreme test conditions and suggest what may have happened. With extended reaction times, the virus particles to be neutralized late in the course of the process will, before meeting a neutralizing antibody, be coated with a high number of the various predominating non-neutralizing antibodies, which, with increasing reaction time, will create a growing barrier or blockage for the access of neutralizing antibodies to

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their antigenic determinant on these virus particles [6,9]. It is worth noting *that such virions, loaded with non-neutralizing antibodies, remain infectious as long as they have not 1) been bound to a neutralizing antibody or 2) been included in aggregates.* This condition conflicts with the unsubstantiated occupancy theory of neutralization.

It was mentioned in earlier discussions [6,9] that *steric hindrance* by non-neutralizing antibodies has been argued to explain the so-called persistent fraction of virus (Ashe and Notkins, 1966 [14]; see also the review by *Burton*, 2023 [15]). However, steric hindrance is a complex phenomenon in chemistry; a simple, gradual increase in a physical blockage for neutralizing antibodies by non-neutralizing antibodies under extreme conditions, as seen in Figure 6, is a logical and acceptable explanation that does not require reference to a specific chemical condition to be understood. In this context, it should be noted that antibodies are flexible molecules: their binding forces bend and draw an attracted arm toward its corresponding reactant.

Non-artificial, late-infection samples had been selected for the 1978 investigations [4]. However, it appeared that although the champion bull delivering that problematic sample (Figure 6) was naturally infected, he had been given a high dose of culture virus parenterally to determine whether this would diminish the risk of virus shedding in later periods (which it didn't). Therefore, the sample in question was from a partially experimental animal, and the second artificial parenteral immunization might have altered neutralization conditions to an unnatural extent, for example, by changing the relative concentrations of neutralizing and non-neutralizing antibodies [6,9]. To conclude with certainty that new observations from experimental animal investigations are natural will require analyses or examinations to rule out that they are related to the experimental conditions. This is not fulfilled for the observation by Andrewes and Elford.

Figure 6 from the 1978 study shows that a serum sample, unintentionally classified as partially experimental, neutralizes a residual portion of the virus at a lower rate. However, this was under extreme reaction conditions, with the highest virus concentration (i.e., an exceptionally high virus dose) and the longest reaction times, illustrating that virions neutralized late would first bind to a large number of non-neutralizing antibodies. In later analyses [6,9], it was concluded that a persistent, or rather *a residual* fraction of virus neutralized at a lower rate, is an irregular result that might well be an artefact not expected to occur under usual testing conditions with natural antibodies, e.g., with ordinary relative concentrations of neutralizing and non-neutralizing antibodies [6,9].

Main conclusions recapitulated

1. Influence of virus concentration on the rate of neutralization.

The regular lines of virus neutralization, not including aggregations, are shown in the standard formula above. The effect of a change in virus concentration on the reaction rate is, although significant, disproportionately small, which may explain why the authors reached an incorrect conclusion.

The 1978 study documented that, with extended reaction time, the rate of virus neutralization (virus-antibody binding) follows the formula, implying a regular and substantial effect of antigen concentration. The rate of neutralization depends on the antigen concentration, to a degree further determined by the factor q.

In this context, the *Percentage Law is erroneous*. The term "neutralization rate constant" is also incorrect and should be replaced with "neutralization rate" or "neutralization rate factor."

2. A fraction of the virus resistant to neutralization, "a persistent fraction".

The statement by Andrewes and Elford that a fraction of the virus is regularly neutralization-resistant and that this fraction constitutes a certain percentage of the virus was not confirmed in the 1978 study.

The *Percentage Law* is also incorrect in this context, and the term "*a persistent fraction"* shall be avoided.

However, they observed that virus neutralization by an experimental antibody preparation was irregular, resulting in partial neutralization [1]. In the 1978 study, a similar effect, a reduced neutralization rate for a *residual* part of the virus under extreme reaction conditions, was observed as an irregular course of neutralization for a partially experimental antibody sample [6,9]. Andrewes and Elford's observation is certainly important and would require further investigation to be understood. It is logically related to the over-time formation of a physical barrier by non-neutralizing antibodies, making it gradually more difficult for a residual fraction of virions to be *hit* by a neutralizing antibody.

3.3. The binding forces in antigen-antibody interactions

In their search for understanding antigen-antibody interactions, researchers turned to chemistry. For many decades, the binding of antibodies to their antigens has been considered to be mediated by weak chemical forces, i.e., electrostatic interactions, hydrogen bonds, van der Waals forces, and hydrophobic interactions; see the discussion in *References 6 and 9*. Such weak binding might result in unstable antigen-antibody complexes, but it does not explain antibody specificity.

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What is known with acceptable certainty is that antibodies are characteristically specific, and their binding force is both attractive and consistently strong enough to ensure a firm, irreversible union with their antigenic determinants under physiological conditions. Similarly, the force that binds C1q to the Fc fragment of antigen-antibody complexes appears to be attractive, regularly resulting in irreversible binding under physiological conditions.

The involvement of weak binding forces in antigen-antibody interactions is a hypothesis derived many decades ago from accepted binding forces in chemistry. However, no evidence has been found to support the involvement of such reactions in antigen-antibody bindings, and they definitely do not explain *specificity*. Furthermore, the Law of Mass Action (the Law of Chemical Equilibrium) does not apply to antigen-antibody interactions [4,6,8,9].

Antibody *affinity* and *avidity* are two terms that were used to describe the strength of interactions between antibodies and antigens: affinity refers to the strength of binding between an antibody and its antigenic determinant, and avidity to the overall binding strength of the antibody arising from its di- or polyvalency. It was anticipated that antigen-antibody interactions would be weak, reversible, and would reach equilibrium. However, all results indicate that the antigen-antibody binding is firm and irreversible under physiological conditions.

The di- or polyvalency of antibodies does not strengthen antigen-antibody binding. Its primary function is to aggregate antigens, thereby facilitating inactivation in various ways. The term *antibody avidity* is incorrect. Investigations into the attractive binding forces are urgently needed [8,9].

Conclusions recapitulated

The hypothesis that weak binding forces accepted in chemistry are involved in antigen-antibody interactions in immunobiology has remained unsubstantiated and does not explain antibody specificity.

The nature and functional mechanisms of the two distinct binding forces identified in antigen-antibody interactions remain *unknown*.

The term "antibody avidity" is incorrect and shall be abandoned.

3.4. Broadly neutralizing antibodies

The term ″broadly neutralizing antibodies″ has been used to describe antibodies with virus-inactivating effects that were not recognized under traditional perceptions of virus neutralization, cf. *References 15 and 16*.

However, *in vitro* virus inactivation by non-neutralizing antibodies occurs regularly in antigen-antibody media, and the reaction lines have been satisfactorily clarified (cf. *Section 2*). Viruses are inactivated by non-neutralizing antibodies *in vitro* in two aggregation reactions: 1) simple direct aggregation of the virus by various di- or polyvalent antibodies, and 2) aggregation of infectious virus-antibody complexes by interference with the hexavalent C1q aggregator, which will bind to the Fc fragment of antibodies sensitized by being bound to their antigenic determinant. These reactions are triggered when related binding sites are close enough for their attractive forces to interact. [5,6,8,9].

Conclusions recapitulated

Observations of irregular virus inactivation *in vivo* should be evaluated against the regular lines of virus-antibody interactions *in vitro* (*Section 2*). Observed effects of broadly neutralizing antibodies will include virus inactivation via aggregation by predominantly non-neutralizing antibodies, with and without interaction with C1q, and following their separate reaction lines. Antibodies should preferably be described and named accordingly.

The term ″broadly neutralizing antibodies" should be avoided.

3.5. The inflammatory *antibody-mediated, complement-activated disease complex* in special tissues or organs, CADC

In the SARS-CoV-2 pandemic, it was observed that the infection caused severe cases of lung disease, further characterized as *acute respiratory distress syndrome*, ARDS [17]. The description and designation of this clinical disease syndrome, cf. *Upadhya et al.* 2021 [18] are not incorrect but *incomplete*, as they do not account for the likely, causative host response, i.e., an adverse reaction evoked by early antibodies.

In 2011, this author published systematic analyses of the epidemiology and pathogenesis of the BoHV-2 infection [19]. The conclusions relevant in the present context were: 1) the infection is spread airborne among susceptible individuals, 2) lesions in the skin are associated with a *dermotropic* characteristic for the virus, which, during a viremic phase, will spread to multiple sites in skin areas, where they start multiplying, 3) skin lesions appear almost simultaneously, 7-9 days after infection, at the sites of virus replication, 4) lesions are inflammatory and furthermore remarkably sensitive to touch, 5) lesions are promoted in cold, unhaired skin, 6) the logical explanation for this evolution is the elicitation, by a reached reaction threshold of new IgG and/or IgM antibodies, of the inflammatory complement activation by the classical pathway, locally at the sites of virus replication, and 7) the preferential, in some cases even exclusive, appearance in cold skin is logically explained as a harmful, increased inflammatory reaction due to impaired removal of cell-toxic complement-activated mediators because of the reduced blood circulation.

The inflammatory characteristics of coronavirus lung disease will be understood as an adverse reaction to antibody-mediated complement activation, associated with pronounced lung-tissue tropism for the virus variant, excessive viral multiplication in lungs, and impaired blood circulation. After infection, the inflammatory complement activation will *inevitably* be elicited by new, early antibodies topically in tissues where the antigen is present, usually with beneficial effects. Under extreme conditions, the outcome may exceed the host organism's tolerance. Such conditions may include an excessive presence of the infectious agent in particularly sensitive tissues or organs, resulting in a sudden, massive complement activation and impaired blood circulation, which may increase the effects of released cytotoxic mediators, thereby aggravating the inflammatory disease.

In this way, the lung disease observed in SARS-CoV-2 cases can be understood, not as a primary or direct effect of the virus, but as a secondary, adverse complement-activated response by the host organism, indicating that this particular lung disease is part of the *sudden*, *antibody-mediated, complement-activated disease complex* (CADC) in specific tissues or organs.

It may be of interest to note that the rash in varicella and some poxvirus infections is also readily explained as CADC. The viruses involved are *dermotropic* and will, during a viremic phase, spread to multiple skin sites, where complement activation triggers a sudden inflammatory reaction once antibody levels reach a threshold. One feature of varicella and smallpox is that lesions typically appear first on the face and extremities, which can be explained by lower skin temperature, which will promote clinical complement-activated reactions due to reduced blood circulation. Some herpesviruses, or variants of these, are *neurotropic*. Shingles and cold sore lesions will therefore appear after reactivation of the infection, where the virus encounters antibodies at nerve endings, triggering inflammatory CADC. A special condition observed in horses infected with an EqHV-1 variant, as studied by the author, is paraplegia ranging from light ataxia to full hindquarter paralysis. It is explained as CADC triggered by new antibodies, occurring approximately 7-9 days after natural respiratory infection, shortly after body temperature has returned to normal. The onset of symptoms was sudden, and the full extent of this secondary clinical disease was reached almost immediately in individual cases, i.e., within a few hours after the first symptoms (not published in detail).

It might be relevant to note that many cases of ARDS have been associated with various infections, and further, that two other ways of inflammatory complement activation have been described. This may suggest that many more cases of ARDS could be explained by complement activation.

Conclusions recapitulated

The overall background for the *acute respiratory distress syndrome* associated with SARS-CoV-2 infection is logically explained by the virus's pronounced lung-tissue tropism and a sudden onset of antibody-mediated complement activation triggered when newly formed IgG and/or IgM antibodies reach a reaction threshold.

So, ample reasons exist to include ARDS associated with SARS-CoV-2 infection into the secondary, *antibody-mediated, complement-activated disease complex*, CADC, elicited by early antibodies, i.e., an acquired (adapted), sudden, but adverse response to antibody-mediated complement activation.

4. Discussion

In their search for regular antigen-antibody reaction lines, researchers turned to chemistry, although immunobiology is not a chemical field (cf. the Law of Mass Action and weak chemical forces to explain antigen-antibody binding).

The harmful influence of false hypotheses will naturally depend on the importance of the topic concerned. However, antigen-antibody interactions are a key topic in immunobiology and biomedicine, and misunderstandings can have significant consequences.

Many unfortunate impacts of published, insufficiently substantiated hypotheses and designations are possible. They cause confusion rather than clarity, may prevent or delay the performance of relevant analyses and investigations, and may result in decisions on unsafe grounds. One example is the equilibrium hypothesis of antigen-antibody interactions, which has hindered widespread understanding of 1) the true interactions that enabled the development of high-sensitivity assays demonstrating antigens and antibodies [7] and 2) the significance of non-neutralizing antibodies in virus inactivation and their reaction lines [8]. Hypotheses may cause more harm than good and shall remain hypothetical until acceptably substantiated.

It is unquestionably a primary obligation for research publishers to ensure that all new innovative results are published for the best of science and our entire community. In 1995, the Danish Ministry of Education and Research issued a white paper stating that it is crucial to publish new and even diverging results for further evaluation by the scientific community. This also indicates that editorial insights and peer reviewing cannot be trusted to ensure safe validation of manuscripts.

From personal experience, the primary conditions preventing the publication of relevant new scientific knowledge appear to be the establishment's disinclination to accept research that conflicts with widely accepted perceptions and a lack of impartiality or open-mindedness among reviewers. In research publishing, this tendency should be countered by stringent requirements for editors and peer

reviewers, preferably including their public identification and a declaration of impartiality for each manuscript. This would definitely assure authors. Author-paid, peer-reviewed online publishing, soon after peer review and acceptance, with rights retained by authors, would seem righteous. The competition among publishers is acceptable, and journal impact factors, although not yet clearly and uniformly defined, seem to be a reasonable guide for authors.

The standard requirements for researchers would be their ability to perform: 1) reflective and unbiased mathematical thinking, 2) systematic planning and analysis, and 3) sound, logical deduction, all with respect for science, justice, and other researchers. Talented researchers would possess these capabilities to varying degrees, and others' ability to fulfil them would determine their professional value.

The remaining problems, therefore, associated with insufficiently substantiated conclusions or theories in publications, appear chiefly to be preventing future inadequate publications and, for the existing misconceptions, to identify and eliminate them.

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