SIGNIFICANCE OF BIVALENCE OF ANTIBODY IN VIRAL NEUTRALIZATION*

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The neutralization of bacteriophage by antibody is a convenient experimental model for the study of immunity to viral infections, for quantification of small amounts of antibody, and for investigation of the kinetics of antigen-antibody interactions.

One approach toward investigating the molecular basis of the neutralization reaction is a comparison of the activity of bivalent antibody with that of its univalent fragments. This could establish whether cross-linkage of antigenic determinants, either on a single virus particle or between particles, is essential for neutralization.

Numerous studies of this question have been reported, with highly variable results depending on the system investigated. The relative effectiveness of univalent fragments covers a spectrum from very low activity (1–7) to a significant fraction of that of untreated antibody (8–11). In each investigation, some loss of activity was associated with fragmentation of the molecule.

To investigate this question further we have tested rabbit antisera to virus ϕX174 at different stages of immunization. The plan of immunization and bleeding was designed to yield antibodies of both high and low affinity. Our data show that univalent 3.5S fragments are equally as active as 7S immunoglobulin (IgG) in viral neutralization when derived from "late" antisera, but are virtually inactive when prepared from antisera taken after a short course of immunization. This indicates that bivalent attachment or cross-linkage is not required for neutralization of bacteriophage, provided the combining affinity of the univalent fragments is sufficiently high. It would also appear that the greater size of untreated antibody and the presence of fragment Fc are not significant factors in neutralization. The relatively greater effectiveness

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of bivalent antibody derived from early antisera can be attributed to the increased affinity associated with bivalent attachment (1, 4, 12).

Materials and Methods

Immunization.—Rabbits weighing 3-4 kg each were injected intravenously with 2×10^{11} particles of bacteriophage ϕX174 (generously provided by Dr. R. Sinsheimer) in 1 ml of saline. The animals were bled from the central artery of the ear 2 and 4 wk later. 19 wk after immunization, the animals received a second similar injection of ϕX and were bled 1 wk later.

Preparation of IgG and Its Fragments.—Rabbit IgG was prepared from antisera by two precipitations with sodium sulfate, at final concentrations of 18 and 14% (13), followed by passage through diethylaminoethyl (DEAE)-cellulose in 0.02 M phosphate buffer, pH 7.0 (14). A single band characteristic of IgG was observed on immunoelectrophoresis of each preparation, using a polyvalent goat antiserum directed against whole rabbit serum.

Fragment Fab was prepared by digestion of IgG with papain for 4 hr, as described by Porter (15). The digest was dialyzed against 0.01 M sodium acetate buffer, pH 5.8, and passed through carboxymethyl cellulose equilibrated with the same buffer. Crystals of fragment Fc were removed by centrifugation before chromatography. Fragment Fab, which was eluted in the void volume, was characterized by its reaction with goat antirabbit Fab, its failure to react with anti-Fc, and its sedimentation as a single peak (3.2-3.4S) in isotonic NaCl-borate buffer, pH 8, at 20°C.

Fragment $F(ab')_2$ was prepared by digestion of IgG with pepsin for 8 hr at 37°C at pH 4.2 or 4.3 (16), and was isolated by gel filtration through Sephadex G-150 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) at neutral pH. Fragment Fab′ was prepared from $F(ab')_2$ by reduction with 0.025 M 2-mercaptoethanol at neutral pH, alkylation with excess sodium iodoacetate, and subsequent dialysis. The sedimentation coefficients of fragments F(ab′) and $F(ab')_2$ were approximately 3.4S and 4.6S, respectively, in neutral isotonic buffer at 20°C. Single, symmetrical peaks were obtained with each preparation.

Antibody Assays.—Antibody titrations were carried out by our modification (17) of the conventional method described by Adams (18). This method involves diluting the phage-antibody mixture 1:100 at 0°C to stop further neutralization and then adding bacteria to the mixture and allowing incubation at 37°C for 18 hr in order to develop plaques.

To inhibit infection of bacteria by virus which dissociates from antibody, the decision-tube method of Jerne and Avegno (19) was also employed to quantify antibody to ϕX. Aliquots of the phage-antibody mixture obtained at appropriate intervals were diluted at 24°C and immediately incubated with bacteria for 10 min. At this time, 0.1 ml of diluted rabbit hyperimmune antiserum to ϕX was added to give a k of 0.3 in the mixture. After 5 min of further incubation at 24°C, this mixture was "plated," and the plates were incubated overnight. With this method, phage can only infect bacteria during the 10 min incubation period because the large excess of hyperimmune antibody will neutralize phage that dissociates after this period of time.

RESULTS

Antibody Activity of IgG and Fragments.—The neutralizing activity of IgG and its fragments, prepared from a pool of serum of six rabbits obtained 2 wk after immunization and from four pairs of sera from individual rabbits bled at 4 and 20 wk after primary immunization, are shown in Table I.

As can be seen, univalent fragments, Fab or Fab′, obtained from 2- or 4-wk antisera, were relatively ineffective in neutralization as compared to IgG.

Univalent fragments from the 2 wk pooled serum were 0.02–0.07% as effective as IgG and the relative effectiveness of univalent fragments from the 4-wk sera ranged from 1.3 to 11.7%. In contrast, such fragments obtained from later bleedings (20-wk antisera) appeared virtually as effective in neutralization as the intact molecule. In nearly all experiments, fragment $F(ab')_2$ was at least as active as IgG. The greater activity of $F(ab')_2$ per unit weight[1] observed in several experiments may be explained on the basis that, because of the absence of fragment Fc in IgG, the molar concentration of the $F(ab')_2$ exceeded that of IgG. As expected, in all the paired sera, the k/OD 280 ratio of intact IgG and its fragments increased with immunization, reflecting an increased ratio of antibody to nonantibody IgG, increased binding affinity of antibody, or both. It is also of interest that in five instances Fab′ showed slightly less antibody activity than Fab. One possible explanation is that some reduction of interchain bonds joining heavy and light chains occurred during reduction of $F(ab')_2$ to Fab′ with 0.025 M 2-mercaptoethanol and that this decreased the neutralizing efficiency of the fragment.

These studies indicate that the Fc fragment plays no significant role in neutralization of bacteriophage and that univalent antibody can be as effective as intact IgG. The striking differences between the relative effectiveness of univalent antibodies obtained from "early" and "late" sera may be attributable to differences in affinity.

Dissociability of Rabbit Anti-φX and Its Fragments.—The marked difference between the specific neutralizing activity of univalent antibody obtained from 2–4–wk *versus* 20-wk sera could conceivably be explained by differences in dissociability of bacteriophage from early and late antibody. This possibility was tested in two ways:

(*a*) Neutralization was performed by the decision-tube method (19). In this assay, hyperimmune antiphage serum is added 10 min after the addition of bacteria to the undiluted phage-antibody mixture. This maneuver should prevent infection of bacteria by phage which dissociates from antibody in the conventional assay, in which the phage-antibody mixtures are diluted and then incubated with bacteria overnight.

By this method, Fab fragments of IgG prepared from 4- and 20-wk antisera of rabbit No. 4 were examined and the ratios of k values obtained from decision-tube and conventional methods were compared. The results obtained with the two Fab preparations were identical, i.e., a ratio of 2.7 for the 4 wk and 2.6 for the 20 wk antiserum.

(*b*) Neutralized complexes were diluted to test for differences in dissociability. Dissociation of diphtheria toxin and bacteriophage T4 from early but not late antiserum was first described by Jerne (19, 20). Accordingly, Fab and IgG

[1] The extinction coefficient of fragment $F(ab')_2$ agrees very closely with that of IgG (16).

TABLE I

Antibody Activity to Bacteriophage φX174 of Rabbit IgG and its Fragments

Animal No.	Time after initial immunization	IgG or fragment	k/OD 280	Per cent of specific antibody activity relative to IgG
	wk			
Pool No.				
1–6	2	IgG	70.5	100
		$F(ab')_2$	67.5	96
		Fab′	0.014	0.020
		Fab	0.049	0.069
1	4	IgG	12.1	100
		$F(ab')_2$	5.7	47
		Fab	0.16	1.3
	20	IgG	107	100
		$F(ab')_2$	118	110
		Fab′	124	116
		Fab	106	99
3	4	IgG	1.43	100
		$F(ab')_2$	1.80	126
		Fab′	0.081	5.6
		Fab	0.122	8.5
	20	IgG	75.7	100
		$F(ab')_2$	82.9	109
		Fab′	55.4	73
		Fab	76.6	101
4	4	IgG	28.2	100
		$F(ab')_2$	33.2	118
		Fab′	0.85	3.0
		Fab	2.01	7.1
	20	IgG	157	100
		$F(ab')_2$	200	127
		Fab′	150	96
		Fab	190	121
6	4	IgG	2.23	100
		$F(ab')_2$	3.99	179
		Fab′	0.26	11.7
	20	IgG	144	100
		$F(ab')_2$	114	79
		Fab′	117	81

antibody from 4- and 20-wk sera were incubated with ϕX and, at the time that approximately 30–50% of the phage was neutralized, the mixtures were diluted 1:1000 and incubated at 37°C for 30 min. A representative experiment is shown in Fig. 1. As can be seen, no appreciable dissociation occurred in any of the mixtures.

A second set of experiments was therefore performed in which the diluted

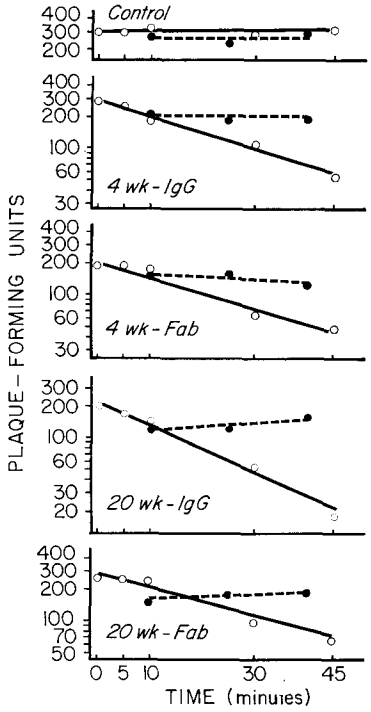

FIG. 1. Dissociation of phage-antibody complexes after dilution at 37°C. See Materials and Methods. O——O, conventional titration; ●----●, 1:1000 dilution.

mixtures were incubated at 47°C in an attempt to increase the rate of dissociation.[2] A representative experiment is shown in Fig. 2. As can be seen, phage was reactivated by this technique; however, the proportion reactivated was small and no difference was detected between Fab obtained from 4- and 20-wk sera.

[2] In these experiments the decision-tube, rather than the conventional assay, was performed. This was done to exclude the possibility that a large degree of dissociation, occurring during the plating procedure, was masking dissociation which occurred upon dilution of the primary reaction mixture.

No evidence was obtained, therefore, by either method to indicate that differences in dissociability of antibody from phage could explain the marked difference in neutralizing efficiency between Fab obtained from early and late antisera. It could be argued that the methods used are both indirect; however, they rely on the same technique, phage neutralization, as the one that detected the differences in question.

DISCUSSION

Previous reports have indicated that univalent 3.5S fragments of antibody produced by enzymatic digestion, or univalent 7S antibodies formed by hy-

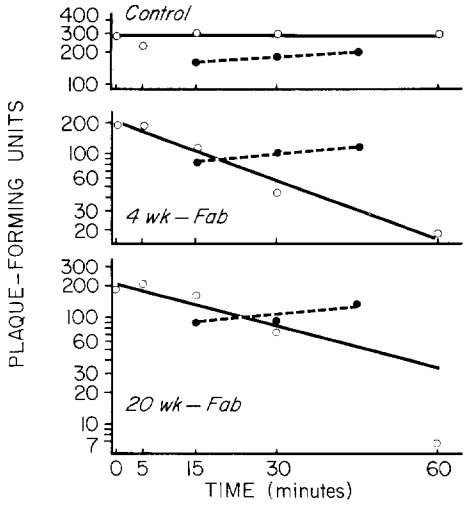

FIG. 2. Dissociation of phage-antibody complexes after dilution at 47°C. Dilution mixtures were plated by the decision-tube method. See Materials and Methods. O——O, conventional titration; ●----●, 1:100 dilution.

bridization of a half-molecule of antibody with a half-molecule of nonspecific IgG, are generally less effective on a molar basis than bivalent antibody in the neutralization of viruses that infect animal or bacterial cells. However, wide variations have been reported in the relative effectiveness of univalent fragments as compared to intact antibody (1–11). The data presented here may account for some of the variation observed.

Univalent 3.5S fragments of rabbit antibody to the virus ϕX174 were virtually as effective as bivalent antibody, even at high dilution, when the antibody was prepared from a late antiserum, taken from a rabbit after 20 wk of immunization. In contrast, when the IgG was isolated from bleedings taken after only 2–4 wk, univalent Fab or Fab′ fragments were markedly reduced in activity compared to intact IgG.

These results indicate that cross-linkage is not essential for viral neutralization. They are also consistent with the possibility that the lower activity of univalent, as compared to bivalent, antibody in antiviral antibodies that have previously been investigated is attributable to a greater combining affinity of the bivalent antibody. This in turn may be attributable to a greater combining energy associated with the attachment of two sites of a single molecule to a particle (1, 4, 12).[3] Our data provide strong support for this hypothesis and indicate that a single site of attachment can suffice for neutralization. The results can be explained on the basis that combining affinity increases during prolonged immunization (19, 21), and that a minimum threshold of combining affinity is necessary for effective viral neutralization. When the association constant of a single site exceeds this threshold, bivalency is no longer required.

Attempts to demonstrate directly the relatively low affinity of early antibody or its fragments, by dissociation at high dilution and elevated temperature (47°C), were not successful (Figs. 1 and 2). Although slight reactivation of virus occurred at 47°C there were no significant differences between early and late antibodies or between univalent fragments and bivalent antibody. Since attachment of antibody or its fragments to the virus particle is not readily reversible at neutral pH, the relatively high neutralization constant of Fab fragments from later bleedings either reflects a higher velocity constant for association, and is independent of the rate constant for dissociation, or a secondary reaction which is not rate determining takes place after attachment of the antibody or its fragments and prevents dissociation upon subsequent dilution.

The failure to observe dissociation upon dilution is in agreement with the results of Vogt et al. (9) and of Keller (10), who worked with univalent fragments of antibody directed to polio virus. Keller did find, however, that univalent antibody dissociated more rapidly than bivalent antibody at low pH.

Our results contrast with those obtained with antibodies to influenza virus (1). Univalent antibody fragments were found to dissociate to a large extent from that virus upon dilution, whereas bivalent antibody, after being in contact with virus for some time, did not.

With each antiserum we tested, the 5S bivalent fragment of antibody produced by peptic digestion was as effective as the untreated 7S molecule. This is in agreement with the findings of Klinman et al., obtained with bacteriophage R17 (4), and supports the absence of any significant role for fragment Fc in these systems. Several other investigators have found that the 5S fragment retains a substantial amount of activity, although in each system intact IgG was somewhat more effective (2, 5, 7, 10, 22).

It should be noted that complement was not present in our test system, and in any event, univalent fragments do not fix complement (23, 24). Data con-

[3] Doubling the standard free energy of interaction is equivalent to squaring the association constant for the interaction.

cerning the role of complement may, however, be relevant to the mechanism of neutralization of virus. Complement (25, 26), and in particular its first four components (27–30), has been shown to enhance the viral-neutralizing capacity of antibodies. Because the complete complement system is not needed, it was proposed that complement enhances activity sterically by contributing bulk to the virus-antibody complex (27, 28). Alternatively, we would suggest that complement may act by stabilizing the interaction of antibody with virus since it is known to be capable of cross-linking antigen-antibody complexes (31, 32). In effect, cross-linkage could increase the effective valence of antibody and therefore its avidity. A similar argument based on increased effective valence can be advanced to account for the observed enhancement of the activity of antiviral antibody, or of its fragments or polypeptide chains, by antiglobulin reagents (6, 7, 33–36). Alternatively, anti-antibodies may act by contributing bulk and thus blocking critical sites (7, 35), by stabilizing the virus-antibody complex through cross-linkage (34, 35), or possibly by distorting the structure of the viral protein coat (7). It is relevant that both complement (37–39) and antiglobulin reagents (36, 39, 40) generally provide maximal enhancement when used in conjunction with antibodies from early bleedings.

Support for the viewpoint that a steric effect of antibody rather than destruction of the virus is the mechanism underlying neutralization in many systems is the fact that the neutralization is frequently reversible; the activity of virus-antibody complexes has been restored by proteolytic digestion of the antibody (41), by acidification (42), or, as indicated above, by dilution. It is evident that in such experiments contact with antibody did not destroy the virus.

It should be of interest to extend these studies to other viruses, by comparing the effectiveness of univalent fragments and untreated antibody after brief and prolonged immunization.

SUMMARY

The role of bivalence of antibody in its capacity to neutralize virus was studied with rabbit antibodies to the bacteriophage, ϕX174. Univalent Fab or Fab′ fragments of IgG isolated from antiviral antisera obtained early in the immunization schedule had virtually no activity compared to that of the intact IgG. When the antibodies were isolated from antisera of the same rabbits several months later, the univalent fragments and IgG were essentially equal in activity. The results are interpreted on the basis that an IgG molecule, because of its bivalence, has a higher effective combining affinity (avidity) than a univalent fragment. After prolonged immunization, however, the affinity of univalent antibody becomes sufficiently high that it exceeds a threshold value, above which further increase in affinity, through bivalence, is no longer significant. The results could explain the variability in relative effectiveness of univalent antibodies observed in previous studies. These data, and the fact

that $F(ab')_2$ fragments from either "early" or "late" antisera were as effective as IgG, indicate that fragment Fc is not a significant factor in neutralization.

No differences in dissociation from the virus of univalent antibody from early and late antisera could be demonstrated by dilution at temperatures up to 47°C. The attachment at sites of neutralization on the virus appears to be functionally almost irreversible in this system.

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