The effect of an agglutogen on virus infection: biotinylated filamentous phages and avidin as a model

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Abstract To address the effect of an agglutogen on virus infection, we studied the avidin-associated inhibition of infection by biotinylated M13 phages (BIO-phages). Microscopic observation of mixtures of BIO-phages and avidin–fluorescein conjugates revealed many aggregates. Even at low phage concentrations, avidin induced inhibition of infection significantly. Anti-M13 phage antibody also made aggregates and inhibited the infection but in a different manner from avidin. The inhibition by avidin was at ≥ 2 μg/ml, time dependent and marked until 10 min after the mixing of the BIO-phages and *Escherichia coli*. On the other hand, antibody inhibited the infection at ≥ 0.1 μg/ml dose dependently, and the inhibition was time dependent and marked until 45 min after the mixing at moderate and low phage concentrations. These results indicate that avidin against BIO-phages and antibodies are agglutogens, and the inhibition of the BIO-phages by avidin is closely related to the tetramerization of avidin. Agglutogens may be novel alternative antiviral drugs. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Biotin; Inhibitor; Aggregation; Antibody; Antiviral drug; Tetramerization

1. Introduction

Developing effective antiviral drugs is an intriguing challenge for many researchers, especially from therapeutic viewpoints. For example, combination drug therapy against human immunodeficiency virus-1 (HIV-1) targets two virus-specific enzymes, HIV-1 protease and reverse transcriptase [1]. In a new strategy, peptide and protein inhibitors that target the gp41 coiled-coil pocket have been developed to inhibit HIV entry [2–4]. A recombinant immunotoxin directed against the HIV-1 envelope glycoprotein has been developed to kill infected cells specifically [5–7]. However, these drugs all target virus-infected cells or factors required for virus entry, not the virus itself. Agglutogens may be candidate antiviral agents for direct inhibition of the virus.

To address the effects of agglutogens on virus infection, we prepared biotinylated filamentous bacteriophages (BIO-phages) [8] and investigated the influence of avidin on their infection of *Escherichia coli*. We found that avidin connected the BIO-phages to each other, causing aggregation and leading to a strong inhibition of infection. In addition, we discuss the characteristics and mechanisms of this inhibition and describe the potential use of agglutogens as virus inhibitors.

2. Materials and methods

2.1. Materials

D-Biotin-*N*-hydroxysuccinimide ester (BIO) was purchased from Roche Molecular Biochemicals (Tokyo, Japan), avidin–horseradish peroxidase conjugate (avidin–HRP) from ProZyme (San Leandro, CA, USA), fluorescein avidin D from Vector Laboratories (Burlingame, CA, USA), and avidin from Wako Fine Chemicals Inc. (Osaka, Japan). Anti-M13 monoclonal antibody was purchased from Amersham Pharmacia Biotech UK Ltd. (Buckinghamshire, UK). This antibody is a subclass IgG2a mouse monoclonal antibody purified from BALB/c mouse ascites fluid and reacts specifically with the bacteriophage M13 major coat protein product of gene VIII. All other reagents were of biochemical research grade.

2.2. Assessment of the ability of avidin to bind BIO-phages

BIO-phages were prepared as described [8]. BIO-phages were detected by using quantitative dot blotting as described previously [8,9] with some modification. Aliquots of BIO-phages and avidin–HRP were bound to polyvinyldifluoride membranes. The membranes were washed once in phosphate-buffered saline (PBS), blocked with PBS containing 2% skim milk, and incubated with 2000-fold diluted avidin–HRP for 60 min at room temperature. After washing with PBS containing 0.1% Tween-20, the blots were developed by using a chemiluminescent substrate and analyzed by using a luminescent image analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan).

2.3. Confocal microscopy

We mixed 5 μl of BIO-phages (6.0×10^7 cfu) or M13KO7 phages (2.2×10^7 cfu) with 5 μl of fluorescein avidin D (0 to 1 mg/ml in PBS) at room temperature. Just after the mixing, the mixtures were mounted on glass slides, covered with cover glasses, and viewed through a confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany). To observe the antibody-associated inhibition, M13KO7 phages (8.0×10^7 cfu/μl) and anti-M13 antibody–fluorescein conjugate (1 mg/ml) were used instead of BIO-phages and avidin, respectively.

2.4. Assays of inhibition of phage infection

E. coli JM109 cells were grown at 37°C overnight in LB medium. We combined 490 μl of the overnight culture with 5 μl of an avidin solution (0–1 mg/ml in PBS) and then added 5 μl of a solution containing BIO-phages (7×10^0, 9×10^0, 2.3×10^1, 4.5×10^1, 1.2×10^2, 2.8×10^2, 7.5×10^2, 5.3×10^3, 1.6×10^5, 4.3×10^6 cfu/μl) or unlabeled M13KO7 phages (1.5×10^6 cfu/μl). The reaction mixture was incubated for 1 h at 37°C, after which it was plated and incubated overnight, and the bacterial colonies were counted. The data are presented as the means ± 1 standard deviation from three experiments. To assess the antibody-associated inhibition of infection, 5 μl anti-M13 antibody (0–1 mg/ml in PBS) and 5 μl of M13KO7 phages (2.3×10^1,

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6.1×10^2, 3.0×10^4, 7.4×10^5, 1.0×10^7 cfu/μl) were used instead of avidin and BIO-phages, respectively.

We assessed the time-dependency of the avidin-associated inhibition of infection. At time 0, 490 μl of the overnight cultured *E. coli* JM109 cells solution and 5 μl of BIO-phages (1.2×10^2, 6.3×10^4, 3.2×10^6 cfu/μl) were combined and incubated at 37°C. After various times, 5 μl avidin (1 mg/ml) was added to the culture solutions, which were then incubated at 37°C until 1 h after the start of the assay. After incubation it was plated and incubated overnight, and the bacterial colonies were counted. The data are presented as the means ± 1 standard deviation from three experiments. To assess the time-dependency of the antibody-associated inhibition, 5 μl of M13KO7 phages (2.4×10^2, 5.2×10^4, 2.1×10^6 cfu/μl) and 5 μl of anti-M13 (1 mg/ml in PBS) were used instead of BIO-phages and avidin, respectively.

3. Results

3.1. Assessment of the binding of BIO-phages and avidin

To assess their ability to bind to avidin, BIO-phages and avidin–HRP were blotted on membranes and reacted with avidin–HRP. The peroxidase activities of the directly blotted avidin–HRP and the BIO-phage-binding avidin–HRP were related (Fig. 1). According to this result, the signal intensity from 1.0×10^5 BIO-phages corresponded with that of 4.5×10^9 molecules of avidin–HRP (molecular weight, ~110 kDa).

3.2. Microscopic observation of BIO-phage aggregates

We observed the mixtures of BIO-phages and an avidin–fluorescein conjugate using fluorescence microscopy. These mixtures contained many large avidin–BIO-phage aggregates (Fig. 2a) when the final concentration of the avidin–fluorescein conjugate was ≥ 50 μg/ml; these large aggregates were not observed at conjugate concentrations of < 10 μg/ml. In a control experiment, we mixed unbiotinylated phages (2.2×10^7 cfu) with the avidin–fluorescein conjugate (1 mg/ml) but observed no aggregates (Fig. 2b). We also observed the mixtures of unbiotinylated phages (8.0×10^7 cfu/μl) and anti-M13 antibody–fluorescein conjugate (1 mg/ml) and the mixture revealed many aggregates (data not shown).

3.3. Avidin-associated inhibition of the infection of E. coli by BIO-phages

Various titrations of BIO-phage were added to *E. coli* JM109 cultures containing various concentrations of avidin, and the number of phage-infected bacterial colonies was determined. Avidin reduced the number of BIO-phage-infected colonies. In contrast, 5 and 10 μg/ml avidin did not influence

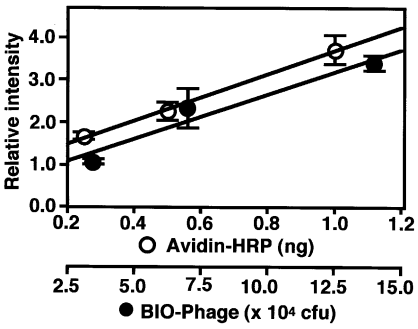

Fig. 1. Assessment of the ability of avidin to bind BIO-phages. Aliquots of BIO-phages (open circles) and avidin–HRP (solid circles) were bound to polyvinyldifluoride membranes, reacted with avidin–HRP, and made visible by using a chemiluminescent substrate. Relative intensity was expressed as a ratio with the intensity of 3.5×10^4 cfu of BIO-phages and is presented as the means ± 1 standard deviation (bars) of three experiments.

infection by unbiotinylated phages (1.5×10^6 cfu/μl) (data not shown). When the solution of BIO-phages was of high titration ($>7.5\times10^2$ cfu/μl) (Fig. 3a), the colonies began to decrease at 2 μg/ml avidin, became marked at 3 μg/ml, and was almost 0 at 5 μg/ml. Their decrease patterns were similar to each other. These results indicate that the decreases in the number of infectious BIO-phages corresponded to the avidin concentration mainly and not to the ratio of BIO-phages to avidin molecules at high titration of phage. However, the antibody-associated inhibition of phage infection is dependent upon the antibody concentration (Fig. 4), and the profile of this inhibition was quite different from that of avidin–high titration BIO-phages. Together, these results suggest the avidin's cooperative inhibition of infection by BIO-phages. To put it concretely, the tetramerization of avidin may cause marked inhibition of infection by BIO-phages, which may inhibit their attachment to *E. coli* due to their aggregation.

When the solution of BIO-phages was of low titration ($\leq 2.8\times10^2$ cfu/μl), the colonies began to decrease ≤ 1 μg/ml avidin, gradually (Fig. 3b). These findings were similar to that of antibody–phages; however, the inhibition was weaker than that of antibody. These results suggested that monomer avidin may cause weak inhibition by low titration BIO-phage.

3.4. Time-dependent inhibition of BIO-phage infection by avidin

To investigate the time-dependency of the associated inhi-

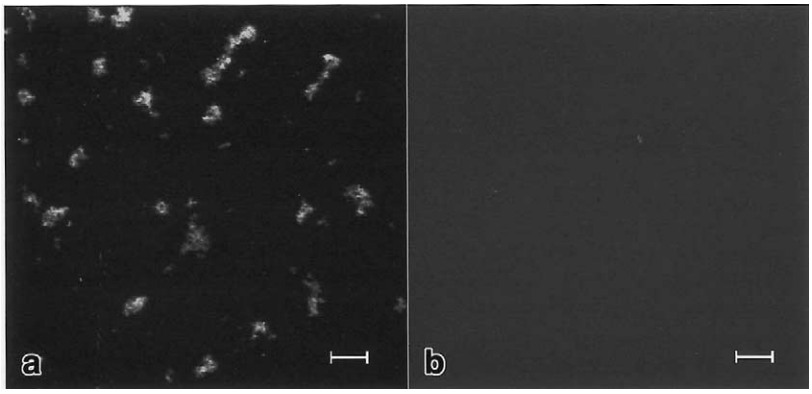

Fig. 2. Confocal microscopy of mixtures of BIO-phages and fluorescein avidin D. a: Mixtures of BIO-phages (6.0×10^7 cfu) and an avidin–fluorescein conjugate (final concentration, 0.5 mg/ml) showed many large aggregates. b: In a control experiment, we mixed unbiotinylated M13KO7 phages (2.2×10^7 cfu) with the avidin–fluorescein conjugate, but no aggregates were observed. Bar, 10 μm.

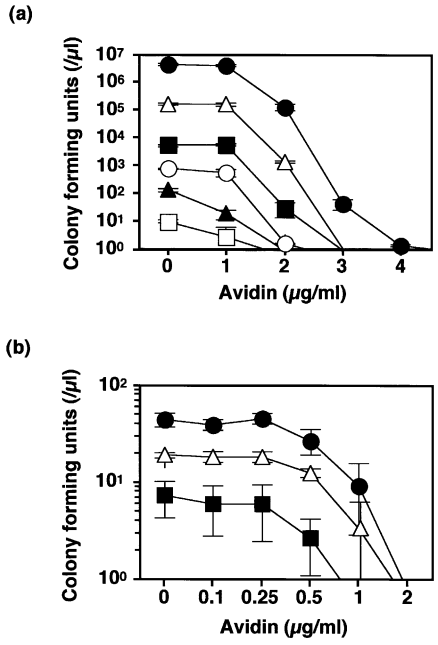

Fig. 3. Inhibition of the infection of various concentrations of BIO-phages into *E. coli* by avidin. A mixture of 490 μl of an overnight culture of *E. coli* JM109 and 5 μl of a solution containing various concentrations of avidin was combined with 5 μl of a solution containing various concentrations of BIO-phages solutions (a: 9×10^0 cfu/μl, open boxes; 1.2×10^2 cfu/μl, solid triangles; 7.5×10^2 cfu/μl, open circles; 5.3×10^3 cfu/μl, solid boxes; 1.6×10^5 cfu/μl, open triangles; 4.3×10^6 cfu/μl, solid circles. b: 7.0×10^0 cfu/μl, solid boxes; 2.3×10^1 cfu/μl, open triangles; 4.5×10^1 cfu/μl, solid circles) and incubated for 1 h at 37°C. After incubation, the solutions were plated and incubated overnight, after which the bacterial colonies were counted. Data are presented as the means ± 1 standard deviation (bars) from three experiments.

bition of infection, avidin (final concentration, 10 μg/ml) was added at various times to *E. coli* JM109 cultures containing various titrations of BIO-phages; the titers were determined 1 h after the start of the assay. When avidin was added immediately before the BIO-phages were added, infection was almost inhibited. Noteworthy inhibition occurred when avidin was added during the first 10 min of the assay, but avidin had lost most of its inhibitory effect on infection by the 20-min timepoint at each titration (Fig. 5). In contrast, anti-M13 antibody (final concentration, 10 μg/ml) inhibited infection of low or moderate titration of phages when added at any point during the first 45 min after the start of the assay; however, the inhibition was weak against high titration BIO-phages (Fig. 5).

4. Discussion

In the present report, we show that BIO-phages were aggregated by avidin and the infection of JM109 cells by BIO-phages was inhibited by the addition of avidin to the culture medium in two different manners. Fluorescence microscopy revealed many large aggregates in mixtures of BIO-phages and an avidin–fluorescein conjugate (final concentration, 50 μg/ml to 1 mg/ml). These large aggregates were not observed at conjugate concentrations of <10 μg/ml. Inhibition of the infection could be observed at conjugate concentrations of <10 μg/ml. These results suggest that assembly of several phage particles (i.e. microaggregates), not formation of large aggregates, mainly drives inhibition of the infection. Microaggregates may have formed, which might not have been visible by fluorescence microscopy because of their small size.

Assays of inhibition of phage infection using BIO-phage and avidin revealed many characteristic findings. At low concentration of BIO-phage, avidin inhibited its infection dose dependently like that of antibody. However, the inhibition ability was weaker than that of antibody and was not observed at high concentration of BIO-phage. At high concentrations of phages the remarkable inhibition was induced by ≥ 2 μg/ml avidin, suggesting that the inhibition was related to the concentration of avidin and not to the ratio of avidin to phages. In addition, avidin added before or within 10 min after the addition of BIO-phages to *E. coli* cultures markedly inhibited infection of the cells by the phages. However, the inhibitory effect of avidin was decreased when it was added to the phage-containing cultures after 10 min.

The binding avidity of avidin to biotin is very high (K_d, $\sim10^{-15}$ M) [10]. Results of a recent protein engineering study have indicated that monomeric avidin recognizes biotin at a K_d of $\sim10^{-8}$ M [11]. In the present study, 1 μg/ml avidin corresponded to 10^{-8} M, a concentration that did not lead to inhibition of infection except for low concentration of phage. These results suggest that the aggregates we observed are formed via the following steps: (1) avidin monomers bind biotinylated capsid proteins; (2) tetramerization of avidin monomer is induced in a concentration-dependent manner; (3) tetrameric avidin binds the intercellular BIO-phages; and (4) aggregates of BIO-phages are formed. Because inhibition of infection did not occur at 1 μg/ml avidin at high titration of phage, not many and simple binding of monomeric avidin with BIO-phages likely did not influence the interaction of the pIII with the F-pilus and subsequent infection. Thus, the tetramerization of avidin may cause marked inhibition of infection by BIO-phages, which may inhibit their attachment to *E. coli*. In addition, because no clear aggregate could be observed at the avidin–fluorescein conjugate of <10 μg/ml on microscopy, large aggregate formation may not be essential for loss of infectivity.

Our results have demonstrated that both avidin against BIO-phages and antibodies against phages are agglutogen

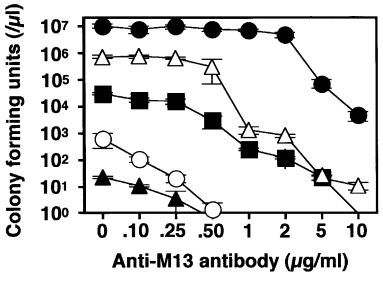

Fig. 4. Antibody-associated inhibition of infection of *E. coli* by BIO-phages. A mixture of 490 μl of an overnight culture of *E. coli* JM109 and 5 μl of a solution containing various concentrations of anti-M13 antibody was combined with 5 μl of a solution containing M13KO7 phages (2.3×10^1 cfu/μl, solid triangles; 6.1×10^2 cfu/μl, open circles; 3.0×10^4 cfu/μl, solid boxes; 7.4×10^5 cfu/μl, open triangles; 1.0×10^7 cfu/μl, solid circles) and incubated for 1 h at 37°C. After incubation, the solutions were plated and incubated overnight, after which the bacterial colonies were counted. Data are presented as the means ± 1 standard deviation (bars) from three experiments.

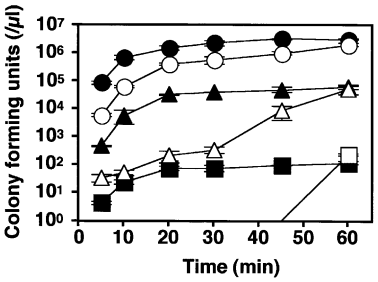

Fig. 5. Time-sensitivity of inhibition of the infection of *E. coli* by BIO-phages. 490 μl of an overnight culture of *E. coli* JM109 was mixed with 5 μl of a solution containing BIO-phages (1.2×10^2 cfu/μl, solid boxes; 6.3×10^4 cfu/μl, solid triangles; 3.2×10^6 cfu/μll solid circles) or M13KO7 phages (2.4×10^2 cfu/μl, open boxes; 5.2×10^4 cfu/μl, open triangles; 2.1×10^6 cfu/μl, open circles) and incubated at 37°C. After various times, 5 μl of a solution containing 1 mg/ml avidin or 5 μl of a solution containing 1 mg/ml anti-M13 antibody was added, and the mixture was incubated for a total of 1 h. After incubation, the solutions were plated and incubated overnight, and then the bacterial colonies were counted. Data are presented as the means ± 1 standard deviation (bars) from three experiments.

and inhibit the phage infection significantly. Avidin, however, inhibits the high titration phage infection in a different manner from antibody in time-dependency. The antibody activity against virions most often considered is neutralization including aggregation, inhibition of viral entry by inhibition of attachment and inhibition of fusion with the target cell [12–17], as well as post-entry mechanism, such as interference with primary and secondary uncoating of the genetic information of the virus [15]. The decrease of time-dependent inhibition after 10 min of avidin would indicate that avidin had only a part of neutralization activity of antibody.

Both additions of avidin before or within 10 min after infection and antibody before or within 45 min of infection markedly inhibited phage infection. The time-dependent aspect of the agglutogen inhibition of infection suggests that agglutogens may be effective for preventing their infection of cells. At least pre-existing of agglutogen inhibited virus infection significantly. Therefore agglutogen would be an effective disinfecting and prophylactic agent against virus infection and expansion from infected cells to uninfected cells.

The development of an agglutogen against a specific virus and subsequent agglutogen therapy would be an alternative strategy for the design of antiviral drugs. It might be difficult, however, to find and to make agglutogens that combine the required high specificity and avidity. Recently the direct evolution of antibody fragments with femtomolar antigen-binding affinity has been reported [18] and tetravalent antibody technologies have been developed for tumor therapy [19–22]. These technologies would provide strong agglutogens against virus. Ligand-induced multimerization of virus particles by novel reagents would likely be an effective strategy for the development of antiviral drugs.

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