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Formation and Activity of Covalent Conjugates of Poliovirus and Ligands Binding to Cell Surface Structures

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Disulfide-linked conjugates of poliovirus with streptavidin or concanavalin A were formed and the binding of the conjugates to mouse L cells that lack natural poliovirus receptors was studied. The conjugate with streptavidin was specifically bound to biotinylated L cells, but not to unmodified L cells. The conjugate with conA was bound to L cells in the absence of, but not in the presence of α-methyl mannoside. Incubation of L cells with bound conjugates did not produce virus, although the conjugates were highly infectious in HeLa cells, containing natural poliovirus receptors. This suggests that the artificially bound virus was unable to penetrate the L cells and start replication. The possibility that binding of the virus to the natural receptor is required for efficient infection is discussed.

Poliovirus binds to receptors that are present only on cells of primate origin. At low virus concentrations this binding is essential for infection [1, 2]. Recent findings at this laboratory indicate that the penetration of the poliovirus genome into the cytosol takes place in acidified endocytic vesicles where the low pH exposes hydrophobic domains in the virus capsid [3, 4]. The possibility was considered that the sole function of the virus receptor is to increase the local concentration of the virus at the cell surface and thus augment the efficiency of virus uptake by endocytosis. If this were so, it might be possible to replace the specific binding to the poliovirus receptor by conjugating the virus to a ligand that binds to cell surface structures other than the normal receptor. In fact, in the case of enveloped viruses, binding by alternative receptors, has led to productive infection in cells lacking the naturally occurring receptor [5, 6].

The specific binding of picornaviruses to their receptors could, however, also play a more direct role in virus penetration. This may be the case with diphtheria toxin where specific interaction with the anion antiporter appears to be essential for efficient entry [7, 8]. In the case of poliovirus and other picornaviruses it has been suggested that the cellular receptor binds to the canyon surrounding the twelve vertices at the virus surface [9, 10]. Insertion of the specific receptor into this canyon could act as a wedge and facilitate the conformational change which is triggered by low pH and which exposes hydrophobic domains on the virus

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surface [3, 4]. In fact, the exposure of hydrophobic domains occurs at less acidic pH when the virus binds to cell surface receptors than when the virus is free in solution [3].

In order to differentiate between these two possibilities, we have conjugated poliovirus to streptavidin and conA (concanavalin A) and measured the ability of the conjugates to bind to and to infect mouse L cells that lack poliovirus receptors. In the experiments with the streptavidin conjugate we labelled the L cells with biotin to generate binding sites for streptavidin. It has been repeatedly shown that isolated RNA of picornaviruses, such as poliovirus and mengovirus, is able to replicate in various mammalian cell lines that lack the specific virus receptor if the RNA is artificially brought into the cytosol [11–13].

MATERIALS AND METHODS

Materials

Iminobiotin-agarose, biotin-*N*-hydroxysuccinimide ester, streptavidin, α-methyl mannoside, and concanavalin A were obtained from Sigma Chemical Co., St. Louis, Mo., USA. SPDP (*N*-succinimidyl 3-(2-pyridyldithio)propionate) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Anti-poliovirus serum (from rabbits) was prepared as earlier described [14]. [^{35}S]Methionine (sp. act. 1480 Ci/mmol), [^{3}H]leucine (sp. act. 133 Ci/mmol) and ^{125}I were obtained from The Radiochemical Centre, Amersham, Bucks, England.

Virus

Poliovirus type 1 (Sabin) was obtained from the National Institute for Public Health, Oslo, Norway. The virus was propagated in HeLa S3 cells and purified as earlier described [14]. Production of [^{35}S]methionine-labelled virus was as described [14]. The specific activity of the virus was 7.7×10^{5} virus particles per cpm in one experiment and 1.83×10^{6} virus particle per cpm in another.

Cell Culture

HeLa OHIO cells and L cells were grown as monolayer cultures in minimal essential medium with 10% fetal calf serum (FCS) as described [15]. HeLa OHIO cells used in the titration experiments were seeded out the day before the experiment into 24-well microtitre plates, usually at a density of 10^{4} cells/well. The concentration of FCS was as indicated in the figure captions.

Conjugation of Poliovirus to Streptavidin and ConA

Streptavidin or conA in PBS was reacted with a 4–5-fold molar excess of SPDP for 30 min at 23°C with occasional stirring. The reaction was carried out as described by Carlsson et al. [16] with minor modifications [17]. The solution was dialysed overnight against PBS at 4°C. The extent of substitution was estimated from the absorbance at 286 and 343 nm measured in aliquots taken before and after reduction with 100 mM dithiothreitol, as described [16, 17]. The substitution was approx. 1½ molecule of SPDP per molecule of protein.

In the formation of conjugates with poliovirus we took advantage of the fact that there are free SH groups on the virus [18, 19]. 2-Pyridyl-dithiopropionate-substituted streptavidin or conA was therefore mixed with virus and allowed to react for 24 h at room temperature with careful shaking. To the virus preparation used was added some virus that had been metabolically labelled with [^{35}S]methionine [14] to simplify the detection of the virus during the subsequent purification steps.

The conjugate of virus with streptavidin was applied to a 300 µl column of iminobiotin–agarose equilibrated with 450 mM NaCl, 50 mM NH_4Cl, 50 mM Na_2CO_3, 1% Triton X-100, pH 9.5. The column was washed with the same buffer to remove unreacted virus. Conjugate and free streptavidin were both subsequently eluted with 450 mM NaCl, 50 mM ammonium acetate, 1% Triton X-100, pH 4.5. The radioactivity in aliquots of the fractions was determined.

The conjugate with conA was applied to a 2.5 ml column of Sephacryl S300 equilibrated with PBS. After washing the column with PBS to remove unreacted virus, the conjugate and free conA were both eluted with 0.25 M α-methyl mannoside in the same buffer.

Finally, unreacted streptavidin and conA were removed by banding the virus in CsCl gradients, as described earlier [14].

Biotinylation of Cells

L cells in 24-well microtitre plates were washed twice with PBS containing 0.5 mM $CaCl_2$ and adjusted to pH 8.0, and the cells were then incubated with the same buffer for 10 min at 23°C. Biotin-*N*-hydroxysuccinimide was added to a final concentration of 0.2 mM and the incubation was continued for another 30 min. Excess reagent was then removed by washing the cells twice with HEPES medium, pH 7.3, containing 0.1 mg/ml bovine serum albumin (BSA).

Solutions

HEPES *medium*. Minimum essential medium where the bicarbonate was replaced by 20 mM HEPES. PBS (phosphate-buffered saline): 140 mM NaCl, 10 mM Na-phosphate, pH 7.4.

RESULTS

Formation of Disulfide-linked Conjugates of Poliovirus and Streptavidin or ConA

To test if poliovirus can infect cells when bound to an alternative receptor, we first decided to prepare a conjugate of streptavidin and poliovirus and test if it was infectious in biotinylated mouse L cells that lack natural poliovirus receptors. For this purpose we first reacted streptavidin with SPDP to obtain the 2-pyridyl-dithiopropionate derivative of streptavidin. This derivative was then reacted with poliovirus. It has been well documented that free SH groups are exposed on poliovirus [18, 19] and in accordance with this, 2-pyridyl-dithiopropionate-derivatized streptavidin reacted in good yield with the native virus (see below).

Streptavidin binds less strongly to iminobiotin than to biotin and the binding is weak at low pH [20, 21]. The conjugate was therefore purified by adsorbing it to an iminobiotin–Sepharose column at alkaline pH and it was subsequently eluted at pH 4.5. The data in fig. 1*A* show that approx. 10% of the labelled virus was obtained in the fraction eluted at the acidic pH. In control experiments where unmodified virus was passed through an iminobiotin column at alkaline pH and the column was then eluted at pH 4.5, there was no evidence of binding of the virus to the column (data not shown).

The eluted conjugate was further purified by CsCl-gradient centrifugation to remove unreacted streptavidin. As shown in Fig. 1*B*, most of the radioactive material banded at a buoyant density of $\varrho = 1.34$, indicating that the density of the virus had not been measurably altered by the conjugation. In most cases a small peak of radioactive material with buoyant density of $\varrho = 1.42$ was also found. This material was not analysed further.

When the purified conjugated virus was titrated on the poliovirus-sensitive HeLa OHIO cells, it was found highly infectious (data not shown). Clearly

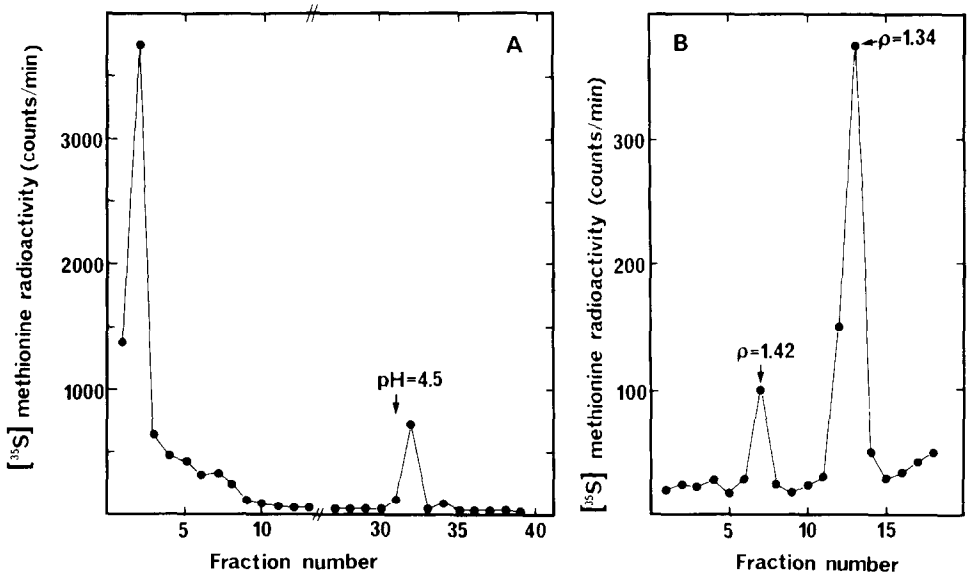

Fig. 1. Purification of streptavidin–poliovirus conjugate. (*A*) Streptavidin and [^{35}S]methionine-labelled poliovirus was conjugated as described in Materials and Methods and applied to a 300-μl column of iminobiotin–agarose equilibrated with 450 mM NaCl, 50 mM NH_4Cl, 50 mM Na_2CO_3, 1% Triton X-100, pH 9.5. The column was washed with the same buffer to remove free unreacted virus, after which the conjugate was eluted with 450 mM NaCl, 50 mM ammonium acetate, 1% Triton X-100, pH 4.5. Finally, the radioactivity in aliquots of each fraction was measured. (*B*) Conjugate eluted at pH 4.5 was separated from free streptavidin by banding in a CsCl gradient, as described in Materials and Methods. Aliquots of the different fractions from the gradient were taken and the density and radioactivity was measured.

therefore, the conjugation of streptavidin to the virus does not interfere greatly with the normal entry mechanism of the virus.

The formation of a conjugate between poliovirus and conA was carried out essentially as described for the conjugation with streptavidin. The purification to eliminate free virus was carried out on a Sephacryl 300 column to which conA binds and can be eluted with α-methyl mannoside.

In experiments using the conA labelled with ^{125}I, SDS–PAGE revealed the presence of conjugates of conA subunits SS-linked to virus capsid proteins (data not shown).

Altogether, it may be concluded that disulfide-linked conjugates of poliovirus can be formed with a good yield, using either streptavidin or conA and that the infectivity of the virus on poliovirus-sensitive cells is not greatly weakened by the process.

Ability of Conjugated Poliovirus to Bind to L Cells Lacking Poliovirus Receptors

ConA binds to a large number of cell surface glycoproteins present on most cells, including L cells [22]. On the other hand, there are no naturally occurring

cell surface-binding sites for streptavidin. To generate such sites, cells were reacted with biotin-*N*-hydroxysuccinimide ester to form covalently bound biotin groups on reactive groups at the cell surface, such as free amino groups in surface proteins. This treatment did not impair the viability of the cells (data not shown).

To test whether the conjugate of poliovirus and streptavidin was bound to biotinylated L cells, biotinylated cells as well as untreated cells were incubated with the conjugate for 30 min at 23°C. Unbound conjugate was then washed away and the cells were disrupted to release the bound conjugate. The lysate was titrated on unmodified HeLa OHIO cells to assess the amount of virus that had been associated with the L cells. The data in fig. 2 show that the extract from the biotinylated cells did indeed contain virus, while there was no evidence of the presence of virus in the extract from unmodified cells. This indicates that biotin–streptavidin-dependent binding of virus did take place on the biotinylated cells.

We then tested whether the bound conjugate was able to induce productive infection. For this purpose, L cells with surface-bound conjugate were incubated for 26 h at 37°C, and the cells were then disrupted and the lysate was titrated as above. The data in fig. 2 show that there was essentially no increase in the amount of infectious virus associated with the cells, as compared with cells disrupted immediately after the binding step. This indicates that the artificially bound virus was essentially unable to enter the cells productively.

In a similar way we tested whether the conjugate of poliovirus and conA would bind to L cells. As shown in fig. 3, the lysate from cells treated with the conjugated virus did contain virus, while the lysate from cells treated with conjugate that had been reduced with dithiothreitol to split the disulfide link between conA and the virus did not contain measurable amounts of virus. Also, when the cells were exposed to the intact conjugate in the presence of α-methyl mannoside, which inhibits the binding of conA to cells, there was no evidence of virus binding. Clearly therefore, in the absence of the competing sugar the intact conjugate became bound to cells lacking natural poliovirus receptors. In this case too the amount of virus in the disrupted cells was essentially the same whether or not the cells with bound conjugate had been incubated at 37°C overnight (fig. 3). It therefore appears that although both conjugates are able to bind to L cells via the virus-linked binding protein, the binding is not efficient in inducing virus infection.

DISCUSSION

In this paper we have demonstrated that it is possible to form covalent conjugates of poliovirus and proteins that bind to cell surface markers without radically impairing the infectivity of the virus. We estimated the amount of conjugated virus bound to L cells by disrupting the cells and titrating the lysate for cytopathogenic effect on the sensitive HeLa OHIO cells. Even though the conjugates did bind to the L cells, they were apparently unable to infect the cells.

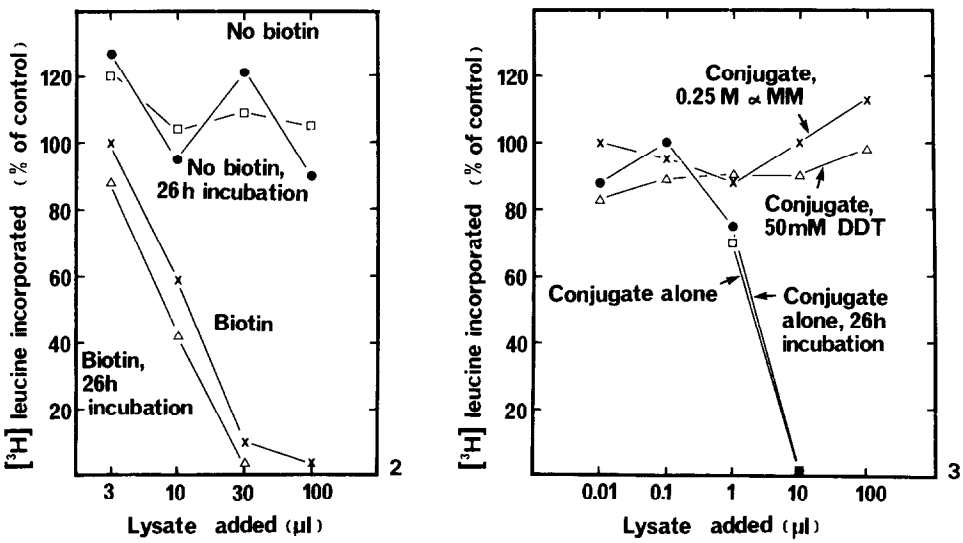

Fig. 2. Binding of poliovirus–streptavidin conjugate to unmodified and to biotinylated L cells. L cells were biotinylated by treatment with biotin-*N*-hydroxysuccinimide as described in Materials and Methods. Poliovirus–streptavidin conjugate was added to biotinylated and to unmodified cells and incubated at 23°C for 30 min. Then the cells were washed to remove unbound conjugate and part of the cells were disrupted by three cycles of freezing and thawing. To another part of the cells was added minimum essential medium with 2% FCS and the cells were incubated at 37°C for 26 h and subsequently disrupted. The lysates were filtered and then titrated on HeLa OHIO cells. After 2 days the ability of the HeLa OHIO cells to incorporate [^{3}H]leucine was measured. □, No biotin, no incubation; ●, no biotin, 26 h incubation; ×, biotin, no incubation; △, biotin, 26 h incubation.

Fig. 3. Binding of poliovirus–conA conjugate to L cells. L cells were incubated for 15 min at 0°C in serum- and glucose-free medium or in medium containing 0.25 M methyl mannoside. Conjugate was added, and the incubation was continued for 1 h at 0°C. In one case, as indicated, the conjugate was pretreated with 50 mM dithiothreitol. The cells were then washed with 0.14 M NaCl, 20 mM Na phosphate, pH 7.4, and further incubated in minimum essential medium containing 1% FCS for 26 h at 37°C. Finally the cells were frozen and thawed three times and the lysates were titrated on poliovirus-sensitive HeLa OHIO cells. After 3 days the ability of the cells to incorporate [^{3}H]leucine during 15 min was measured. ×, Conjugate, α-methylmannoside; △, conjugate pretreated with dithiothreitol; ●, conjugate, 26 h incubation; □, conjugate, no incubation.

The reason why the bound conjugates did not infect L cells could be that the binding of the conjugate differs from the binding of the virus to its specific cell surface receptors. If a single conA or streptavidin molecule is sufficient to retain the virus on the columns used for the purification, the fact that only ~10% of the virus particles treated with conA or streptavidin became bound to the respective affinity columns, indicates that only a fraction of the virus particles were modified. It is therefore likely that essentially all modified particles have bound only one molecule of conA or streptavidin. When the unmodified virus binds to sensitive receptor-bearing cells, the virus probably interacts with a number of receptor molecules. As a result of such multiple binding, a close interaction between the virus particle and the cell membrane should occur. Such close

interaction could be crucial for entry of the viral genome when the bound virus is exposed to low pH.

The possibility should also be considered that the picornavirus receptor plays a particularly important role in virus entry. It has been speculated that the canyon surrounding the twelve vertices at the virus surface represents the receptor-binding region on the virion. If the cellular receptor is bound within this cleft, it is possible that the binding plays a role in the induction of the conformational change required for entry. This binding, together with low pH, may be the requirement for exposure of the hydrophobic domains in the virion necessary for the transfer of the viral RNA to the cytosol [23].

Obviously, one possible reason why virus particles bound to surface markers other than the virus receptor does not induce infection could be that such complexes are not transported to an acidic compartment. This possibility is unlikely, however. Thus, the conjugates should bind to a large number of different cell surface molecules, and at least some of them are likely to be endocytosed by the coated vesicle pathway and directed to acidic endosomes. In fact, conjugates of diphtheria toxin and conA were toxic to L cells that lack natural, functioning diphtheria toxin-binding sites [17]. Furthermore, attempts to induce entry of poliovirus into L cells by exposing cells with bound conjugates to medium adjusted to pH 4.0 were not successful.

In the case of enveloped viruses it appears that alternative modes of binding can induce infection. Thus, it was shown that mutant Sendai virus that lacks the hemagglutinin–neuraminidase attachment protein was able to infect a line of hepatoma cells that express the asialoglycoprotein receptor [6]. Essential for the infection was the interaction of the F-glycoprotein of the virus with the asialoglycoprotein receptor. The mutant virus was also able to induce cell–cell fusion in the hepatoma cultures. Sendai virus was also shown to induce fusion of desialylated erythrocytes in the presence of hybrid antibodies that had affinity both for red blood cells and for Sendai virus [24]. Finally, Fuller et al. [5] showed that vesicular stomatitis virus and Semliki Forest virus that normally infect MDCK cells only from the basolateral side, were able to infect also from the apical side in cells that expressed influenza hemagglutinin on the apical side. The hemagglutinin on the apical side bound glycoproteins on the viruses and thus induced virus entry and infection. Also BHK21 cells that are normally resistant to the corona virus, mouse hepatitis virus MHV-A59, could be infected with this virus via the hemagglutinin–sialic acid interaction.

In all these cases it is possible that multiple attachment points between virus and cell were present, and the systems are therefore not directly comparable to our system where a single point interaction may have occurred. It is also possible that in the case of the enveloped viruses the effect of the receptor binding consists only in bringing the membranes of the virus and the cell in sufficient proximity to allow fusion to occur, while in the case of the picornaviruses the receptor may play a more active role in the entry process.

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REFERENCES

1. Holland, J J, Virology 15 (1961) 312.
2. McLaren, L C, Holland, J J & Syverton, J T, J exp med 109 (1985) 475.
3. Madshus, I H, Olsnes, S & Sandvig, K, EMBO j 3 (1984) 1945.
4. — Virology 139 (1984) 346.
5. Fuller, S D, von Bonsdorff, C-H & Simons, K, EMBO j 4 (1985) 2475.
6. Markwell, M A K, Portner, A & Schwartz, A L, Proc natl acad sci US 82 (1985) 978.
7. Olsnes, S, Madshus, I H, Sandvig, K & Sundan, A, Biochem soc symp 50 (1985) 171.
8. Olsnes, S & Sandvig, K, J biol chem 261 (1986) 1553.
9. Hogle, J M, Chow, M & Filman, D J, Science 229 (1985) 1358.
10. Rossman, M G, Arnold, E, Erickson, J W, Frankenberger, E A, Griffith, J P, Hecht, H-J, Johnson, J E, Kamer, G, Luo, M, Mosser, A G, Rueckert, R R, Sherry, B & Vriend, G, Nature 317 (1985) 145.
11. Holland, J J & Hoyer, B H, Cold Spring Harbor symp quant biol 27 (1962) 101.
12. Soloviev, V D, Krispin, T I, Zaslawsky, V G & Agol V I, J virol 2 (1968) 553.
13. Wilson, T, Papahadjopoulos, D & Taber, R, Proc natl acad sci US 74 (1977) 3471.
14. Madshus, I H, Olsnes, S & Sandvig, K, J cell biol 98 (1984) 1194.
15. Sandvig, K & Olsnes, S, J biol chem 257 (1982) 7495.
16. Carlsson, J, Drevin, H & Axén, R, Biochem j 173 (1978) 723.
17. Guillemot, J C, Sundan, A, Olsnes, S & Sandvig, K, J cell physiol 122 (1985) 193.
18. Choppin, P W & Philipson, L, J exp med 113 (1961) 713.
19. Philipson, L & Choppin, P W, J exp med 112 (1960) 455.
20. Hofmann, K, Wood, S W, Brinton, C C, Montibeller, J A & Finn, F M, Proc natl acad sci US 77 (1980) 4666.
21. Orr, G A, J biol chem 256 (1981) 761.
22. Sharon, N & Lis, H, Science 177 (1972) 949.
23. Olsnes, S, Madshus, I H & Sandvig, K, *in:* Virus attachment and entry into cells (ed R L Crowell & K Lonberg-Holm) p. 171. American Society for Microbiology (1986).
24. Chejanovsky, N, Fridlender, B & Loyter, A, Biochim biophys acta 812 (1985) 353.

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