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Vaccinia Virus and the EGF Receptor: A Portal for Infectivity?

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We previously demonstrated that occupancy of the epidermal growth factor (EGF) receptor reduced the ability of vaccinia virus to infect L cells [Eppstein et al: Nature 318:663, 1985]. This result suggested that vaccinia virus was utilizing the EGF receptor as one pathway to infect cells. We have studied this system further, and now find that antibodies to the EGF receptor also reduce the ability of vaccinia virus to infect cells productively. Inclusion of both EGF and antibodies to the EGF receptor did not cause inhibition over that obtained by EGF alone, providing another line of evidence that the antiviral effects on vaccinia virus were at the level of the EGF receptor. The antiviral effects of EGF or synthetic peptides corresponding to the third disulfide loop of TGF-α or the vaccinia virus growth factor were specific to vaccinia virus and did not inhibit replication of herpes simplex virus type 2 or vesicular stomatitis virus. The inhibitory effects on replication of vaccinia virus were obtained when EGF (but not insulin or growth hormone) was present prior to, but not after, productive viral adsorption. These results provided further evidence that the antivaccinia viral effects of EGF were at the level of initial receptor occupancy. As interferon (IFN) treatment has been shown to interfere with the action of some growth factors, including EGF, we examined the effects of IFN treatment of cells on the antivaccinia viral activity of EGF. Our results show that the antivaccinia effect of IFN-β either interfered with or partially coalesced with the inhibitory effects of EGF. The former interpretation is consistent with the report that IFN treatment results in a decrease both in the apparent number and affinity of cell-surface receptors for EGF [Zoon et al: Proc Natl Acad Sci USA 83:8226, 1986].

Key words: interferon, viral inhibition, vaccinia virus, EGF receptor

Cellular receptors for several viruses have been identified as being receptors for other specific physiological ligands. For example, Epstein-Barr virus infects B-lymphocytes via the complement receptor CR2 [1]; the AIDS virus (HIV-1) receptor on T-lymphocytes contains the T4 antigen [2–4]; rabies virus may utilize the acetylcholine receptor [5]; and the reovirus receptor has recently been identified as the cellular β-adrenergic receptor [6]. We asked the question of whether vaccinia virus (VV) utilizes the epidermal growth factor (EGF) receptor to bind to and infect cells,

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in light of the observed sequence homology between an early protein encoded by VV (vaccinia growth factor, VGF) and EGF or transforming growth factor-α (TGF-α) [7–9], all of which bind to the cellular EGF receptor [10–13].

Treatment of murine L-cells with EGF and synthetic decapeptides [14,15] corresponding to the third disulfide loop of TGF-α resulted in a dose-dependent reduction of vaccinia virus replication, as monitored by a plaque reduction assay [16]. The ability of EGF to inhibit VV replication correlated with its affinity for the EGF receptor on the L-cells. These results indicated that occupancy of the EGF receptor reduced infectivity by VV, and suggested that VV was utilizing the EGF receptor as one pathway to infect cells. We have now further characterized the interaction of VV with the EGF receptor, and our results provide additional evidence that interaction of VV with the EGF receptor aids viral infectivity.

MATERIALS AND METHODS

Chemicals

Eagle's minimal essential medium (EMEM) was from K.C. Biological (Lenexa, KS); fetal bovine serum was from Hyclone (Logan, UT); Sea Plaque agarose was from Marine Colloid Division, FMC Corp. (Rockland, ME); penicillin (10,000 U/ml)-streptomycin (10,000 U/ml), neutral red, and insulin were from GIBCO (Grand Island, NY); EGF was from Collaborative Research (Lexington, MA); TGF-α was a generous gift from Genentech, Inc. (S. San Francisco, CA); monoclonal antibody (MAb) 29.1.1 (an IgG_1 raised against A431 cell EGF receptor) was from International Diagnostic Laboratories, Inc. (Chesterfield, MO); MAb 528 (an IgG_{2a} raised against EGF receptors of A431 cells [17]) was from Oncogene Science, Inc. (Mineola, NY); murine interferon-β (MuIFN-β) was from Lee Biomolecular (San Diego, CA); and synthetic decapeptides were prepared by J. Nestor, Jr. (Syntex Research, Palo Alto, CA) [14,15].

Cells and Viruses

Vero cells, vaccinia virus (strain Western Reserve), herpes simplex virus type 2, strain G [HSV-2(G)], and A431 cells were from American Type Culture Collection (Rockville, MD); L cells and vesicular stomatitis virus (VSV), Indiana strain, were obtained from Dr. C. Samuel, University of California (Santa Barbara, CA).

Preparation of Vaccinia Virus Stock

Confluent monolayers of L cells seeded in T-75 flasks were washed and inoculated with vaccinia virus at a multiplicity of infection (MOI) of 0.05 plaque forming units (PFU)/cell. Virus inoculum was aspirated off and cells were washed after 1-hr incubation at 37°C. Twenty-five milliliters of EMEM + 2% FCS was then added to the flask, and cells were incubated for 2–3 days until cytopathic effect (CPE) reached 80–90%. Cells were scraped off the flask, homogenized with a Dounce homogenizer, and cell debris was removed by centrifugation at 2,000 rpm for 10 min. Virus stock was stored at −80°C.

Plaque Reduction Assay

Plaque reduction assay was performed in 24-well dishes, employing Vero cells (5×10^4 cells/well) for HSV-2(G), and L cells (10^5 cells/well) for vesicular stomatitis

virus (VSV) and vaccinia virus. Antibodies as specified were added to cells 4 hr before as well as during viral adsorption; EGF and other compounds were added 3 hr before viral adsorption. Cells were then inoculated with 30–40 PFU of virus per well. Virus inocula were aspirated off after 1 hr adsorption at 37°C in 5% CO_2 atmosphere, and the cells were washed and overlaid with 1 ml/well of EMEM with 1.5% Sea Plaque agarose, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in 5% CO_2 until plaque formation became apparent under light microscope observation (approximately 3 days for HSV-2(G), 1 day for VSV and 4 days for vaccinia virus). Wells were overlaid with 0.5 ml/well EMEM with 1.5% Sea Plaque agarose and 0.01% neutral red. Plaques were counted after ~24 hr of incubation with stain [18]. Statistical analyses were performed using Student's t-test.

Single-Cycle Virus Yield Assay

Confluent monolayers of murine L-cells plated in Costar 24-well dishes (10^5 cells/well, incubated 48 hr before use) were treated with EGF (1 μg/ml = 1.7×10^{-7}M) or control media 3 hr prior to as well as during the 1 hr vaccinia viral adsorption (MOI of 1 PFU/cell). After 1-hr adsorption at 37°C in 5% CO_2 atmosphere, cells were washed, and EGF or control media was added again as indicated to triplicate test wells. In one set of virus control wells, viral adsorption was prolonged to 3 hr. In two other sets of virus control wells, cells were washed (in addition to the wash after 1 hr of adsorption) a second time, at 2 or 3 hr after initial addition of virus. Virus yield was determined 7 hr post-adsorption, by plaque assay in L-cells.

RESULTS

The specificity of the inhibition of vaccinia virus replication by EGF and homologous synthetic peptides is illustrated in Table I. First, two polypeptides unrelated to EGF, ie, insulin and bovine growth hormone (BGH), which bind to their respective receptors on L-cells, were unable to inhibit vaccinia viral plaque formation. Second, replication of two unrelated viruses—VSV, an RNA virus; and HSV-2(G), a

TABLE I. Effects of Peptides on Replication of Vaccinia Virus, VSV, and HSV-2

	No. plaques, ± SD (% of control)				
Virus (no. control plaques)	Ac-TGF-α[34–43] NHEt (3×10^{-5} M)	Ac-VGF[71–80] NH_2 (3×10^{-5}M)	EGF (0.17 μM[a])	Insulin (0.2 μM[a])	BGH (0.05 μM[a])
Vaccinia (39 ± 4)	26 ± 1* (68)	28 ± 3* (71)	22 ± 3* (43)	35 ± 2 (90)	35 ± 3 (91)
VSV (31 ± 2)	ND[b]	29 ± 2 (92)	30 ± 3 (96)	ND	ND
HSV-2 (19 ± 1)	ND	20 ± 2 (105)	20 ± 2 (105)	ND	ND

[a]EGF, insulin, and BGH were tested at 1 μg/ml.
[b]ND = not determined.
*$P < 0.001$, vs virus control, Student's t-test. All other values were not significantly different from the corresponding virus control.

DNA virus—was not inhibited by EGF or the related synthetic peptide. In addition, TGF-α, which also binds to the EGF receptor, inhibited VV replication to the same degree as did EGF (data not shown).

Analyses of single-cycle growth studies (Table II) showed that 60–70% of vaccinia virions productively adhered to cells during the first hour of adsorption; additional washing of cells 2 or 3 hr after initial addition of virus reduced viral yield by 30–40% (Table II, B, C, vs A, $P \leqslant 0.01$). When 1.7×10^{-7}M EGF (= 1 μg/ml) was added 3 hr prior to as well as being present during the initial 1-hr viral adsorption period, virus yield was reduced by 50% (Table II, E, $P < 0.001$). However, when addition of EGF was delayed until immediately after the initial 1-hr viral adsorption period, virus yield was reduced by 45% (Table II, F, $P = 0.04$). This latter reduction of virus yield was eliminated when EGF addition was delayed by 2 hr (Table II, G vs control C, $P = 0.5$), by which time viral adsorption was complete.

Incubation of L-cells with MAb 29.1.1 resulted in a dose-dependent inhibition of VV plaque formation ($P < 0.001$ vs control for [Ab] $\geqslant 1$ μg/ml); unrelated IgG_1 MAb's had no inhibitory effect ($P > 0.1$) (Fig. 1). Similar results were obtained in A431 cells with MAb 29.1.1 as well as MAb 528 against vaccinia replication (data not shown). Pretreatment with EGF (1 μg/ml) resulted in approximately 50% inhibition of viral replication ($P < 0.001$); no further inhibition was obtained by simultaneous incubation with EGF plus the antibody to the EGF receptor ($P \geqslant 0.3$), suggesting that both EGF and the antireceptor antibody likely act through the same inhibitory pathway. These results are consistent with the hypothesis that VV can productively bind to cells via the EGF receptor.

Figure 2 summarizes the effects of MuIFN-β and EGF on VV plaque formation. Antiviral activity of IFN was obtained by a 26-hr ($P = 0.04$ for 0.1 U/ml; $P \leqslant 0.001$

TABLE II. Single-Cycle Yield of Vaccinia Virus

Sample	Treatment schedule	Virus yield (PFU/ml)	% of std control	% of own control
	Hr: −3 −2 −1 0 1 2 3 4 5 6 7 (Harvest ↓)			
A	W; Virus ↑ ——— ↓	$(7.6 \pm 0.6) \times 10^3$	100	
B	W W; Virus ↑ ↑ ——— ↓	$(5.3 \pm 0.6) \times 10^3$*	70	
C	W W; Virus ↑ ↑ ——— ↓	$(5.0 \pm 0.5) \times 10^3$*	66	100
D	W; Virus ↑ ——— ↓	$(8.7 \pm 0.6) \times 10^3$	115	
E	W; EGF (1.7×10^{-7}M) Virus ↑ ——— ↓	$(3.8 \pm 0.6) \times 10^3$*	49	
F	W; Virus ↑ EGF ——— ↓	$(4.2 \pm 1.5) \times 10^3$*	55	
G	W W; Virus ↑ ↑ EGF ——— ↓	$(4.5 \pm 0.9) \times 10^3$*	59	90**

*$P < 0.05$ vs A.
**$P = 0.5$ vs C.

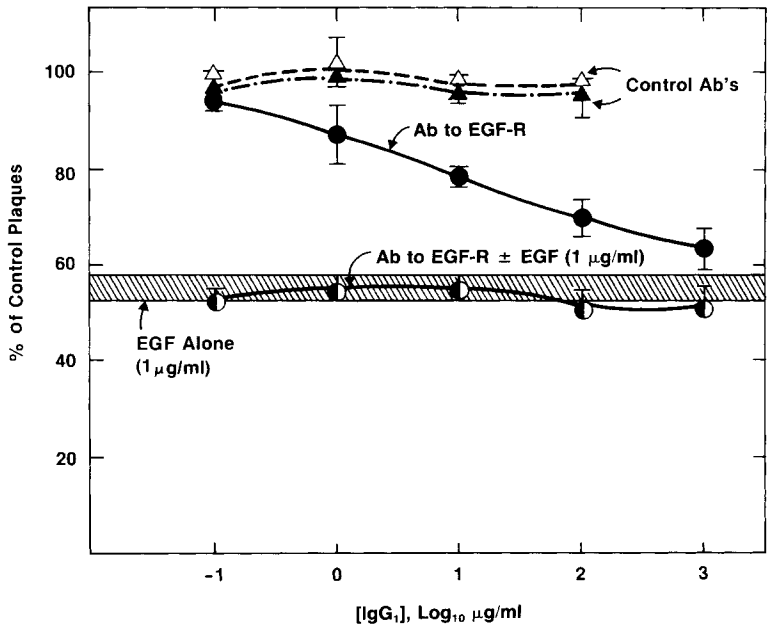

Fig. 1. Antivaccinia viral activity of EGF and antibody to the EGF receptor. L-cells were incubated with antibody to the EGF receptor (MAb 29.1.1) 4 hr prior to as well as during viral adsorption in a standard plaque reduction assay. EGF incubations were 3 hr before and during adsorption with vaccinia virus. The hatched area shows the level of viral plaques obtained by treatment with 1 μg/ml (1.7×10^{-7}M) EGF. (●), MAb 29.1.1; (◐), MAb 29.1.1 and EGF (1 μg/ml); (△) and (▲), control MAb's of IgG_1 subclass made against unrelated antigens.

for $\geqslant$ 1 U/ml) but not a 3-hr pretreatment ($P > 0.1$), and its inhibitory effect did not increase with addition of EGF ($P \geqslant 0.4$). These results suggest either that IFN and EGF may utilize at least in part a common mechanism in the inhibition of VV growth, or that IFN may have interfered with the antivaccinia viral activity of EGF.

DISCUSSION

We have studied the vaccinia viral/EGF receptor system further and have obtained additional results showing that vaccinia virus can productively bind to cells via the EGF receptor. Two monoclonal antibodies made against the EGF receptor reduced the ability of VV to replicate. Inclusion of both EGF and antibody to the EGF receptor did not further increase the inhibition over that obtained by either EGF alone, or by the antibody alone, consistent with the hypothesis that the antivaccinia effects of EGF involved interaction at the EGF receptor. The activity of MAb's to the EGF receptor was specific as shown by inability of two control MAb's of the same IgG_1 subclass, made against unrelated antigens, to block vaccinia replication (Fig. 1). We have also shown that the antiviral effects of EGF and synthetic decapeptide analogs of the third disulfide loop of TGF-α or VGF were specific for vaccinia virus but not VSV or HSV-2 (Table I). In addition to inhibition of VV replication by pretreatment with EGF, we have found that TGF-α similarly inhibited vaccinia virus replication. Single-cycle virus growth studies indicated that the inhibitory effect of

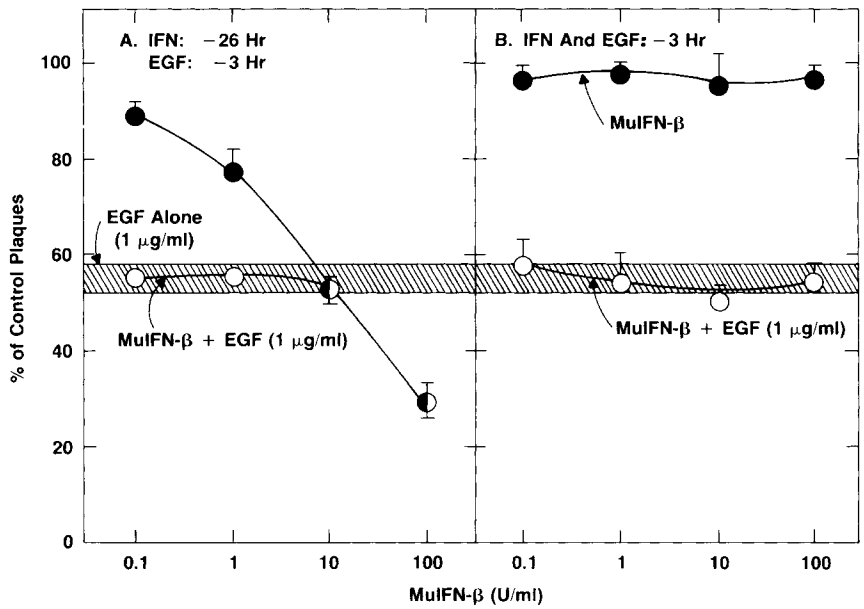

Fig. 2. Antivaccinia viral activity of MuIFN-β and EGF. L-cells were incubated with MuIFN-β either **(A)** 26 hr, or **(B)** 3 hr before viral adsorption in a standard plaque reduction assay. EGF (1 μg/ml) was added 3 hr before and during vaccinia virus adsorption. The hatched area shows the level of viral plaques obtained by treatment with 1 μg/ml (1.7×10^{-7}M) EGF. (●), MuIFN-β; (○), MuIFN-β + EGF (1 μg/ml); (◐), points at which MuIFN-β and MuIFN-β + EGF coincide.

EGF was at the early stages of the vaccinia viral replication cycle, acting mainly by inhibiting productive viral adsorption (Table II).

We have demonstrated that a relationship exists between vaccinia virus and the cellular receptor for EGF. EGF pretreatment of cells has an antiviral effect on VV replication. Such a pretreatment resulting in antiviral activity is reminiscent of the antiviral activity of IFN. In addition to its antiviral properties, IFN has inhibitory effects on growth of cells. IFN-β has been shown specifically to inhibit the action of some growth factors, including platelet-derived growth factor (PDGF) [19,20] and EGF [21]. In light of the interaction observed between VV and the EGF receptor, we were thus interested in determining if any relationship existed between the antivaccinia viral activity of IFN and EGF, and the inhibitory effect of IFN on EGF activity. Dose-dependent antivaccinia activity for IFN was obtained with a 26- but not a 3-hr pretreatment of L-cells, consistent with the known need for IFN to establish an antiviral state in the cells to exert its effects. EGF alone (1 μg/ml) resulted in approximately 50% inhibition of VV replication. Additional antiviral activity was not observed with combined IFN-EGF treatment, suggesting that either these two inhibitory pathways interfered with each other, or that the two pathways at least partially coalesced. Recently, Zoon et al [23] have shown that IFN treatment of cells resulted in a decrease both in the number and affinity of cell surface receptors for EGF. Our results using combinations of IFN and EGF treatments are consistent with these findings, ie, that IFN treatment interfered with the inhibitory activity of EGF.

Studies with monoclonal antibodies to the EGF receptor have provided further support for the hypothesis that vaccinia virus can have an initial productive interaction

with cells through the EGF receptor. Binding of the MAb to the EGF receptor can result in competition for binding of VV to the same receptor, thus reducing the viral infectivity. The fact that complete inhibition of vaccinia virus infection was not obtained with saturating concentrations (in terms of EGF receptor binding) of EGF suggests that the EGF receptor is not the only pathway by which VV can bind to and infect L-cells. This is consistent with the report of Stroobant et al [22] that vaccinia virus was able to infect NR-6 cells which lack detectable EGF receptors. However, our results suggests that in cells bearing the EGF receptor, an interaction of VV with the EGF receptor enhances the ability of VV to productively infect the cells.

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