Fibroblast Growth Factor Receptor: Does It Have a Role in the Binding of Herpes Simplex Virus?

R. J. Kaner *et al.* (*1*), report that Chinese hamster ovary (CHO) cells expressing a transfected receptor for fibroblast growth factor (FGF) bound and internalized greater amounts of radiolabeled herpes simplex virus (HSV) than did the FGF receptor–deficient parental cells or control cells. The authors conclude that the FGF receptor is a "portal of entry" into cells for HSV. Neither the receptor-positive nor the receptor-deficient cells exhibited morphological evidence of viral infection after exposure to HSV. However, in this study the authors did not determine whether expression of an FGF receptor facilitated entry of HSV into the cells for the initiation of infection. We addressed this question, using the same cell lines studied by Kaner *et al.* We also repeated some of their experiments and obtained significantly different results.

CHO cells do not support HSV replication (*2*). Little attention has been focused on determining whether HSV can enter CHO cells to initiate viral gene expression. Tackney *et al.* (*3*) showed that UV-irradiated HSV could transduce genes to CHO cells. We found (*4*) that CHO-K1 cells (obtained from J. D. Esko, University of Alabama, Birmingham) could be abortively infected with HSV. After exposure to virus at different input doses, as many as 50% of the CHO cells expressed immediate-early or α viral proteins. Fewer cells (about 30%) expressed early (β) viral proteins, and cells expressing late (γ) viral proteins were rare. We have no explanation at present for the high multiplicity of HSV required for this abortive infection (about 10 to 50 times more virus is needed to maximize expression of viral proteins in CHO cells than is required to infect 100% of permissive cells, such as human HEp-2 cells) or for the fact that only a fraction of CHO cells expressed viral proteins. The amount of radiolabeled virus that could bind to the CHO cells under saturating conditions was close to that which bound to fully permissive HEp-2 cells. This suggests that penetration of HSV into CHO cells may be inefficient. Alternatively, expression of immediate-early HSV genes may be inefficient, requiring that multiple viral genomes be introduced into a cell in order to initiate the abortive infection.

In order to test whether expression of a transfected FGF receptor could enhance the susceptibility of CHO cells to abortive infection with HSV, we obtained two of the cell lines used in (*1*) from C. Basilico (New York University). One of these transformed CHO derivatives (4-1 cell line) expressed a mouse FGF receptor. The other (A-1 cell line) was a matched control cell line, which carried the FGF cDNA sequence in antisense orientation (*5*). Expression of the FGF receptor by 4-1 cells conferred the ability to bind basic FGF with high affinity and responsiveness to FGF, properties that were absent or deficient in the parental and control CHO cell lines (*5*).

We found that purified radiolabeled HSV could bind as well or better to the FGF receptor-deficient A-1 cells as they could to the FGF receptor-positive 4-1 cells (Fig. 1). At every concentration, about twice as much virus bound per cell to the A-1 cells as to the 4-1 cells, in the absence of heparin. The binding was inhibited by heparin, as has been shown previously for the binding of HSV to permissive HEp-2 cells (*6*). Because the adsorption of HSV to cells requires the presence of cell surface heparan sulfate (*6*), these results suggest that the faster growing 4-1 cells may express less cell surface heparan sulfate than do the control A-1 cells. At the highest dose of virus tested in Fig. 1, which was not sufficient to saturate receptors for HSV, the amount of virus bound to the A-1 cells was about 1,800 plaque-forming units (PFU) per cell or 36,000 particles per cell. We estimate that the amount of virus required to saturate HSV receptors on permissive HEp-2 cells is 60,000 particles per cell (*7*). Therefore, the ability of the FGF receptor-deficient CHO cells to bind HSV may be similar to that of permissive HEp-2 cells.

We also quantitated the adsorption of radiolabeled HSV to both cell lines at 37°C and determined the susceptibility of the cells to abortive infection by monitoring expression of the immediate-early HSV protein designated ICP4. Adsorption of virus was similar for the A-1 and the 4-1 cells, and the two cell lines were indistinguishable with respect to the fraction of cells that expressed ICP4 (Fig. 2). In other experiments, we found that as many as 48% of cells (both A-1 and 4-1) expressed ICP4 at an input viral multiplicity of about 800 PFU per cell. Because only about 4% of input virus actually adsorbed to the cells under the conditions used, the effective multiplicity was about 30 PFU bound on average per cell.

Our results with respect to HSV adsorption to CHO transformants were different from the results published by Kaner *et al.* (*1*) even though we used the same cell lines. They reported that, at both 37° and 4°C, significantly more label from radiolabeled virus preparations bound to the FGF receptor–positive cells than to the FGF receptor–deficient cells. We suggest that their virus preparations may have been contaminated with labeled DNA not associated with virions. It can be difficult to purify HSV virions (labeled in the DNA) that are free of contaminating labeled DNA. When cell lysates have been prepared by Dounce homogenization of concentrated infected cell suspensions and care has been taken not to break nuclei, Dextran gradient centrifugation of the lysates have yielded virus bands containing 10^9 to 10^{10} PFU/ml and negligible DNA contamination (*8*). The titers of the virus preparations used by Kaner *et al.* were low (10^8 PFU/ml) and the specific radioactivities (10^{-1} disintegrations per minute per PFU) were higher than can be achieved in practice for highly purified [^{3}H]thymidine-labeled HSV. Because Kaner *et al.* detected significant quantities of basic FGF in their virus preparations, it is possible that they were measuring the binding to cells of FGF complexed with labeled DNA.

Heparin can bind to HSV virions and effectively blocks the adsorption of HSV to cells (*6*). Heparin-binding proteins such as platelet factor 4 can also inhibit the adsorp-

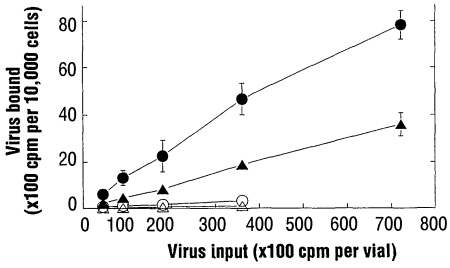

Fig. 1. Adsorption of purified HSV virions to FGF receptor-positive 4-1 cells (triangles) and FGF receptor–deficient A-1 cells (circles) at 4°C. The virus used was HSV type 1, strain KOS. The virus was produced in HEp-2 cells, labeled with [^{3}H]thymidine, and purified as described previously (7). The radioactivity of the purified virus was 4.3×10^{-4} cpm/PFU. The cells were plated on the bottoms of glass scintillation vials at 5×10^4 cells per vial. After overnight incubation, the cells were exposed to purified virus at the input doses indicated, and in the absence (closed symbols) and presence (open symbols) of heparin at 10 μg/ml, in a total volume of 0.1 ml. After 5 hours at 4°C (the time required at this temperature for virus binding to approach equilibrium), the cells were washed and scintillation fluid was added for the quantitation of virus bound to the cells. The cell numbers were 1.9×10^4 per vial for the A-1 cells and 6.4×10^4 per vial for the 4-1 cells. Over this range of cell numbers, the amount of virus bound is proportional to cell number for a single cell type. Each point represents the mean of duplicate determinations and the error bars represent standard deviation.

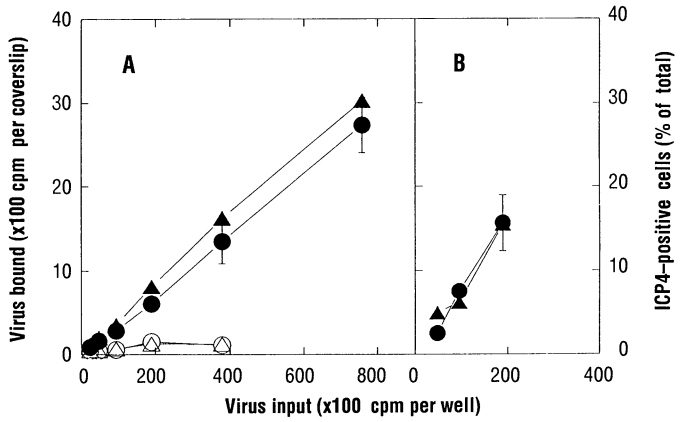

Fig. 2. Adsorption of purified HSV virions to FGF receptor-positive 4-1 cells (triangles) and FGF receptor-deficient A-1 cells (circles) at 37°C and percentage of cells expressing ICP4. The purified virus was prepared as described in Fig. 1 and had a radioactivity of 3.4×10^{-4} cpm/PFU. The cells were plated on cover slips in 24-well plates at 10^5 cells per well. After overnight incubation, the cells were exposed to purified virus at the input doses indicated, in the absence (closed symbols) or presence (open symbols) of heparin at 10 μg/ml. After incubation of the virus with the cells for 2 hours at 37°C, some of the cover slips were washed and immediately transferred to scintillation vials for the quantitation in duplicate of radioactivity bound to the cells (**A**). Other cover slips were incubated for an additional 22 hours and then fixed and stained with an anti-ICP4 monoclonal antibody (*12*) to quantitate the percentage of cells expressing ICP4 (**B**). Each point in (B) represents a count of five to eight microscopic fields and approximately 200 cells total. The error bars represent standard deviation.

tion of HSV to cells (*6*), presumably by occupying sites on the cell surface heparan sulfate to which virions normally bind. Kaner *et al.* (*1*) showed that basic FGF could inhibit plaque formation by HSV. Their interpretation was that FGF blocked the binding of virus to an FGF receptor. The interpretation we favor is that basic FGF, a heparin-binding protein (*9*), blocks the binding of virus to cell surface heparan sulfate. The concentrations of basic FGF required to inhibit HSV plaque formation (*1*) were closer to the dissociation constant (K_d) for the low-affinity FGF receptors, thought to be cell surface heparan sulfate, than to the K_d for the high-affinity protein receptor (*10*). Moreover, heparin at 10 μg/ml can completely block HSV adsorption and infection (*6*) as well as the binding of FGF to heparan sulfate, but does not block the binding of basic FGF to the receptor transfected into the CHO cells (*5*).

The possibility exists that a cell surface receptor in addition to heparan sulfate may be required for HSV entry into cells (*11*). Our results indicate that the identity of this putative receptor remains to be determined.

Note added in proof: We have found recently that CHO cells appear to be much more susceptible to infection by HSV-1(F), the virus strain used by Kaner *et al.* (*1*) than to HSV-1(KOS). CHO cells transfected with the mouse FGF receptor and control cells were equally susceptible to HSV-1(F), with 100% of cells expressing ICP4 after exposure to virus at multiplicities of between 10 and 50 PFU per cell. We have also studied several more transformed CHO cell lines provided by C. Basilico. Sent as coded samples, these cell lines included one transformed with the *flg* FGF receptor, two with the *bek* FGF receptor, and one control transformed with an antisense *flg* construct. All four cell lines were equally susceptible to infection with HSV-1(KOS) and HSV-1(F).

MEI-TSU SHIEH
PATRICIA G. SPEAR
Department of Microbiology-Immunology, Northwestern University Medical and Dental Schools, 303 East Chicago Avenue, Chicago, IL 60611

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Response: Our paper should not be construed to imply that heparan sulfate is not essential to viral adsorption and infectivity. Our study was designed to show that the virus is capable of binding to an FGF receptor, but we did not establish that this is the only mechanism of viral attachment to the target cell.

Shieh and Spear argue that if the high-affinity receptor is involved, lower concentrations of FGF should inhibit infection. We would not expect the concentration of ligand necessary to inhibit infection to be near the FGF dissociation constant (K_d) for the receptor, since the affinity of the virus for the receptor is not known. The concentration of ligand may have to be much higher to ensure more than 99% occupancy of the receptors. Thus, nanomolar concentrations of basic FGF could be necessary to inhibit infection.

Experiments recently done in our laboratory with several transfected CHO cell lines show that both parental and nonsense transfected cells take up a radioactively labeled virus in quantities comparable to those taken up by the cells overexpressing the *flg* gene product. Southern analysis (DNA) confirms that all of these cell lines contain HSV-specific DNA when exposed to HSV-1. Thus, our current data support the conclusion of Shieh and Spear that CHO cells are capable of taking up HSV-1.

One possible explanation for this observation is that the virus preparation we used originally was in some way different from our recent preparations. For example, the preparations may have had different amounts of FGF, and we are investigating this. The situation is complicated by the presence of low-affinity FGF receptors that participate in the "high-affinity" response and also apparently in HSV-1 adsorption to the cell surface. Thus, a complex interaction between these elements could play a role in HSV-1 uptake.

We have found that conditioned media from CHO cells, transfected with a truncated form of the *flg* gene that produces a soluble receptor protein, can inhibit HSV-1 plaque formation by 40 to 60% in both NIH 3T3 fibroblasts and smooth muscle cells. The binding of HSV-1 in vitro to immobilized recombinant purified extracellular domain (FGF binding region) of the *flg* gene product should also be investigated as this reagent is now available.

ROBERT J. KANER
Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021
ANDREW BAIRD
ROBERT Z. FLORKLEWICZ
Department of Molecular and Cellular Growth Biology, Whittier Institute for Diabetes and Endocrinology, La Jolla, CA 92037
ALKA MANSUKHANI
CLAUDIO BASILICO
Department of Microbiology,

New York University School of Medicine, New York, NY 10016
DAVID P. HAJJAR
Department of Pathology, Cornell University Medical College, New York, NY 10021

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Vaccination, Immunopathology, and Immunity

The idea that certain recombinant virus vaccines could be dangerous is close to the fears of both careful scientists and doubting laymen. S. Oehen *et al.* (*1*) interpret their results as showing that the worsening of disease after the use of a vaccine.

> happens usually not with whole virus vaccines exhibiting multiple protective T cell epitomes but may be induced when only one or few of the virus epitomes are used for vaccination, as is the case in the newer types of peptide or recombinant vaccines. . . .

We disagree with this reading of both the authors' results and of our own (*2, 3*). We showed more than 25 years ago that, with some strains of lymphocytic choriomeningitis virus (LCMV), peripheral inoculation and subsequent central challenge to the immune system could greatly increase sickness and death. Although one of our papers (*2*) is cited by Oehen *et al.*, they do not mention that we did indeed find "paradoxical effects" of vaccination with this whole virus vaccine.

With certain viral diseases (of which LCMV is the prime example), the viral infection itself is virtually harmless to cells, which subsequently recover and become virus-free (*4*). Disease, if it occurs at all, is mediated by autoimmune mechanisms, rather than intrinsic viral cytotoxicity. Initial sensitization of a specific cellular immune response by appropriate antigens (including both recombinant virus vaccines and nonlethal doses of intact whole virus) can—under certain conditions of timing, dose, and strain—cause marked exacerbation of the disease and increased mortality. This "paradoxical effect" is present not only during the early days after inoculation but also in waning immunity after wild-type viral infection (*5*). The phenomenon is intrinsic to the basic immunology of LCMV infection and no doubt of other viral diseases with a similar pathogenic mechanism.

It is unrealistic to impute these undesirable effects of vaccination to modern genetic techniques; they are as old as the viruses. If a vaccine can induce enhanced disease, this is, like immunity, good evidence of its efficacy. We should not confuse the vagaries of viral pathogenesis with advanced genetic procedures or we may foolishly condemn perfectly good weapons in the fight against human diseases.

JOHN HOTCHIN
LOIS (BENSON) HOTCHIN
18 Paxwood Road, Delmar, NY 12054

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Response: Hotchin *et al.* found accelerated disease when the immune system was challenged during the induction phase. Most mice challenged on the fifth day or thereafter were fully protected. In contrast, we evaluated an immunological memory model. We were interested in how different vaccines could influence susceptibility to LCM disease. Vaccination with whole virus three or more weeks before challenge infection usually did not accelerate disease. Under some conditions, with recombinant vaccines that expressed only a limited number of T cell epitopes, vaccination 3 weeks or less before intracerebral (i.c.) challenge accelerated LCM disease.

In this model the i.c. challenge infection occurred during an immune memory state that was characterized by increased amount of cytotoxic T lymphocyte precursor (CTLp). Frequencies of CTLp in LCMV-immune mice were 10 to 20 times higher than those in mice vaccinated with recombinant vaccinia virus. This could make the difference between prevention and aggravation of disease. Mice that were immune to LCMV showed a secondary immune response against LCMV isolate WE 3 to 4 days sooner than did mice that were vaccinated with a recombinant vaccinia virus. Vaccination with wild-type LCVM protected against high-dose i.c. challenge, whereas vaccination with some of the recombinant vaccinia viruses apparently shifted the equilibrium between immunosuppression and immune response only to a modest extent. This led to lethal LCM disease. Absence of vaccination would have ensured survival in the face of high zone immune paralysis.

The aim of our paper was not to discredit the development of recombinant vaccines. The "new" types of vaccines are promising, but the minimal safety requirements must be fulfilled. Multiple T or B cell epitopes, or both, induction of high neutralizing antibody titers, or induction of consistently high CTLp frequencies must be demonstrated. Recombinant hepatitis B vaccine serves as a good example of a vaccine that has met these requirements.

Our animal model demonstrated that, unless these requirements are met, a vaccine may not work as desired. The nonrecombinant, formalin-inactivated respiratory syncytial virus has also been found to induce damaging T cell responses (*1*). Even if a vaccine expresses a neutralizing determinant (as in the case of vaccinia-LCMV-GP recombinant virus), it may still not perform as expected. If limitations of efficacy or unwanted effects can be demonstrated in a vaccine model, such results should be studied carefully. Thus we caution that vaccination with recombinant vaccinia vaccines expressing only some T cell epitopes (but not wild-type virus) may enhance T cell–mediated immunopathology in the presence of a noncytopathic virus.

STEPHAN OEHEN
HANS HENGARTNER
ROLF M. ZINKERNAGEL
Institut für Pathologie der Universität Zürich, Universitätsspital, Zürich CH-8091, Switzerland

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