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A Soluble Receptor for Interleukin-1β Encoded by Vaccinia Virus: A Novel Mechanism of Virus Modulation of the Host Response to Infection

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Summary

Vaccinia virus gene B15R is shown to encode an abundant, secretory glycoprotein that functions as a soluble interleukin-1 (IL-1) receptor. This IL-1 receptor has novel specificity since, in contrast with cellular counterparts, it binds only IL-1β and not IL-1α or the natural competitor IL-1 receptor antagonist. The vaccinia IL-1β receptor is secreted when expressed in a baculovirus system and competitively inhibited binding of IL-1β to the natural receptor on T cells. Deletion of B15R from vaccinia virus accelerated the appearance of symptoms of illness and mortality in intranasally infected mice, suggesting that the blockade of IL-1β by vaccinia virus can diminish the systemic acute phase response to infection and modulate the severity of the disease. The IL-1β binding activity is present in other orthopoxviruses.

Introduction

Interleukin-1 (IL-1), a cytokine produced in response to infection and tissue injury, is involved in the regulation of the inflammatory and immune responses and in the activation of a broad spectrum of systemic effects that contribute to host defense (Dinarello, 1988, 1989; Di Giovine and Duff, 1990). The two forms of IL-1 (α and β) produce similar biological effects that are mediated by interaction with specific receptors in different cells. Two classes of IL-1 receptors have been identified that bind both IL-1α and IL-1β with similar affinities (Dower and Urdal, 1987). The 80 kd type I IL-1 receptor is found on T cells and fibroblasts, while the type II IL-1 receptor of 60 kd is present in B cells and macrophages. Sequence of cDNA clones of both receptors revealed that they belong to the immunoglobulin superfamily (Sims et al., 1988, 1989; McMahan et al., 1991). Binding studies have shown heterogeneity in the IL-1 receptor on B cells concerning the affinity for IL-1α or IL-1β (Benjamin et al., 1990), and the existence of a secreted receptor from Raji cells, which binds only IL-1β, has been reported (Giri et al., 1990; Symons and Duff, 1990; Symons et al., 1991).

Different natural inhibitors that modulate the biological effect of IL-1 have been reported (Larrick, 1989; Shields and Mazzei, 1991). The best-characterized natural inhibitor is designated IL-1 receptor antagonist (IL-1RA), which competes with IL-1 for binding to the receptor but cannot trigger the cellular responses of IL-1 (Carter et al., 1990; Eisenberg et al., 1990; Hannum et al., 1990). The blockade of the interaction of IL-1 with its receptor by IL-1RA, a soluble extracellular binding domain of the receptor, or monoclonal antibodies against the receptor has been shown to inhibit the biological effects of IL-1 in vivo (Fanslow et al., 1990; Gershenwald et al., 1990; Ohlsson et al., 1990; Alexander et al., 1991; Dinarello and Thompson, 1991; McIntyre et al., 1991).

Vaccinia virus is the representative member of the poxvirus family of cytoplasmic DNA viruses (Moss, 1990a). The virus genome encodes a broad range of enzymes necessary for cytoplasmic replication (Moss, 1990a; Trakman, 1990) and, in addition, encodes a variety of proteins that are nonessential for virus replication in tissue culture but that influence pathogenesis in vivo (Turner and Moyer, 1990; Buller and Palumbo, 1991). Some of these are enzymes, while others interfere with different aspects of the immune response to infection (for references see Moore and Smith, 1992). Previously, two vaccinia virus open reading frames (ORFs) of the Western Reserve (WR) strain, named B15R and B18R, were predicted to encode proteins of the immunoglobulin superfamily that show homology to the IL-1 receptor and the immunoglobulin-like domain of the IL-6 receptor (Smith and Chan, 1991), suggesting that they can interfere with the immune response by blocking the effects of these cytokines. Sequence of the type II IL-1 receptor revealed a stronger homology of B15R to this type of receptor (McMahan et al., 1991). B18R encodes the surface or soluble early antigen of vaccinia virus, which is present in the membrane of infected cells and in supernatants (Ueda et al., 1972, 1990).

We report here that both B15R and B18R ORFs are actively transcribed, translated, and secreted to the medium during the vaccinia virus replication cycle. The B15R gene product is shown to bind IL-1β when expressed from vaccinia or from recombinant baculovirus. The role of the IL-1β binding activity in the biology of vaccinia virus was investigated by deleting the gene from the virus genome and analyzing the biological effects on infected mice. The presence of the binding activity in other orthopoxviruses is also presented.

Results

Transcriptional Analysis of B15R and B18R

Transcription of genes from vaccinia virus is regulated in a temporal fashion, and genes are classified as early or late on the basis of their requirement for viral DNA synthesis (Moss, 1990b). S1 nuclease protection experiments were performed to detect and map B15R- and B18R-specific messenger RNAs (mRNAs) produced during infection. Figure 1A shows that the B15R-specific probe was partially protected from S1 nuclease digestion by late viral RNA, and the size of the protected fragment mapped the transcriptional start site to the TAAAAT motif at the 5′ end of B15R. Although the sequence TAAAT(G) has been shown to constitute a late promoter consensus sequence for vaccinia virus, a few exceptions have been found that possess an additional A (Moss, 1990b). The B18R-specific

probe was protected from S1 nuclease digestion by early viral RNA that initiated 16–18 nt upstream of the ORF (Figure 1B). This is consistent with the presence of vaccinia virus early transcriptional terminator signal TTTTTNT 13 nt downstream of the ORF (Smith and Chan, 1991) and is in agreement with results obtained by primer extension in the Lister strain (Ueda et al., 1990), although analysis of late RNA was not included in the previous report. The weak signal detected at late times probably corresponds to early transcripts still present in the late viral RNA sample since, for constitutively expressed genes, the early and late transcripts initiate at different positions (Moss, 1990b). These data show that both B15R and B18R are actively transcribed during the vaccinia virus replication cycle from positions indicating that the first codon of each ORF is likely to be used as the translation initiation site. The transcription of the genes at different times of infection suggests that the gene products will have different functions.

Identification of Proteins Encoded by B15R and B18R

Vaccinia virus recombinants overexpressing the proteins or lacking the coding regions were constructed to identify the gene products and to study the biological activity of the proteins. Overexpression of the proteins was achieved by cloning a second copy of B15R or B18R, transcribed under the control of the late 4b promoter, in the thymidine kinase (TK) locus. The genomic structure of the recombinant viruses, called vB15R and vB18R, was confirmed by Southern blot hybridization (Figure 2A) and by polymerase chain reaction (PCR) using oligonucleotides specific for the 5′ and 3′ ends of the TK gene (data not shown). Deletion of 72% of B15R or 92% of B18R from the viral genome was carried out by transient dominant selection (Falkner and Moss, 1990; Isaacs et al., 1990). This method allows construction of a virus that only differs from the wild type in the deleted sequence and does not contain any selectable marker that could affect the new phenotype. The genomic structure of the deletion mutants, named vΔB15R and vΔB18R, was confirmed by Southern blot hybridization (Figure 2B). The isolation of deletion mutants for B15R or B18R that grow normally in tissue culture confirmed that both genes are dispensable for virus replication in vitro (Perkus et al., 1991; Ueda et al., 1990).

To identify the gene products, labeling experiments with ^{35}S-labeled methionine and cysteine in the presence (early) or absence (late) of an inhibitor of DNA synthesis (cytosine arabinoside) were performed in cells infected with different vaccinia virus recombinants. Extracts from cells or culture supernatants were immunoprecipitated and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Specific antiserum against B15R immunoprecipitated a broad band of 50–60 kd in supernatants from WR-infected cells at late times of infection (Figure 3A), confirming the transcriptional analysis. An intermediate glycosylated form of 47 kd was de-

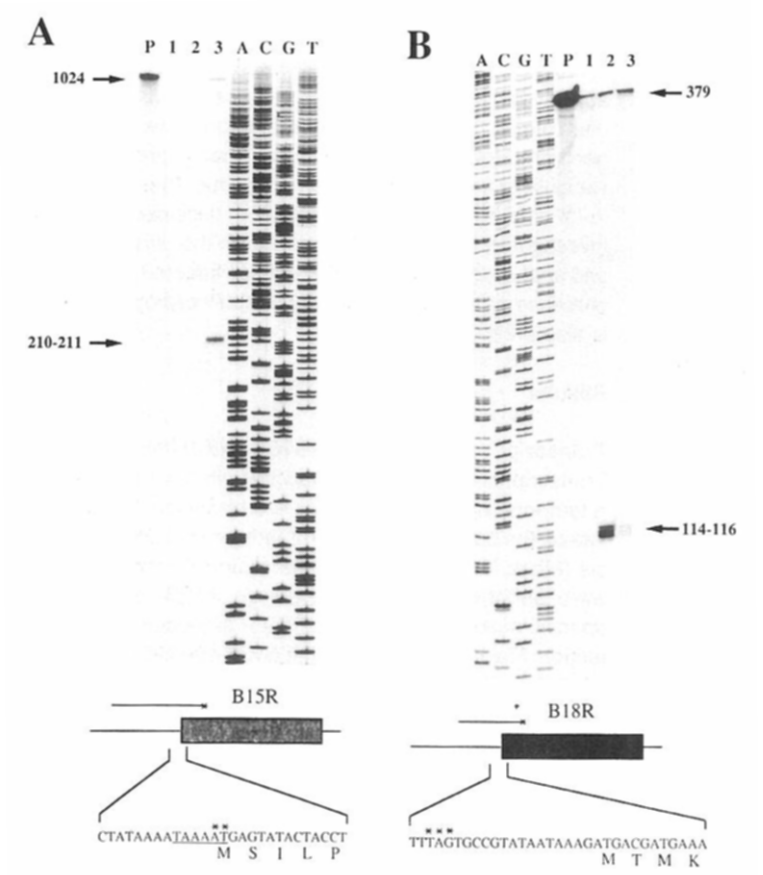


Figure 1. S1 Mapping of 5′ Ends of mRNAs Coding for B15R and B18R

Specific 5′ radiolabeled probes (lane P), prepared as described in Experimental Procedures, were hybridized with yeast transfer RNA (lane 1) or vaccinia virus early (lane 2) or late (lane 3) RNA and digested with S1 nuclease. Nuclease-resistant fragments were resolved on a sequencing gel alongside an M13 sequencing ladder (lanes A, C, G, and T). Autoradiographs corresponding to B15R (A) and B18R (B) probes are shown. The sizes of the probes and fragments protected are indicated in bases. Indicated below the autoradiographs are the probe position relative to the ORFs (underline and asterisks), the nucleotide and the deduced amino acid sequence at the 5′ end of the ORFs, the vaccinia late promoter consensus sequence (underline), and the sites of transcriptional initiation (asterisks).

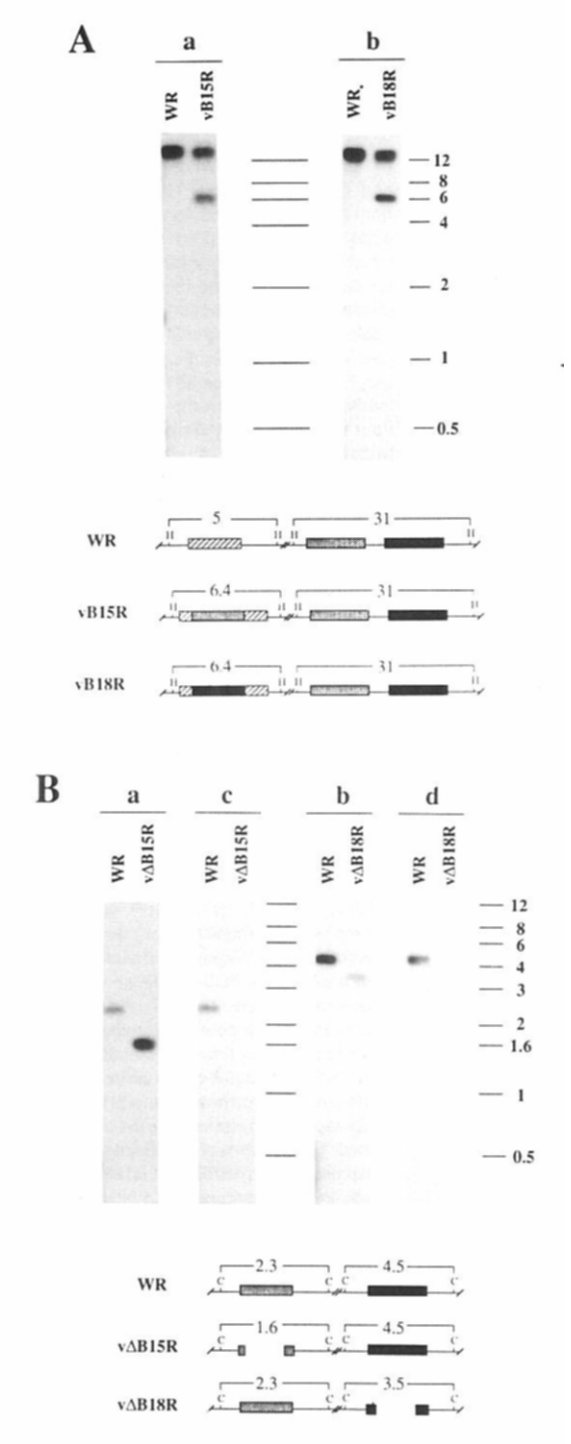


Figure 2. Structure of Recombinant Vaccinia Virus Genomes

Vaccinia virus DNA was digested with HindIII (A) or ClaI (B), and fragments were resolved on an agarose gel and transferred to nitrocellulose. Filters were probed with fragments containing the gene and flanking sequences of B15R (lane a) or B18R (lane b), with an internal oligonucleotide to B15R (lane c) or an internal fragment to B18R (lane d). Sizes in kilobases are indicated. Schematic representations of the structure and sizes of the relevant HindIII (H) or ClaI (C) fragments containing the TK gene (hatched box), B15R gene (stippled box), or B18R gene (closed box) in the different viruses are included.

(A) Recombinant viruses overexpressing the ORFs, vB15R and vB18R, compared with WR.

(B) Deletion mutants vΔB15R and vΔB18R compared with WR. The ClaI fragments containing the genes either decreased to the expected size or were not detected in the deletion mutants when hybridized to probes that consisted of the ORF and flanking regions (lanes a and b) or probes corresponding to internal sequences (lanes c and d), respectively.

tected in infected cells. As observed by immunoprecipitation (Figure 3A) and in the whole extract (data not shown), the expression of the protein either was increased or was not detected in vB15R- or vΔB15R-infected cells, respectively. The presence of tunicamycin inhibited the secretion, indicating that a correct glycosylation and/or folding of the protein is required for this process, and reduced the size to 35 kd, close to the predicted molecular weight of 36,500. The other bands detected in the presence of tunicamycin were not specific for B15R since they were also immunoprecipitated by an antiserum against B18R (data not shown). Thus, a high degree of glycosylation accounts for 30%–40% of the size of the secreted protein encoded by B15R.

The protein encoded by B18R was detected in vaccinia virus–infected cells when overexpressed at late times of infection under the strong 4b promoter but was not detected in the deletion mutant (Figure 3B). Two forms of the protein (52 kd and 60–65 kd) were detected in cell extracts, and only the 60–65 kd protein, presumably containing a higher degree of glycosylation, was secreted to the medium. Since translation of B18R-specific mRNA in rabbit reticulocyte lysates produces a protein of the predicted size of 40 kd (Ueda et al., 1990), the carbohydrate component of the secreted B18R gene product accounts for 33%–38% of the size of the protein. Previous results have identified a 40 kd polypeptide immunoprecipitated from infected cells as the early surface antigen of vaccinia (Ikuta et al., 1980), later shown to be encoded by B18R (Ueda et al., 1990). The use of an antiserum against early cell surface antigens of vaccinia virus, and not antibodies specific for the B18R gene product, might explain the discrepancy. Alternatively, the antiserum raised against B18R expressed in insect cells might not recognize that form of the protein.

Expression of B15R and B18R in Baculovirus

To characterize further the products of genes B15R and B18R and to produce greater amounts of material for functional analysis, the proteins were expressed in Spodoptera frugiperda (Sf) insect cells infected with Autographa californica nuclear polyhedrosis virus (AcNPV) under the control of the polyhedrin promoter. The recombinant viruses constructed were termed AcB15R and AcB18R.

As shown in pulse-label experiments (Figure 3C), both B15R and B18R proteins were secreted to the medium by insect cells as 40–44 kd and 48 kd polypeptides, respectively. An incomplete glycosylation of the polypeptides in insect cells (Luckow and Summers, 1988) might explain the lower size of the proteins when compared with the vaccinia virus expression. The 45 kd protein in AcB15R-infected insect cell extracts might correspond to a glycosylated form with a signal sequence still bound to the polypeptide, possibly owing to the inability of insect cells to

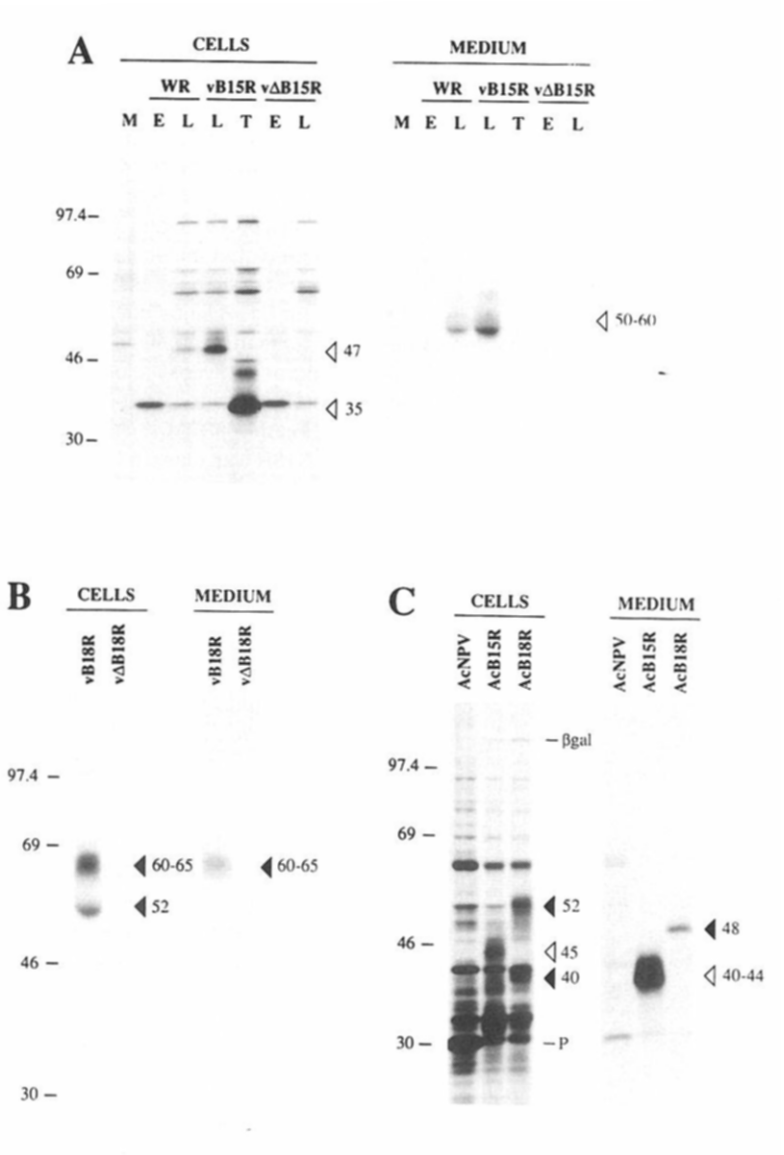


Figure 3. Identification of B15R and B18R Gene Products in Vaccinia- and Baculovirus-Infected Cells

(A) Identification of B15R from vaccinia virus–infected cells. BS-C-1 cells were mock infected (M) or infected with WR, vB15R, or vΔB15R and pulse-labeled with ^{35}STrans-label either from 2 to 4 hr after infection in the presence of cytosine arabinoside (E) or from 6 to 8 hr after infection in the absence (L) or presence (T) of tunicamycin. Cells and media were immunoprecipitated with rabbit antiserum raised against AcB15R-infected Sf cells, and the samples were analyzed by SDS–PAGE. A fluorograph is shown. Twice as much material was loaded onto gels from the supernatants than from cells, and the fluorograph was exposed twice as long. Molecular size markers and the size of the B15R gene products (open arrowhead) are indicated in kilodaltons.

(B) Identification of B18R from vaccinia virus–infected cells. BS-C-1 cells were infected with vB18R or vΔB18R and pulse-labeled with ^{35}STrans-label from 6 to 8 hr after infection, the cell extracts and media were immunoprecipitated with antiserum raised against B18R expressed in baculovirus-infected cells, and the samples were resolved by SDS–PAGE. A fluorograph is shown. As in (A), the quantity of sample from medium was estimated to correspond to about four times the amount analyzed from cells. Molecular size markers and the size of the B18R gene products (closed arrowhead) are indicated in kilodaltons.

(C) Expression of B15R and B18R in baculovirus-infected insect cells. Sf cells infected with AcNPV, AcB15R, or AcB18R were pulse-labeled with ^{35}STrans-label for 2 hr after 24 hr of infection. Proteins present in cells and media were analyzed by SDS–PAGE and visualized by autoradiography. As in (A), the quantity of sample from medium was estimated to correspond to about four times the amount analyzed from cells. The B15R (open arrowhead) and B18R (closed arrowhead) gene products and the molecular size markers are indicated in kilodaltons. The positions of β-galactosidase (βgal), coexpressed with B15R and B18R in the recombinant baculoviruses, and polyhedrin (P), expressed only in AcNPV, are shown.

process properly the high amount of B15R protein expressed under the strong polyhedrin promoter. Interestingly, two B18R proteins of 40 kd and 52 kd were detected in insect cell extracts, the smaller size corresponding to that predicted from the amino acid sequence and found in in vitro translation of specific mRNA (Ueda et al., 1990). This also correlates with two polypeptides in cells infected with vaccinia virus (vB18R), although the sizes are different, probably owing to a different posttranslational processing of the polypeptide in insect cells. Time course experiments revealed that the expression levels of both recombinant proteins reached maximum between 1 and 2 days after infection, and pulse–chase experiments showed that the proteins were stable in the medium during at least 20 hr (data not shown).

IL-1 Binding Activity Expressed by Vaccinia Virus and Recombinant Baculoviruses

Since the sequences of B15R and B18R are related to IL-1 and IL-6 receptors, the presence of binding activity to human recombinant IL-1α, IL-1β, or IL-6 in detergent-solubilized cell extracts and medium from vaccinia virus– or recombinant baculovirus-infected cells was examined in a solid phase binding assay (Figure 4A). Binding of radioiodinated IL-1α or IL-6 to vaccinia virus or baculovirus was not detected while these ligands bound to EL4 6.1 C10 and U266 cells, which overexpress IL-1 and IL-6 receptors, respectively. IL-1β binding activity was clearly found in vaccinia virus–infected cells and supernatants harvested at 24 hr after infection and in the baculovirus recombinant expressing B15R 3 days after infection. The low binding

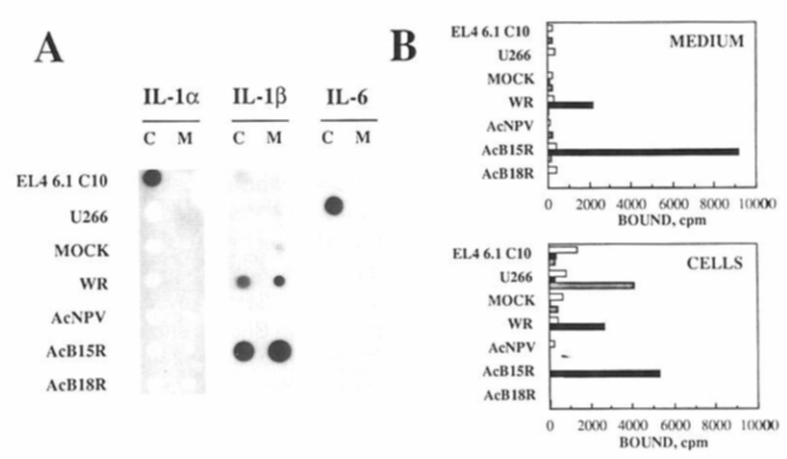


Figure 4. Binding Assays to IL-1α, IL-1β, and IL-6

(A) Nitrocellulose binding assay. Triton X-100 cell extracts (C) (5 μl) or concentrated medium (M) (10 μl) from EL4 6.1 C10 cells, U266 cells, mock- or vaccinia (WR)-infected TK⁻143 cells, and Sf cells infected with AcNPV, AcB15R, or AcB18R were dotted onto nitrocellulose filters, and the membranes were incubated with radioiodinated IL-1α (120 pM), IL-1β (200 pM), or IL-6 (120 pM). The volume added to the assay corresponded to 5×10^5 cell equivalents of cell extract or medium, except for EL4 6.1 C10 and U266 cell extracts in which 2×10^6 cell equivalents were used. An autoradiograph is shown.

(B) Soluble receptor binding assay. Tissue culture supernatants (MEDIUM) or Triton X-100 cell extracts (CELLS) from the sources indicated were incubated with 100 pM of ^{125}I-IL-1α (open box), ^{125}I-IL-1β (closed box), or ^{125}I-IL-6 (stippled box) in solution, and the binding was determined by the polyethylene glycol precipitation method. The amount of medium used corresponded to 1×10^5 cell equivalents. In the case of the detergent-solubilized cell extracts, 5×10^5 cell equivalents were added, except for EL4 6.1 C10 and U266 cells in which 2×10^6 cell equivalents were used. The bound radioactivity is shown.

to EL4 6.1 C10 cells at this dose of ^{125}I-IL-1β probably reflects a 6-fold lower affinity for the β form compared with IL-1α, described for the type I receptor expressed in this cell line (Sims et al., 1988). These results were corroborated in a more quantitative binding assay in solution, which differentiates bound from free ligand by precipitating the ligand–receptor complex with polyethylene glycol (Figure 4B). The finding of a higher binding activity for IL-1β in the supernatants compared with the cell extracts, despite using material from 5-fold more cells, is in agreement with B15R being secreted from the cell. The cell-associated binding activity may correspond to protein present in the secretory pathway or in the plasma membrane. The first possibility was supported by comparing ^{125}I-IL-1β binding with detergent-solubilized cell extracts (Figure 4B) and intact cells in suspension, which showed that only 6%–9% of the cell-associated binding activity is detected on the cell surface (data not shown).

The kinetics of production of soluble IL-1β receptor from vaccinia virus–infected cells was examined by soluble binding assay and showed that no IL-1β receptor was detected, above the background attributable to virus inoculum, in the presence of cytosine arabinoside. In contrast, in the absence of the drug, IL-1β accumulated in the supernatant and reached 80% of total by 24 hr (data not shown). These data are consistent with the transcriptional and polypeptide analyses and show that the IL-1β receptor is expressed late during infection.

Figure 5 shows the binding of radioiodinated IL-1β to medium from different recombinants using 1×10^4 cell equivalents, conditions that allowed a better quantitation of the binding activity. The fact that the ^{125}I-IL-1β binding increased in the vaccinia recombinant containing two copies of B15R (vB15R) and was absent in the mutant containing a deleted version of the gene (vΔB15R) demonstrates that B15R is the only gene product from vaccinia virus responsible for the IL-1β binding activity. In agreement with this, overexpression (vB18R) or deletion (vΔB18R) of B18R, a secreted and structurally related protein, in vaccinia virus did not affect the binding activity. The result obtained with medium from insect cells infected with AcB15R clearly reflects the high expression level of the baculovirus system.

Binding Properties of the Vaccinia IL-1β Receptor

The specific binding of only IL-1β to B15R demonstrated a novel specificity compared with other cloned IL-1 receptors. In view of this, we wished to exclude the possibility that radioiodination of IL-1α or IL-6 had prevented their binding to the vaccinia receptor. This was determined by assaying the ability of unlabeled ILs, including IL-1RA, to compete with ^{125}I-IL-1β for the binding to the vaccinia IL-1 receptor. As shown in Figure 6A, the interaction of radioiodinated IL-1β with the soluble receptor present in vaccinia virus supernatants was competed in a dose-dependent

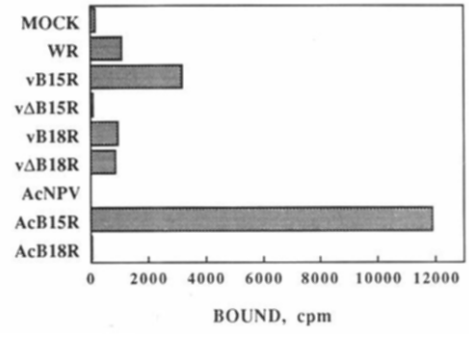


Figure 5. Binding Assay in Solution to Recombinant Viruses

A volume of medium corresponding to 1×10^4 cell equivalents from uninfected TK⁻143 cells (MOCK) or from TK⁻143 or Sf cells infected with the indicated vaccinia virus or baculovirus recombinants, respectively, was incubated with 180 pM of ^{125}I-IL-1β in the soluble receptor binding assay. The radioactivity bound to soluble receptor present in supernatants is represented.

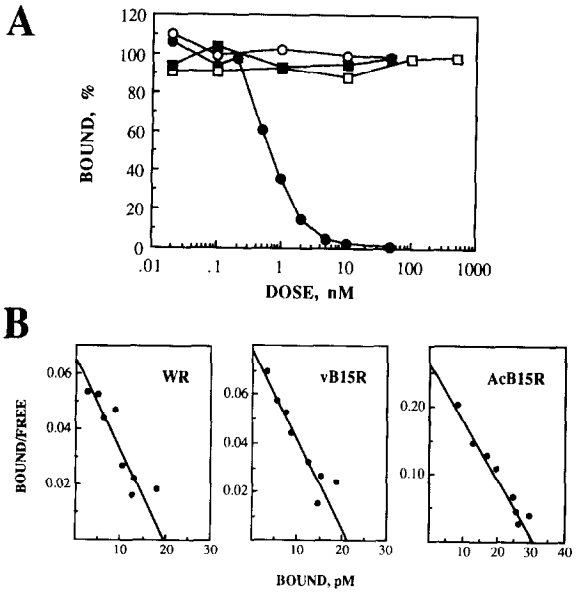


Figure 6. Binding Characteristics of Vaccinia IL-1β Receptor

(A) Competition for binding of ^{125}I-IL-1β to soluble receptor. Medium from WR-infected cells, corresponding to 4 × 10^4 cell equivalents, was incubated with 100 pM of ^{125}I-IL-1β in the presence of the indicated concentrations of unlabeled IL-1α (open circle), IL-1β (closed circle), IL-1RA (open square), or IL-6 (closed square), and the radioactivity bound to the soluble receptor was determined by the polyethylene glycol precipitation method. Binding is expressed as a percentage of the binding occurring in the absence of unlabeled IL (1477 cpm).

(B) Scatchard analysis of ^{125}I-IL-1β binding to medium from vaccinia virus– or baculovirus-infected cells. Medium from cultures infected with WR (1.8 μl, 8 × 10^3 cell equivalents), vB15R (0.56 μl, 2.5 × 10^3 cell equivalents), or AcB15R (0.15 μl, 250 cell equivalents) was incubated with different concentrations (25–1000 pM) of radiolabeled IL-1β for 2 hr at room temperature, and the radioactivity bound was determined by the polyethylene glycol precipitation method. Data were converted to the Scatchard coordinate system. Binding shown represents specific binding. The data were analyzed by the LIGAND program.

manner by unlabeled IL-1β but not by IL-1α or IL-6. The natural competitor IL-1RA did not block the binding of labeled IL-1β to vaccinia IL-1 receptor, even when added at higher concentrations that are required to compete the binding of ^{125}I-IL-1 to the type II IL-1 receptor on polymorphonuclear leukocytes or a pre-B lymphocyte line (Dripps et al., 1991; Granowitz et al., 1991; McIntyre et al., 1991). As a control, the doses of unlabeled ILs used competed the binding of the corresponding radioiodinated IL to its natural receptor on EL4 6.1 C10 or U266 cells (data not shown). The receptor expressed in the baculovirus system showed similar properties. The binding of 100 pM of ^{125}I-IL-1β to 8 × 10^3 cell equivalents of medium from AcB15R-infected cells (6610 cpm) was 94.1%, 7.4%, 99.0%, and 110.1% in the presence of 10 nM of IL-1α, 10 nM of IL-1β, 100 nM of IL-1RA, and 10 nM of IL-6, respectively.

Scatchard analysis of binding of ^{125}I-IL-1β to soluble receptor secreted from vaccinia-infected cells or insect cells infected with AcB15R was performed to estimate the affinity and the number of receptors (Figure 6B). High affinity binding sites for IL-1β were detected in supernatants from WR-infected cultures, with a dissociation constant (K_D) of 234 ± 49 pM. The estimated number of binding sites secreted after 24 hr per WR-infected cell was 1.1 ± 0.1 × 10^5. This is extraordinarily high considering that the number of receptors in IL-1 responsive primary cultures or cell lines varies from <100 receptors per cell to a maximum of 1 × 10^4 receptors per cell (Dower and Urdal, 1987). Cultures infected with vB15R secreted 3.8 ± 0.4 × 10^5 binding sites per cell after 24 hr with a similar affinity (K_D 226 ± 38 pM). In spite of a different glycosylation, the receptor secreted from infected Sf cells showed a similar affinity for IL-1β (K_D 117 ± 10 pM). The number of receptors produced in insect cells after 3 days of infection (6.0 ± 0.2 × 10^6 sites per cell) reflects the high level of expression of the baculovirus system.

Competition of IL Binding to Cells

The specificity for IL-1β was also tested in competition experiments of binding of labeled cytokines to their natural receptors on cell lines overexpressing IL-1 and IL-6 receptors. As shown in Figure 7, the binding of labeled IL-1α to EL4 6.1 C10 cells (A) and of IL-6 to U266 cells (C) was not competed by medium from baculovirus-infected insect cells expressing B15R or B18R, while the binding was competed by the corresponding unlabeled ILs. In contrast, the interaction of IL-1β with EL4 6.1 C10 cells was specifically blocked by supernatants containing B15R and not by B18R (Figure 7B). The percentage of competition correlated with the amount of ^{125}I-IL-1β bound to different doses of receptor in solution (data not shown). Supernatants from WR-infected cells, and not from vΔB15R-infected cells, competed the binding of ^{125}I-IL-1β to cells in culture, while they did not affect binding of IL-1α or IL-6 (data not shown). Altogether, these results provide independent evidence that B15R binds specifically IL-1β and also show that the vaccinia IL-1 receptor is capable of competing with cells for IL-1β binding. This indicates that B15R might function as an inhibitor of the biological activities mediated by IL-1β.

Pathogenicity of Vaccinia Virus Lacking B15R in Mice

Deletion of B15R had no effect on the growth of vaccinia virus in tissue culture. However, since the expression of an IL-1β binding activity might interfere with the inflammatory and/or immune responses in vivo, the pathogenicity of the deletion mutant vΔB15R was compared with that of the parent virus in a mouse model. The intranasal inoculation of the WR strain of vaccinia virus in mice produces an extensive respiratory infection followed by viremia that leads to infection of the central nervous system and death of the animals (Turner, 1967; Williamson et al., 1990).

Figure 8A shows that, although there were not significant differences in the final number of mortalities following infection with different doses of WR or vΔB15R, 70% of the mortalities in vΔB15R-infected animals occurred 1 day sooner than control (panels f). This unexpected and enhanced pathogenicity of vΔB15R was also demonstrated by the clearly accelerated onset of symptoms with doses of virus from 10^5 to 3 × 10^7 plaque-forming units (pfu) (Figure 8A, panels a–d). On day 5 after infection with

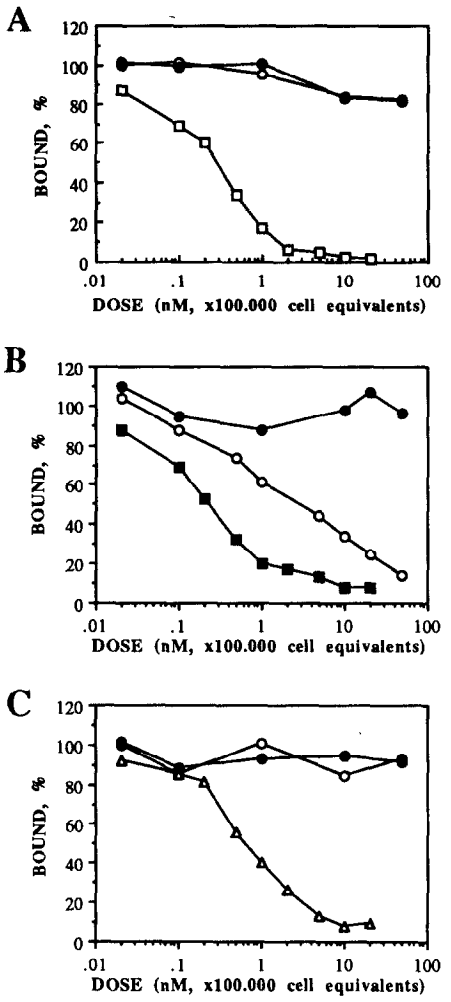


Figure 7. Competition Experiments to EL4 6.1 C10 and U266 Cells

Different cell equivalents of medium from baculovirus-infected cells expressing B15R (AcB15R; open circle) or B18R (AcB18R; closed circle) were incubated with 130 pM of ^{125}I-IL-1α (A), 180 pM of ^{125}I-IL-1β (B), or 100 pM of ^{125}I-IL-6 (C) for 1 hr at 4°C. At the end of the incubation period, 2.5 × 10^6 EL4 6.1 C10 cells were added to the samples containing radiolabeled IL-1α (A) and IL-1β (B), and 2.5 × 10^6 U266 cells were added to those containing radioiodinated IL-6 (C). Samples were incubated at 4°C for 2 hr, and the radioactivity bound to the cells was determined by phthalate oil centrifugation. Competition of binding with unlabeled IL-1α (open square), IL-1β (closed square), or IL-6 (open triangle), expressed in nanomolars, was included as a control. The percentages refer to the binding in the absence of competitor, which was 3720 cpm for ^{125}I-IL-1α and EL4 6.1 C10 cells (A), 2040 cpm for ^{125}I-IL-1β and EL4 6.1 C10 cells (B), and 4963 cpm for ^{125}I-IL-6 and U266 cells (C).

vΔB15R, all 20 animals showed clear disease symptoms (ruffled fur, arched backs, and reduced mobility), while none of the comparable WR-infected animals did so (Figure 8A, panels f). The early onset of symptoms in vΔB15R-infected animals was very clear at a dose of 10^5 pfu (Figure 8A, panels d), where they appeared 2 or 3 days sooner than in WR-infected animals. In contrast, only the WR-infected animals developed symptoms at a low dose of virus (10^4 pfu; Figure 8A, panels e), suggesting a possible effect of B15R on the progression of the infection.

A second experiment was performed in which the symptoms of illness were quantified by measuring the weight of the animals, since in this animal model these symptoms parallel development of cachexia (Moore and Smith, 1992). As shown in Figures 8B and 9, an early onset of symptoms was again observed in animals infected with vΔB15R, and this correlated with accelerated weight loss. The apparent attenuation of vΔB15R compared with WR at 10^4 pfu was not confirmed in this experiment; in contrast, it corroborated that vΔB15R induced earlier and, according to the weight, more severe symptoms of illness.

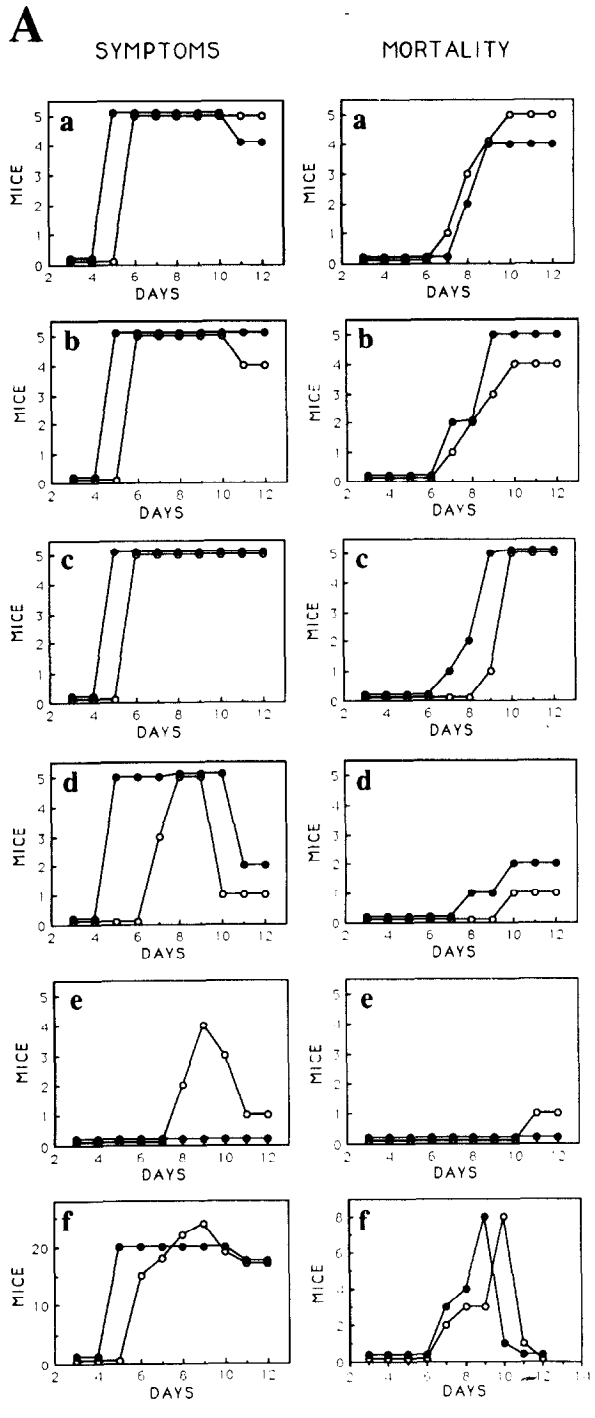
The earlier appearance of symptoms and weight loss probably reflect systemic effects mediated by the IL-1β induced in response to the infection that were neutralized by an active secretion of B15R by the wild-type virus. The accelerated onset of symptoms and mortality indicated that the secreted IL-1β receptor can moderate the severity of the infection.

IL-1 Binding Activity in Other Orthopoxviruses

The IL-1α, IL-1β, and IL-6 binding activity was investigated in a soluble receptor binding assay on supernatants from cultures infected with different strains of vaccinia virus (Copenhagen, IHD-J, IHD-W, Wyeth, Lister, Tian-Tan, and Tashkent) and the related orthopoxviruses rabbitpox and cowpox and compared with the WR strain. The binding to labeled murine IL-1β (mIL-1β) was also investigated to confirm that B15R is able to sequester IL-1β in infected mice. No binding to human ^{125}I-IL-1α or ^{125}I-IL-6 was detected (data not shown). However, binding to labeled IL-1β and mIL-1β was found in all viruses except for rabbitpox, Tashkent, and Copenhagen strains (Figure 10). The failure of Copenhagen to express an IL-1 receptor is in agreement with sequencing data that showed a nonsense mutation at codon 31 of the ORF (Goebel et al., 1990). Even if translation reinitiated from the next methionine codon, which seems unlikely given its distance from the mRNA 5′ end, the protein would lack a signal peptide and therefore would not be secreted but would probably be degraded within the cytoplasm. Binding experiments of labeled IL-1β to detergent extracts from cells infected with the different viruses gave similar results to those obtained with medium (data not shown), indicating that no intracellular receptor is produced in rabbitpox, Tashkent, or Copenhagen strains. Interestingly, the ratios of binding of murine versus human IL-1β varied from 1.6 for the Wyeth strain to 10 for the Tian-Tan strain (Figure 10). The specific binding of ^{125}I-mIL-1β to medium from AcB15R-infected cells indicates that the B15R protein is also responsible for binding mIL-1 (data not shown). The specific binding for IL-1β in other vaccinia virus strains and cowpox and the inability of IL-1RA to compete this binding (data not shown) indicate that the receptor encoded by other orthopoxviruses possesses similar binding properties.

Discussion

Two vaccinia virus ORFs, B15R and B18R, that encode proteins of the immunoglobulin superfamily related to the extracellular domains of the IL-1 and IL-6 receptors have been characterized. Both ORFs are transcribed, but at



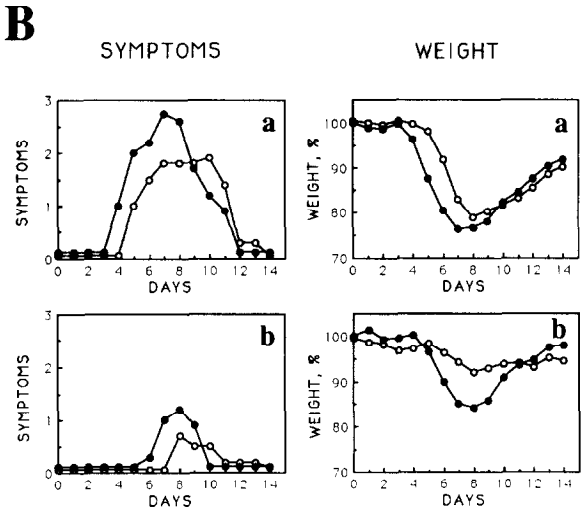


Figure 8. Effect of the Deletion of B15R from Vaccinia Virus on the Infection of Mice

(A) Groups of five mice were intranasally infected with 3×10^7 (panels a), 10^7 (panels b), 10^6 (panels c), 10^5 (panels d), or 10^4 (panels e) pfu of WR (open circle) or vΔB15R (closed circle) and examined daily for symptoms of illness or death. The number of animals that presented strong symptoms of illness (including death) and the accumulated number of mortalities are represented for each dose of virus at different days of infection. No differences were observed between day 12 and day 17. The (f) panels summarize the onset of symptoms (left) and the number of mortalities (right) that occurred at different days after infection.

(B) Groups of 10 mice were intranasally infected with 10^5 (panels a) or 10^4 (panels b) pfu of WR (open circle) or vΔB15R (closed circle). Symptoms of illness were scored from zero to four, and the mean value of each group was represented. Animals were weighed individually each day, and the mean group weight was expressed as the percentage of the mean weight of that group of animals immediately prior to infection. No mortalities occurred at these doses of virus.

different phases of the virus replication cycle, suggesting functional differences. The gene products are glycosylated and secreted from infected cells, in agreement with the absence of transmembrane anchor sequences that are present in the cellular IL-1 and IL-6 receptors. The dispensability for virus replication in tissue culture, the secretion to the extracellular space, and the predicted receptor-like structure of the proteins suggest that both are involved in interference with host defense mechanisms in vivo.

The B18R gene product is shown not to bind IL-1α, IL-1β, or IL-6, despite the homology with the receptors for these cytokines (McMahan et al., 1991; Smith and Chan, 1991). Two forms of the protein (52 kd and 60–65 kd) were detected, the larger of which is found in supernatants while the smaller might represent a membrane-associated molecule. This would be in agreement with previous reports showing immunofluorescence in the plasma membrane of infected cells using antiserum specific for proteins secreted at early times of infection (Ueda et al., 1972), which

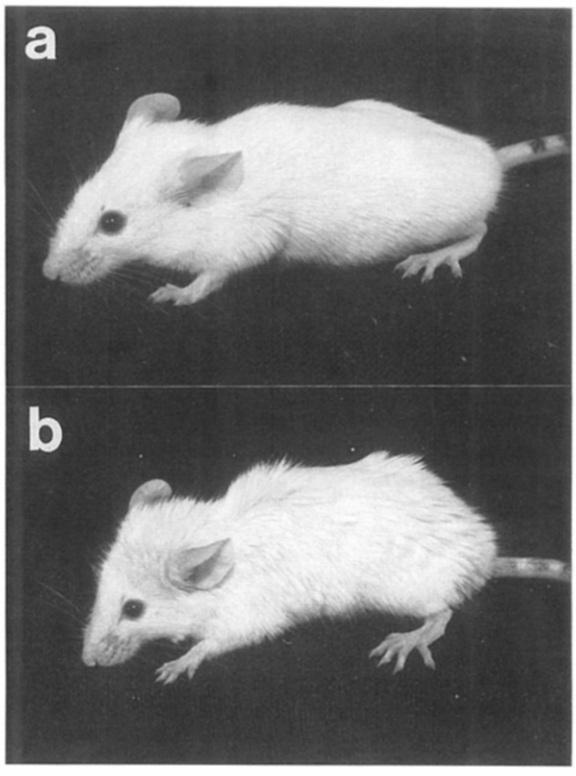


Figure 9. Effect of Expression of the Vaccinia IL-1β Receptor on Mice Infected with Vaccinia Virus

A representative mouse 5 days after infection with 10^5 pfu of WR (a) or vΔB15R (b) is shown. Note the ruffled fur in (b), which correlated with accelerated weight loss (see Figure 8B, panels a).

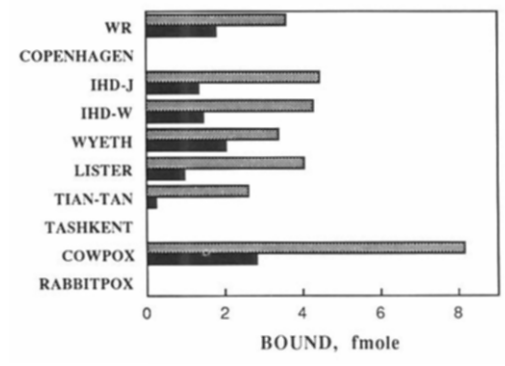


Figure 10. Binding of Murine and Human IL-1β to Different Strains of Vaccinia Virus, Rabbitpox, and Cowpox

Tissue culture medium (1×10^5 cell equivalents) from TK⁻143 cells infected with the indicated viruses was incubated in a binding assay in solution with 100 pM of radioiodinated IL-1β or mIL-1β. The bound human (closed box) or murine (stippled box) ^{125}I-IL-1β, expressed in femtomoles, is shown. One femtomole corresponded to 935 or 535 cpm for IL-1β or mIL-1β, respectively.

was attributed later to reactivity against B18R (Ueda et al., 1990). An intriguing possibility is that B18R, a secreted glycoprotein structurally related to cytokine receptors, might bind a soluble mediator of the immune response not included in this study and, perhaps, for which the cellular receptor counterpart has not yet been sequenced.

In contrast with B18R, B15R ORF is shown to encode an IL-1β binding activity present in the supernatants of vaccinia virus–infected cells and to represent a novel soluble IL-1 receptor. The high affinity for IL-1β binding (K_D 234 pM) is similar to those reported for the cellular receptors (Sims et al., 1988, 1989; McMahan et al., 1991) and is consistent with the retention of full binding activity by the extracellular domain of the IL-1 receptor (Dower et al., 1989). The size (50–60 kd) and high carbohydrate content of the mature vaccinia IL-1β receptor are in agreement with those reported for the truncated and complete versions of the cellular receptor, respectively (Urdal et al., 1988; Dower et al., 1989). The secretion of a biologically active 40–44 kd protein from insect cells suggests that the carbohydrate is not an essential component for the IL-1 binding.

The vaccinia IL-1 receptor constitutes a novel receptor for IL-1 because of the specificity for IL-1β. This was shown in binding experiments to radioiodinated ILs and was corroborated in competition assays with unlabeled cytokines and by blocking the interaction of the ILs with the natural receptor on cells in culture. A cellular receptor for IL-1β that is present in the membrane and secreted from Raji cells, which has a molecular weight similar to the type II receptor, has been described but not cloned (Benjamin et al., 1990; Giri et al., 1990; Symons and Duff, 1990; Symons et al., 1991). B15R might constitute a viral homolog of a new type of IL-1 receptor that remains to be sequenced. Alternatively, since B15R has a higher similarity to the cellular type II receptor than to the type I receptor (McMahan et al., 1991; Smith and Chan, 1991) and since this similarity is comparable with those found between other vaccinia virus proteins and their cellular counterparts (Smith et al., 1991a), B15R is more likely to be derived from the type II IL-1 receptor or a variant thereof.

Unfortunately, the comparison of the sequence of the vaccinia IL-1β receptor with the type I and type II IL-1 receptors does not permit identification of the amino acids that confer specificity for IL-1β since the sequences are quite divergent. However, the availability of the vaccinia virus gene will allow mutagenesis studies to identify these positions. Furthermore, the sequence of B15R ORF in other vaccinia virus strains that show different affinities for the human and murine IL-1β may provide structural information on the binding domain. In this case, the comparison of the sequence may be more useful since genes from different orthopoxviruses are highly conserved.

The vaccinia IL-1β receptor might be useful as a tool to investigate the function of IL-1α and IL-1β in vivo in different models. In contrast, the other IL-1 inhibitors available (IL-1RA, a soluble truncated IL-1 receptor, and monoclonal antibodies against the receptor) block the binding of both forms of IL-1 (Fanslow et al., 1990; Gershenwald et al., 1990; Ohlsson et al., 1990; Alexander et al., 1991; McIntyre et al., 1991). The molecule could also be a potential therapeutic agent to regulate responses normally controlled by IL-1β, and, since it does not bind IL-1α or IL-1RA, it might offer advantages over the other inhibitors. The failure of the vaccinia IL-1β receptor to bind IL-1RA illus-

trates the adaptation of the virus to the physiological response of the host by preventing interference with the natural antagonist.

The number of IL-1β binding sites secreted from vaccinia virus–infected cells (about 10^5 receptors per cell 24 hr after infection) is without precedent and makes the supernatants from cultures infected with vaccinia virus the most concentrated naturally occurring soluble IL-1 binding activity. An excess of soluble receptors must be required to block the effects of IL-1β in vivo, since only a few cellular IL-1 receptors need to be occupied to elicit a biological response. This was illustrated in the competition of IL-1β binding to T cells, which also indicates that the vaccinia IL-1β receptor will probably block the biological effects induced in cells expressing IL-1 receptors.

The blockade of IL-1 by a virus is interesting since this cytokine orchestrates the host response to infection, inducing a broad spectrum of systemic effects and playing an important role in initiating the inflammatory and immune responses. But more interesting is the specificity of the blockade for IL-1β. To date, both IL-1α and IL-1β have been found to induce similar activities in a number of model systems (Dinarello, 1989). The fact that vaccinia virus secretes a protein that specifically blocks the effects of IL-1β suggests that soluble IL-1β plays a more important role than IL-1α in the host response to orthopoxvirus infections.

The deletion of B15R ORF from the WR strain of vaccinia virus does not greatly affect virulence in intranasally infected BALB/c mice, in which virus virulence is defined according to the number of mortalities. However, two observations revealed that the vaccinia IL-1β receptor does play an important role in vaccinia virus infection in vivo. First, the animals infected with vΔB15R developed symptoms and lost weight more rapidly than the corresponding control group. The early onset of symptoms is very likely to represent systemic effects induced by circulating IL-1β produced in response to vaccinia virus infection. IL-1 is known to function as a hormone mediating multiple effects such as fever, headache, and sleep and at high doses can induce hypotension and a shocklike state (Dinarello, 1988, 1989). The vaccinia IL-1β receptor, expressed in the wild-type virus, may thus limit the systemic acute phase response otherwise initiated by increased levels of IL-1β. The finding that weight loss, which can be induced by IL-1 (Di Giovine and Duff, 1990), occurred earlier in animals infected with vΔB15R supports this view. A generalized response can contribute to host defense; for example, temperature typical of fever has been reported to enhance the proliferation of T cells that might facilitate a T cell–dependent immune response (Duff and Durum, 1983). However, an increased systemic reaction to infection did not affect the outcome of infection by vΔB15R. Second, although the absolute number of mortalities are indistinguishable, 70% of them occurred 1 day sooner in animals inoculated with the deletion mutant. This would be consistent with the vaccinia IL-1β receptor reducing the pathological effects mediated by excessive IL-1β production that are detrimental to the host (Dinarello, 1988, 1989) and thus moderating the severity of the disease.

The consequence of secretion of a specific inhibitor of IL-1β in the progression of vaccinia virus infection may be dual, since IL-1β triggers local and systemic effects necessary for an efficient host response to infection, but also contributes to the pathological process. Therefore, B15R might function as a virulence or attenuation factor for the virus. It is unclear whether the effects of deleting B15R from the WR strain of vaccinia virus, which was selected for high neurovirulence by passage in mouse brain, are representative of infections with other orthopoxviruses that also express the IL-1β binding activity. Other animal models for localized and systemic orthopoxvirus infections (Buller and Palumbo, 1991) may illustrate the different roles of B15R in infection. The importance of the route of virus inoculation for virus pathogenesis is illustrated by the parallel study by Spriggs et al. (1992 [this issue of *Cell*]). These authors show that a WR-based B15R deletion mutant had a 100-fold increase in lethal dose 50 compared with WR when administered by intracranial injection.

Interestingly, another orthopoxvirus mechanism to inhibit specifically IL-1β action and to diminish the inflammatory response has just been reported (Ray et al., 1992). In this case, the protein encoded by the cowpox virus *crmA* gene (related to serine protease inhibitors) was shown to inhibit the IL-1β converting enzyme, which cleaves pro-IL-1β to generate active IL-1β. The intracellular location and the kinetics of synthesis of this protein indicate that this mechanism is restricted to infected cells during early phases of virus replication. The secretion of an abundant IL-1β receptor at late times of vaccinia virus infection, which we show here is also active in cowpox virus, is an ideal complement to the inhibitory role of the crmA protein, since it is effective extracellularly and would bind IL-1β released by both infected and uninfected cells recruited to the site of infection. However, with vaccinia virus the systemic response induced by IL-1β in infected mice seems mainly to be controlled by the soluble receptor, since the ORF corresponding to the *crmA* gene (B13R) is still present in the deletion mutant vΔB15R that induces more severe symptoms of disease.

B15R ORF is one of a few virus genes that has been shown to increase the pathogenicity or the severity of the infection when deleted from the virus genome (Ginsberg et al., 1989; Romanczuk and Howley, 1992). Virus attenuation can result from deletion or inactivation of genes encoding proteins that interfere with host defense mechanisms, but, as shown here, some virus proteins might also be devised to diminish the detrimental effects that the infection produces in the host. This would help host survival and thereby be beneficial for the virus. Consistent with this view, in a 21.8 kb region of the genome of the highly pathogenic Harvey strain of variola major virus, 7 out of 32 ORFs are disrupted into small fragments, and 2 ORFs are partially or totally deleted, compared with vaccinia virus (Aguado et al., 1992). Another interesting correlation is that the vaccine strains of vaccinia virus that gave higher frequencies of postvaccinial complications (Copenhagen, Tashkent, and Tian-Tan; Fenner et al., 1988) fail to express the IL-1β binding activity or, as in Tian-Tan, recog-

nize human IL-1β poorly. It is tempting to speculate that active expression of B15R might be to some extent responsible for the lower degree of pathogenicity of the other vaccine strains (Lister and Wyeth).

The reduction of systemic effects possibly attributed to IL-1 by neutralization of the IL-1β activity by vaccinia virus presented in this report suggests that the β form of IL-1, and not IL-1α, is mediating the endocrine, long range effects in the host in response to vaccinia virus infection. This view is supported by previous observations. It has been reported that IL-1β is the predominant form of IL-1 secreted from human monocytes (Hazuda et al., 1988), and the release of adrenocorticotropic hormone, one of the neuroendocrine actions of IL-1, is exclusively induced by IL-1β (Uehara et al., 1987). Similarly, IL-1β is more potent than IL-1α in the induction of fever, and the effect is mediated through different mechanisms (Busbridge et al., 1989), which correlates with the discovery of IL-1β (Breder et al., 1988) and receptors specific for IL-1β (Katsuura et al., 1988) in the brain.

B15R is the second soluble cytokine receptor to be identified in a virus. A soluble receptor for tumor necrosis factor (TNF) has been shown to be active in Leporipoxviruses and to increase the pathogenicity of the virus (Smith et al., 1991b; Upton et al., 1991). The WR and Copenhagen strains of vaccinia virus contain one and two homologs, respectively, to the TNF receptor, but the presence of frameshifts and stop codons make expression of active proteins unlikely (Howard et al., 1991; Upton et al., 1991). The presence of soluble receptors for either TNF or IL-1 in different genera of poxviruses, which produce very different patterns of disease, is interesting since these cytokines share many biological properties. The soluble IL-1β receptor is one of the increasing number of activities encoded by vaccinia virus that aid evasion from the host immune system (for references see Moore and Smith, 1992) and, in particular, is another viral-encoded protein that interferes with cytokine functions. Besides the TNF receptor of leporipoxvirus and the crmA protein of cowpox virus, other examples found are the 14.7 kd protein of adenovirus that inhibits cytolysis by TNF (Gooding et al., 1988), the IL-10 activity encoded by Epstein–Barr virus (Hsu et al., 1990), and the presence of IL-6 binding sites in the envelope protein of hepatitis B virus (Neurath et al., 1992).

In summary, the vaccinia IL-1β receptor may be a useful tool to discriminate the physiological roles of IL-1α and IL-1β and might be used as an anti-inflammatory therapeutic reagent. The expression of this activity by vaccinia virus and other orthopoxviruses represents a novel mechanism of virus evasion from the immune system. Here we show that the IL-1β receptor is modulating the systemic response to infection and the severity of the disease, which suggests that IL-1β, and not IL-1α, is the main mediator of the endocrine effects of the IL-1 produced in response to vaccinia virus infection in mice. These observations illustrate how the understanding of the mechanisms devised by viruses to modulate the host defenses allow us to learn about the contributions of different molecules in the generation of an efficient host response to infection.

Experimental Procedures

Cells and Viruses

The cell line EL4 6.1 C10, a subclone of the mouse thymoma EL4 that expresses a high number of IL-1 binding sites (MacDonald et al., 1985), was a gift of H. R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). U266 cells overexpressing IL-6 receptors (Taga et al., 1987) were obtained from the Cell Bank of the Sir William Dunn School of Pathology (University of Oxford). These cell lines were grown in suspension in RPMI 1640 medium containing 10% fetal calf serum.

Sf 21 insect cells and AcNPV were obtained from R. Possee (Natural Environmental Research Council Institute of Virology and Environmental Microbiology, Oxford) and were cultured in TC100 medium (GIBCO) containing 10% fetal calf serum (Brown and Faulkner, 1977).

Vaccinia virus strain WR and recombinants derived from it were grown in CV-1 or BS-C-1 cells. TK^-143 and D98 cells were used for the selection of recombinants. The WR strain was obtained from B. Moss (National Institutes of Health, Bethesda, Maryland) and cells were obtained from the American Type Culture Collection. Cells were grown in minimal essential medium (GIBCO) supplemented with 10% fetal calf serum. Purified virus stocks were prepared by sedimentation through a sucrose cushion (Mackett et al., 1985). The Tashkent, IHD-J, and IHD-W strains of vaccinia virus and cowpox virus were obtained from M. Mackett (Paterson Institute for Cancer Research, Manchester, England) and J. D. Williamson (St. Mary's Hospital Medical School, London). The New York City Board of Health vaccine strain (Wyeth) was obtained from Wyeth Laboratories, and the Lister strain was obtained from Vestric Limited. Rabbitpox virus was provided by R. W. Moyer (University of Florida, Gainesville, Florida). The Temple of Heaven strain (Tian-Tan) and a temperature-sensitive mutant of the Copenhagen strain were obtained from J. Zhou (Princess Alexandra Hospital, Brisbane, Australia) and R. Drillien (University Louis Pasteur, Strasbourg, France), respectively.

Reagents

Radioiodinated human recombinant IL-1α and IL-1β proteins and recombinant mIL-1β were obtained from Du Pont–New England Nuclear, and the radioiodinated human recombinant IL-6 was purchased from Amersham. IL-1α had been radioiodinated using the chloramine-T procedure to a specific activity of 70–120 μCi/μg. IL-1β and IL-6 had been labeled with Bolton Hunter reagent to a specific activity of 80–180 μCi/μg and 800–1200 Ci/mmol, respectively. Unlabeled human recombinant IL-1α (code 86/632), IL-1β (code 86/680), and IL-6 (code 88/514) were obtained from the National Institute for Biological Standards and Control (South Mimms, Hertfordshire, England). The activity for IL-1α and IL-1β was 10^5 U/μg and for IL-6 was 5×10^3 U/μg. Unlabeled human recombinant IL-1RA, specific activity 1×10^5 to 1.4×10^5 U/mg, was purchased from British Bio-technology.

Plasmid Constructions

Restriction endonuclease digestions, PCR, DNA ligations, and plasmid DNA preparations were performed according to standard procedures (Sambrook et al., 1989).

Cloning of B15R into pUC118

A derivative of plasmid pUC118 (Vieira and Messing, 1987) was constructed that contained the entire B15R ORF, lacking most of the flanking regions, and with convenient restriction sites at each end. A combination of subcloning and PCR was used. The left end SalI–XbaI fragment of the SalII fragment of vaccinia DNA containing B15R was inserted into SalI- and BamHI-cut pUC118, and the resultant plasmid was called pAA1. To remove most of the 3′ flanking region of B15R, pAA1 was digested with EcoRV and XbaI, and the largest fragment was gel purified, end filled with Klenow fragment, and self-ligated to form pAA3, which contains 348 bp of the 3′ flanking region. To introduce restriction sites close to the initiator methionine of B15R, a PCR copy of 5′ region of the ORF was constructed, using plasmid pAA1 as template and an oligonucleotide containing the sequence of the first 21 nt of B15R and the recognition sequence for BamHI and NcoI (B15R-1; 5′-CCCGGATCCACCATGGGTATACTACCTGTTATA-3′) and an oligonucleotide that hybridizes to an internal sequence of B15R, corresponding to nucleotides 1008–1025 of the SalII fragment (B15R-

2; 5′-CCGCTCCTCGTTTTTCCC-3′). The fourth nucleotide of B15R in the PCR fragment was G instead of A to create a NcoI recognition sequence, giving rise to a serine to glycine substitution in the second amino acid of the protein. The 222 bp PCR fragment was digested with AccI, forming a 20 bp fragment containing the BamHI and NcoI restriction sites bound to the first 8 nt of B15R, and was treated with polynucleotide kinase. This fragment was cloned into pAA3 digested with HincII and AccI, which removed the 5′ flanking region, to render plasmid pAA4. This construct was confirmed by DNA sequencing.

Cloning of B18R into pUC4K

The XbaI–SalI right end fragment of the SalII fragment of vaccinia DNA, which contains B18R, was cloned into XbaI- and SalI-cut pUC118 to form plasmid pAA2. A SspI fragment, containing the whole B18R ORF and 22 5′ and 263 3′ nucleotides, was excised from pAA2 and cloned into pUC4K (Vieira and Messing, 1982) digested with SalI and end filled with Klenow fragment to create blunt termini, and the resulting plasmid was termed pAA5.

Vectors for Overexpression in Vaccinia Virus

The transfer vector used for overexpression of B15R and B18R in vaccinia virus was pRK19 (Kent, 1988), which contains the vaccinia virus 4b promoter to control the transcription of the inserted gene, flanked by sequences of the TK gene that allow insertion in the TK locus of the virus genome. BamHI fragments containing the ORFs were excised from pAA4 and pAA5 and cloned into the BamHI site of pRK19, and the resulting plasmids were named pAA10 and pAA11, respectively.

Vectors for Expression in Baculovirus

The transfer vector for construction of baculovirus recombinants was pAcDZ1, which uses the polyhedrin promoter to drive the transcription of foreign genes and coexpresses Escherichia coli β-galactosidase for selection of the recombinants (Zuidema et al., 1990). This vector was provided by J. M. Vlak (Department of Virology, Agricultural University, Wageningen, The Netherlands). The genes were excised from pAA4 and pAA5 with BamHI and inserted into BamHI-cut pAcDZ1, forming pAA14 and pAA15, respectively.

Vectors for Deletion of the Genes in Vaccinia Virus by Transient Dominant Selection

The flanking sequences of B15R and B18R were excised from pAA1 or clones from a M13 library containing random subfragments of the SalII fragment of vaccinia DNA, which were used to sequence this region of the vaccinia virus genome (Smith et al., 1991a), and were cloned into pSJH7 (Hughes et al., 1991). The 5′ flanking region of B15R was obtained by digestion of the replicative form of the M13 clone SalII.144 with EcoRI and SphI, and the 3′ flanking region was excised from pAA1 by digestion with SphI and BamHI. Both fragments were cloned in one step into EcoRI- and BamHI-cut pSJH7. The resultant plasmid, called pAA16, contained 360 and 1316 nt of the 5′ and 3′ flanking sequence, including 17 and 252 nt of the coding sequence, respectively, so that 72% of the B15R coding sequence was deleted. DNA fragments containing the flanking sequences of B18R were obtained by BamHI and EcoRI digestion of the replicative form of the M13 clones SalII.44 and SalII.81. Both fragments, of 400 and 449 bp, were cloned in one step into EcoRI-cut pSJH7, and the plasmid in which the sequence of B18R was transcribed in the same direction as the Ecogpt gene present in pSHJ7 was chosen and called pAA17. This construct lacked 18 nt of the 5′ flanking region and 92% of the ORF and retained only 78 nt of the 3′ end of the coding region.

S1 Mapping of the 5′ End of mRNAs

To prepare the ^{32}P-labeled DNA probe used to identify the 5′ end of the mRNA coding for B15R, a PCR fragment was obtained with the oligonucleotide B15R-2 (above) and the 17-mer sequencing primer (−20), using pAA1 as template. The PCR product was purified, labeled with [γ-^{32}P]ATP and polynucleotide kinase, and subsequently digested with SalI, giving rise to a 1024 bp fragment containing 211 nt of the 5′ coding region of B15R.

To map the 5′ end of the B18R transcript, pAA2 was digested with EcoRI, a band of 505 bp was purified and dephosphorylated with calf intestinal alkaline phosphatase, and the 5′ ends were labeled with [γ-^{32}P]ATP using polynucleotide kinase. After digestion with DraI, a fragment of 379 bp was isolated that contained 98 nt corresponding to the 5′ coding region of B18R.

Both ^{32}P-labeled fragments specific for B15R and B18R were hybridized to 10 μg of vaccinia virus RNA (provided by J. B. Moore) obtained at 8 hr after infection from cells infected in the presence (early) or absence (late) of cycloheximide or yeast transfer RNA. The hybrids were digested with S1 nuclease, and the protected fragments were separated on 6% polyacrylamide sequencing gel and detected by autoradiography as described (Moore and Smith, 1992). An M13 sequencing ladder was used as size markers.

Transfection and Selection of Recombinant Viruses

Sf cells were cotransfected with purified AcNPV DNA and pAA14 or pAA15 using the calcium phosphate precipitation technique, and the recombinant viruses were identified by staining with X-Gal as described (Zuidema et al., 1990). The insertion of foreign genes (B15R and B18R) into the baculovirus genome was confirmed by Southern blot hybridization of ^{32}P-labeled specific probes on viral DNA digested with HindIII (data not shown). The recombinant viruses expressing B15R and B18R were plaque purified five times and called AcAA3 and AcAA4, respectively, and are referred to here as AcB15R and AcB18R.

Recombinant vaccinia viruses were constructed by standard procedures (Mackett et al., 1985). The genomes of viruses containing a second copy of B15R or B18R in the TK locus of the vaccinia DNA were analyzed by Southern blotting, using viral DNA extracted from virus cores (Esposito et al., 1981). These structures were confirmed by PCR using oligonucleotides that hybridized to the 5′ and 3′ ends of the TK gene, which gives rise to a longer PCR product in the recombinant viruses compared with WR owing to the insertion of foreign DNA in the TK locus (data not shown). The recombinant vaccinia viruses containing a second copy of B15R and B18R were called vAA1 and vAA4, respectively, and are referred to here as vB15R and vB18R. Vaccinia virus deletion mutants were constructed by transient dominant selection as described elsewhere (Falkner and Moss, 1990; Isaacs et al., 1990). Vaccinia viruses containing deleted versions of B15R and B18R were termed vAA5 and vAA6, respectively, and are referred to here as vΔB15R and vΔB18R.

The B15R-specific probe containing the ORF and 348 bp of the 3′ flanking region was excised from pAA4 by digestion with BamHI and used for Southern blot hybridization. The oligonucleotide B15R-2 was used as an internal probe for B15R ORF. A BamHI fragment excised from pAA5, containing B18R ORF and 263 bp of the 3′ flanking region, was used as a B18R-specific probe. The internal probe for B18R was obtained by excision of a 424 bp EcoRI fragment from pAA2.

Preparation of Antisera

Rabbit sera specific for B15R or B18R were obtained by immunization with AcB15R- or AcB18R-infected Sf cell extracts and concentrated medium according to standard procedures (Harlow and Lane, 1988).

Metabolic Labeling of Proteins and Immunoprecipitation

Sf or BS-C-1 cells were infected with the baculovirus or vaccinia virus recombinants, respectively, at high multiplicity of infection (20–40 pfu/cell). At the indicated times of infection, infected cells were pulse-labeled with 750 μCi/ml ^{35}STrans-label (ICN Biomedicals; a mixture of ~80% [^{35}S]methionine and ~20% [^{35}S]cysteine, 1200 Ci/mmol) in methionine-free TC100 medium or methionine- and cysteine-free minimal essential medium, respectively, in the absence of serum. Cytosine arabinoside (40 μg/ml) or tunicamycin (1 μg/ml) was added to the medium throughout the infection and during the pulse period when indicated.

Medium or cells were incubated in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride and immunoprecipitated by the indicated rabbit serum and protein A–Sepharose (Harlow and Lane, 1988). For the electrophoretic analysis, whole extracts or immune complexes were dissociated in sample buffer (0.4 M Tris–HCl, [pH 6.3], 2.3% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and analyzed by SDS–PAGE in 10% or 10%–20% acrylamide gels as described (Laemmli, 1970). Radioactive bands were detected by autoradiography or fluorography with salicilate (Harlow and Lane, 1988).

Preparation of Cell Extracts and Medium for IL-1 Binding Assays

Sf cells and TK$^-$143 cells, grown in 175 cm^2 or 80 cm^2 flasks, were infected at a density of 1.5 × 10^5 to 2 × 10^5 cells/cm^2 with a multiplicity

of infection of 5–10 pfu per cell in serum-free medium. Cells and medium were harvested from vaccinia- or baculovirus-infected cells at 1 or 3 days after infection, respectively. The final concentration of the supernatants was 1×10^6 to 5×10^6 cell equivalents per milliliter. The medium was centrifuged at 3000 rpm for 10 min at 4°C, the pellet discarded, and supernatants made 20 mM HEPES (pH 7.4) and 0.1% sodium azide. Supernatants were stored at −70°C until used in binding assays in solution or concentrated and dialized against phosphate-buffered saline (PBS) at 4°C in a Micro-ProDiCon (Bio-Molecular Dynamics) with PA-10 ProDiMen dialysis membranes (MW 10,000) to a final concentration of 5×10^7 cell equivalents per milliliter. The concentrated medium was made 1% in sodium azide and stored at −70°C. Cells were detached from the plastic by incubation with 0.5 mM EDTA in PBS and washed twice with PBS, and the pellet was resuspended in 1% Triton X-100 in PBS containing 1 mM phenylmethylsulfonyl fluoride to a final concentration of 1×10^8 cells per milliliter. Samples were incubated on ice for 15 min and centrifuged at 12,000 × g for 30 min at 4°C as described (Urdal et al., 1988). The cell extracts were made 1% in sodium azide and stored at −70°C. Detergent-solubilized lysates of EL4 6.1 C10 and U266 cells were prepared in the same way to a final concentration of 4×10^8 cells per milliliter. The supernatants were harvested from cells seeded at a cellular density of 5×10^5 cells per milliliter and grown in culture over a period of 3 days. The medium was concentrated in a Micro-ProDiCon to 5×10^7 cell equivalents per milliliter.

Sf and TK⁻143 cells were harvested for binding assays to intact cells by treatment with PBS containing 0.5 mM EDTA. EL4 6.1 C10, U266, Sf, and TK⁻143 cells were washed twice in serum-free medium and resuspended in binding medium.

Binding Assays

The binding medium used in the different assays was RPMI 1640 containing 20 mM HEPES (pH 7.4), 1% bovine serum albumin, and 0.1% sodium azide. Solid phase binding assays on nitrocellulose were performed as described (Urdal et al., 1988). Binding to intact cells was carried out in duplicate in 150 μl of binding medium for 2 hr at 4°C, and bound ^{125}I-IL was determined by phthlate oil centrifugation as described (Dower et al., 1985). In the competition assays of labeled ILs to intact cells, samples were preincubated with the ILs in 125 μl for 1 hr at 4°C. Subsequently, 2.5×10^6 EL4 6.1 C10 or U266 cells were added in 25 μl and incubated for 2 hr at 4°C.

Soluble receptor binding assays were performed by precipitating the ligand–receptor complexes with polyethylene glycol and filtration through Whatman GF/C filters as described by Symons et al. (1990). Supernatants were incubated in duplicate with labeled ILs in a final volume of 150 μl for 2 hr at room temperature. Background radioactivity precipitated in the presence of binding medium was subtracted. Kinetics experiments of ^{125}I-IL-1β binding to vaccinia virus supernatants showed that maximum binding was reached after 5 min at room temperature (data not shown). The saturation experiments to soluble receptor from WR, vB15R, and AcB15R were performed in a final volume of 75 μl in the same conditions. The binding of ^{125}I-IL-1β to supernatants from vΔB15R or AcB18R were considered as nonspecific binding and subtracted from total binding. In previous experiments we determined that these values are similar to those obtained in the presence of 100-fold excess of cold IL-1β (data not shown). Binding data were analyzed using the LIGAND program (Munson and Rodbard, 1980).

Animals

Female BALB/c mice (5 to 6 weeks old) were anesthetized and infected intranasally with 20 μl of the diluted virus in 1 mM Tris–HCl (pH 9.0). Mice were weighed daily and monitored for signs of illness or death (Turner, 1967; Williamson et al., 1990). As a control, an aliquot of the dilutions of vΔB15R or WR used to inoculate the animals was grown in TK⁻143 cells, and the absence or the presence of IL-1β binding activity in the medium at 24 hr after infection was confirmed in a binding assay in solution (data not shown).

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