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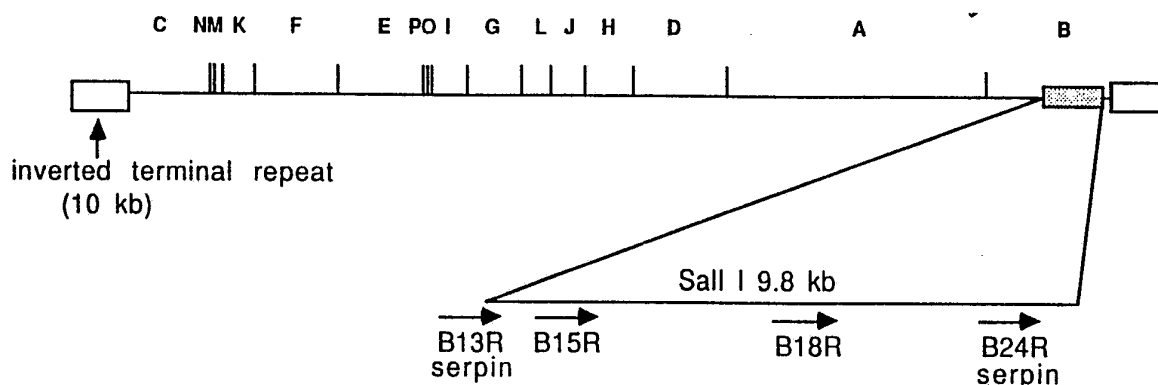
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(54) Title: VACCINIA VIRUS B15R USED IN INTERLEUKIN B1-INVOLVING CONDITION



(57) Abstract

This invention relates to proteins encoded by vaccinia virus and which function to interfere with the host's immune system. In particular, the invention provides for use of the nucleotide sequence designated herein as B15R or of a nucleotide sequence coding for an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide encoded by the B15R nucleotide sequence in a process relating to the manufacture of a medicament for the treatment of a condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition. The invention also provides an anti-fever medicament which comprises a polypeptide which is encoded by the B15R gene product or an allele of, or functionally equivalent fragment, derivative or variant of that product.

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VACCINIA VIRUS B15R USED IN INTERLEUKIN B1-INVOLVING CONDITION.

5 The present invention relates to vaccinia virus proteins, uses and products relating thereto.

Where the term IL-1 β receptor is used in the text as referring to the vaccinia virus B15R polypeptide or allele or functionally equivalent fragment, derivative or variant thereof, the word "receptor" should be
10 interpreted as referring to a substantially soluble binding protein.

Live vaccinia virus was used as the vaccine to immunise against, and eradicate smallpox. Vaccinia virus (VV) is the prototypical member of the poxvirus family
15 and therefore it has been extensively studied. It is a large DNA-containing virus which replicates in the cytoplasm of the host cell. The linear double-stranded genome of approximately 185,000 base pairs has the potential to encode at least 200 proteins (Moss, B.
20 (1990a)). The cytoplasmic site of replication requires that vaccinia virus encodes many enzymes and protein factors necessary for transcription and replication of its genome. The virus also encodes a variety of factors which modulate virus replication in the multicellular
25 host and aid evasion of the host immune system (Moss, B. (1990a)). Advances in molecular genetics have made possible the construction of recombinant vaccinia viruses that contain and express genes derived from other organisms (for review see Mackett, M. & Smith G.L.
30 (1986), J. Gen. Virol., 67, 2067-2082). The recombinant viruses retain their infectivity and express the foreign gene (or genes) during the normal replicative cycle of the virus. Immunisation of animals with the recombinant viruses has resulted in specific immune responses against
35 the protein(s) expressed by the vaccinia virus, including those protein(s) expressed by the foreign gene(s) and in several cases has conferred protection against the pathogenic organism from which the foreign gene was

derived.

Recombinant vaccinia viruses have, therefore, potential application as new live vaccines in human or veterinary medicine. Advantages of this type of new vaccine include the low cost of vaccine manufacture and administration (because the virus is self-replicating), the induction of both humoral and cell-mediated immune responses, the stability of the viral vaccine without refrigeration and the practicality of inserting multiple foreign genes from different organisms into vaccinia virus, to construct polyvalent vaccines effective against multiple pathogens. A disadvantage of this approach, is the use of a virus vaccine that has been recognised as causing rare vaccine-related complications.

It would therefore be of considerable value to produce a vaccinia virus with an attenuated phenotype as a basis for the production of a new generation of live recombinant vaccinia virus vaccines.

Vaccinia virus contains two genes designated B15R and B18R from near the right inverted terminal repeat (ITR) which are each predicted to encode a soluble glycoprotein that has three immunoglobulin-like (Ig) domains, but no transmembrane anchor sequence of cytoplasmic tail (Smith, G.L. and Chan Y.S. 1991).

The product of B18R is apparently expressed on the surface of the infected cell early during infection and antibodies directed against a mixture of it and other proteins confer resistance to virus infection without directly neutralizing infectivity (Ueda, Y., Morikawa, S. and Matsuura, Y. (1990); Ueda and Tagaya, I. (1973) J. Exp. Med. 138, 1033-1043; Ikuta, K., Miyamoto, H. and Kato, S. (1980). On the basis of studies investigating homology of these gene products with other proteins, the applicants recognised limited similarity of these gene products to receptors for certain interleukin (IL) molecules and they surmised that either of these gene products may bind ILs and prevent these cytokines reaching their natural receptors. In consequence, the

inflammatory response may be diminished and virus replication enhanced to constitute a novel method of immune evasion. The applicants present data herein which shows that gene product B18R does not bind interleukin-1 (IL-1) or interleukin-6 (IL-6). However, the gene product B15R does bind IL-1, though not IL-6. Surprisingly, this binding is specific for IL- β as IL- α and the IL-1 receptor antagonist protein (IL-1ra) do not bind to the B15R gene product.

The applicants propose that one or more of the gene sequences B15R and B18R may be inactivated, or part or all of one or more of these gene sequences may be deleted from the viral genome to allow (i) greater attenuation of the virus; and/or (ii) enhancement of immunogenicity of recombinant vaccinia virus; and/or (iii) further gene sequences insertion sites, so that more foreign DNA may be included in the virus. Inactivation of the gene sequences may be by mutation or the insertion of foreign DNA. Alternatively, one or more of the gene sequences B15R and B18R may be changed to alter the function of a protein product encoded by the nucleotide sequence. Mutation of the nucleotide sequence may be effected by the deletion, addition, substitution or inversion of one or more nucleotides.

DNA sequences encoding one or more heterologous polypeptides may be incorporated in the viral genome. The DNA sequences encoding the heterologous peptides may be inserted into one or more ligation sites created by the deletion or deletions from the viral genome. A heterologous peptide is one not normally coded for by wild type vaccinia virus. Typically the heterologous nucleotide sequence will encode an immunogen or a desirable polypeptide product. An immunogenic polypeptide will be substantially homologous to an epitope expressed by a pathogenic organism during infection, and which is seen by the infected individual as foreign.

The recombinant vaccinia viruses as described have

the potential for enhanced immunogenicity. This may result from the deletion of vaccinia genes B15R and/or B18R which cause immunosuppression.

The recombinant vaccinia vectors as described may be
5 used as immunogens for the production of monoclonal and polyclonal antibodies or T-cells with specificity for heterologous peptides encoded by DNA sequences ligated into the viral genome. The term antibody as used above should be construed as also covering antibody fragments
10 and derivatives of a parent antibody and which have the same specificity as the parent antibody.

The monoclonal antibodies, polyclonal antibodies, antisera and/or T-cells produced by the use of the recombinant virus vectors hereof can be used in the
15 diagnostic tests and procedures, for example, in detecting antigen in a clinical sample. They can also be used therapeutically or prophylactically for administration by way of passive immunisation.

Diagnostic test kits may comprise monoclonal
20 antibodies, polyclonal antibodies, antisera and/or T-cells obtained by the use of the recombinant vaccinia vectors described.

Vaccines and medicaments may comprise a recombinant vaccinia virus as described. These may have enhanced
25 safety and immunogenicity over current vaccinia virus strains for the reasons indicated.

A method of attenuating a vaccinia virus vector may comprise: a) deleting part or all of one or more of the following nucleotide sequences from the viral genome;
30 and/or b) inactivating one or more of said nucleotide sequences by mutating said nucleotide sequences or by inserting foreign DNA; and/or c) changing said one or more nucleotide sequences to alter the function of a protein product encoded by said nucleotide sequence;
35 which nucleotide sequences are sequences designated herein as: i) B15R, ii) B18R.

A vaccinia virus vector as described may be used to prepare a vaccine or a medicament.

The translation products encoded by the nucleotide sequences B15R and B18R may have pharmaceutical utility. In particular, they may have utility as anti-inflammatory medicaments.

5 These genes and/or translation products thereof may be used in processes relating to the preparation of an anti-inflammatory medicament. As stated earlier, the gene product B15R binds IL-1, though not IL-6. Surprisingly, this binding is specific for IL-1 β as IL-1 α and IL-1ra do not bind to the B15R gene product.

10 Therefore, the B15R translation product or functional equivalents thereof will have useful pharmaceutical applications. Although many cell types synthesise both IL-1 α and IL-1 β it has been shown that
15 most IL-1 α remains in the cytosol of the cells, while much of the IL-1 β is released into the extracellular space and circulation. In humans circulating IL-1 β can be seen in the plasma of patients with sepsis, acute organ rejection and rheumatoid arthritis, while IL-1 α is
20 rarely detected in such patients. This evidence would support the view that IL-1 β is the IL-1 form which is released by the cell and acts on other cell types in pathological situations (Dinarello, C.A., & Wolff S.M., 1993 New Eng. J. Med. vol.328 p106-113). IL-1 β is
25 implicated in chronic inflammatory conditions such as rheumatoid arthritis and it has the following functions in relation to inflammation: i) it acts as a T-cell activator; ii) it induces fibroblast proliferation; iii) it increases the production of mediators of the
30 inflammatory response e.g. prostaglandin-E2 from fibroblasts and iv) it increases the production of collagenase from chondrocytes (Digiovine F.S. et al, 1990, Immunol. Today Vol.11 p13-20). Therefore the gene product of B15R which can bind to IL-1 β provides a means
35 of blocking the activity of IL-1 β in order to control and/or reduce the symptoms of inflammation.

Furthermore, IL-1 β acts as a mediator in septic shock and it is a growth factor for certain malignant

cells e.g. leukaemic cells. Therefore the gene product of B15R provides a means for controlling septic shock and growth of certain tumour cells.

5 Data presented in this application demonstrate the key role of IL-1 β in controlling fever. Thus the IL-1 β receptor may be used to control disease mediated by excessive fever.

10 Therefore, the present invention provides use of the nucleotide sequence designated herein as B15R, or of a nucleotide sequence coding for an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide encoded by the B15R nucleotide sequence, in a process relating to the manufacture of a medicament for the treatment of a condition in which
15 IL-1 β is involved in the mediation of one or more symptoms associated with the condition. Also provided is use of a polypeptide which is encoded by the nucleotide sequence designated herein as B15R or of an allele of, or functionally equivalent fragment, derivative or variant
20 of the polypeptide, to manufacture a medicament for the treatment of a condition in which IL-1 β is involved in the mediation of one or more symptoms associated with the condition.

25 An allele of, or functionally equivalent fragment, derivative or variant of the B15R polypeptide may be any polypeptide with substantial homology to part or all of the B15R polypeptide and which also has the ability to bind IL-1 β .

30 Conditions may be fever, inflammation, diseases such as rheumatoid arthritis in which inflammation is presented as a symptom, septic shock, and certain leukaemias and/or cancers, inflammatory bowel disease, graft versus host disease and diabetes. The results disclosed hereafter also demonstrate that surprisingly
35 the cytokine IL-1 β is the primary mediator of fever. Previous results have shown that a number of different cytokines including IL-1 α , IL-1 β , IL-6 and TNF α can cause fever (Rothwell, N.J. 1990, European Cytokine Network,

Vol.1, p211-213; Kluger M.J. 1991, Physiological Reviews Vol.71 p93-127). However, in infection with vaccinia virus, inhibition of IL-1 β activity alone can clearly abolish the fever response. Therefore, the present invention particularly provides use of the nucleotide sequence designated herein as B15R, or a nucleotide sequence coding for an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide encoded by the B15R nucleotide sequence in a process relating to the manufacture of a medicament for the treatment of fever. Also provided is use of a polypeptide encoded by the nucleotide sequence designated herein as B15R or of an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide to manufacture such a medicament. Also provided is a pharmaceutical which is an anti-fever medicament. Also provided is a pharmaceutical which is an anti-fever medicament comprising a polypeptide which is encoded by the nucleotide sequence designated herein as B15R, or an allele of, or functionally equivalent fragment derivative or variant of the polypeptide.

Also provided is a diagnostic reagent for the detection or measurement of interleukin-1 β in a sample which reagent comprises a polypeptide encoded by the nucleotide sequence designated herein as B15R, or of an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide. Also provided is a diagnostic kit which comprises a diagnostic reagent as described above and one or more ancillary kit components for making the detection or measurement of interleukin-1 β .

Also provided is a method of diagnosing a patient's condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition, which method comprises testing a sample of biological material obtained from the patient using a diagnostic reagent or kit as described above.

Also provided is a method of treating a patient

suffering from a condition in which interleukin- 1β is involved in the mediation of one or more symptoms associated with the condition, which method comprises administering a medicament prepared as described above.

5 Also provided is a method of treating a patient for fever which comprises administering a medicament prepared as described above, or a pharmaceutical as described above.

10 The proteins may be produced in a recombinant system according to techniques well known in the art. Thus the nucleotide sequences provided herein could be inserted into a suitable expression vector (not necessarily vaccinia, for example the baculovirus system described herein). Such vectors can then be used to infect or
15 transform a cell line suitable for the production of these particular proteins.

 Reagents comprising polypeptides such as the B15R gene product, or alleles of or functionally equivalent fragments, derivatives or variants of that gene product
20 may be used as research tools as a means to study the function of IL- 1β versus IL- 1α .

 The applicants have provided herein sequence information for B15R and B18R and identified the location of their nucleotide sequences in the viral genome.
25 Having done this, it is within the capacity of one skilled in the art to either inactivate these sequences in or delete these sequences from the VV genome or change them to alter the function of the encoded protein product. All the necessary standard procedures are
30 described in Molecular Cloning, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbour Laboratory Press 1989.

 Furthermore, it would be within the normal capacity of one skilled in the art to use the sequence information provided herein to develop useful pharmaceuticals and
35 immunogens as herein provided; to use the immunogens to produce antibodies and the like; to use the antibodies in kits and pharmaceuticals.

Brief Description of the Drawings

In order that the present invention may be understood more clearly, the identified gene sequences will be described more fully with reference to the figures listed below.

Figure 1(A). HindIII restriction map of the 186kb VV genome. The 9.8kb SalI I fragment is expanded to show the position and direction of transcription of the genes B15R and B18R and the serpin genes (Smith, G.L., Howard, S.T. and Chan, Y.S. (1989). J. Gen. Virol. 70, 2333-2343) (this nomenclature indicates the genes are the fifteenth and eighteenth ORFs starting from the left end of the HindIII B and are transcribed rightwards towards the genomic terminus).

Figure 1(B). Nucleotide sequence and deduced amino acid sequence of gene B15R. Potential transcriptional control signals are underlined and a possible signal peptide at the N-terminus is boxed. Sites for the addition of N-linked carbohydrate (NXS/T) are boxed and the cys residues likely to form disulphide bonds within Ig-like domains are stippled.

Figure 1(C). Nucleotide sequence and deduced amino acid sequence of gene B18R. The three amino acid positions at which the sequence differs from the published sequence of this gene from another strain of VV (Ueda, Y., Morikawa, S. and Matsuura, Y. (1990)) are shown. Other features as marked in (B).

Figure 2. Amino acid alignment of the Ig domains from B15R and B18R with the Ig-like domains of the human and murine IL-1RI, the human IL-6R, the VV haemagglutinin (VV HA), domain 1 of the fasciclin II, domain 3 of myelin-associated glycoprotein and the V-domain of Ig kappa. The regions predicted to form the β -strand structures of Ig-like domains are indicated above the alignment. Residues identical in 6 or more sequences are boxed. A few residues between the β -strands B and C have been omitted. Also omitted for brevity are β -strands D and, where appropriate, C' and C". Higher numbers of

residues (about 30 or more) between strands C and E are indicative of the V domains.

Figure 3. Amino acid alignment of B15R with the external regions of the IL-1RI from human and mouse and the signal sequence and single Ig domain of human IL-6R. Gaps have been introduced to maximise the sequence alignments and are indicated by dashes. Where 4 sequences are aligned, the boxes indicate identical amino acids in three sequences, otherwise boxes indicate complete conservation in all aligned sequences. Potential sites for addition of N-linked carbohydrate are underlined. Arrows and numbers mark the cysteines predicted to form intradomain disulphide bonds of Ig-like domains.

Figure 4. 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 5. 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 v B15R and v B18R 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.

Figure 6. 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 v B15R and pulse-labeled with ³⁵STrans-label either from 2 to 4hr 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 arrow-head) are indicated in kilodaltons.

(B) Identification of B18R from vaccinia virus-infected cells. BS-C-1 cells were infected with vB18R or v B18R and pulse-labeled with ³⁵STrans-label from 6 to 8h 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.

5 (C) Expression of B15R and B18R in baculovirus-infected insects cells. Sf cells infected with AcNPV, AcB15R, or AcB18R were pulse-labeled with ^{35}S Trans-label for 2 hr after 24 hr of infection. Proteins present in cells and media were analyzed by SDS-PAGE and visualized
10 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.
15 The positions of β -galactosidase (β gal), coexpressed with B15R and B18R in the recombinant baculoviruses, and polyhedrin (P), expressed only in AcNPV, are shown.

Figure 7. Binding Assays to IL-1 α , IL-1 β and IL-6.

(A) Nitrocellulose binding assay. Triton X-100 cell
20 extracts (C) (5 μ l) or concentrated medium (M) (10 μ l) 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
25 radioiodinated IL-1 α (120 pM), IL-1 β (200 pM) or IL-6 (120 pM). The volume added to the assay corresponded to 5×10^6 cell equivalents were used. An autoradiograph is shown.

(B) Soluble receptor binding assay. Tissue culture
30 supernatants (MEDIUM) or Triton X-100 cell extracts (CELLS) from the sources indicated were incubated with 100 pM of ^{125}I -IL-1 α (open box), ^{125}I -IL-1 β (closed box), or ^{125}I -IL-6 (stippled box) in solution, and the binding was determined by the polyethylene glycol precipitation
35 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 $2 \times$

10⁶ cell equivalents were used. The bound radioactivity is shown.

Figure 8. Binding Assay in Solution to Recombinant Viruses. A volume of medium corresponding to 1 x 10⁴ 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 ¹²⁵I-IL-1 β in the soluble receptor binding assay. The radioactivity bound to soluble receptor present in supernatants is represented.

Figure 9. Binding Characteristics of Vaccinia IL-1 β Receptor.

(A) Competition for binding of ¹²⁵I-IL-1 β to soluble receptor. Medium from WR-infected cells, corresponding to 4 x 10⁴ cell equivalents, was incubated with 100 pM of ¹²⁵I-IL-1 β in the presence of the indicated concentrations of unlabeled IL-1 α (open circle), IL-1 β (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 ¹²⁵I-IL-1 β binding to medium from vaccinia virus- or baculovirus-infected cells. Medium from cultures infected with WR (1.8 μ l, 8 x 10³ cell equivalents), vB15R (0.56 μ l, 2.5 x 10³ cell equivalents), or AcB15R (0.15 μ l, 250 cell equivalents) was incubated with different concentrations (25-1000 pM) of radiolabeled IL-1 β for 2 hr at room temperature, and the radioactivity bound was determined by the polyethylene glycol precipitation method. Data were converted to the Scatchard co-ordinate system. Binding shown represents specific binding. The data were analyzed by the LIGAND program.

Figure 10. 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-1 α (A), 180 pM of ^{125}I -IL-1 β (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-1 α (A) and IL-1 β (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-1 α (open square), IL-1 β (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-1 α and EL4 6.1 C10 cells (A), 2040 cpm for ^{125}I -IL-1 β and EL4 6.1 C10 cells (B), and 4963 cpm for ^{125}I -IL-6 and U266 cells (C).

Figure 11. 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 v B15R (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 v B15R (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.

Figure 12. Effect of Expression of the Vaccinia IL-1 β Receptor on Mice Infected with Vaccinia Virus. A representative mouse 5 days after infection with 10⁵ pfu of WR (a) or v B15R (b) is shown. Note the ruffled fur in (b), which correlated with accelerated weight loss (see Figure 8B, panels a).

Figure 13. Binding of Murine and Human IL-1 β to Different Strains of Vaccinia Virus, Rabbitpox, and Cowpox. Tissue culture medium (1 x 10⁵ 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-1 β or mIL-1 β , expressed in femtomoles, is shown. One femtomole corresponded to 935 or 535 cpm for IL-1 β or mIL-1 β , respectively.

Figure 14. Kinetics of symptoms of illness and mortality in mice infected with recombinant vaccinia viruses. BALB/c mice were intranasally infected with WR, v B15R or v B18R as described in Table 2.

(A) The total number of deaths in animals infected with different recombinant viruses is represented as a function of days post-inoculation.

(B) Animals inoculated with the different recombinants were examined for illness and the number of animals with symptoms (including mortality) in each group were represented as a function of time. Symptoms scored were (a) possession of ruffled fur (b) hunching and (c) immobility.

Figure 15. Temperature of BALB/c mice following intranasal infection with 10⁵ pfu of either wild type vaccinia virus (strain WR: open circles), or a deletion mutant lacking the B15R ORF encoding a soluble receptor for interleukin-1 β (IL-1 β : closed circles), or a revertant virus in which the B15R gene has been re-introduced into the deletion mutant virus (triangles). These data represent the mean temperature of groups of 10 animals.

Figure 16. Temperatures of BALB/c mice following intranasal infection with 10^5 plaque forming units of vaccinia virus strain Copenhagen (A), Tashkent (B) or Tian-Tan (C). These data represent the mean temperature of groups of 10 animals. Strain Tian-Tan expresses a soluble receptor for IL- 1β while strains Copenhagen and Taskent do not. In panel D the temperature profiles of animals infected with Copenhagen or Tian-Tan are compared. Data are expressed showing the net increase or decrease in temperature following infection.

All the genetic manipulations described below were carried out according to standard procedures (Molecular cloning, eds. Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory Press, 1989) and the conditions used for enzymatic reactions were as recommended by the manufacturer (GIBCO-BRL Life Technologies).

Determination of the Nucleotide and Amino Acid Sequences

The nucleotide sequence of the SalI I restriction fragment of the vaccinia virus genome (strain WR) were determined by established methods (Sanger, F. et al. (1980), J. Mol. Biol., 143, 161-178) and Bankier, A. and Barrell, B.G. (1983) Techniques in Life Sciences B508., 1-34, Elsevier).

For example, the 9.8kb SalI I fragment of vaccinia virus (strain WR) was isolated from cosmid 6, which contains virus DNA derived from a rifampicin resistant mutant (Baldick, C.J. & Moss, B. (1987) Virology 156, 138-145), and was cloned into SalI cut pUC13 to form plasmid pSalI I. The SalI fragment was separated from plasmid sequences and self-ligated with T4 DNA ligase. Circular molecules were randomly sheared by sonication, end-repaired with T4 DNA polymerase and Klenow enzyme, and fragments of greater than 300 nucleotides cloned into SmaI cut M13mpl8. Single stranded DNA was prepared and sequenced using the dideoxynucleotide chain termination method (Sanger, F., Nicklen, S. & Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA. 74, 5463-5467), using [^{35}S]-dATP and buffer gradient polyacrylamide gels

(Biggin, M.D., Gibson, T.J. & Hong, G.F. (1983), Proc. Natl. Acad. Sci. USA, 80, 3693-3695). For further details see (Bankier, A.T., Western, K.M. & Barrell, B.G. (1987) in Wu R. (ed) Methods in Enzymology 155, 51-93. Academic Press, London). The 6.3 kb SalI L fragment was similarly treated.

Computer Analysis

Nucleotide sequence data were read from autoradiographs by sonic digitiser and assembled into contiguous sequences using programmes DBAUTO and DBUTIL (Staden, R. (1980) Nucleic Acids Res. 8, 3673-3694; Staden, R. (1982) Nucleic Acids Res. 10, 4731-4751) on a VAX 8350 computer. The consensus sequence was translated in 6 frames using programmes ORFFILE and DELIB (M. Boursnell, Institute of Animal Health, Houghton, UK). Open reading frames were compared against SWISSPROT protein database version 14 and against the applicant's own database of vaccinia amino acid sequences using programme FASTP (Lipman, D.J. & Pearson, W.R. (1985) Science 227, 1435-1441). Alignments of multiple protein sequences were performed using programme MULTALIGN (Barton, G.J. & Sternberg, M.J.E. (1987) J. Mol. Biol. 198, 327-337).

There follows a detailed description of the individual gene sequences B15R and B18R and their relationship to IL-1 receptor.

IL-1 Receptors

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, IL-1 α and IL-1 β produce similar biological effects that are mediated by interaction with specific receptors in different cells.

There are two classes of IL-1 receptors, and both bind IL-1 α and IL-1 β with similar affinities (Dower, S.K., and Urdal, D.L. (1987)). The 80 kDa type I IL-1

receptor is found on T cells and fibroblasts (Bird, T.A., and Saklatvala, J. (1986) Nature 324, 263-266), while the 60 kDa type II IL-1 receptor is present in B cells and macrophages (Matsushima, K. et al (1986) J. Immunol. 136, 4496-4508). Sequence of cDNA clones of both receptors revealed that they belong to the Ig superfamily and each have three Ig-like domains followed by a transmembrane anchor sequence and a cytoplasmic tail (Sims, J.E. et al (1988) and (1989); MacMahan et al (1991)). Binding studies have shown heterogeneity in the IL-1 receptor on B cells concerning the affinity of IL-1 α or IL-1 β (Benjamin, D., et al, (1990)). The existence of a secreted receptor on Raji cells, which binds only IL-1 β , has been reported (Symons, J.A., et al, (1991)).

There are different natural inhibitors that modulate the biological effect of IL-1 (Larrick, J.W. (1989); Shields, J., and Mazzei, G.J. (1991)). The best characterized natural inhibitor is designated IL-1 receptor antagonist (IL-1ra), which competes with both IL-1 α and IL-1 β for binding to the receptor but cannot trigger the cellular responses of IL-1 (Hannum et al (1990); Eisenberg S.P. et al, (1990); Carter, D.B. et al, (1990)). Many studies have demonstrated the ability of IL-1ra to block the biological effects of IL-1 in vitro and in vivo (Dinarello, C.A., and Thompson, R.C. (1991)).

Genes B15R and B18R

Genes B15R and B18R (Figure 1) from near the right hand inverted terminal repeat (ITR) are predicted to encode proteins of 36.5 kDa and 40.7 kDa, respectively, that have an N-terminal hydrophobic sequence, possible attachment sites for N-linked carbohydrate and hydrophobic residues near the C-terminus. These properties are consistent with the mature proteins being either virion, cell-surface or secretory glycoproteins.

The nucleotide sequence and deduced amino acid sequence around the gene designated B15R is shown in figure 1B. the nucleotide sequence shown is 11462-12664 nucleotides from the left end of the vaccinia virus

HindIII B fragment and the coding region for B15R is at nucleotide positions 11584-12561 (or at nucleotides 815 to 1792 from the left end of the SalI I fragment). Similarly, the nucleotide sequence and deduced amino acid sequence around the gene designated B18R is shown in figure 1c. The nucleotide sequence shown is 15448-16741 nucleotides from the left end of the vaccinia virus HindIII B fragment and the coding region for B18R is at nucleotide positions 15568-16621 (or at nucleotides 4799 to 5851 from the left end of the SalI I fragment).

The single letter code is used for the designation of amino acids.

B15R and B18R each possess three domains with characteristics of the immunoglobulin (Ig) superfamily (Williams, A.F. and Barclay, A.N. (1988). Ann. Rev. Immunol. 6, 381-405) namely a pair of cysteines forming an intradomain disulphide bridge, sequences predicted to form β -strand structures and an invariant tryptophan in β -strand C. In B15R these cysteines are present at positions 48 and 99, 143, and 194, and 242 and 309. In B18R the corresponding cysteines are at positions 73 and 129, 172 and 221, and 272 and 333. The distance between these cysteine pairs in B15R (51, 51 and 67 residues) suggest the first two domains are C regions while the third may be a V-domain. For B18R the distances (56, 49 and 61) suggest these are C-domains. These regions are aligned with selected Ig-like domains of IL-1R (IL-1 receptor and IL-6 receptor), VV haemagglutinin and Ig kappa (Hilschman, N. and Hoppe-Seyer's, Z. (1967) Physiol. Chem. 348, 1077-1080), fasciclin II (Harrelson, A.L. and Goodman, C.S. (1988). Science 242, 700-708), chicken neural cell adhesion molecule (NCAM) (Hemperley, J.J. Murray, B.A., Edelman, G.M. and Cunningham, B.A. (1986). Proc. Natl. Acad. Sci. USA 83, 3037-3041) and myelin-associated glycoprotein (Salzer, J.L., Holmes, W.P. and Colman, D.R. (1987). J. Cell Biol. 104, 957-965) (Figure 2). In this alignment the β -strands C', C'' and D have been omitted for brevity. The cysteines forming the

intradomain disulphide bridge and the tryptophan in β -strand C are completely conserved. The relationship between the vaccinia proteins and the Ig family was confirmed by statistical computational analysis using the programme ALIGN (Dayhoff, M.O., Barker, W.C. and Hunt, L.T. (1983). Meth. Enzymol. 91, 524-545) (Table 1). With B15R the highest individual scores are found against the human and murine IL-1RI domains. Domain 2 of B18R also scores well against the IL-1RI domains and overall there are highly significant scores against a wide range of Ig domains.

An alignment of B15R with the extracellular regions of IL-1Rs and IL-6R (Figure 3) and the above alignment of individual domains (Figure 2) indicates a closer relationship between IL-1Rs, IL-6R and the VV Ig domains than other Ig members. This is exemplified by the following observations.

- (1) B15R and the external region of IL-1Rs have a very similar length.
- (2) There are additional conserved cysteines in B15R, B18R and the IL-1Rs located near the beginning of β -strands A and G in domain 1 and at similar positions in domain 2 of the IL-1Rs and B18R. These cysteines lie within the 3-dimensional structure of an Ig C domain in positions probably allowing another intradomain disulphide bond.
- (3) In B15R, both IL-1Rs and IL-6R there is a proline following the invariant cysteine in β -strand B of domains 1 and 2, an unusual residue in this position. B18R domain 1 also contains proline at this position.
- (4) In β -strand F of domain 3 of B15R and both IL-1R sequences, the glycine typical of other Ig domains is absent. Moreover, the otherwise invariant tyrosine is replaced in both IL-1Rs and in B15R with phenylalanine.
- (5) A glycosylation site is conserved in domain 1, β -strand F of IL-1Rs and B15R despite divergence of amino acid sequence.
- (6) Domain 3 does not contain additional cysteines

and is longer than 1 and 2 in B15R, B18R and the IL-1Rs.

Protein sequence comparisons show that these VV proteins are related to each other (22.5% identity), to the human and murine IL-1R, the human IL-6R (Yamasaki, K., Taga, T., Hirat, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T. and Kishimoto, T. (1988). Proc. Jpn. Acad, 64, 209-211) and the immunoglobulin (Ig) superfamily (Williams, A.F. and Barclay, A.N. (1988). Ann. Rev. Immunol. 6, 381-405). Members of this family contain varying numbers of structurally similar domains (Ig domains) and perform diverse functions, although a unifying theme is surface interactions between cells or by binding cytokines. The VV haemagglutinin is another member of this superfamily (Jin, D., Li, Z., Jin, Q., Yuwen, H. and Hou, Y. (1989). J. Exp. Med. 170, 571-576). The B18R sequence from VV strain IHD was recently reported but the relationship to interleukin receptors and the Ig superfamily was not described (Ueda, Y., Morikawa, S. and Matsuura, Y. (1990). Virology 177, 588-594).

Cytokines IL-1 and IL-6 by binding to their respective natural receptors mediate immune responses against an invading pathogen and cause inflammation. VV may be combatting this part of the immune response by producing proteins which mimic the receptors for IL-1 and IL-6.

Recently the sequences of additional (type II) human and murine interleukin-1 receptors were reported (McMahan et al., (1991)). These are more closely related to the vaccinia virus B15R gene product than are the formerly described type I IL-1 receptors. Indeed vaccinia B15R is more closely related to human and murine type II IL-1 receptors than either of these are to the type I IL-1 receptors. Since both type I and II IL-1 receptors have been shown to bind IL-1, the applicants have investigated whether or not the products of either B15R and/or B18R will also bind this cytokine.

The applicants 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-1 β when expressed from vaccinia or from recombinant baculovirus.

5 The role of the IL-1 β 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 biological effects on infected mice were also investigated for vaccinia virus without the
10 B18R coding sequence. The presence of the binding activity in other orthopoxviruses is also presented.

EXPERIMENTAL PROCEDURES

Cells and Viruses

The cell line EL4 6.1 C10, a subclone of the mouse
15 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
20 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.
25 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
30 from it were grown in CV-1 or BS-C-1 cells. TK⁻143 and HeLa D98 cells were used for the selection of recombinants. The WR strain was obtained from B. Moss (National Institute of Health, Bethesda, Maryland) and cells were obtained from the American Type Culture
35 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).

- 5 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
10 (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.

15 Reagents

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

30 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

- 35 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 SalI I fragment of vaccinia DNA containing B15R was inserted into SalI- and BamHI-cut pUC118, and the resultant plasmid was called pAA1. To
5 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
10 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;
15 5'-CCCGGATCCACCATGGGTTACTACCTGTTATA-3') and an oligonucleotide that hybridizes to an internal sequence of B15R, corresponding to nucleotides 1008-1025 of the SalI I fragment (B15R-2; 5'-CCGCTCCTCGTTTTTCCC-3'). The fourth nucleotide of B15R in the PCR fragment was G
20 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
25 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.

30 Cloning of B18R into pUC4K

The XbaI-SalI right end fragment of the SalI I 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'
35 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, R.K. 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 EcoRpt gene present in pSJH7 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.

10 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 [^{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.

20 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 [^{32}P]ATP using polynucleotide kinase. After digestion with DraI, 25 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 obtained at 8 hr after infection from cells infected in the presence (early) or absence (late) of cycloheximide 30 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.

35 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
5 Southern blot hybridization of ³²P-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
10 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
15 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
20 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
25 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 v B15R and v B18R.

30 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
35 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 μ Ci/ml 35 S Trans-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 μ g/ml) or tunicamycin (1 μ g/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 salicylate (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 millilitre. The medium was centrifugated 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 millilitre. 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 millilitre. Samples were incubated on ice for 15 min and centrifuged at 12,000 x 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 millilitre. The supernatants were harvested from cells seeded at a cellular density of 5×10^5 cells per millilitre 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 millilitre.

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 μ l 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 μ l for 1 hr at 4°C. Subsequently, 2.5×10^6 EL4 6.1 C10 or U266 cells were added in 25 μ l 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 μ l 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 μ l in the same conditions. The binding of 125 I-IL- 1β to supernatants from v B15R 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).

In vivo Experiments

(1) Female BALB/c mice (5 to 6 weeks old) were anesthetized and infected intranasally with 20 μ l 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 v B15R 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).

(2) 4-6 weeks old BALB/c mice were intranasally infected with doses of WR, v B15R or v B18R as shown in Table 2. The table also shows the mortality of animals after 15 days.

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-specific messenger RNAs (mRNAs) produced during infection. Figure 4A 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 4B). This is consistent with the presence of vaccinia virus early transcriptional terminator signal TTTTNT 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

5 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, 10 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 5A) and by polymerase chain reaction (PCR) using oligonucleotides 15 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 20 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 v B15R and v B18R, was confirmed by Southern blot hybridization (Figure 5B). The isolation 25 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).

30 To identify the gene products, labelling experiments with ³⁵S-labelled 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 35 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 6A), confirming the transcriptional analysis. An intermediate glycosylated form of 47 kd was detected in infected cells. As observed by immunoprecipitation (Figure 6A) and in the whole extract (data not shown), the expression of the protein either was increased or was not detected in vB15R- or v B15R-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 6B). Two forms of the protein (52kd and 60-65kd) 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 recognise 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 6C), 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 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-1 α , IL-1 β , 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 7A). Binding of radioiodinated IL-1 α or IL-6 to vaccinia virus or baculovirus was not detected while these ligands bound to EL46.1C10 and U266 cells, which overexpress IL-1 and IL-6 receptors, respectively. IL-1 β 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 to EL4 6.1C10 cells at this dose of ^{125}I -IL-1 β probably reflects a 6-fold lower affinity for the β form compared with IL-1 α , 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 7B). The finding of a higher binding activity for IL-1 β 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-1 β binding with detergent-solubilised cell extracts (Figure 7B) 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-1 β receptor from vaccinia virus-infected cells was examined by soluble binding assay and showed that no IL-1 β 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-1 β 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-1 β receptor is expressed late during infection.

Figure 8 shows the binding of radioiodinated IL-1 β 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-1 β 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 (v B15R) demonstrates that B15R is the only gene product from vaccinia virus responsible for the IL-1 β binding activity. In agreement with this, overexpression (vB18R) or deletion (v B18R) 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-1 β Receptor

The specific binding of only IL-1 β to B15R demonstrated a novel specificity compared with other cloned IL-1 receptors. In view of this, the applicants wanted to exclude the possibility that radioiodination of IL-1 α or IL-6 has prevented their binding to the vaccinia receptor. This was determined by assaying the ability of unlabelled ILs, including IL-1RA, to compete with ^{125}I -IL-1 β for the binding to the vaccinia IL-1 receptor. As shown in Figure 9A, the interaction of radioiodinated IL-1 β with the soluble receptor present in vaccinia virus supernatants was competed in a dose-dependent manner by unlabelled IL-1 β but not by IL-1 α or IL-6. The natural competitor IL-1RA did not block the binding of labelled IL-1 β 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 unlabelled ILs
5 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 100pM of ^{125}I -IL- 1β to 8×10^3 cell
10 equivalents of medium from AcB15R-infected cells (6610 cpm) was 94.1%, 7.4%, 99.0% and 110.1% in the presence of 10nM of IL- 1α , 10nM of IL- 1β , 100nM of IL- 1α , and 10nM of IL-6, respectively.

Scatchard analysis of binding of ^{125}I -IL- 1β to
15 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 9B). High affinity binding sites for IL- 1β were detected in supernatants from WR-infected cultures, with a
20 dissociation constant (K_D) of 234 ± 49 pM. The estimated number of binding sites secreted after 24h per WR-infected cell was $1.1 \pm 0.1 \times 10^5$. This is
extraordinarily high considering that the number of receptors in IL-1 responsive primary cultures or cell
25 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 $3.8 \pm 0.4 \times 10^5$ binding sites per cell after 24 hr with a similar affinity (K_D 226 ± 35 pM). In spite of a different
30 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 ($6.0 \pm 0.2 \times 10^6$ sites per cell) reflects the high level of expression of the
35 baculovirus system.

Competition of IL Binding to Cells

The specificity for IL- 1β was also tested in competition experiments of binding of labelled cytokines

to their natural receptors on cell lines overexpressing IL-1 and IL-6 receptors. As shown in Figure 10, the binding of labelled IL-1 α 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 unlabelled ILs. In contrast, the interaction of IL-1 β with EL4 6.1 C10 cells was specifically blocked by supernatants containing B15R and not by B18R (Figure 10B). The percentage of competition correlated with the amount of ^{125}I -IL-1 β bound to different doses of receptor in solution (data not shown). Supernatants from WR-infected cells, and not from v B15R-infected cells, competed the binding of ^{125}I -IL-1 β to cells in culture, while they did not affect binding of IL-1 α to cells in culture, while they did not affect binding of ^{125}I -IL-1 β to cells in culture, while they did not affect the binding of IL-1 α or IL-6 (data not shown). Altogether, these results provide independent evidence that B15R binds specifically IL-1 β and also show that the vaccinia IL-1 receptor is capable of competing with cells for IL-1 β binding. This indicates that B15R might function as an inhibitor of the biological activities mediated by IL-1 β .

Pathogenicity of Vaccinia Virus Lacking B15R or B18R in Mice

(1) Deletion of B15R had no effect on the growth of vaccinia virus in tissue culture. However, since the expression of an IL-1 β binding activity might interfere with the inflammatory and/or immune responses in vivo, the pathogenicity of the deletion mutant v B15R 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 viraemia that leads to infection of the central nervous system and death of the animals (Turner, 1967; Williamson et al., 1990).

Figure 11A shows that, although there were not

significant differences in the final number of mortalities following infection with different doses of WR or v B15R, 70% of the mortalities on v B15R-infected animals occurred 1 day sooner than control (panels f).

5 This unexpected and enhanced pathogenicity of v B15R 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 11A, panels a-d). On day 5 after infection with v B15R, all 20 animals showed clear
10 disease symptoms (ruffled fur, arched backs, and reduced mobility), while none of the comparable WR-infected animals did so (Figure 11A, panels f). The early onset of symptoms in v B15R-infected animals was very clear at a dose of 10^5 pfu (Figure 11A, panels d), where they
15 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 11A, panels e), suggesting a possible effect of B15R of the progression of the infection.

20 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 11B and 12, an early
25 onset of symptoms was again observed in animals infected with v B15R, and this correlated with accelerated weight loss. The apparent attenuation of v B15R compared with WR at 10^4 pfu was not confirmed in this experiment; in contrast, it corroborated that v B15R induced earlier
30 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-1 β induced in response to the infection that were
35 neutralized by an active secretion of B15R by the wild-type virus. The accelerated onset of symptoms and mortality indicated that the secreted IL-1 β receptor can moderate the severity of the infection.

Table 2 and Figure 14 show the results for the in vivo experiment in which mice were intranasally infected with doses of WR, v B15R or v B18R. The results show that groups of mice infected with a vaccinia virus unable to produce the B18R gene product had a lower incidence of mortality than equivalent groups of mice infected with either wild-type vaccinia virus or a vaccinia virus unable to produce the B15R gene product. In Figure 14, mortality and symptoms of illness are presented as a function of days post-inoculation. The incidence of mortality was similar for groups of mice infected with either wild-type vaccinia virus or a vaccinia virus unable to produce the B15R gene product. However, symptoms of illness appeared sooner in groups of mice infected with vaccinia virus unable to produce the B15R gene product as compared to equivalent groups of mice infected with the wild-type vaccinia virus.

These data shown in Table 2 indicate that deletion of the B18R gene causes a lower mortality rate than deletion of the B15R gene. Therefore this discovery supports the provision of attenuated vaccinia virus by deletion of part or all of the B18R or by mutation of the B18R gene. Since removal of the B18R gene attenuates vaccinia virus and as discussed herein, the B18R gene product does not bind the cytokines discussed, the B18R gene product may be interfering with the hosts defense mechanisms in some other way.

IL-1 Binding Activity in Other Orthopoxviruses

The IL-1 α , IL-1 β , 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 compared with the WR strain. The binding to labelled murine IL-1 β (mIL-1 β) was also investigated to confirm that B15R is able to sequester IL-1 β in infected mice. No binding to human ¹²⁵I-IL-1 α or ¹²⁵I-IL-6 was detected (data not shown).

However, binding to labelled IL-1 β and mIL-1 β was found in all viruses except for rabbitpox, Tashkent and Copenhagen strains (Figure 13). 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 labelled IL-1 β 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-1 β varied from 1.6 for the Wyeth strain to 10 for the Tian-Tan strain (Figure 13). The specific binding of ¹²⁵I-mIL-1 β 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-1 β 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.

Temperature Regulation by B15R

The results shown in Figures 15 and 16 demonstrate the ability of the B15R protein to regulate the temperature of the infected animal. In Figure 15 the B15R protein is shown to suppress the temperature of the infected mouse over the first six days of infection. Animals infected with the wild-type (WT) virus (B15R +ve) have a reduced temperature compared with those infected with the deletion mutant (B15R -ve) and re-insertion of the B15R gene into the deletion mutant virus restored the temperature profile of the infected animals to that typical of WT virus.

In Figure 16 the applicants have compared the temperatures of mice infected with different strains of vaccinia virus, which had been characterised previously with respect to the expression or non-expression of the IL-1 β receptor (Alcami and Smith, Cell 71, 153-167, 1992). These data show that mice infected with the Tian-Tan strain, which expresses the IL-1 β receptor, have reduced temperatures following infection, while those animals infected with strains lacking the receptor (Copenhagen and Tashkent) develop fever.

These data support the original proposals that the IL-1 β receptor is functional in vivo and specifically show that it is IL-1 β not IL-1 α which is the predominant cytokine regulating the temperature of the infected animal.

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 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-1 α , IL-1 β 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 was attributed later to reactivity against B18R (Ueda et al, 1990).

5 In contrast with B18R, B15R ORF is shown to encode an IL-1 β 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-1 β binding (K_D 234 pM) is similar to those reported for the
10 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-1 β
15 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
20 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-1 β . This was shown in binding experiments to radioiodinated ILs and was corroborated in competition assays with
25 unlabeled cytokines and by blocking the interaction of the ILs with the natural receptor on cells in culture. A cellular receptor for IL-1 β that is present in the membrane and secreted from Raji cells, which has a molecular weight similar to the type II receptor, has
30 been described but not cloned (Benjamin et al, 1990; Giri et al, 1990; Symons and Duff, 1990; Symons et al, 1991). 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
35 comparable with those found between other vaccinia virus proteins and their cellular counterparts (Smith et al, 1991a), B15R may derive from the type II IL-1 receptor or a variant thereof.

Unfortunately, the comparison of the sequence of the vaccinia IL-1 β receptor with the type I and type II IL-1 receptors does not permit identification of the amino acids that confer specificity for IL-1 β 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-1 β 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-1 β receptor might be useful as a tool to investigate the function of IL-1 α and IL-1 β 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). As a therapeutic agent, the molecule may regulate responses normally controlled by IL-1 β , and, since it does not bind IL-1 α or IL-1RA, it might offer advantages over the other inhibitors. The failure of the vaccinia IL-1 β receptor to bind IL-1RA illustrates the adaptation of the virus to the physiological response of the host by preventing interference with the natural antagonist.

The number of IL-1 β binding sites secreted from vaccinia virus-infected cells (about 10⁵ 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-1 β 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-1 β binding to T-

cells, which also indicates that the vaccinia IL-1 β 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
5 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-1 β . To date,
10 both IL-1 α and IL-1 β 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-1 β indicates that soluble IL-1 β plays a more important role than
15 IL-1 α 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
20 mortalities. However, two observations revealed that the vaccinia IL-1 β receptor does play an important role in vaccinia virus infection in vivo. First, the animals infected with v B15R developed symptoms and lost weight more rapidly than the corresponding control group. The
25 early onset of symptoms is very likely to represent systemic effects induced by circulating IL-1 β 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
30 hypotension and a shocklike state (Dinarello, 1988, 1989). The vaccinia IL-1 β receptor, expressed in the wild-type virus, may thus limit the systemic acute phase response otherwise initiated by increased levels of IL-1 β . The finding that weight loss, which can be induced
35 by IL-1 (Di Giovine and Duff, 1990), occurred earlier in animals infected with v B15R 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 v B15R. 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-1 β receptor reducing the pathological effects mediated by excessive IL-1 β 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-1 β in the progression of vaccinia virus infection may be dual, since IL-1 β 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-1 β 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 study of Spriggs et al. (Cell 71, 145-152, 1992) in which 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-1 β 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-1 β converting enzyme, which

cleaves pro-IL-1 β to generate active IL-1 β . 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-1 β 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-1 β released by both infected and uninfected cells recruited to the site of infection. However, with vaccinia virus the systemic response induced by IL-1 β in infected mice seems mainly to be controlled by the soluble receptor, since the ORF corresponding to the crmA gene (B15R) is still present in the deletion mutant v B15R 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 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-1 β binding activity or, as in Tian-Tin, recognize human IL-1 β 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 attribute to IL-1 by neutralization of the IL-1 β activity by
5 vaccinia virus presented in this report suggests that the β form of IL-1, and not IL-1 α , 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-1 β
10 is the predominant form of IL-1 secreted from human monocytes (Hazuda et al, 1988), and the release of adrenocorticotrophic hormone, one of the neuroendocrine actions of IL-1, is exclusively induced by IL-1 β (Uehara et al, 1987). Similarly, IL-1 β is more potent than IL-1 α
15 in the induction of fever, and the effect is mediated through different mechanisms (Busbridge et al, 1989), which correlates with the discovery of IL-1 β (Breder et al, 1988) and receptors specific for IL-1 β (Katsuura et al, 1988) in the brain.

20 B15R is the second soluble cytokine receptor to be identified in a virus. A soluble récepteur 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
25 and Copenhagen strains of vaccinia virus contain one and two homologs, respectively, of 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
30 TNF or IL-1 in different genera of poxviruses, which produce very different pattens of disease, is interesting since these cytokines share many biological properties. The soluble IL-1 β receptor is one of the increasing number of activities encoded by vaccinia virus that aid
35 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 (Hse et al, 1990),
5 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-1 β receptor may be a useful tool to discriminate the physiological roles of IL-1 α and IL-1 β and might be used as an anti-inflammatory
10 therapeutic reagent. The expression of this activity by vaccinia virus and other orthopoxviruses represents a novel mechanism of virus evasion from the immune system. It is shown that the IL-1 β receptor is modulating the systemic response to infection and the severity of the
15 disease, which suggests that IL-1 β , and not IL-1 α , is the main mediator of the endocrine effects of the IL-1 produced in response to vaccinia virus infection in mice.

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Table 1

		B15R			B18R		
		1	2	3	1	2	3
5	B15R	1					
		2	6.71				
		3	1.63	2.44			
	B18R	1	4.81	1.88	0.58		
		2	4.13	2.75	2.25	2.70	
		3	1.94	2.64	3.51	3.67	-0.21
10	IL-1R Mouse	1	7.77	4.32	3.27	3.87	4.64
		2	4.70	7.62	2.20	0.39	6.42
		3	2.03	1.74	2.68	2.96	1.91
	IL-1R Man	1	7.62	2.37	3.21	2.53	4.60
		2	4.07	8.29	1.27	0.31	5.88
		3	1.07	1.29	4.64	1.73	1.65
15							
	IL-6R Man		5.87	3.98	5.28	3.24	6.30
	L1CAM	6	4.40	4.13	6.44	2.79	5.43
	CHNCAM	1	4.12	4.01	4.07	3.19	5.54
	MAG	3	4.04	4.67	6.17	1.22	3.10
20	PDGFR	3	2.84	2.81	5.14	3.88	4.86
	TCRCD3		5.65	4.95	1.75	2.38	4.64
	LAR	2	5.33	6.52	4.46	3.26	5.32
25	CEA	1	3.42	3.58	3.18	1.24	6.33
	RPIgR	5	3.92	4.47	4.35	1.67	1.80

Table 2

<u>DOSE (PFU)</u>	<u>WR</u>	<u>vΔB15R</u>	<u>vΔB18R</u>
3×10^7	5/5	4/5	3/5
10^7	4/5	5/5	0/5
10^6	5/5	5/5	1/5
10^5	1/5	2/5	0/5
10^4	1/5	0/5	0/4

NB 4/5 = 4 deaths in a group of 5

4-6 week old Balb/c mice were infected intranasally with the indicated doses of WR, B15R-deleted (v Δ B15R) or B18R-deleted (v Δ B18R) virus. The mortality of animals after 15 days is shown.

Table 1. Similarity scores for the Ig domains of vaccinia virus proteins B15R and B18R against selected domains from other Ig superfamily members computed using the ALIGN programme (Dayhoff, M.O., Barker, W.C. and
5 Hunt, L.T. (1983). Meth. Enzymol. 91, 524-545). The programme compares to the best alignment score for two domains with the mean score of 100 alignments of the randomly scrambled sequences. The score for the best
10 alignment of the real sequences is presented as the number of standard deviations from the mean score of the randomised sequences. Values of greater than 3.1 are significant (probability 10^{-3}), while values of 4.8, 6.0 and 7.9 indicate probabilities of 10^{-6} , 10^{-9} and 10^{-15} , respectively. The domains illustrated are from B15R
15 amino acids 28-119 (1), 121-214 (2), 222-end (3); B18R 53-149 (1), 152-241 (2), 252-end (3); murine IL-1R precursor (Sims, J.E., March, C.J., Widmer, M.B., MacDonald, H.R., McMahan, D.J., Grubin, C.E., Wignall, J.M., Jackson, J.L., Call, S.M. Friend, D., Alpert, A.R.
20 Gillis, S., Urdal, D.L. and Dower, S.K. (1988). Science 241, 585-589) 26-119 (1), 125-219 (2), 231-335 (3); human IL-1R (Sims, J.E., Acres, R.B., Grubin, C.E., McMahan, C.J., Wignall, J.M. (1989). Proc Natl. Acad. Sci USA 86, 8946-8950) 24-116 (1), 122-216 (2), 228-332 (3); human
25 IL-6R (Yamasaki, K., Taga, T., Hirat, Y., Yawata, H., Kawanishi, Y., Seed, B., Taniguchi, T., Hirano, T and

- Kishimoto, T. (1988). Proc. Jpn. Acad. 64, 209-211) 27-116; murine neural cell adhesion molecule L1 precursor (L1CAM) (Moos, M., Tacke, R., Scherer, H., Teplow, D., Freuth, K., Schachner, M. (1988). Nature 334, 701-703)
- 5 518-610 (6); chicken neural cell adhesion molecule (CHNCAM) (Hemperley, J.J., Murray, B.A., Edelman, G.M. and Cunningham, B.A. (1986). Proc. Natl. Acad. Sci. USA 83, 3037-3041) 2-97; myelin-associated glycoprotein (MAG) (Salzer, J.L., Holmes, W.P. and Colman, D.R. (1987). J.
- 10 Cell Biol. 104, 957-965) 225-309; platelet derived growth factor receptor (PDGFR) (Yarden, Y., Escobedo, J.A., Kuang, W.J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y., Ando, M.E., Harkins, R.N., Francke, U., Friend, V.A., Ullrich, A. and Williams L.T. (1986).
- 15 Nature 323, 226-232) 183-279; T cell receptor CD3 epsilon chain (TCRC3) (Clevers, H., Dunlap, S., Saito, H., Georgopoulos, K., Wileman, T. and Terhorst, C., (1988). Proc. Natl. Acad. Sci. USA 85, 8623-8627) 1-82; leukocyte antigen receptor protein (LAR) (Streuli, M., Krueger,
- 20 N.X., Hall, L.R., Schlossman, S.F. and Saito, H. (1988). J. Exp. Med. 168, 1553-1562) 125-216; carcinoembryonic antigen precursor (CEA) (Oikawa, S., Nakazato, H. and Kosaki, G. (1987). Biochem. Biophys. Res. Commun. 142, 511-518) 113-201; rabbit poly-Ig receptor (RPIgR)
- 25 (Mostov, K.E., Friedlander, M. and Blobel, G. (1984). Nature 308, 37-43) 458-558.

CLAIMS:

1. Use of the nucleotide sequence designated herein as B15R, or of a nucleotide sequence coding for an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide encoded by the B15R nucleotide sequence, in a process relating to the manufacture of a medicament for the treatment of a condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition.

2. Use of a polypeptide encoded by the nucleotide sequence designated herein as B15R, or of an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide, to manufacture a medicament for the treatment of a condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition.

3. Use according to claim 1 or claim 2 wherein the condition is selected from the group consisting of: inflammation, rheumatoid arthritis, septic shock, cancer, fever; inflammatory bowel disease; graft versus host disease and diabetes.

4. Use according to claim 3 wherein the condition is fever.

5. A pharmaceutical which is an anti-fever medicament comprising a polypeptide which is encoded by the nucleotide sequence designated herein as B15R, or an allele of, or functionally equivalent fragment, derivative or variant of said polypeptide.

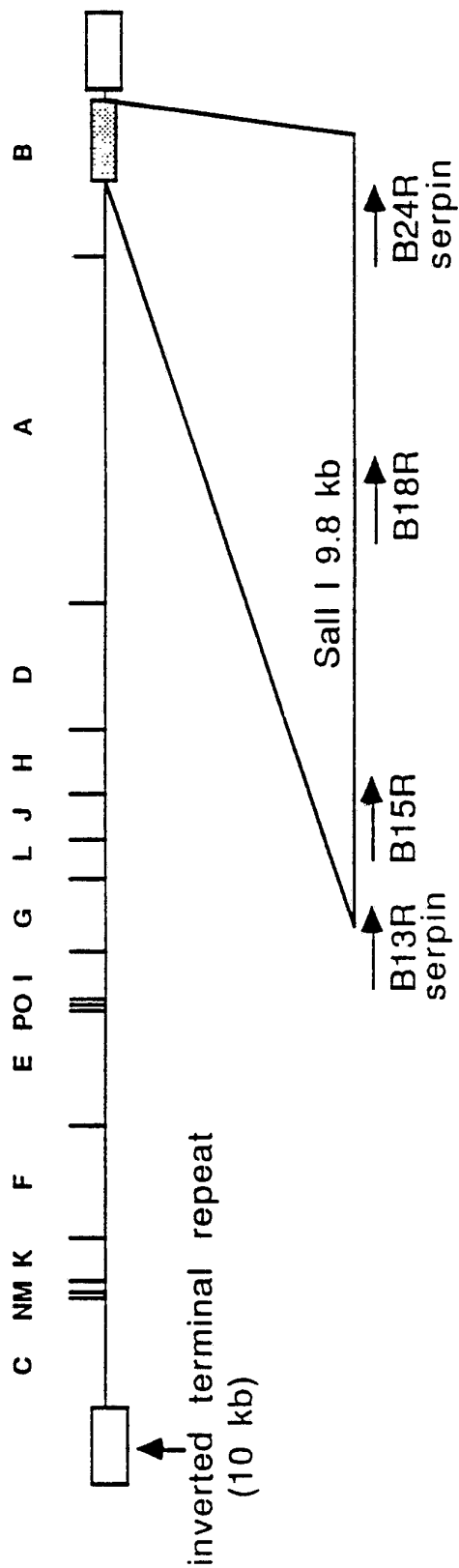
6. A diagnostic reagent for the detection or measurement of interleukin-1 β in a sample which reagent comprises a polypeptide encoded by the nucleotide

sequence designated herein as B15R, or of an allele of, or functionally equivalent fragment, derivative or variant of the polypeptide.

- 5 7. A diagnostic kit which comprises a diagnostic reagent according to claim 6 and one or more ancillary kit components for making the detection or measurement of interleukin-1 β .
- 10 8. A method of diagnosing a patient's condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition, which method comprises testing a sample of biological material obtained from the patient using a diagnostic reagent
15 according to claim 6 or a diagnostic kit according to claim 7.
- 20 9. A method of treating a patient suffering from a condition in which interleukin-1 β is involved in the mediation of one or more symptoms associated with the condition which method comprises administering a medicament prepared according to claim 1 or claim 2.
- 25 10. A method of treating a patient for fever which comprises administering a medicament prepared according to claim 3 or a pharmaceutical according to claim 5.

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Fig. 1A



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Fig. 1B

CGTGGATTATAACATCATATCCGGCGTATGAATTGATGAGTTGTACATCTTGACATTTTCTTT

M S I L P V I F L S I F F Y S S F V Q T F

ATGAGTATACTACCTGTTATATTCTTCTATATTTTATTTTATCTTCATTCGTTTCAGACTTTT

N E P V I L P C P Q I N T L S S G Y N I L

AACGAGCCAGTAATCTTACCATGTCTCAATAAATACGCTATCATCCGGATATAATATATTA

G S N M L I L N P T Q S D S G I Y I C I T

GGTAGCAATATGCTAATTCTGAACCCGACACAAATCAGACTCTGGTATTATATATGCATTACC

E S N I D L I S Y P Q I V N E R S T G E M

GAATCAAAATAGATCTTATCTCGTATCCACAAATAGTAAATGAGAGATCTACTGGCGAAATG

S G H R R L R N K R L K Q R T P G I I T I

AGCGGCATCGACGCCCTTAGAAATAAGAGACTTAAACAACGGACACCTGGAATTATTACCATA

G G K T Y N V T R I V K L E V R D K I I P

GGTGGCAAAACATATAACGTAACCCAGAAATTGTAAATTAGAGGTACGGGATAAAATAATACCT

A C R V S L R P P T T D A D V F W I S N G

GCATGCAGAGTATCGTTGAGACCTCCACACGGATGCAGACGTCTTTTGGATAAGTAATGGT

I Y M T D K R R V I T S R L N I N P V K E

ATCTATATGACCGATAAGAGACGTGTTATTACATCCCGGTTAAACATTAATCCTGTCAAGGAA

V T V S I T

GTTACTGTTAGTATAACGTGAATGTATGTTGTACATTTCCATGTCAATTGAGTTTATAAGAA

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Fig. 1B cont.

CTTTCTTCTCTCCCTTCCAGAAACAACTTTTACCCTATAAAATAAA 120
 N A P E C I D K G Q Y F A S F M E L E 40
 AACGCGCCTGAATGTATCGACAAAGGCAATATTTGCATCATTCATGGAGTTAGAA 240
 D I L W E K R G A D N D R I I P I D N 80
 GATATTTATGGGAAAACGAGGAGCGGATAATGATAGAAATTATACCGATAGATAAT 360
 T N E T Y C D M M S L N L T I V S V S 120
 ACGAACGAAACCTACTGTGACATGATGTCGTTAAATTTGACAAATCGTGTCTCTCA 480
 V C P N I N A F I A S N V N A D I I W 160
 GTATGTCCCAATATTAATGCATTTATGCTAGTAACGTAACGCAGATATTATATGG 600
 E D V R K N D A G Y Y T C V L E Y I Y 200
 GAAGATGTTAGAAAAAATGATGCTGGTTATTATACATGTGTTTAGAATATATATAC 720
 S T M Q L P D G I V T S I G S N L T I 240
 TCTACTATGCAATTACCAGATGGCATTTGTAACCTCAATAGGTAGTAATTTGACTATT 840
 M Y Y E E D D G D G N G R I S V A N K 280
 ATGTATTACGAAGAAGATGATGGGACGGAAACGGTAGAATAAGTGTAGCAAAATAAA 960
 E D A T T F T C M A F T I P S I S K T 320
 GAAGATGCTACAACGTTTACGTGTATGGCGTTTACTATTCTCCTAGCATCAGTAAACA 1080
 TTTTATACATTATCTTCCAACAAGCAATTGACGAACGTATTGCTATGATTAACCTCC 326
 TTTTATACATTATCTTCCAACAAGCAATTGACGAACGTATTGCTATGATTAACCTCC 1200

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Fig. 1C

GACAAATAACGATCTTTATAATATATCGTATCCACCTACCAAGTATAGTTGTATTTTCTCATGCGATGTGT
 M T M K M M V H I Y F V S L L L L L F H S Y A I
 ATGACGATGAAAATGATGGTACATATATTTTCGTATCATATTTGTATTTGCTATTCACAGTTACGCCATAG
 L P A K D S K W L N P A C M F G G T M N D I A A
 CTACCAGCTAAAGACTCTAAATGGTTGAATCCAGCATGTATGTTCCGAGGCACAATGAATGATATAGCCGCTC
 L S H R Y K D Y V V K W E R L E K N R R Q V S
 TTATCGCACAGATATAAAGACTATGTGGTTAAATGGAAAGGCTAGAAAAAATAGACGGCAGGTTTCTA
 K F S N R R Y L T V T T K N G D C V Q G I V R
 AAATTCAGTAACCGTAGGTATTTGTGCACCGTAACCTACAAAGAAATGGTGACTGTGTTCAAGGTATAGTTAGAT
 G T H D K Y G I D L Y G I L Y A K H Y N N I T
 GGTACTCATGATAAGTATGGCATAGACTTATCTGTGGAATTTCTTACGCCAAACATTATAATAATACTT
 Q T G K E L I I H N P E L E D S G R Y D Y V H
 CAAACGGGAAGGAATTAATTATTCATAATCCAGAGTTAGAAGATAGCGGAAGATACGACTGTACGTTTCATT
 I L T V I P S Q D H R F K L I L D P K I N V T I
 ATACTTACGGTTATACCGTCACAAGACCACAGGTTTAAACTAATACTAGATCCAAAAATCAACGTAACGATAG
 I D D V L I E W E N P S G W L I G F D F D V Y S
 ATTGACGATGTACTGATTGAATGGGAAAATCCATCCGGATGGCTTATAGGATTCGATTTTGATGTACTCTG
 N V T E E Y I G N T Y K R G H N Y Y F E K T L
 AATGTTACTGAAGAATATATAGGTAATACATATAAATGTGCGTGGACACAACCTATTATTGAAAAAACCCCTTA
 TTACTGAATAATTATTATTATTATATCGTATTTGTGCTATAACGCGACTATCTAGGTATTGTATCTCA

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Fig. 1C cont.

GTAAAAAACTGATATTATATAAAATTTAGTGCCGTATAATAAG	120
D I E N E I T E F F N K M R D T	40
ACATCGAAAAATGAAATCACAGAAATCTTCAATAAAATGAGAGATACT	240
L G E P F S A K <u>C</u> P P I E D S L	80
TAGGAGAGCCATTTCAGCGCAAAGTGTCCTCCTATTGAAGACAGTCTT	360
N K R V K H G D L W I A <u>N Y T</u> S	120
ATAAACGTGTTAAACATGGTGATTTATGGATAGCCCAACTATACATCT	480
S H I R K P P S C I P K T Y E L	160
CTCATATTAGAAAAACCTCCTTCATGCATTCCAAAAACATATGAACTA	600
W Y K D N K E I N I D D I K Y S	200
GGTATAAAGATAATAAGGAAATTAATATCGACGACATTAAAGTATCA	720
Y D D V R I K N D I V V S R C K	240
ACGACGACGTTAGAATCAAGAAATGATATCGTAGTATCAAGATGTAAA	840
G E P A <u>N I T</u> <u>C</u> T A V S T S L L	280
GAGAACCTGCCAAATATAACATGCACCTGCTGTGTCAACGTCATTATG	960
V L T S R G G I T E A T L Y F E	320
TTTTAACTAGTAGAGCGGTATTACCGAGCGACCTTGTTACTTTGAA	1080
T T T V V L E	351
CAACTACAGTAGTATTGGAGTAAATATACAATGCATTTTATATACA	1200
CCGATAGAGAACATATAAAT	1293

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Fig. 2.

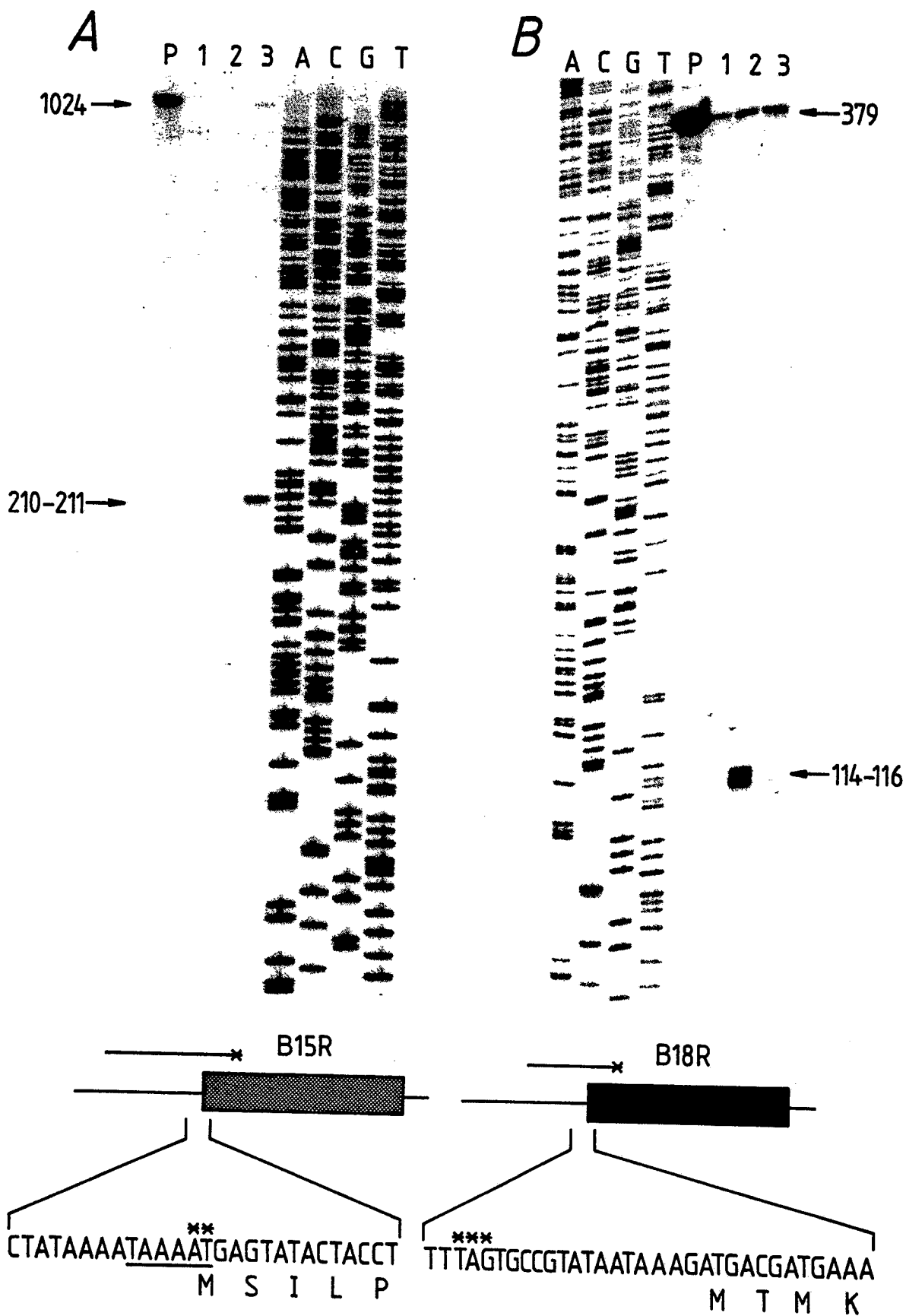
	A	B	C
B15R 1	Y I L G K L E A A N A R A P N V M S	P V A K Y T P V I K V I T S L L T	G R R G E N S D D C G
B15R 2	F S P G I D L I L Q M E F L R L A S K K I S A	L M I A L I R L L R L L L L L F L I	R H N E L D P D D C
B15R 3	A S P G I D L I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	K G S R L N P D D C
B18R 1	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	E S I E Y K N Y Y K W K W L E D S R Q
B18R 2	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	W W W W W W W W W W W W W W W W W
B18R 3	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	L I F F K T E T Q Y I Q Y H A R S T N
H-IL-1R 1	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	I I V V I I I L A I V V V S L L L L
H-IL-1R 2	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	D D D V V N L T I A I V V V S L L L L
H-IL-1R 3	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	L A A Y N V G P D P D A V S K P I
M-IL-1R 1	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(8) A A Y N V G P D P D A V S K P I
M-IL-1R 2	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(8) A A Y N V G P D P D A V S K P I
M-IL-1R 3	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(6) A A Y N V G P D P D A V S K P I
H-IL-6R	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(10) A A Y N V G P D P D A V S K P I
VV HA	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(4) A A Y N V G P D P D A V S K P I
FAS 1	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(7) A A Y N V G P D P D A V S K P I
CHNCAM 1	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(4) A A Y N V G P D P D A V S K P I
MAG 3	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(9) A A Y N V G P D P D A V S K P I
Ig Kappa	F A Y D M N E K I L Q M E F L R L A S K K I S A	C C C C C C C C C C C C C C C C C C C	(3) A A Y N V G P D P D A V S K P I

Fig. 3.

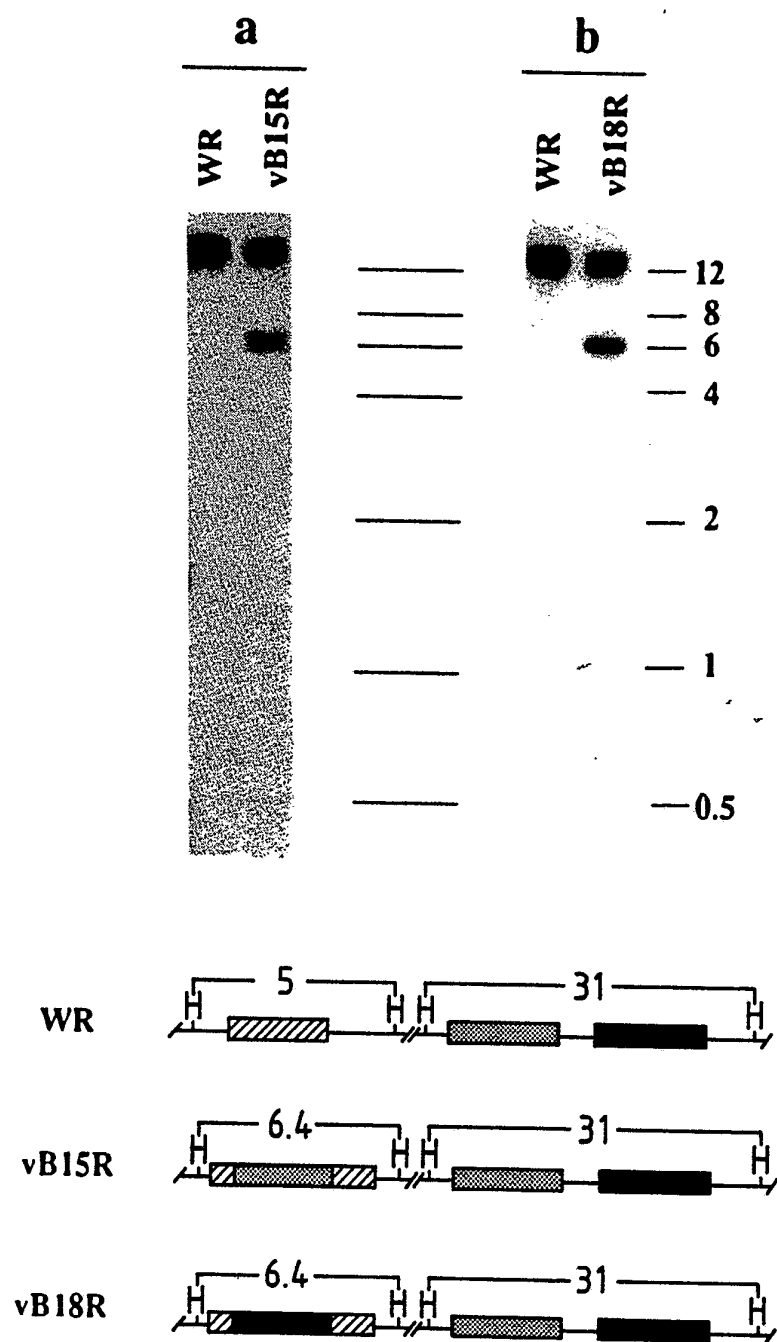
IL1R-H	MKVIIRLTCFIALLISSIEADKCKEREKIIILVSS-ANEIDVRPCPLNENE-----HKGTITWYRDDSKTFVSTEQASRTIHQHKE	1→
IL1R-M	MENMKVILCLICLMVPLLS-LEIDVCTEYPNQIVLFLS-VNEIDIRKCPLTENKM-----HGDTJIWYKNDSTKTEISADRDSRTIHQONE	
B15R	MSILPMIFLSIFFYSSFVQTFNAPECIDKGQYFASFEMELENPEVILPCH-QINLTSSGYNILDIILWEK-----RGADNDRIIPIDNGSN	
IL6R-H	MLAVGCALITAAALLAAPGAALAPRRCPAQEVARGVLTSLPGDSVTLTICE-GVEPE-----DNATVHWVL---RKPAAGSHFSRWAGMGR	
IL1R-H	KIWFVPAKVE DSGHYCYVVRNSSCYQLRIKISAKFVENEPNICYNAQAIFKCKLPVAGDCGIVCPYMEEFKNNENNELPKLCWYKDCCKPL	2→
IL1R-M	HIWFVPAKVEDSGHYCYIVRNSSTCYQLKTKVTVTVIENDPQLCYSTQATFPQRLHIAGDCSIVCPYVSYFKDENNELPEVQWYKNCCKPI	
B15R	MIILNPTQ-SDSGHYCYITTNETCYQDMMSI.NLTIIVSVSES--NIDLISYPCIVNERSTCEMVCPNINAEI-ASN VNADIIMSGHRR--	
IL6R-H	RIILRSVQLIDSGNYSCYRAGRPAAGTVHLLVDVPPPE-EPQL	
IL1R-H	IIDNLIHESGVKDRLI-VMNVAEKHRCNYITCHIASYTYIGKQYPI TRVITIEFITLEENKPTRPVIVSPANETMEVDIGSQIQLICNMTGQL	3→
IL1R-M	IIDNVSFFGVKDKLL-VRNVAAEEHRCDYICRMSYTFRGKQYPTVTRIQFITIDENKRDPRVILSPRNETIEADPGSMIQLICNMTGQF	
B15R	-IRNKRRLKQRTPGIITIEDVRKNDACGYTQVLEHYCYGKTYNVNTR-IJVKLEV-RIKIIPSTMQIEDG--IVTSIGSNLTIACTVSIIRP	
IL1R-H	-----SDIAYMKWNGSVIDEDDPVLGEDYYSVENP--ANKRRSTIIT-VLNISEIESRFYKHPETCFAKNTHIGIDAAYIQI.IYPVTN	↓
IL1R-M	-----SDLVYMKWNGSEIEWNDPFLAEDYQFVEHP--STKRKYTLIT-TLNISEVKSQFYRYPFICVVKNNTNIFESAHVQLIYPVPD	
B15R	PTTDAD-VEWISNGMYEEDDGDGNGRISVANKIYMTDKRR--VLTSLRNLINPVKEE-DATTEICMAFTIPSI-SKTVTVTSIT	

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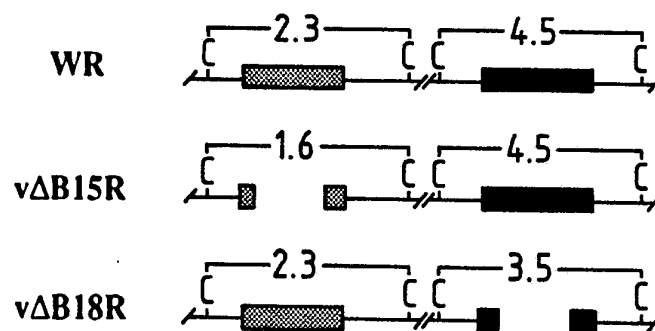
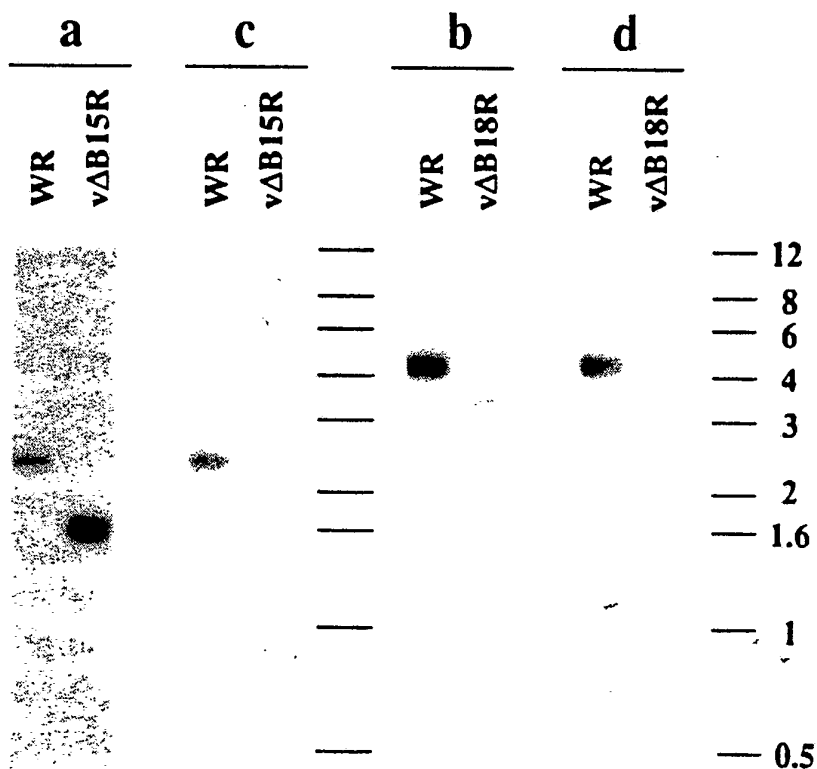
Fig.4.



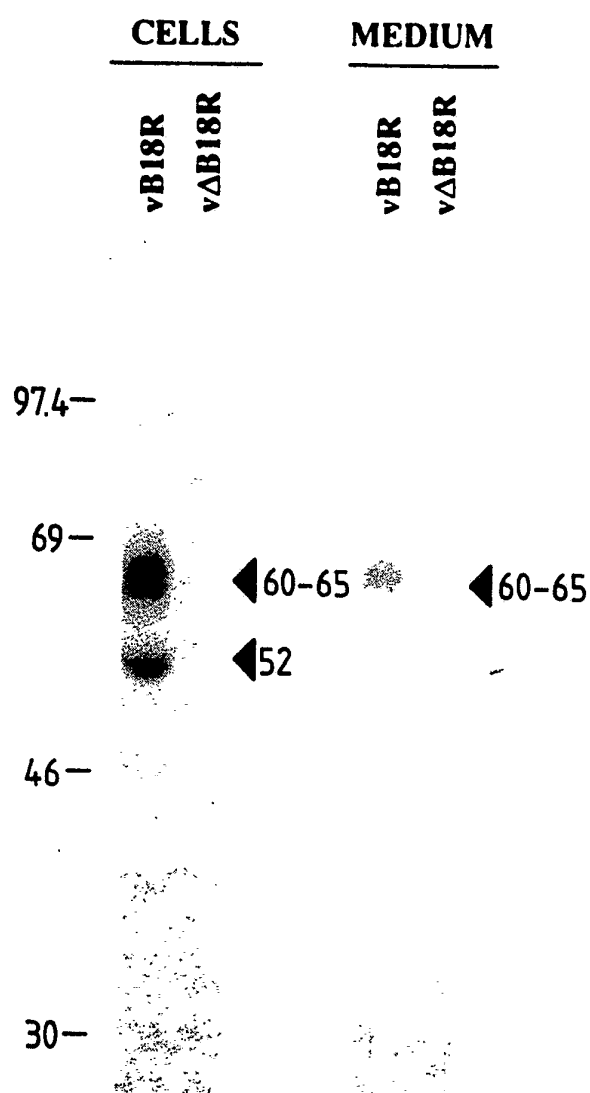
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Fig. 5A.

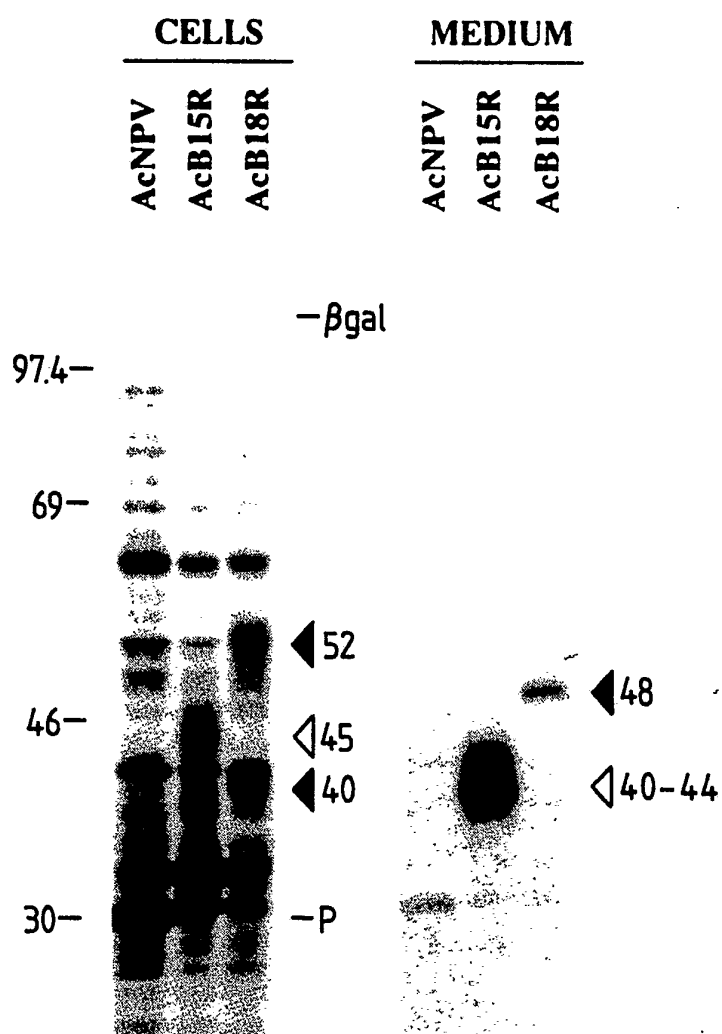
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Fig. 5B.

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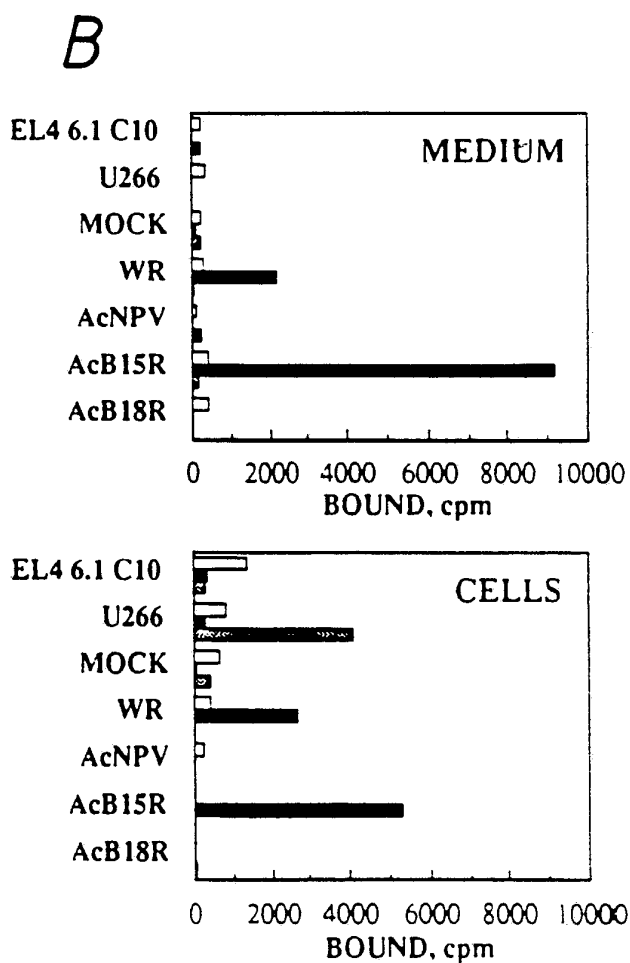
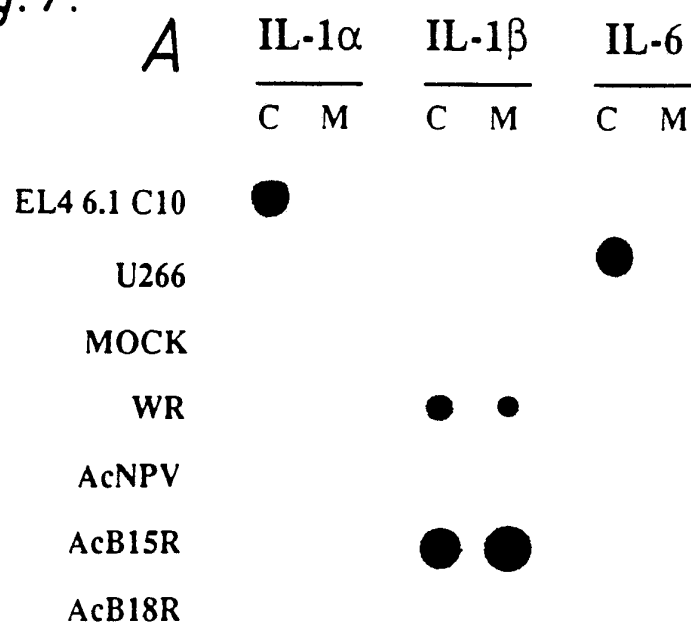
Fig. 6B.

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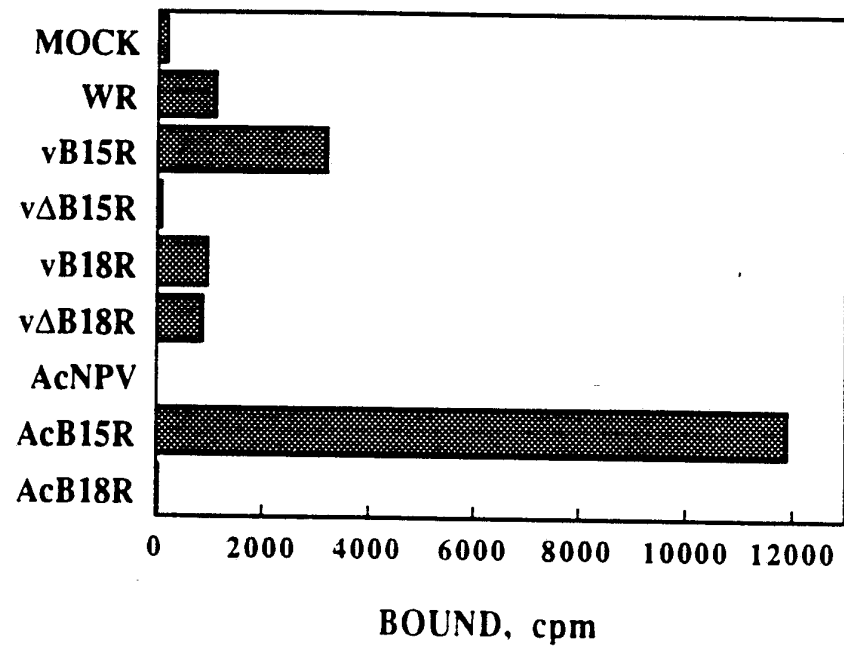
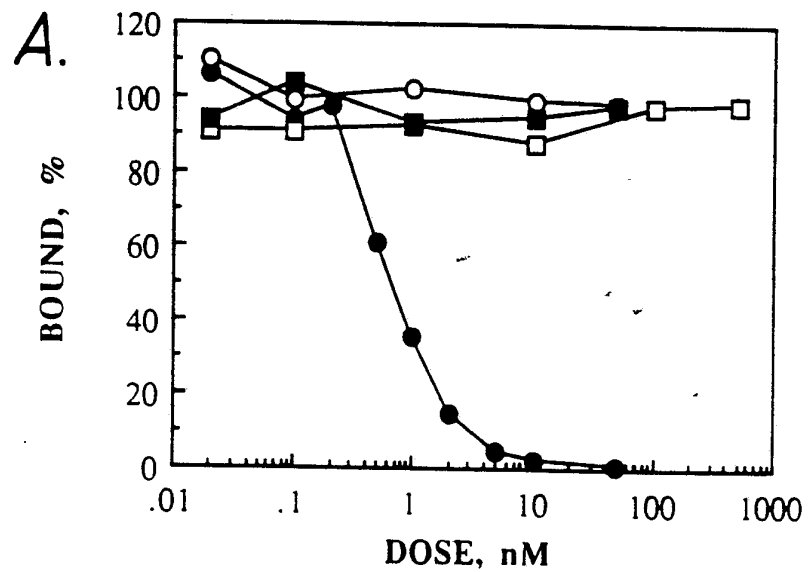
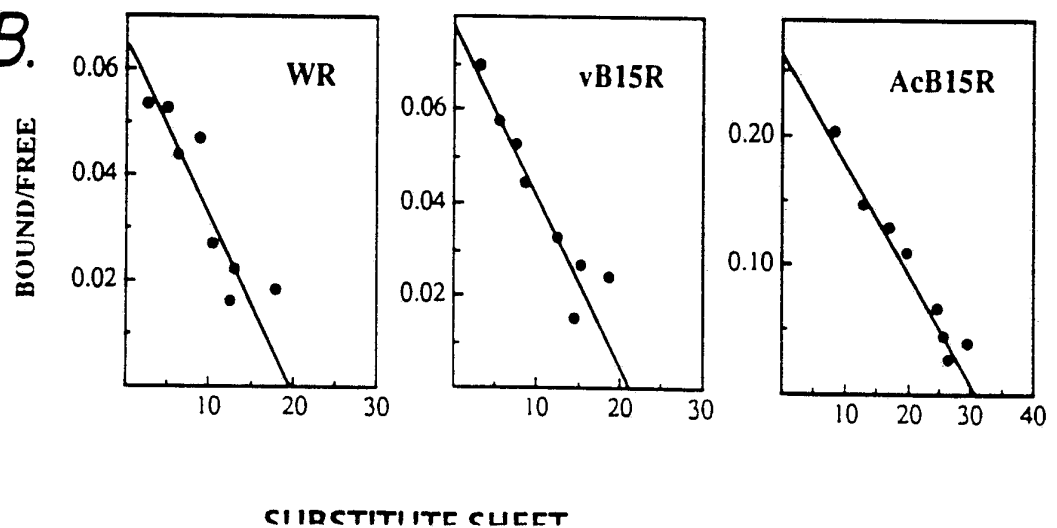
Fig. 6C.

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Fig. 7.



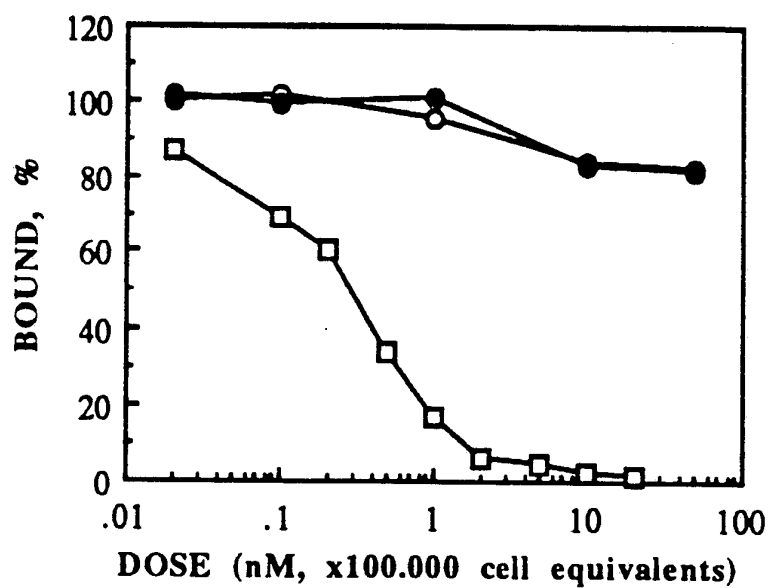
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Fig. 8.*Fig. 9.**B.*

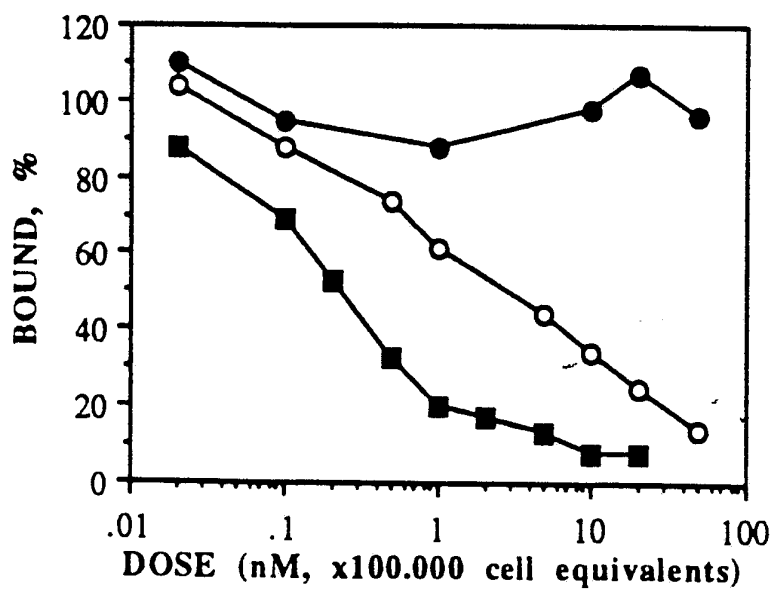
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Fig.10.

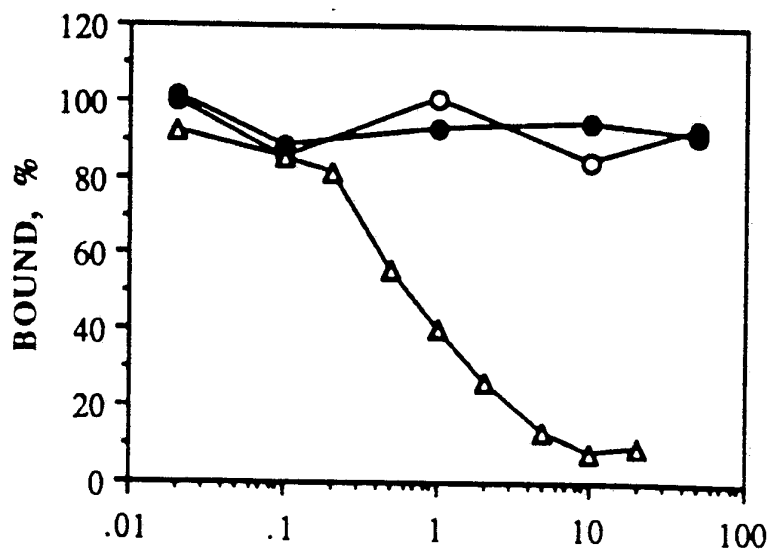
A



B

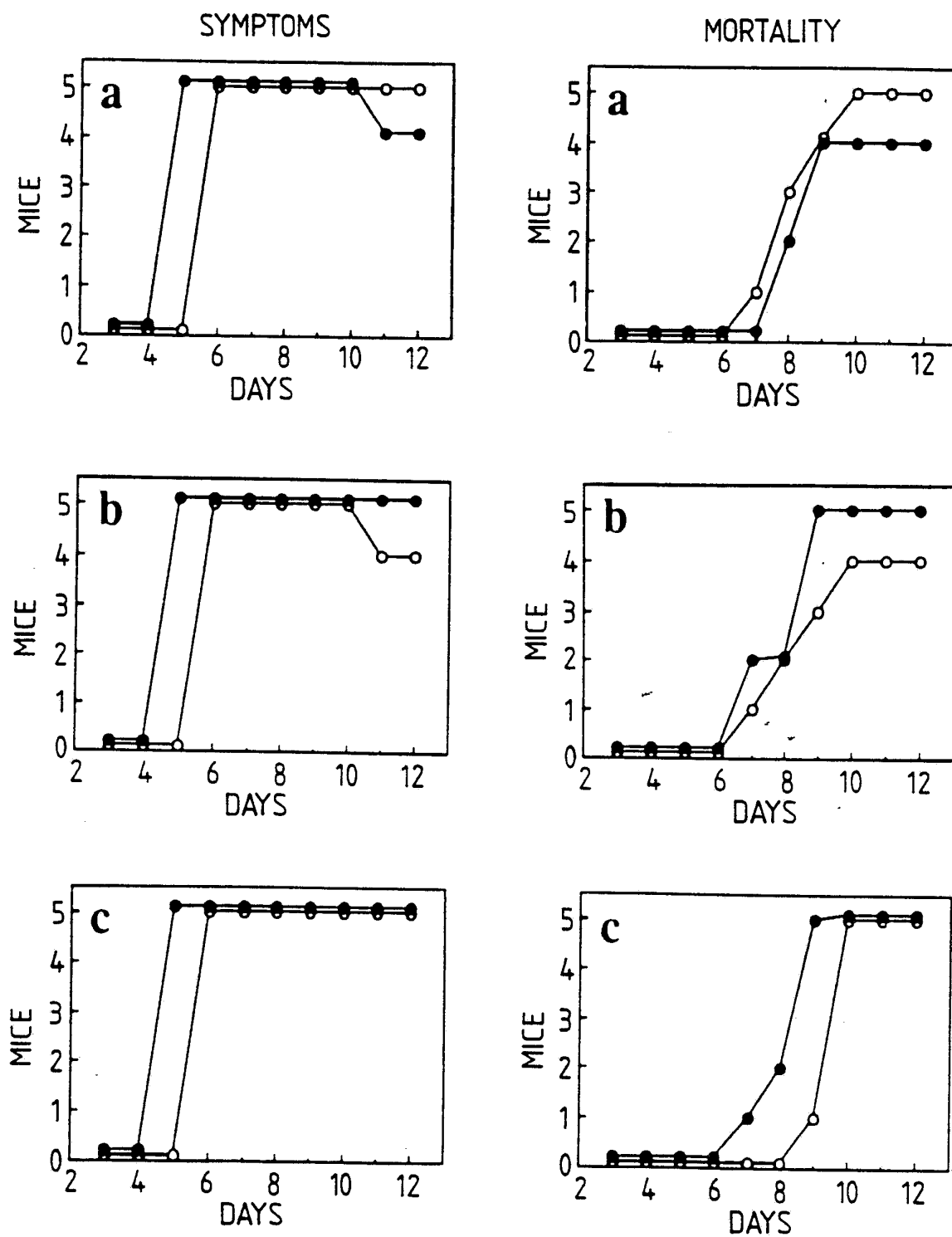


C

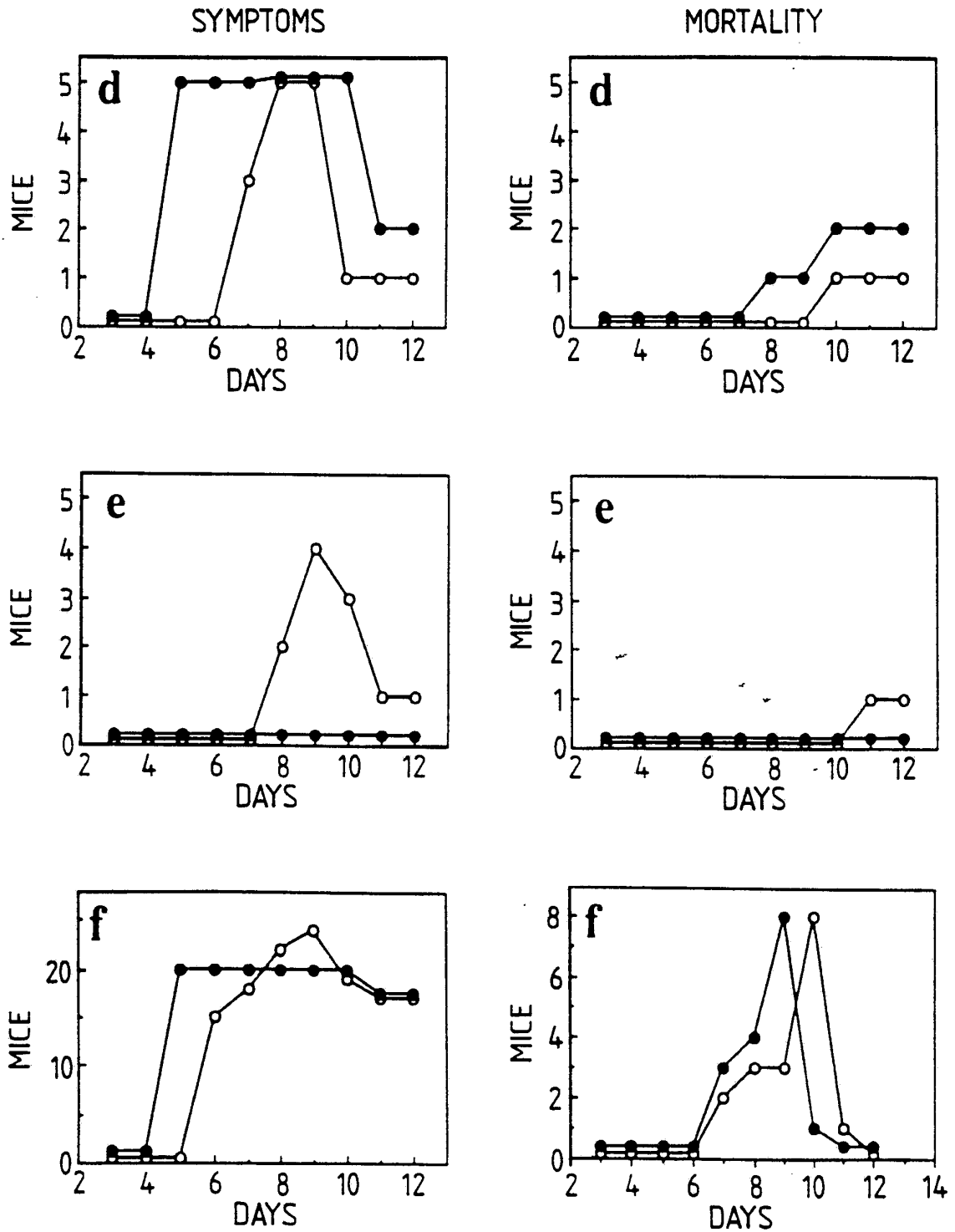


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Fig.11A.

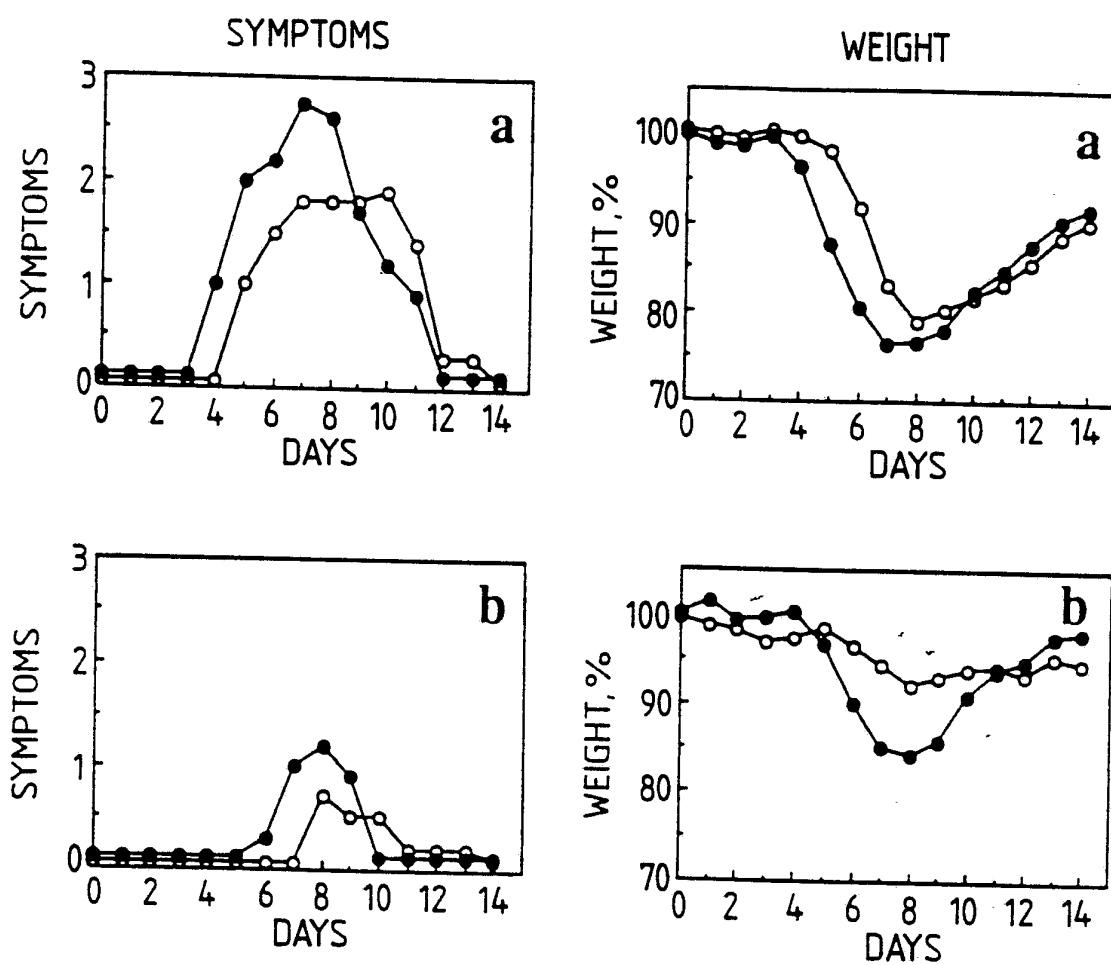


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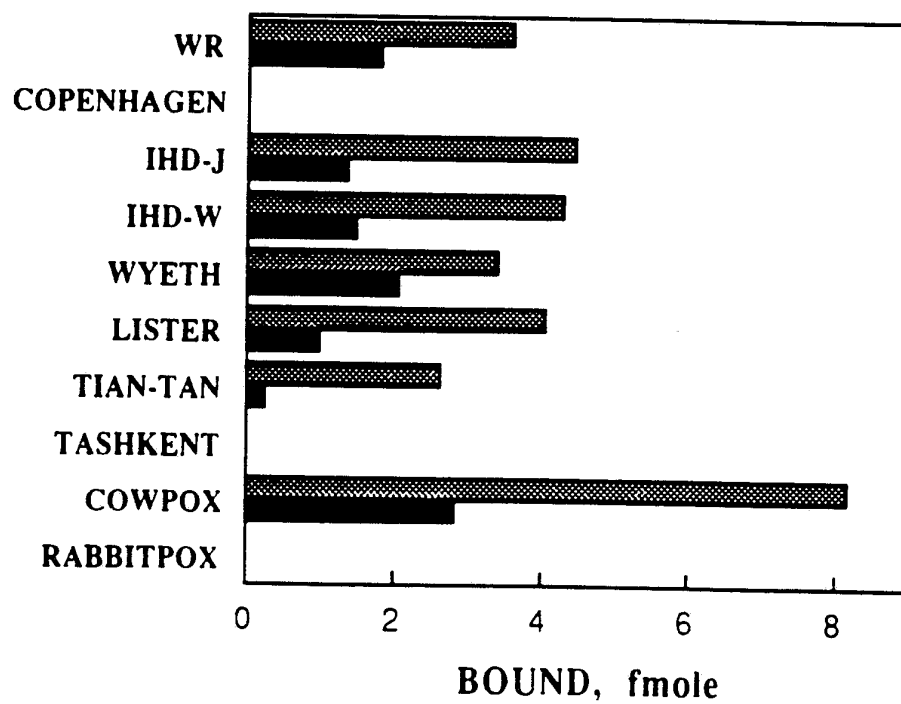
Fig.11A(Cont).

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Fig.11B.



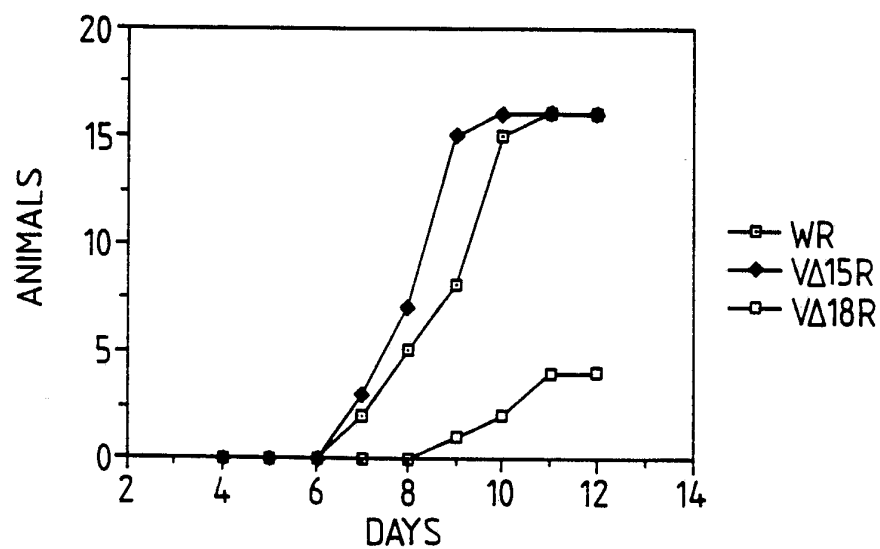
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Fig. 12.*Fig. 13.*

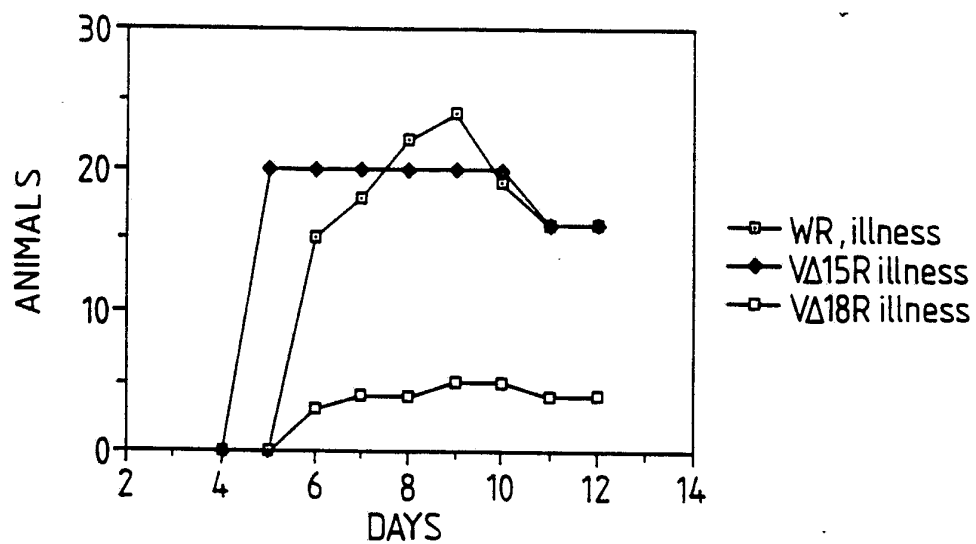
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Fig.14.

A. MORTALITY



B. SYMPTOMS



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Fig.15.

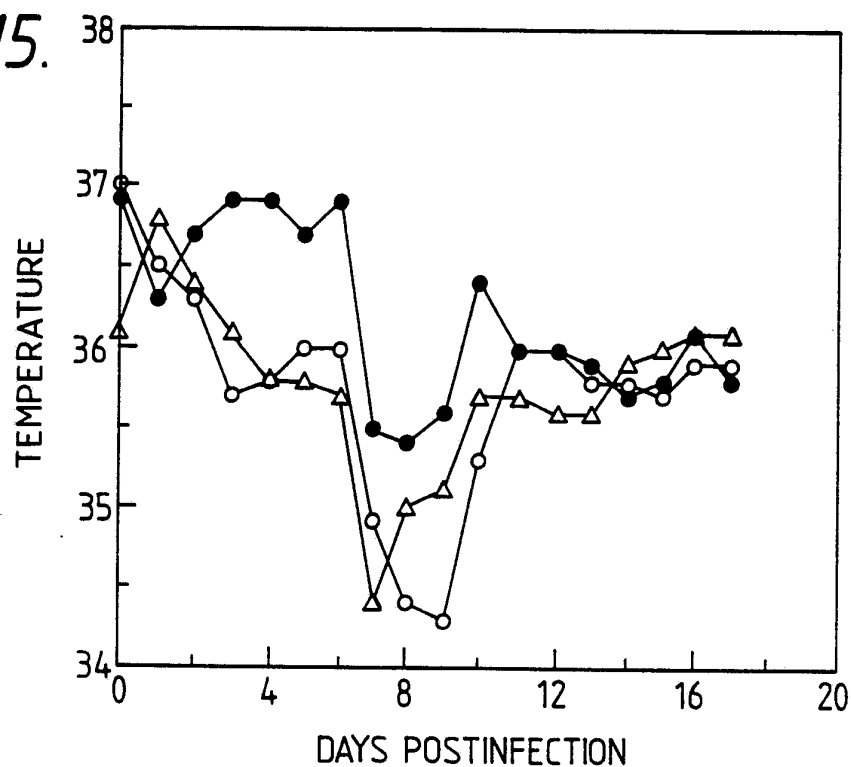
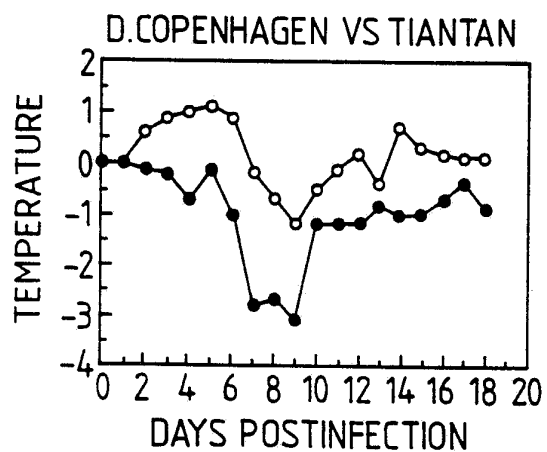
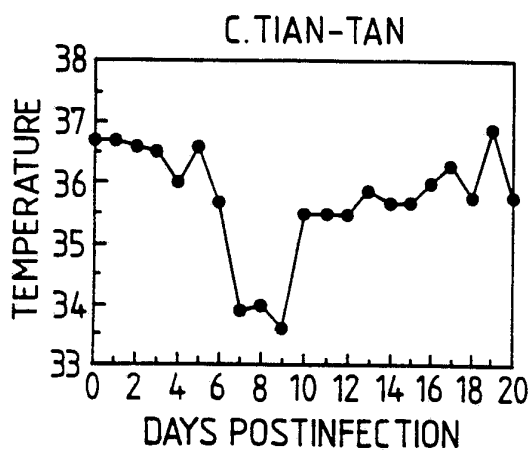
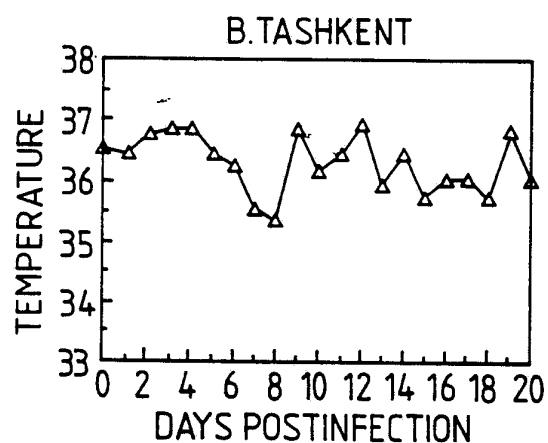
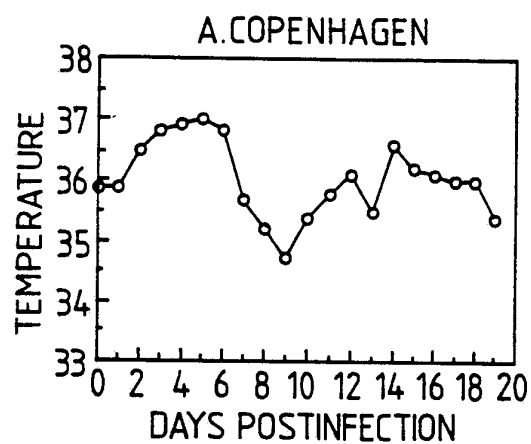


Fig.16.



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 93/00460

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/39; A61K39/285		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,Y	WO,A,9 207 944 (LYNXVALE LIMITED) 14 May 1992 see claims 17-20	1-10
A	--- JOURNAL OF GENERAL VIROLOGY vol. 72, no. 3, March 1991, GB pages 511 - 518 SMITH, G.L. ET CHAN, Y. S. 'Two vaccinia virus proteins structurally related to the interleukin-1 receptor and the immunoglobulin superfamily' cited in the application see the whole document --- -/-	1-10
¹⁰ Special categories of cited documents : ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
24 JUNE 1993	21. 07. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,Y	CELL vol. 71, no. 1, October 1992, CAMBRIDGE, NA US pages 153 - 167 ALCAMI, A. ET SMITH, G.L. 'A soluble receptor for interleukin-1-beta encoded by vaccinia virus a novel mechanism of virus modulation of the host response to infection' see the whole document -----	1-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB93/00460

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9 and 10 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

GB 9300460
SA 70981

24/06/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9207944	14-05-92	None	
