PATENT REQUEST: CONVENTION PATENT

We, BOEHRINGER INGELHEIM PHARMACEUTICALS, INC., being the person identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification. Full application details follow:-

**Applicant:** BOEHRINGER INGELHEIM PHARMACEUTICALS, INC.

**Address:** 900 Ridgebury Road, Ridgefield, Connecticut 06877-0368, United States of America

**Nominated Person:** BOEHRINGER INGELHEIM PHARMACEUTICALS, INC.

**Address:** 900 Ridgebury Road, Ridgefield, Connecticut 06877-0368, United States of America

**Invention Title:** DETECTION OF VIRUSES

**Name(s) of actual Inventor(s):** Kathleen Last-Barney, Vincent J. Merluzzi, Ronald Faanes and Steven D. Marlin

**Address for service in Australia:** CALLINAN LAWRIE, 278 High Street, Kew 3101, Victoria, Australia

**Attorney Code:** CL

---

**Convention Details**

<table>
<thead>
<tr>
<th>Application Number</th>
<th>Country</th>
<th>Country Code</th>
<th>Date of Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>689407</td>
<td>USA</td>
<td>US</td>
<td>22 April, 1991</td>
</tr>
</tbody>
</table>

Drawing number recommended to accompany the abstract - Fig. 1.

DATED this 22nd day of April 1992.

BOEHRINGER INGELHEIM PHARMACEUTICALS, INC.

By their Patent Attorneys:

CALLINAN LAWRIE
We, BOEHRINGER INGELHEIM PHARMACEUTICALS, INC., being the applicant and person nominated for grant of patent in respect of the Application for an invention entitled "DETECTION OF VIRUSES", state the following:-

STANDARD CONVENTION FILING

(a) The person nominated for the grant of the patent has entitlement from the actual inventors who are the applicants of the basic application by virtue of being a person who would, if a patent were to be granted upon an application made by the said inventors, be entitled to have the patent assigned to it; and

(b) The basic application listed on the request form is the first application made in a Convention country in respect of the invention.

DATED this 22nd day of April 1992.

Michael J. Houlihan
Regd. Patent Attorney

Date: 22 April 1992.
A method for the detection of a virus or a related group of viruses, in a fluid sample, using a sandwich assay comprising an immobilized primary cell surface receptor for the virus or related group of viruses, and a soluble labelled primary cell surface receptor for the virus or related group of viruses.

Claim

1. A method for the determination of the presence of a virus or a related group of viruses, in a fluid sample, which comprises the steps of:
   A (a) incubating the fluid sample with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a first insoluble complex of the virus or one or more of the related group of viruses, and the immobilized primary receptor; and
   (b) incubating the first insoluble complex with a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor;
or B (a) incubating the fluid sample with a labelled soluble primary receptor for the virus or the related group of viruses, to form a soluble complex of the virus or one or more of the related group of viruses, and labelled soluble receptor; and

    (b) incubating the soluble complex of (a) with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related viruses, and the immobilized primary receptor;

or C (a) incubating the fluid sample with (i) an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample; and (ii) a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor.
I certify that the following twenty-two (22) pages are a true and correct copy of the description and claims of the original complete specification in respect of an invention entitled

"DETECTION OF VIRUSES"

Name of Applicant: BOEHRINGER INGELHEIM PHARMACEUTICALS, INC.

Signature: [Signature]

(Note: An exact copy of the material attached to Form 10 must be attached to this form).
DETECTION OF VIRUSES

This invention relates to a method for the detection of a virus or a related group of viruses, in a fluid sample, using one or more primary cell surface receptors for the virus. More particularly, this invention relates to a sandwich assay for detecting the presence of a virus or a related group of viruses, in a fluid sample, using an immobilized primary cell surface receptor for the virus or the group of related viruses, and a soluble labelled primary cell surface receptor for the virus or the related viruses.

Viruses are important etiological agents of a wide variety of diseases. However, unlike antibiotic treatment for bacterial infections, no general rational therapy has yet been developed for viral infections. In general, vaccines and therapeutic treatments for one virus do not have broad applicability against other viruses or sometimes even against strains of the same virus. Accordingly, determining if a patient is infected with a particular virus or group of viruses, is desirable for designing prophylactic and therapeutic treatment.

For viral infection, a virus must initially attach itself to the surface of the target cell. Receptors on the cell surface facilitate this attachment. Two types of receptors have been reported in the art. One type of receptor, referred to as a primary receptor, mediates the attachment of the virus to the host cell. A primary receptor is present on the specific cell type which is prone to infection with a particular species of virus and, in general, binds only to that particular species of virus [see T. Lentz, J. of Gen. Virol. 71: 751 (1990) and T. Lentz, TIPS 9: 247 (July 1988)]. The second reported receptor for viruses, referred to as a
secondary receptor, is not specific to any particular species of virus. The secondary receptor is present on many cells and is involved in the penetration of the virus into the host cell after attachment to the primary receptor (see US-A-4859769).

Primary receptors for several viruses have been reported in the art. For example, Fisher et al., Nature 331: 76 (1988), report that a primary receptor for HIV is CD4; Weiss et al., Nature 333: 426 (1988), report that a primary receptor for influenza virus haemagglutinin is sialic acid; and Mendelsohn et al., Cell 56: 855 (1989), discuss the primary receptor for poliovirus.

Recently, it has been shown that soluble intercellular adhesion molecule-1 (sICAM-1), a soluble form of the primary receptor for the major subgroup of rhinoviruses, is capable of inhibiting binding and subsequent infection of target cells by major subgroup rhinoviruses [see Marlin et al., Nature 344: 70 (1990) (hereinafter referred to as "Marlin et al")]. Since this major subgroup of rhinoviruses is responsible for about 45-50% of common cold infections, the use of sICAM-1 or derivatives thereof may be beneficial for prophylaxis and treatment. It would therefore be beneficial to have a method of detecting major group rhinoviruses.

Assays for the detection of viruses and diagnosis of viral infections using antibodies and cDNA probes are known (see Al-Nakib et al., J. of Med. Virol. 20: 289 (1986) and Al-Nakib et al., J. of Med. Virol. 29: 268 (1989)). These assays, however, frequently have drawbacks either in specificity or in usage and are often not suitable for routine diagnosis.


Accordingly, an object of the present invention is
to provide a rapid and reproducible method for the
detection of the presence of viruses in fluids, such as
the detection of the presence of the major subgroup of
rhinoviruses, using primary receptors in a "sandwich"
configuration.

Viewed from one aspect the invention provides a
process for the determination of the presence of a virus
or a related group of viruses, in a fluid sample, which
comprises the steps of:

(a) incubating the fluid sample with an
immobilized primary receptor for the virus or the
related group of viruses, the immobilized primary
receptor being insoluble in the fluid sample, to form a
first insoluble complex of the virus or one or more of
the related group of viruses, and the immobilized
primary receptor;

(b) incubating the first insoluble complex with a
labelled soluble primary receptor for the virus or the
related group of viruses, to form a final insoluble
complex of the labelled soluble receptor, the virus or
one or more of the related group of viruses, and the
immobilized primary receptor;

(c) separating the final insoluble complex from
the fluid sample and any unreacted labelled soluble
primary receptor;

(d) measuring either the amount of label
associated with the immobilized primary receptor or the
amount of unreacted label; and

(e) determining the presence of the virus or
related group of viruses, in the fluid sample, by
relating the measurement of (d) to a measurement of a
reference sample free of the virus or the related group
of viruses.

Viewed from a further aspect the present invention
provides a process for the determination of the presence
of a virus or a related group of viruses, in a fluid
sample, which comprises the steps of:
(a) incubating the fluid sample with a labelled soluble primary receptor for the virus or the related group of viruses, to form a soluble complex of the virus or one or more of the related group of viruses, and the labelled soluble receptor;
(b) incubating the soluble complex of (a) with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor;
(c) separating the final insoluble complex from the fluid sample and any unreacted labelled soluble receptor;
(d) measuring either the amount of label associated with the immobilized primary receptor or the amount of unreacted label; and
(e) determining the presence of the virus or the related group of viruses, in the fluid sample by relating the measurement of (d) to a measurement of a reference sample free of the virus or the related group of viruses.

Viewed from a yet further aspect the present invention provides a process for the determination of the presence of a virus or a related group of viruses, in a fluid sample, which comprises the steps of:
(a) incubating the fluid sample with (i) an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample; and (ii) a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor;
(b) separating the final insoluble complex from
the fluid sample and any unreacted labelled soluble primary receptor;

(c) measuring either the amount of label associated with the immobilized primary receptor or the amount of unreacted label; and

(d) determining the presence of the virus or the related group of viruses, in the fluid sample by relating the measurement of (c) to a measurement of a reference sample free of the virus or the related group of viruses.

The term "primary receptor" as used herein, means the specific cell surface recognition structure which facilitates initial cell adherence of a virus or a related group of viruses.

The terms "major group rhinoviruses" and "major subgroup of rhinoviruses" as used herein, mean the human rhinoviruses which use intercellular adhesion molecule-1 (ICAM-1) as a primary receptor. For a general discussion of the ICAM-1 molecule, see Rothlein et al., J. Immunol. 137: 1270 (1986).

The terms "minor group rhinoviruses" and "minor subgroup of rhinoviruses" as used herein, mean the human rhinoviruses which use a primary receptor other than ICAM-1.

The term "related group of viruses" as used herein, means any group of viruses with at least one common primary receptor.

Particularly preferred embodiments of the individual steps of the processes of the invention will now be described in further detail.

The immobilized primary receptor is preferably immobilized on a solid support and is preferably in soluble form prior to its immobilization onto the solid support. The labelled soluble primary receptor is preferably in soluble form prior to being labelled. The soluble primary receptor can be prepared either by chemical or enzymatic cleavage of the receptor from the
cell surface, or by truncation via recombinant DNA methodology (see Marlin et al).

The solid support can be any of the known support materials useful in prior art assays, such as for example cellulose, sepharose, polystyrene, nylon, polyacrylamide, latex, glass, magnetizable particles or nitrocellulose. Most preferably, the solid support is polystyrene. A primary receptor can be immobilised onto the solid support by any procedure which produces an immobilized primary receptor capable of being bound by the target virus. For example, the primary receptor can be adsorbed in microtiter wells as described in Example 1 below and in Erlich et al, Methods in Enzymology 68: 443 (1979), or can be immobilized on a solid support using a bifunctional reagent as described in Kagedal et al, Clinica Chimica Acta 78: 103 (1977).

The amount of immobilized primary receptor utilized in the method of the invention should be sufficient to bind a detectable quantity of the target virus. This amount will vary depending upon the target virus, primary receptor, label used, etc., and should be determined empirically. In general, it is preferred that about 10 µg/ml to about 20 µg/ml of primary receptor immobilized on the solid support per 50 µl of fluid sample, be utilized.

A primary receptor can be labelled by known means (e.g., with enzymatic, fluorogenic, radiometric, bioluminescent, chemiluminescent, colorimetric, etc., labels and markers), provided that the label does not have a deleterious effect on the binding of the primary receptor to the target virus. For example, the procedure described in Example 1 below can be used in biotinylating a primary receptor for the purposes of this invention, or a procedure such as the one described in Woodhead et al, Clinical Chemistry 29(8): 1474 (1983), can be used in labelling a primary receptor for the purposes of this invention. The amount of labelled
soluble receptor utilized in the method of this invention should be sufficient to permit the detection of the virus bound by the labelled soluble receptor. This amount will vary depending primarily upon the type of label used and should be determined empirically. Preferably, the primary receptor is biotinylated using the procedure of Example 1 below. The bound biotinylated primary receptor is then detected by using a streptavidin enzyme conjugate, such as a streptavidin peroxidase conjugate.

The final insoluble complex of labelled soluble receptor, virus and immobilized receptor bound to a solid support, can be produced using three assay procedures, referred to as a forward assay, a reverse assay, and a simultaneous assay.

In the forward assay, the fluid sample is first incubated with an immobilized receptor for an appropriate period of time to form a first insoluble complex and then the first insoluble complex so formed is incubated with a labelled soluble receptor for an appropriate period of time to form the final insoluble complex. In the forward assay, the immobilised receptor and the labelled soluble receptor can be the same primary receptor or two different primary receptors for the same target virus or related group of viruses, which do not interfere with the binding of each other to the target virus or related group of viruses.

In the reverse assay, the fluid sample is first incubated with a labelled soluble receptor for an appropriate period of time to form a soluble complex of labelled soluble receptor and virus. The soluble complex so formed is then incubated with an immobilized receptor for an appropriate period of time to form the final insoluble complex. In the reverse assay, the immobilized receptor and the labelled soluble receptor should be two primary receptors for the same target virus or related group of viruses, which do not
interfere with the binding of each other to the target virus or related group of viruses.

In the simultaneous assay, the fluid sample is incubated with a labelled soluble receptor and an immobilized receptor at the same time to form the final insoluble complex. In the simultaneous assay, the immobilized receptor and the labelled soluble receptor should be two primary receptors for the same target virus or related group of viruses, which do not interfere with the binding of each other to the target virus or related group of viruses.

The incubation conditions for each of the steps of the forward, reverse and simultaneous assays may be varied, depending on time, temperature, and final incubation volume, but, preferably, each incubation step should be conducted for at least 15 minutes, preferably for 30 minutes or longer, at ambient temperature.

Following incubation, the final insoluble complex is separated from the incubation medium (i.e., fluid sample, unbound labelled soluble receptor, etc.). Since the final insoluble complex is insoluble in the incubation medium, it can be separated from the incubation medium by conventional means.

The uptake of labelled soluble receptor is directly related to the presence of the target virus bound to the complex. The amount of label associated with the final insoluble complex can be determined by at least two methods: (1) direct quantitation of the label associated with the final insoluble complex, or (2) indirect quantitation of the label remaining in the incubation medium after separation and then subtraction of this amount from the total label offered. The appropriate quantitation procedure will depend largely on the label used.

The amount of label determined to be associated with the final insoluble complex in the method of this invention is then compared to the amount of label
determined to be associated with the final insoluble complex of a reference sample obtained using the same label, procedure and reaction conditions that are used to obtain the signal from the fluid sample. The reference sample is free of the target virus or any member of the target group of related viruses, and any label detected in the reference sample is considered background signal. The detection of signal in the fluid sample over the background signal of the reference sample indicates the presence of the target virus or of a member of the target group of related viruses in the fluid sample.

In a preferred embodiment of the method of this invention, the target group of related viruses are major group rhinoviruses, the immobilized primary receptor is sICAM-1 immobilized on polystyrene, the labelled soluble primary receptor is biotinylated sICAM-1, and the assay utilized is the forward assay.

Preferred embodiments of the invention will now be described by way of the following example and with reference to the accompanying drawings in which:

Figure 1 shows the ability of biotinylated sICAM-1 to detect a major rhinovirus 51 using several dose concentrations of biotinylated sICAM-1 with overnight incubation of virus at 30,000 TCID₅₀ [●] and overnight incubation at 10,000 TCID₅₀ [●]. The results are expressed as the mean ± SD of three (3) replicate cultures.

Figure 2 shows the effect of virus incubation time on the ability of sICAM-1 (100 μg/ml) to detect Rhinovirus 54 in ELISA. A 6,000 TCID₅₀ titer of rhinovirus 54 was used for all incubation times shown. The results are expressed as the Mean ± SD of three (3) replicate cultures.

Figure 3 shows the effect of virus concentration on the detection of rhinovirus 54 [●] and rhinovirus 2 [●]. The concentration of biotinylated sICAM-1 was 100 μg/ml.
The results are expressed as the Mean ± SD of three (3) replicate cultures.

Figure 4 shows the use of an ABC peroxidase kit for the enhancement of detection of rhinovirus 34. The concentration of sICAM-1 used was 100 μg/ml. SA-HRP alone (Ο); ABC peroxidase kit utilizing biotinylated horseradish peroxidase (●). The results are expressed as the Mean ± SD of three (3) replicate cultures.

EXAMPLE 1
Detection of Major Group Rhinoviruses Using sICAM

A. Cell Lines

HeLa cells (human carcinoma of the cervix; CCL2 F 6333), were obtained from The American Type Culture Collection (ATCC), Rockville, MD. The cells were maintained in RPMI-1640 culture medium supplemented with 50 μg/ml gentamycin, 1 mM L-glutamine (GIBCO, Grand Island, NY) and 10% heat-inactivated (56°C, 30 minutes) fetal calf serum (FCS) specifically screened for mycoplasma and viruses (Whittaker, M.A. Bioproducts, Walkersville, MD). The cells were seeded into 75cm² tissue culture flasks (Corning) and were removed by incubating the monolayers when 90% confluent with 0.25% trypsin and 0.1mM EDTA (GIBCO) at 37°C for 5 minutes. After trypsinization, the cells were washed once by centrifugation (500 x g) and plated into 96 well flat-bottom microtiter plates (Linbro No. 76-003-05) at 20,500 cells per culture well. Twenty-four hours later the cells reached 80-90% confluence and were challenged with the pre-titered viruses described below.

B. Virus Stocks

The viruses used are listed in Table 1 below.
Table 1
Summary of Virus Stocks

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Source</th>
<th>HRV-Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coxsackie A13</td>
<td>Picornavirus</td>
<td>ATCC VR-1019</td>
<td>Major</td>
</tr>
<tr>
<td>Rhinovirus 1B</td>
<td>Picornavirus</td>
<td>ATCC VR-481</td>
<td>Minor</td>
</tr>
<tr>
<td>Rhinovirus 2</td>
<td>Picornavirus</td>
<td>ATCC VR-482</td>
<td>Minor</td>
</tr>
<tr>
<td>Rhinovirus 34</td>
<td>Picornavirus</td>
<td>ATCC VR-1144</td>
<td>Major</td>
</tr>
<tr>
<td>Rhinovirus 49</td>
<td>Picornavirus</td>
<td>ATCC VR-516</td>
<td>Minor</td>
</tr>
<tr>
<td>Rhinovirus 54</td>
<td>Picornavirus</td>
<td>ATCC VR-521</td>
<td>Major</td>
</tr>
<tr>
<td>Rhinovirus 89</td>
<td>Picornavirus</td>
<td>ATCC VR-1199</td>
<td>Major</td>
</tr>
</tbody>
</table>

*Major: ICAM-1
Minor: non-ICAM-1 receptor

The virus stocks were amplified and expanded in the HeLa cells prepared in A, by infecting HeLa cells with the virus in RPMI-1640 media supplemented with 5% FCS and then freezing the infected cells at -80°C. For rhinoviruses, the medium was further supplemented with 20mM MgCl₂.

All the virus stocks except Coxsackie A13, were concentrated using a modification of the method described in Abraham et al, J. of Virol. 54: 409 (1984). Briefly, HeLa cells were infected with a particular rhinovirus for 4-6 hours in RPMI-1640 media containing 1% fetal bovine serum followed by incubation in media containing 2% fetal bovine serum. The culture media so produced was then frozen at -80°C after a generalized cytopathic effect was observed (18-48 hours) and thawed at 33°C. The supernatant virus was then precipitated with polyethylene glycol and pelleted through a 30% sucrose cushion (34,900 rpm for 2 hours in a Beckman SW41 rotor). The bottom 0.5ml was then vortexed,
pooled, and stored in aliquots at -80°C.

C. Cytopathogenic Effect (CPE) Assay

For all viruses, HeLa cells were plated at 20,500 cells per culture well. Twenty-four (24) hours later the medium was removed and replaced with culture medium (RPMI-1640 supplemented with 5% FCS; rhinovirus medium was further supplemented with 20mM MgCl₂) containing dilutions of the expanded HeLa-virus stocks prepared in B. The plates were incubated at 37°C (Coxsackie) or 33°C (rhinoviruses) in 5% CO₂-humidified air and were stationery for 96 hours. CPE becomes apparent at 24-72 hours by inspection using an inverted microscope. CPE was scored visually at these points in a qualitative manner. The quantitative read-out was analyzed at 96 hours after direct virus challenge in the following manner: the medium was aspirated off and 45 µl of 0.5% crystal violet in 20% methanol was added to each culture well for 5 minutes. The plates were then vigorously washed with water, air dried, and read on microplate reader (Dynatech MR600) at 570 nM. A standard curve plot of cell number vs. optical density (O.D.) was used to determine the number of viable cells left in each culture well. Percent reduction of CPE was determined as follows:

\[
\text{Percent Reduction} = \left( \frac{\text{O.D. (Medium alone)} - \text{O.D. (Virus alone)}}{\text{O.D. (Medium alone)} - \text{O.D. (Virus alone)}} \right) \times 100
\]

The titers of virus stocks were analyzed at 96 hours by determining the dose of virus that produces a 50% CPE as determined by linear regression. Unless otherwise specified, 100 times the tissue culture infectious dose that causes a 50% CPE (100 TCID₅₀) was
CPE Assay Using Biotinylated sICAM-1

sICAM-1 was prepared as described in Marlin et al.

sICAM-1 was prepared as described in Marlin et al. 4200µg of sICAM-1 was dissolved in 2.8ml of 100mM sodium acetate buffer, pH 5.5, and dialysed at 4°C overnight. The dialysed sICAM-1 solution was recovered and measured, and then incubated with a 5mM final sodium periodate solution for 30 minutes at 0°C. The resultant sICAM-1/periodate solution was dialysed overnight at 4°C. The dialysed sICAM-1 solution was recovered and measured, and then incubated with 1mM final biotin-LC-hydrazine (Pierce #21340) for 5 hours at room temperature. The biotinylated sICAM-1 so produced was dialysed overnight vs. PBS at 4°C. The dialysed biotinylated sICAM-1 was recovered, measured, aliquoted and then stored at -80°C.

Biotinylated sICAM-1 was tested in a viral CPE assay using the procedure described in C above, using various minor and major rhinoviruses at 100 TCID₅₀, with and without 25µg/ml of sICAM-1.

The results of this testing are listed in Table 2 below.
Table 2
Effect of Biotinylated sICAM-1 on Inhibition of Picornavirus Cytopathogenicity

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Receptor Group</th>
<th>a Inhibition CPEa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinovirus 54</td>
<td>Major</td>
<td>92</td>
</tr>
<tr>
<td>Rhinovirus 34</td>
<td>Major</td>
<td>100</td>
</tr>
<tr>
<td>Rhinovirus 89</td>
<td>Major</td>
<td>56</td>
</tr>
<tr>
<td>Coxsackie A13</td>
<td>Major</td>
<td>100</td>
</tr>
<tr>
<td>Rhinovirus 2</td>
<td>Minor</td>
<td>0</td>
</tr>
<tr>
<td>Rhinovirus 1B</td>
<td>Minor</td>
<td>0</td>
</tr>
<tr>
<td>Rhinovirus 49</td>
<td>Minor</td>
<td>0</td>
</tr>
</tbody>
</table>

*aCPE determined on Hela cell monolayers with 100 TCID50 of all virus strains: biotinylated sICAM-1 at 50μg/ml.

These results demonstrate that the biotinylated sICAM-1 was active in inhibiting major group rhinoviruses and was not active against minor group viruses.

E. Rhinovirus Detection Using a Forward Assay
sICAM-1 was immobilized in the following manner: 50μl of sICAM-1 solution (20μg/ml dissolved in PBS) was pipetted into the bottom of each of NUNC maxisorp 96-wells and incubated at 37°C for 60 minutes. Each well was then washed with 200μl of phosphate buffered saline (PBS), pH7.5.

i) A forward assay was conducted in the following manner: 200μl of 2% bovine serum albumin/phosphate buffered saline (BSA-PBS) was added to each of the NUNC maxisorp 96-wells containing the immobilized sICAM-1 and incubated at 37°C for 60 minutes. This constitutes the
blocking step. Virus, as prepared in B above, was
diluted in appropriate diluents, added to the wells, and
incubated at 4°C or at ambient temperature for 2-5
hours. The wells were then washed 5 times with 200μl
PBS. 50μl of the biotinylated sICAM-1 prepared above,
was then added to each well and incubated at room
temperature for 30 minutes. 50μl of streptavidin
peroxidase conjugate (SA-HRP) (1:4000 suggested working
stock) (Zymed #43-4323) was added to each well and
incubated at room temperature for 30 minutes. The wells
were then washed in 200μl of PBS. 200μl of the
substrate, 2,2-azino-di(3-ethylbenzthiazoline) sulfonic
acid (ABTS) (manufacturer's solution) (Zymed #00-2011),
was then added to each well and incubated at room
temperature for 10-240 minutes. The optical density of
each of the wells was then determined at 410nM on a
Dynatech MR600 plate reader.

The results of this assay are listed in Table 3
below.

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Receptor Group</th>
<th>Optical Densitya Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinovirus 54</td>
<td>Major</td>
<td>0.586 ± 0.043</td>
</tr>
<tr>
<td>Rhinovirus 34</td>
<td>Major</td>
<td>1.014 ± 0.132</td>
</tr>
<tr>
<td>Rhinovirus 89</td>
<td>Major</td>
<td>0.120 ± 0.014</td>
</tr>
<tr>
<td>Rhinovirus 1B</td>
<td>Minor</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>Rhinovirus 2</td>
<td>Minor</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>Rhinovirus 49</td>
<td>Minor</td>
<td>&lt;0.050</td>
</tr>
</tbody>
</table>
Viruses were incubated overnight at 10,000 TCID<sub>50</sub> and detected with biotinylated sICAM-1 at 100 µg/ml as described above. Optical Density expressed as the Mean ± SD of three (3) replicate cultures.

The data shows that three (3) major group rhinoviruses (34, 54 and 89) were detected while three (3) minor group rhinoviruses (1B, 2 and 49) did not show any significant colour development.

ii) A variation of the above-described forward assay procedure was used in which an ABC peroxidase kit (Pierce #32052) was used instead of the SA-HRP. The kit was used according to manufacture instructions except that the dilutions were made in 1% BSA-PBS.

The results of this assay are listed in Table 4 below.

<table>
<thead>
<tr>
<th>Virus Incubation Time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Optical Density&lt;sup&gt;b&lt;/sup&gt; SA-HRP</th>
<th>Optical Density&lt;sup&gt;b&lt;/sup&gt; SA-Biotin-HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 Hours</td>
<td>0.110</td>
<td>0.372</td>
</tr>
<tr>
<td>3.0 Hours</td>
<td>0.243</td>
<td>0.762</td>
</tr>
<tr>
<td>6.0 Hours</td>
<td>0.431</td>
<td>1.420</td>
</tr>
<tr>
<td>24.0 Hours</td>
<td>0.504</td>
<td>1.692</td>
</tr>
</tbody>
</table>

<sup>a</sup>Rhinovirus 34 at 100 TCID<sub>50</sub> and biotinylated sICAM-1 at 75 µg/ml. <sup>b</sup>SA-HRP = Streptavidin horseradish peroxidase; SA-Biotin-HRP = Streptavidin biotinylated horseradish peroxidase (ABC kit).

The ABC peroxidase reagent (SA-Biotin-HRP) amplified the signal at all virus trapping incubation
times and enhanced the response at the shortest incubation times, allowing for a higher signal sooner.

iii) An assay was performed with a 3 hour virus (rhinovirus 34) incubation period but with varied concentrations, using the procedure described in E(i) and E(ii) above. The results of this assay are shown in Figure 4.

iv) HRV54, at two different concentrations, was trapped after an overnight incubation by immobilized sICAM-1 and detected by the addition of biotinylated sICAM-1, SA-HRP and substrate using the procedure described in E(i) above. The results of this assay are shown in Figure 1. The data shows that the results were dependent upon virus concentration.

v) Rhinovirus 54 was detected at times as short as 1 hour after being trapped by sICAM-1 using the procedure described in E(i) above. The results of this assay are shown in figure 2.

vi) Rhinovirus 54 was detected at a concentration as low as 1000 TCID$_{50}$ and rhinovirus 2 was not detected at any tested concentration. Both rhinovirus 54 and rhinovirus 2 were trapped after a 3 hour incubation with sICAM-1 using the procedure described in E(i) above. The results of this assay are shown in Figure 3.
The claims defining the invention are as follows:

1. A method for the determination of the presence of a virus or a related group of viruses, in a fluid sample, which comprises the steps of:
   A (a) incubating the fluid sample with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a first insoluble complex of the virus or one or more of the related group of viruses, and the immobilized primary receptor; and
   (b) incubating the first insoluble complex with a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor;

or B (a) incubating the fluid sample with a labelled soluble primary receptor for the virus or the related group of viruses, to form a soluble complex of the virus or one or more of the related group of viruses, and labelled soluble receptor; and
   (b) incubating the soluble complex of (a) with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related viruses, and the immobilized primary receptor;

or C (a) incubating the fluid sample with (i) an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample; and (ii) a labelled soluble primary receptor for the virus or the
related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor.

2. A method as claimed in claim 1 which comprises the steps of:

(a) incubating the fluid sample with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a first insoluble complex of the virus or one or more of the related group of viruses, and the immobilized primary receptor; and

(b) incubating the first insoluble complex with a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor.

3. A method as claimed in claim 1 which comprises the steps of:

(a) incubating the fluid sample with a labelled soluble primary receptor for the virus or the related group of viruses, to form a soluble complex of the virus or one or more of the related group of viruses, and labelled soluble receptor; and

(b) incubating the soluble complex of (a) with an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related viruses, and the immobilized primary receptor.
4. A method as claimed in claim 1 which comprises the step of:

(a) incubating the fluid sample with (i) an immobilized primary receptor for the virus or the related group of viruses, the immobilized primary receptor being insoluble in the fluid sample; and (ii) a labelled soluble primary receptor for the virus or the related group of viruses, to form a final insoluble complex of the labelled soluble receptor, the virus or one or more of the related group of viruses, and the immobilized primary receptor.

5. A method as claimed in any one of the preceding claims further comprising the steps of:

(c) separating the final insoluble complex from the fluid sample and any unreacted labelled soluble receptor;

(d) measuring either the amount of label associated with the immobilized primary receptor or the amount of unreacted label; and

(e) determining the presence of the virus or the related group of viruses, in the fluid sample, by relating the measurement of (d) to a measurement of a reference sample free of the virus or the related group of viruses.

6. A method as claimed in any one of the preceding claims wherein the immobilized primary receptor is immobilized on a solid support.

7. A method as claimed in claim 6 wherein the solid support comprises polystyrene.

8. A method as claimed in any preceding claim wherein the related group of viruses is the major subgroup of
rhinoviruses.

9. A method as claimed in any preceding claim wherein the immobilized primary receptor is immobilized sICAM-1.

10. A method as claimed in any preceding claim wherein the labelled soluble primary receptor is labelled sICAM-1.

11. A method as claimed in claim 10 wherein the labelled sICAM-1 is biotinylated sICAM-1.

12. A method as claimed in any preceding claim substantially as herein described in the Example.

13. Each and every novel compound, composition, process or method herein disclosed.

DATED this 22nd day of April 1992.

BOEHRINGER INGELHEIM PHARMACEUTICALS, INC.
By their Patent Attorneys:
CALLINAN LAWRIE
Biotinylated sICAM-1 (µg/ml)
Rhinovirus 54 Incubation Times

(assay at room temp.)