Herpes Simplex Virus-induced Changes of the Keratin Type Intermediate Filament in Rat Epithelial Cells

By L. NØRREGÅRD NIELSEN, 1 J. FORCHHAMMER, 2 E. DABELSTEEN, 3 A. JEPSEN, 4 C. STUBBE TEGLBÆRG 1
AND B. NORRILD 1.

1 Institute of Medical Microbiology, University of Copenhagen, Juliane Maries Vej 22, DK-2100 Copenhagen, 2 The Fibiger Institute, Ndr. Frihavnsgade 70, DK-2100 Copenhagen, 3 Department of Oral Diagnosis, The Royal Dental College, DK-2100 Copenhagen and 4 Tissue Culture Laboratory, Department of Oral Pathology, The Royal Dental School, DK-8000 Århus, Denmark

(Accepted 17 November 1986)

SUMMARY

Herpes simplex virus type 1 (HSV-1) infection of human fibroblast cells grown in culture induces reorganization of the cytoskeleton fibrillar structures. Normal transport and insertion of HSV glycoproteins into the plasma membrane of the cells depend on the integrity of the microtubules. The natural host cells for HSV are epithelial cells, and an epithelial cell line established from rat palate was used in the present study. The effect of virus on the structure of the intermediate filaments and especially on the keratin proteins was studied. Two-dimensional gel electrophoresis of total cell extracts identified in uninfected cells two major acidic keratin proteins with apparent molecular weights of 44000 (44K) and 48K (pI 5.45 to 5.30, 5.50 to 5.35). A new keratin protein of 46K (pI 5.40 to 5.25) appeared in infected cells between 8 h and 12 h post-infection. Pulse-chase experiments identified the 46K protein as a processed form of the 48K keratin component, which was also cleaved in uninfected cells grown in the presence of cycloheximide. Partial proteolysis of the 46K and 48K keratins with Staphylococcus aureus V8 protease showed that the 48K and the 46K proteins differed in only one oligopeptide. The significance of the changed keratin composition of HSV-infected cells is discussed.

INTRODUCTION

In cultured human fibroblasts and epithelial cells, the organization of the fibrillar cytoskeletal elements, the actin filaments, the microtubules and the intermediate type filaments has been studied extensively. The three fibrillar structures were identified by indirect immunofluorescence staining with either monoclonal or polyclonal, monospecific antibodies made against each of the proteins (Norrild et al., 1986; Sun & Green, 1978a; Weber & Osborn, 1981). Distinct functions are associated with the different cytoskeleton filaments. The actin microfilaments determine cell morphology (Tilney, 1983), microtubules are involved in organelle movement and perhaps also in secretory processes (Dustin, 1978; Pollard et al., 1976; Schliwa, 1984), but the function of intermediate filaments is unknown at present. The vimentin type of intermediate filament present in fibroblasts may be involved mainly in anchorage of the cell nuclei (Lehto et al., 1978; Menko et al., 1983). The function of the keratin type filaments of epithelial cells is unknown, but they apparently play a role in the differentiation and maturation of the epithelial tissue (Fuchs & Green, 1980; Sun et al., 1984).

The proteins of the different filament structures in human cells and tissue are well known. Actin has a molecular weight of 43000 (43K), tubulin is present in two molecular forms, alpha and beta of molecular weights 58K and 55K respectively. The fibroblast intermediate filament protein vimentin has a molecular weight of 57K (Osborn & Weber, 1983; Sun & Green, 1978a),...
but the keratins of the intermediate filaments of epithelial cells are heterogeneous and at least 19 different proteins have been identified in human tissue. The molecular weight range is from 40K to 68K (Franke et al., 1981; Moll et al., 1982; Sun et al., 1984), and the subset of keratins that is expressed in a cell is unique for the specific tissue and for the particular layer in the epithelium (Osborn & Weber, 1983; Sun et al., 1984). The keratin expression changes that occur during terminal differentiation of cells seems to be programmed by the differentiation process (Sun et al., 1983, 1984), and the keratins are translated from different mRNA molecules (Fuchs & Green, 1979).

The functions of the cytoskeletal fibrillar structures in cell morphology and intracellular transport of secretory proteins or particles might be further elucidated by analysis of virus-infected cells. It has been observed that actin microfilaments are often reorganized, or changed quantitatively, after infection with both DNA and RNA viruses (Bedows & Welsh, 1983; Fagraeus et al., 1978; Heeg et al., 1981; Meyer et al., 1981; Rutter & Mannweiler, 1977), and direct interaction between the paramyxovirus M protein and actin has also been suggested (Giuffre et al., 1982). We have shown that a reorganization of both actin filaments and microtubules occurs after herpes simplex virus (HSV) infection whereas the vimentin type of intermediate filament remains intact during the first 9 h of infection (Norrid et al., 1986). Several virus infections, e.g. frog virus 3 and reovirus, lead to collapse of the vimentin type of intermediate filament (Murti & Goorha, 1983; Sharpe et al., 1982).

Epithelial cells are the natural host cells for HSV and for human papillomavirus (HPV). HPV has been reported to induce a decrease in the 67K keratin polypeptide present in human epidermis and to induce the appearance of an additional polypeptide (Staquet et al., 1981). Human epithelial cells transformed by simian virus 40 have been shown to lose a series of keratins as a result of the transformation and keratins characteristic of less differentiated cells are expressed (Hronis et al., 1984).

In the present study we report the modification of keratins in HSV-infected rat epithelial cells.

METHODS

Cell cultures and virus infection. Rat palate epithelial cells established by Jepsen et al. (1980) were grown in Eagle's MEM supplemented with 5% (v/v) foetal calf serum, 1% (v/v) amino acids, 1% (v/v) non-essential amino acids, 1% (v/v) vitamins, 1% (v/v) glutamine, 1% (v/v) penicillin and streptomycin, 44% (w/v) NaHCO₃, 0.5% (v/v) DMSO (Jepsen et al., 1980). The medium was changed every 3 to 4 days. The cells were maintained at 30 °C. Infection with HSV-1 strain F was done at a multiplicity of infection of 10 p.f.u. per cell in MEM with 1% (v/v) foetal calf serum (maintenance medium). After 1 h of infection the virus inoculum was removed and 5 ml of maintenance medium was added. Mock infection was done in parallel.

Labelling of cells with [³⁵S]methionine and [³²P]. Before labelling of the cells with [³⁵S]methionine, cultures were transferred to MEM with one-tenth of the normal amount of methionine for 1 h. [³⁴S]Methionine (sp. act. > 800 Ci/mmol, New England Nuclear) was added at 100 μCi/ml medium, and the cells were labelled for different time intervals as specified in the legends to figures. When the labelled cells were chased, methionine was added in tenfold excess of the standard concentration used in the growth medium.

Labelling with [³²P] (New England Nuclear) was done in medium with a tenfold reduced phosphorus concentration, supplemented with 1 mCi of the isotope per ml.

Preparation of cell extracts. Cells from a 25 cm² tissue culture flask were washed three times in 5 ml phosphate-buffered saline (PBS). The cells were scraped into 3 ml PBS and the cell pellet formed after centrifugation was solubilized in 250 μl of lysis buffer (Bravo et al., 1982). The cell extract was prepared by sonication for 15 s and loaded onto the first dimension gel as specified in legends to figures.

Two-dimensional gel electrophoresis (2D gels). The method was mainly as described by O'Farrell et al. (1977) but with minor modifications (Bravo et al., 1982).

First dimension gel (isoelectric focusing gel, IEF). The 9.1 ml gel mix for the first dimension contained 50 g urea, 1.21 ml of a solution containing 28.38% (w/v) acrylamide, 1.62% bisacrylamide, 1.37 ml H₂O, 1.26 ml Ampholines (pH 8.0 to 4.2; Serva, Heidelberg, F.R.G. and LKB), 1.82 ml NP40, 6.35 μl NN'N'N'tetramethyl-ethylenediamine and 9.10 μl of a 10% solution of ammonium persulphate. The gels were allowed to polymerize for at least 2 h and pre-run for 600 volt hours (V h) as follows: 200 V 0.25 h, 300 V 0.5 h, 400 V 1 h. Samples were applied and overlayed as described previously (O'Farrell et al., 1977; Bravo et al., 1982). The gels were run for 18 h at 400 V (7200 V h), removed from the tubes and stored in sample buffer at −20 °C.
**Herpes simplex virus and the cytoskeleton**

Second dimension gel. Standard 9-25% (w/v) polyacrylamide gels (14 × 16 cm) were used for the second dimension electrophoresis. The gels were cross-linked with 0.24% (w/v) diallyltartardiamide (DATD). The electrophoresis was done as described previously (Bravo et al., 1982). The first dimension tube gel was applied and sealed on top of the second gel with 1% (w/v) agarose in SDS sample buffer (Bravo et al., 1982).

Immunoblotting. The proteins were electrophoretically transferred from the gel to nitrocellulose paper by use of a horizontal electroblotting apparatus (JKA Electrophoresis Equipment, Denmark). The transfer was done according to the instructions for the blotting cell, and the buffers used were as follows. Cathode buffer (A) was 25 mM-Tris, 40 mM-6-amino-n-hexanoic acid, 20% methanol; anode buffer (B) was 25 mM-Tris, 20% methanol; anode electrode buffer (C) was 0.3 mM-Tris, 20% methanol. The use of buffer C closest to the anode is important because the blotting cell was constructed without buffer chambers. Only buffer-soaked filter paper was used for the buffer compartments. The electrophoretic transfer was done at 180 mA for 1 h. The transferred proteins were identified by autoradiography of the nitrocellulose paper by exposure to Kodak T-MAT G film for 1 to 14 days, depending on the experimental design. For immunological detection of the keratins the relevant area of the nitrocellulose was identified in the autoradiogram, and an area of 6 × 6 cm was cut from the nitrocellulose and incubated with keratin-specific antibodies as specified in the legends to the figures. Antibody binding was visualized after incubation of the immunoblot with peroxidase-coupled antibodies reactive with the keratin antibodies. 4-Chloro-1-naphthol was used as substrate (Holland et al., 1983). The identification of the tubulin and actin proteins on the transfers was done by sequential incubation of the nitrocellulose paper with each of the specific antibodies. Each reaction was developed and photographed before the next incubation was initiated.

Antibodies. Monoclonal antibodies to alpha-tubulin, beta-tubulin and actin were purchased from Amersham.

Monoclonal antibodies (AE1) to the acidic keratins were kindly donated by Dr T.-T. Sun (Department of Dermatology, New York University School of Medicine, New York, U.S.A.) and their specificity is described elsewhere (Woodcock-Mitchell et al., 1982).

Polyclonal rabbit antibodies to keratins were purchased from Dakopatts, Copenhagen, Denmark (cat. no. A-575). Peroxidase-coupled antibodies to mouse and rabbit immunoglobulins were from Dakopatts (cat. no. P-161 and P-217).

Proteolytic digestion of proteins with Staphylococcus aureus V8 protease. The autoradiographic image of dried, unfixed 2D gels was used for identification of the keratins. Keratins a to c and d to f were cut from the gels and cleaved with *S. aureus* V8 protease according to Cleveland et al. (1977) as follows. The keratins were placed in separate slots in a 2 cm 3% (w/v) polyacrylamide stacking gel on top of a 15% (w/v) polyacrylamide slab gel. To each slot was added 40 μl Tris buffer (0-125 mM-Tris-HCl pH 7.0, 0-1% w/v SDS, 20% w/v glycerol), and the gel was allowed to swell for 30 min. Twenty μg *S. aureus* V8 protease (Miles Laboratories, code 39-900-1) was used per sample. When the proteins had migrated through three-quarters of the stacking gel the power was turned off for 30 min, allowing the protease to digest the protein. Electrophoresis in the slab gel was continued under standard conditions (Cleveland et al., 1977; Norrild et al., 1985).

**RESULTS**

Total protein extracts were prepared from mock- or HSV-1-infected rat epithelial cells and analysed by 2D gel electrophoresis. Cells were labelled with [35S]methionine from 2.5 to 24 h post-infection, the proteins were separated electrophoretically in 2D gels and transferred from the second dimension gel to nitrocellulose membranes. The transferred cytoskeletal proteins were identified by immunoblotting by the use of polyspecific or monoclonal antibodies to keratins and monoclonal antibodies to actin, to alpha-tubulin and to beta-tubulin. Nine keratin proteins, named a to i, were identified in HSV-1-infected cells. All keratins were immunoreactive when incubated with polyspecific keratin antiserum (Fig. 1d). Proteins a and b of mock-infected cells stained intensely, whereas components d to f were absent (Fig. 1b). The three series of keratins, a to c, d to f and g to i, had molecular weights of 48K, 46K and 44K respectively. Proteins c and i were present on the immunoblots, but are barely visible in the figure because the staining faded during the following incubation with antibodies to actin and tubulin. The appearance of three proteins of the same mol. wt. sequentially migrating in a horizontal line indicates that these proteins might represent processed forms of one keratin. It should be noted that with monoclonal antibodies AE1 keratins c, f, h and i were not detected although the others were clearly identified (Fig. 2), confirming the absence of d and e from uninfected cells. The actin and tubulin proteins were immunostained as molecular weight and IEF (pI) markers on the immunoblots, and it should be noted that the HSV infection did not lead to modification of these proteins (Fig. 1a, c).
Fig. 1. Autoradiographic image of the mock- and HSV-1-infected cell keratins analysed in 2D gels and immunoblots with rabbit antibodies to keratins. (a) Autoradiogram of \([^{35}\text{S}]\)methionine-labelled keratins from mock-infected cell extracts. The two series of keratins are marked a to c and g to i. Alpha and beta tubulin (\(\alpha_t, \beta_t\)) and actin (Ac) are used as markers. (b) Immunoblotting of mock-infected cells by use of sequential incubation with polyspecific rabbit antibodies to keratin and monoclonal antibodies to tubulin and actin. (c) Autoradiogram of \([^{35}\text{S}]\)methionine-labelled keratins from HSV-1-infected cell extracts. The three series of keratins are marked a to c, d to f, and g to i. (d) Immunoblotting of HSV-1-infected cell extracts was done as described for (b). The first dimension gel was loaded with extract corresponding to \(2 \times 10^5\) cells. The X-ray film was exposed for 5 days.

Analysis of the time-dependent accumulation of the HSV-1-induced keratin

HSV-1- or mock-infected epithelial cells were labelled with \([^{35}\text{S}]\)methionine at intervals from 2.5 h to 8 h or to 12 h post-infection and analysed by 2D gel electrophoresis and immunoblotting. Incubation of the nitrocellulose filters with polyspecific antibodies to keratin showed that HSV-1 infection induced the conversion of keratins a, b and c to d, e and f between 8 h and 12 h post-infection (Fig. 3). The synthesis of keratins a to c and g to i continued at least until 12 h post-infection as demonstrated in pulse-chase experiments as described below.

Phosphorylation of the keratins

Identification of three separate keratin proteins of the same molecular size but with different charges indicated that the proteins might be processed by phosphorylation to different extents. \(^{32}\text{P}\) labelling of HSV-1- or mock-infected cells from either 4 h to 8 h or from 8 h to 12 h post-infection followed by analysis of the cell extracts in 2D gels showed that only the two most acidic proteins of each molecular weight series were phosphorylated. In infected cells the keratins named b, c, h and i were labelled between 4 h and 8 h, and keratins e and f were identified when the labelling was from 8 h to 12 h post-infection (Fig. 4).

Pulse-labelling of the keratins

HSV-infected cells were labelled with \([^{35}\text{S}]\)methionine in a 10 min pulse and either harvested immediately or chased in excess unlabelled methionine. Mock-infected cells were analysed in parallel cultures but the chase was done either as described for infected cells or in the presence of
Herpes simplex virus and the cytoskeleton

Fig. 2. The autoradiographic image of the mock- and HSV-l-infected cell keratins analysed in 2D gels and immunoblots made with the monoclonal antibody AE1. (a) Autoradiogram of [35S]methionine-labelled keratins from mock-infected cell extracts. (b) Immunoblot made with antibody AE1 which reacts only with keratins a, b and g. (c) Autoradiogram of [35S]methionine-labelled keratins a to i from HSV-l-infected cell extracts. (d) Immunoblot made with AE1 antibodies which react with a, b, d, e and g. The first dimension gel was loaded with extract corresponding to 2 x 10⁵ cells. The X-ray film was exposed for 6 days.

Cycloheximide (CH). In pulse-labelled, infected cells keratins a to c were labelled more than g to i (Fig. 5). During the following chase, a to c were processed into d, e and perhaps f, which was then not resolved in the gel (Fig. 5b). Keratins a to c accumulated also during a 10 min pulse of mock-infected cells (Fig. 6a), but remained stable during a chase in the absence of CH (data not shown). When the chase was done with CH present, keratins a to c were also processed into keratins d, e and perhaps f as in HSV-infected cells (Fig. 6b). When the pulse-labelled infected and uninfected cells were chased for only 4 h under the same conditions as shown in Fig. 5 and 6 no processing of keratins a to c was observed (data not shown).

It should also be noted that HSV proteins of molecular weights 64K (ICP25), 45K and 43K were strongly labelled during the pulse, but were metabolically degraded during the chase.

Oligopeptide analysis by S. aureus V8 proteolytic degradation

Partial proteolysis of keratins a, b and d, e from both HSV- and mock-infected cells was done by incubation of the proteins with the S. aureus V8 protease. The proteins were first identified in dried gels by autoradiography. The keratins were then cut out for enzyme digestion as described in Methods. Keratins a to c were analysed together as were the proteins d to f. The keratins a to c of HSV-infected, pulse-labelled cells showed six distinct oligopeptide bands in a 15% polyacrylamide gel (Fig. 7, lane b). The same series of bands was obtained from the a to c keratins of mock-infected cells labelled for 10 min and chased with or without CH (Fig. 7, lanes e and f), but the quantity of these proteins was reduced when CH was added during the chase.
Fig. 3. Autoradiographic image and immunoblots of keratins from HSV-1-infected cell extracts at different times post-infection analysed in 2D gels. (a) Cells were labelled from 2.5 to 4 h post-infection, (c) from 2.5 to 8 h post-infection and (e) from 2.5 to 12 h post-infection. (a, c, e) Autoradiograms of gel transfers; (b, d, f) immunoblots of transfers (a), (c) and (e) respectively, using AE1 monoclonal antibodies. The first dimension gels were loaded with extract corresponding to $2 \times 10^5$ cells. The X-ray films were exposed for 6 days in (a) and (c) and for 5 days in (e).
Keratins d to f of HSV-infected cells also showed six oligopeptide bands; although the bands are faint in the figure (Fig. 7, lane c), they could be seen after longer exposure of the gel. The same pattern was obtained after proteolysis of keratins d to f of mock-infected cells chased in the presence of CH (Fig. 7, lane d). Although the number of bands obtained by cleavage of proteins a to c and of their processed forms d to f was the same, one oligopeptide band of the latter keratins had an electrophoretic mobility higher than that of the corresponding band obtained from keratins a, b and c. The molecular weight difference was 3K.

DISCUSSION

The structural integrity of the cytoskeletal actin- and tubulin-containing filaments of HSV-infected cells is disturbed from 6 h post-infection (Bedows & Welsh, 1983; Heeg et al., 1981; Norrild et al., 1986) whereas the intermediate filaments remained intact. Epithelial cells are the target for HSV in infected animals and in man, and we therefore initiated the analysis of the influence of HSV on the synthesis of cytoskeleton proteins in a rat epithelial cell line.
Fig. 5. Autoradiogram of keratins from HSV-1-infected cells analysed in 2D gels and immunoblotted with AE1 antibodies. (a) Infected cells labelled 12 h post-infection with [35S]methionine in a 10 min pulse and harvested immediately. (b) as (a) but cells were incubated in excess methionine for 8 h before harvest. The chase of label from keratins a to c into d to f should be noted. Both insets are immunoblots which also demonstrate a chase from a to c into d to f. AC is actin, and the arrowheads illustrate the three proteins 64K (ICP25), 45K and 43K, which turn over during the chase. The numbers on the left side of each panel are infected cell protein numbers (ICPs; Morse et al., 1978). The first dimension gel was loaded with extract corresponding to $5 \times 10^5$ cells. The X-ray film was exposed for 1 day.
Fig. 6. Autoradiogram of keratins from mock-infected cells analysed in 2D gels and immunoblotted with AE1 antibodies. (a) $^{[35]}$S]Methionine-labelled in a 10 min pulse and harvested. (b) Ten min pulse label followed by a 12 h chase in the presence of CH before harvest. The chase of label from keratins a to c into d and e should be noted. The insets are the immunoblots, and AC is the actin marker. The gels were loaded and exposed as described for Fig. 5.
Fig. 7. Analysis of keratins a to c and d to f after proteolytic digestion with *S. aureus* V8 protease. (a) The mol. wt. markers (*× 10^-3*) were components of a HSV-l-infected cell extract labelled with a mixture of [14C]leucine, [14C]isoleucine and [14C]valine. (b) Cleaved keratins a to c from HSV-l-infected cells labelled in a 10 min [35S]methionine pulse. (c) Cleaved keratins d to f from HSV-l-infected cells labelled as in (b), but followed by an 8 h chase. (d) Digested keratins d to f from mock-infected cells pulse-labelled for 10 min and chased for 8 h in the presence of CH. (The arrow indicates an oligopeptide with a changed electrophoretic mobility.) (e) Digested keratins a to c from mock-infected cells labelled as in (d), but chased in the absence of CH. (f) Digested keratins a to c from mock-infected cells labelled and chased as in (d). The keratins were cut from 2D gels loaded with extracts corresponding to 10^5 cells. The X-ray film was exposed for 8 days.

The rat keratins from oesophagus and tongue tissue have previously been characterized. Depending on the tissue studied, two or three acidic keratins were identified (Franke et al., 1981). In rat palate a series of four 44-5K to 58K keratins were isolated at different stages of differentiation (Dale et al., 1982; Shuler & Schwartz, 1982). We have identified two acidic keratins with apparent molecular weights of 44K and 48K in the rat cell line used in the present study, which indicates that palate cells in culture are less differentiated than those in the tissue. Each of the two keratins appeared in three forms with different charges. The two most acidic proteins, b, c and h, i, from each of the two keratins were phosphorylated. Keratins from human epithelial cells in culture (Sun & Green, 1978b) and from both human and rodent tissue are also known to be processed by phosphorylation (Franke et al., 1981; Sun & Green, 1978b).

The pulse–chase experiments showed that the 48K rat keratin was processed to a smaller 46K keratin in HSV-infected cells. The reduction in apparent molecular weight was 2K and the processed form maintained the phosphorylation heterogeneity of the 48K keratin. A similar
reduction in size was observed during terminal differentiation of human epithelia where it occurred in the granular layer, apparently because of increased proteolytic activity (Fuchs & Green, 1980). HSV is known to cause shut-off of host cell protein synthesis 4 to 6 h after infection (Sydiskis & Roizman, 1966; Fenwick & McMenamin, 1984). The finding that the processing of the keratins begins at 8 h and 12 h post-infection might reflect certain proteolytic processes being initiated by HSV. This suggestion is supported by the observation that CH induces a similar proteolysis of keratins in uninfected cells, and it is therefore apparently a host cell enzyme which is activated.

As keratins are known to be conserved among species (Franke et al., 1981; Osborn & Weber, 1983; Sun et al., 1984), the monoclonal antibodies made to the human keratins of subfamily A (Sun et al., 1984; Woodcock-Mitchell et al., 1982) allowed correlation of the rat keratins with the corresponding human keratin proteins. Based on the reactivity of AE1 to the 48K and the 50K keratins of the human subfamily A (Woodcock-Mitchell et al., 1982) and on the knowledge that cultured epithelial cells express keratins characteristic of less differentiated tissue (Fuchs & Green, 1980; Woodcock-Mitchell et al., 1982), it is most likely that the rat 44K and 48K keratins correspond to the human 48K and 50K keratins respectively.

The functional consequence of the cleavage of the 48K keratin is not yet clear. Our data show that HSV interferes with all three cytoskeletal filament structures. What influence the changed cytoskeleton organization has on the intracellular transport of viral proteins and/or virus particles is still unknown. The possible participation of the different filaments in the intracellular sorting of HSV proteins is under current investigation using both rat epithelial cells and human primary epithelial cells.

Lars Norregård Nielsen was a fellow under Medical Research Council grant no. 12-5113. The excellent technical assistance of Ms Ruth Feldborg and Ms Anita Larsen is kindly acknowledged. The photographic work was done with the excellent assistance of Mr Poul Eriksen. We are especially indebted to Dr T.-T. Sun for the donation of the monoclonal antibodies. The work was supported by the Cancer Society grant no. 86-035, the NOVO Foundation, the Danish Foundation for the Advancement of Medical Science and the Danish Health Foundation. Part of this study was presented at the 10th International Herpesvirus Workshop (Ann Arbor, Mich., U.S.A.) in 1985.

REFERENCES


(Received 9 May 1986)