Characterization of carbohydrates linked to rubella virus glycoprotein E2

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Rubella virus contains two envelope glycoproteins, E1 and E2. The amino acid sequence for both glycoproteins is known, as is the number of N-glycosylation sites. This study has demonstrated the presence of O-linked carbohydrates bound to E2 and determined structural characteristics of the N-linked oligosaccharide chains. O-linked sugars were found to be resistant to digestion with N-glycanase but sensitive to beta-elimination with alkaline borohydride. After treatment with neuraminidase, O-linked sugars bound to peanut agglutinin, suggesting the presence of the disaccharide galactose-N-acetylgalactosamine, masked by sialic acid. The N-linked oligosaccharides were large, probably four-branched, and showed a lectin binding pattern suggesting the complex type, with terminal Gal, GlcNAc and sialic acid. No Endo H-sensitive carbohydrates were detected.

Introduction

Rubella virus (RV), the causative agent of German measles, is a small, enveloped, positive-stranded RNA virus and is the only member of the Rubivirus genus in the Togaviridae family. The virion contains a 40S genomic RNA of positive polarity, which together with multiple copies of a capsid (C) protein constitutes the nucleocapsid (Hovi & Vaheri, 1970; Dorsett et al., 1985). The nucleocapsid has icosahedral symmetry and is enclosed in an envelope in which two viral glycoproteins, E1 and E2, are inserted (Dorsett et al., 1985; Oker-Blom et al., 1983). In the infected cell, a 24S subgenomic RNA is produced in addition to the 40S genomic species. The 24S RNA codes for the structural proteins, which are synthesized as a polypeptide precursor with an $M_r$ of 110K (p110) (Oker-Blom et al., 1984). The three structural proteins have been reported to have slightly different $M_r$s depending on the experimental system used. In our system, E1 has an apparent $M_r$ of 61K, E2 is 45K to 52K and the C protein is 34.5K.

Glycoprotein E2 is highly glycosylated with the carbohydrate moiety making up more than one-third of the $M_r$ (Oker-Blom et al., 1983; Bowden & Westaway, 1985). E2 contains three or four N-glycosylation sites depending on the strain (Clarke et al., 1987; Vidgren et al., 1987; Frey & Marr, 1988). No studies have been published determining whether all the N-glycosylation sites are used or reporting the presence of O-linked carbohydrates. The biological role of E2 is unknown, with haemagglutinating activity and most of the neutralizing epitopes localized to E1. The function of highly glycosylated proteins, such as E2, has been shown to be dependent on both the presence and the composition of carbohydrate residues (Woodward et al., 1985; Fukuda et al., 1987; Sjöblom et al., 1987; Takeuchi et al., 1989).

N-linked oligosaccharides are, with few exceptions, present on all the viral glycoproteins that have been characterized. O-linked, or mucin-type carbohydrates, on the other hand, have been demonstrated on a limited number of viral glycoproteins (Holmes et al., 1981; Niemann & Klenk, 1981; Olofsson et al., 1981; Shida & Dales, 1981; Kari & Gehrz, 1988). N-linked oligosaccharides are synthesized as dolichol-bound precursors, which are common for complex-, hybrid- and high mannose-type carbohydrates. The precursor oligosaccharide is transferred, en bloc, to the growing polypeptide in the endoplasmic reticulum (ER) and is subsequently processed in the ER and the Golgi apparatus to yield the different types of oligosaccharides. O-linked oligosaccharides are added monosaccharide by monosaccharide to the polypeptide backbone, starting typically with N-acetylgalactosamine (GalNAc) and galactose (Gal). The available data suggest that the addition of the core GalNAc can take place both in the ER and in the Golgi apparatus (Strous, 1979; Hanover et al., 1980; Johnson & Spear, 1983; Serafini-Cessi et al., 1983; Spielman et al., 1987).

The present study was undertaken to determine the importance of the glycosylation pattern of glycoprotein E2 to the biological properties of RV. It involved
characterization of the carbohydrate moiety of glycoprotein E2 from RV strain M33 in terms of size, type of oligosaccharides (including the nature of the linkage to the polypeptide backbone) and composition of the terminal monosaccharides.

Methods

**Virus and cells.** RV strain M33 (ATCC, VR-315) was maintained in Vero cells. The Indiana strain of vesicular stomatitis virus (VSV) (kindly provided by Dr Frank Tufaro, University of British Columbia) was used in control experiments. Vero cells were obtained from ATCC and propagated in MEM (Gibco) supplemented with 10% foetal calf serum (FCS).

**Preparation of radiolabelled virus.** Monolayer cultures of Vero cells in small cell culture flasks (Becton Dickinson) were grown to a confluence of 60 to 80%, and then infected with virus at an m.o.i. of 10 to 100 in a volume of 1 ml. Virus was allowed to adsorb for 2 h at 37 °C and 4 ml of medium (MEM, containing 1% penicillin/streptomycin and supplemented with 2% FCS) was added. At 10 h post-infection, the medium was removed and replaced with medium low in glucose (ordinary medium:glucose-free medium 1:5, otherwise as above) and 200 μCi [3H]glucosamine (GlcN) (Amersham, specific activity 740 GBq/mmol) was added. The infected cells were incubated at 37 °C for an additional 20 h before harvesting, which was carried out before any c.p.e. was visible. The medium was clarified by centrifugation at 3000 r.p.m. for 15 min and was subsequently ultracentrifuged for 2 h at 25000 r.p.m. in an SW 28 rotor. The pellet was dissolved in 200 μl of TBS (0.02 M-Tris-Cl, pH 7.4 containing 0.2% Triton; 0.5 mM-NaCl) and centrifuged at 20000 r.p.m. for 1 h. VSV glycoprotein G (VSVG) was prepared according to Lundström et al. (1987), and was digested with Pronase (Calbiochem) and N-glycanase as described in this paper for RV glycoproteins.

**Preparation of radiolabelled E2 and E2 glycopeptides.** RV, radiolabelled with [3H]GlcN, was electrophoresed on a 10% SDS-polyacrylamide slab gel according to Laemmli (1970). [35S]Met-labelled RV prepared in the same way was run as a marker. The [35S]Met-labelled lane was cut out, sliced and counted in a scintillation counter and used, together with prestained Mr markers (Bio-Rad), to determine the position of glycoprotein E2. The [3H]GlcN-labeled E2 was cut out and electroeluted in a minigel system electroeluter (Bio-Rad) at 9 mA/sample for 5 h. N-glycanase-resistant E2 (E2α) was precipitated after digestion for 2 days. Digestion for 5 days gave the same results. The protein concentration in the E2 preparation was considered too low to be critical. After digestion, the enzyme was inactivated by heating to 95 °C for 10 min.

**Digestion with glycosidases.** For Endo H digestion, radiolabelled virus was boiled for 10 min in 200 mM-sodium acetate pH 5.8 containing 0.2% SDS. Endo H [Genzyme, final concentration 40 × 10^-3 units (U)/ml] was added, and the sample was incubated at 37 °C for 16 h. N-glycanase treatment was performed essentially as recommended by the manufacturers. Radiolabelled virus in 0.02 M-sodium phosphate, containing 0.5% SDS and 0.1 M-mercaptoethanol pH 8.6, was boiled for 15 min. Double the sample volume of 0.3 M-sodium phosphate pH 8.6, containing 1.5% NP40 and 3.5 mM-phenanthroline hydrate, was added together with 1 mU N-glycanase (Genzyme) and the sample was incubated at 37 °C (for incubation times, see legends to figures). Prior to neuraminidase digestion, radiolabelled virus, or purified E2, was desalted on a prepacked desalting column (Bio-Rad) against 0.05 M-sodium acetate pH 6.3. The glycoprotein-containing fractions were collected and 30 mU neuraminidase (Behring) was added with CaCl₂ to a final concentration of 1 mM. The sample was incubated at 37 °C for 3 h.

**Lectin binding assay.** Purified, [3H]GlcN-labelled E2 or E2α was desalted against TBS containing 0.2% Triton X-100. For concanavalin A (Con A) and lentil lectin assays, TBS was supplemented with CaCl₂, MgCl₂ and MnCl₂ to a final concentration of 1 mM, respectively. The sample (200 to 300 μl) was mixed with 0.1 ml Sepharose- or agarose-bound lectin (Ricinus communis agglutinin (RCA) agaro, lentil lectin–Sepharose, Con A–Sepharose, peanut agglutinin (PNA)–agarose and wheat germ agglutinin (WGA)–Sepharose, Sigma) and was incubated for 20 min at 20 °C with gentle mixing. Beads were then spun down and washed with TBS–Triton. Bound glycoprotein was eluted with the appropriate monosaccharide in TBS–Triton. Eluting sugars were: 500 mM-alpha-methylmannoside for Con A and lentil lectin, 200 mM-N-acetylglucosamine (GlcNAc) for WGA, 50 mM-glucosamine (GalN) for PNA and 100 mM-Gal for RCA. As glycoproteins bind very tightly to Con A, and are difficult to elute, special experimental conditions were established for the Con A assay (see Table 1 and legend to Fig. 4).

**Chromatographic procedures.** Gel filtration was performed on a 1.0 × 120 cm Bio-Rad Econo column, packed with Sephadex G50 superfine (Pharmacia). Glycopeptides/oligosaccharides, in TBS, were eluted with TBS pH 7.5. Fractions (0.5 ml) were analysed for radioactivity in a scintillation counter and the results obtained were presented as the accumulated value of each of two subsequent fractions.

**Ion-exchange chromatography.** [3H]GlcN-labelled glycopeptides from Pronase-digested E2 were desalted as above against 1 mM-phosphate buffer pH 7.4, and were subsequently separated on a Mono Q H5/S column (Pharmacia) using a gradient of 1 to 200 mM-phosphate.

![Fig. 1. SDS-PAGE of [35S]Met-labelled RV before (lane 2) and after (lane 1) treatment with endo H, and [3H]GlcN-labelled RV before (lane 3) and after (lane 4) treatment with neuraminidase. The positions of radiolabelled Mr markers are indicated.](image_url)
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Alkaline borohydride degradation. Alkaline borohydride degradation was performed as a modification of the method described by Iyer & Carlsson (1971). Purified, Pronase-digested E2 and E2N were desalted against water, as described above, and the glycopeptide-containing fractions were collected and lyophilized. The pellet after lyophilization was dissolved in 0.05 M NaOH containing 0.1 M NaBH₄ and was incubated at 45°C for 16 h. After incubation, the sample was neutralized with glacial acetic acid.

Results

Sensitivity of E2 to endoglycosidases

RV, radiolabelled with [35S]Met, was subjected to digestion with endoglycosidases and subsequently analysed by SDS-PAGE (Fig. 1). Endo H, which selectively cleaves high mannose and certain hybrid-type oligosaccharides (Kobata, 1979), did not cause any shift in electrophoretic mobility. N-glycanase, on the other hand, changed the apparent Mr of E2 dramatically. Sequential digestion with N-glycanase showed that the dominant E2

Fig. 2. SDS-PAGE of radiolabelled RV after digestion with N-glycanase. (a) Sequential digestion of [35S]Met-labelled virus. Samples were inactivated after 5 (lane 2), 10 (lane 3), 15 (lane 4), 20 (lane 5), 30 (lane 6), 40 (lane 7), 60 (lane 8) and 120 (lane 9) min. Lane 1 shows untreated RV. (b) Overnight digestion of [35S]Met-labelled virus with N-glycanase (lane 1). Undigested (lane 3) and endo H-digested (lane 2) virus is also shown. (c) Overnight digestion of [3H]GlcN-labelled virus (lane 3). Undigested, [35S]Met-labelled (lane 1) and [3H]GlcN-labelled (lane 2) virus is also shown.
species after 1 h was a band with an apparent $M_r$ of about 38K to 40K (Fig. 2a) and, even after overnight digestion, no species with lower $M_r$ were visible (Fig. 2b). N-glycanase has been shown to release virtually all types of N-linked oligosaccharides (Tarentino et al., 1985). The $M_r$ of unglycosylated E2, however, is about 32K (Hobman & Gillam, 1989), suggesting that not all carbohydrates are removed by N-glycanase. This was further supported by the finding that E2 retained some of the $[^3H]$GlcN label after extensive digestion with N-glycanase (Fig. 2c). E1 in the same preparation, which contains both complex- and high mannose-type glycans (Hobman et al., 1990), was not labelled with $[^3H]$GlcN after N-glycanase treatment, confirming that the N-glycanase digestion was complete (Fig. 2c).

**Demonstration of terminal sialic acid**

RV was digested with neuraminidase and subjected to SDS–PAGE. The apparent $M_r$ of E2 decreased slightly, showing that at least some of the oligosaccharides contained terminal sialic acid (Fig. 1). To investigate further the degree of sialylation of E2 oligosaccharides, E2 purified by electroelution from polyacrylamide gels was digested with Pronase and subsequently analysed by HPLC using an ion-exchange column. The glycopeptides eluted in two peaks, of which one was retarded by the column (Fig. 3). When E2 was treated with neuraminidase and dialysed prior to Pronase digestion and ion-exchange chromatography, all the radiolabelled material eluted in the void volume, confirming that the charge carried by the retarded glycopeptides was due to the presence of terminal sialic acid (Fig. 3). N-glycanase-treated and Pronase-digested E2 showed the same profile on ion-exchange chromatography, one charged and one non-charged peak (results not shown).

### Table 1. Lectin binding of purified glycoprotein E2 before and after digestion with neuraminidase

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Without neuraminidase*</th>
<th>With neuraminidase*</th>
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<tbody>
<tr>
<td>Con A</td>
<td>52 ± 3†</td>
<td>37 ± 11</td>
</tr>
<tr>
<td>Lentil</td>
<td>23 ± 4</td>
<td>ND†</td>
</tr>
<tr>
<td>RCA</td>
<td>44 ± 5</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>WGA</td>
<td>66 ± 5</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>PNA</td>
<td>3 ± 1</td>
<td>30 ± 4</td>
</tr>
</tbody>
</table>

* Values are expressed as the percentage of total radioactivity added that could be eluted with competing sugar. For Con A, however, parallel experiments were performed, one with 100 mM-alpha-methylmannoside present during the binding step and one in the absence of competing sugar. The value is expressed as the amount of E2 bound in the absence of competing sugar minus the amount bound in the presence of competing sugar.
† The data represent mean values (± S.E.M., n = 2 to 4).]
‡ ND, Not determined.

### Lectin affinities, N-glycanase-sensitive and N-glycanase-resistant E2

Purified E2 was subjected to affinity chromatography on gel-bound lectins, before and after treatment with neuraminidase (Table 1). Con A and *Lens culinaris* lectin, which both bind to the core structure in complex- and hybrid-type oligosaccharides but with slightly different structural specificities (for a review, see Osawa & Tsuji, 1987), bound well to E2 both before and after neuraminidase treatment, suggesting that complex-, or possibly hybrid-type carbohydrates were present. Con A also binds to high mannose oligosaccharides (Ogata et al., 1975; Baenziger & Fiete, 1979). RCA, with specificity for terminal Gal (Osawa & Tsuji, 1987), also bound well to E2. The binding to RCA was enhanced after treatment with neuraminidase, indicating that some of the Gal was masked by sialic acid. WGA, known to bind to terminal sialic acid and GlcNAc (Osawa & Tsuji, 1987), bound about 60% of E2 before neuraminidase treatment and about 30% after. This could indicate that terminal sialic acid was present on over 30% of E2, and that about 30% contained terminal GlcNAc. Terminal Gal, sialic acid and GlcNAc are all found on both N-linked and O-linked oligosaccharides. A significant part of electroeluted E2 bound, after neuraminidase treatment, to PNA, a lectin specific for the disaccharide Gal–GalNAc, the most common core structure in O-linked carbohydrates (Pereira et al., 1976; Goldstein & Hayes, 1978; Momoi et al., 1982; Månsson & Olofsson, 1983; Lundström et al., 1987). In control experiments using VSVG which contains complex-type oligosaccharides (Etchison et al., 1977; Tabas & Kornfeld, 1978), no binding to PNA was observed, either before or after
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Fig. 4. Lectin binding before (●) and after (▲) treatment with neuraminidase. Eluting sugar was added after fraction 7, as indicated by the arrows. (a) N-glycanase-resistant E2 bound to different lectins. (b) VSVG bound to PNA.

neuraminidase treatment (Fig. 4b). To determine the nature of the N-glycanase-resistant carbohydrates, N-glycanase-digested, [3H]GlcN-labelled E2 was electroeluted from polyacrylamide gels and subjected to affinity chromatography on gel-bound lectins. The results were quite different from those obtained with N-glycanase-sensitive E2 (Fig. 4a). Prior to neuraminidase digestion, the only lectin that bound significantly was WGA. After neuraminidase digestion, the N-glycanase-treated E2 did not have any detectable affinity for WGA, but bound to PNA. None of the lectins specific for the core structure in N-linked oligosaccharides bound significantly to N-glycanase-digested E2, either before or after treatment with neuraminidase. These results suggest that the carbohydrates bound to the polypeptide backbone were of the O-linked, rather than the N-linked, type.

Determination of the size of N-glycanase-sensitive oligosaccharides

To determine the size of the N-glycanase-sensitive oligosaccharides, purified [3H]GlcN-labelled E2 was digested with N-glycanase and subsequently analysed by gel filtration on Sephadex G50 superfine (Fig. 5). Most of the radioactivity was eluted close to the position of an oligosaccharide marker obtained from VSVG, which is known to contain two tri-branched, complex-type carbohydrates (Etchison et al., 1977; Tabas & Kornfeld, 1978). The Mr's of the E2 oligosaccharides were slightly higher than those of the VSVG oligosaccharides, which could be interpreted either as tri-branched oligosaccharides with a higher degree of substitution than VSVG carbohydrates, or as four-branched sugars with a fairly low degree of sialylation.
Demonstration of carbohydrates sensitive to alkaline borohydride treatment

The lectin binding pattern and N-glycanase resistance of a fraction of the carbohydrate moiety of E2, suggested that E2 contained O-linked oligosaccharides. Electroeluted E2 and N-glycanase-digested E2 were digested with Pronase and subsequently treated with weak alkali in the presence of NaBH₄. This procedure is known to release O-linked carbohydrates selectively (Iyer & Carlson, 1971; Debray et al., 1984). To minimize the risk of N-linked oligosaccharides being affected by the alkaline borohydride treatment, the NaBH₄ content was kept low (Rasilo & Renkonen, 1981). The resulting preparations, containing glycopeptides and released oligosaccharides, were analysed by gel filtration as above (Fig. 6). When E2 glycopeptides were treated with alkaline borohydride about 25% of the radioactivity was released and eluted as two distinct peaks at Mₜ values significantly lower than that of the tri-branched marker (Fig. 6a). The same peaks appeared after alkaline borohydride treatment of N-glycanase-resistant E2 (Fig. 6b). However, in this case, more than 50% of the radiolabel was cleaved from the glycopeptide backbone.

The results indicate that N-glycanase-resistant E2 contains several O-linked oligosaccharides situated in Pronase-resistant clusters, as had been described earlier for glycoprotein C of herpes simplex virus (Lundström et al., 1987).

Discussion

This study demonstrated that glycoprotein E2 from RV strain M33 contains large, complex-type, N-linked carbohydrates and several smaller, O-linked oligosaccharides.

Few studies have characterized the carbohydrates bound to RV glycoproteins. Several groups have described incorporation of different radiolabelled sugars into E1, E2a and E2b (Bowden & Westaway, 1984; Ho-Terry & Cohen, 1984) and one group (Bowden & Westaway, 1985) has described the size distribution and endo H sensitivity of E1 and E2 glycopeptides. Bowden & Westaway (1985) demonstrated several species of N-linked oligosaccharides, including endo H-sensitive, high mannose-type sugars, bound to E2. No endo H-sensitive carbohydrates were detectable in this study. This discrepancy could be explained by differences in the glycosylation pattern of different RV strains (Bowden and Westaway used the Putnam strain). Small changes in the amino acid sequence might result in the loss of glycosylation sites or alterations in the folding of the protein, in turn changing the availability for enzymes involved in the glycosylation process (Hubbard, 1988). It is possible that Endo H digestion is more efficient when the glycoprotein has been treated previously with Pronase, although this seems less likely, as N-glycanase readily digested all N-linked oligosaccharides from E2 in the absence of prior Pronase digestion. Radiolabelling with [³H]GlcN or [³H]mannose may result in accentuation of different oligosaccharide species in the different studies.

The lectin binding pattern of E2 suggested the presence of complex-type oligosaccharides with terminal Gal and GlcNAc. Some of these may be sialylated as the binding to RCA was slightly enhanced after treatment with neuraminidase. The data obtained from Con A chromatography and from gel filtration were somewhat contradictory in that Con A, which bound well to E2, has been reported to have a low affinity for tri- and four-branched, complex-type oligosaccharides, but binds well to di-branched carbohydrates (Ogata et al., 1975; Baenziger & Fiete, 1979). However, no oligosaccharide species smaller than the tri-branched marker were detectable after either Pronase digestion or N-glycanase treatment. One explanation may be that even though Con A has a higher affinity for complex...
oligosaccharides with a low degree of branching, in this system, the binding to larger carbohydrates may have been significant. The degree of substitution is an important factor in the availability of lectins, and the data from affinity chromatography of E2, using WGA, indicated that E2 contains a significant number of N-linked oligosaccharides with terminal GlcNAc, which in complex-type carbohydrates means a low degree of substitution. These data do not exclude the possibility of hybrid sugars being present. Hybrid oligosaccharides might be endo H-sensitive or -resistant and, in addition to terminal mannose, may contain terminal Gal, GlcNAc and/or sialic acid. However, the N-linked carbohydrates detected appeared too large to be of the hybrid type, suggesting they are of the complex type. The smearing of N-glycanase-sensitive E2 in PAGE suggested that at least one of the N-linked carbohydrates was heterogeneously processed to a polylactosaminoglycan, as had been described earlier for a number of other glycoproteins (Fukuda et al., 1988; Williams & Lamb, 1988). A lectin binding assay with pokeweed agglutinin and treatment with endo-β-galactosidase produced no evidence of such structures (results not shown). The results from gel filtration, endoglycosidase digestion, and lectin binding assays, suggested that E2 contains four-branched, complex-type sugars with a low degree of substitution.

The difference between the high apparent Mr of E2, as determined by PAGE, and the theoretical Mr calculated from the amino acid sequence, has intrigued researchers in the field, and has been assumed to be due to a high degree of glycosylation. No previous reports have demonstrated the presence of O-linked sugars on E2. O-linked oligosaccharides, bound to viral glycoproteins, were first detected in 1981 (Holmes et al., 1981; Niemann & Klenk, 1981; Olofsson et al., 1981; Shida & Dales, 1981). This study presents three independent lines of evidence indicating that E2 contains carbohydrates of the O-linked type. Firstly, [3H]GlcN-labelled N-glycanase-resistant E2 migrated in PAGE at an Mr considerably higher than would be expected from the unglycosylated protein (modified by the addition of covalently bound fatty acid). Secondly, Pronase-resistant glycopeptides obtained from N-glycanase-treated E2 were sensitive to treatment with alkaline borohydride under conditions known to release O-linked carbohydrates selectively (Iyer & Carlson, 1971; Rasilo & Renkonen, 1981; Debray et al., 1984). Thirdly, neuraminidase-treated, N-glycanase-resistant E2 bound significantly to PNA (a lectin with specificity for the most common core disaccharide of O-linked oligosaccharides, Gal-GalNAc), but did not bind to Con A. The affinity for PNA appeared only after digestion with neuraminidase, which was suggestive of extensive sialylation of the O-linked carbohydrates. This was supported by the data from ion-exchange chromatography and could contribute to the multitude of isoelectric species of E2 as demonstrated by Waxham & Wolinsky (1985).

These results, describing the structures of carbohydrates bound to E2 from RV strain M33, provide a basis for comparison of the carbohydrate composition of E2 purified from different RV strains.

We wish to thank Rebecca Ng and Donna MacDermot for excellent assistance in preparation of the manuscript and Dr Elizabeth Hancock and Dr Shirley Gillam for their helpful comments. This work was supported by grants from the Medical Research Council of Canada, the Florence and George Heigham Foundation and the British Columbia Medical Services Foundation.

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(Received 24 September 1990; Accepted 7 January 1991)