Biochem. J. (1991) 278, 441–445 (Printed in Great Britain)

Mammalian cartilage synthesizes both proteoglycan and non-proteoglycan forms of type IX collagen

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Bovine epiphysial cartilage synthesizes both proteoglycan (PG) and non-PG forms of type IX collagen in a ratio of approx. 2:1. The PG form with its attached glycosaminoglycan on the α2(IX) chain is the major form in the medium, whereas both forms are found in the tissue. The results are discussed with regard to cartilage matrix organization.

INTRODUCTION

The function of cartilage to resist both tensile and compressive forces depends largely on the type and precise organization of the collagens and proteoglycans (PGs) that form the major part of this connective tissue. Recent studies have indicated that the collagen fibres are heterotypic, comprising the major type II collagen and the quantitatively minor collagen types IX and XI [1]. Type IX collagen is localized in a D-periodic manner on the outer surface of the assembled 'type II collagen' fibrils [1,2] and has the potential to bridge these fibrils by forming covalent pyridinoline cross-links with more than one type II collagen molecule [3,4]. In addition, the interaction between the *N*-terminal domain of type IX collagen and the major PGs has been proposed [5]. Type IX collagen appears therefore to play an important role in maintaining the structural integrity of the cartilage matrix.

Extensive biosynthetic and DNA-sequencing studies have demonstrated that chick type IX collagen comprises three triple-helical collagenous domains (COL1–3) interspersed with four non-helical domains (NC1–4) [6,7]. The three component chains are genetically distinct and designated α1(IX), α2(IX) and α3(IX). Attached to the α2(IX) chain within the NC3 domain is a glycosaminoglycan (GAG) side chain, allowing type IX collagen to be classified as a special form of PG [8,9]. Human type IX collagen is also synthesized in a PG form [10], and in preliminary experiments with foetal-bovine cartilage explants we have observed that the type IX collagen released into the culture medium is also predominantly in this form. This latter observation contrasts with our previous studies, which showed that at least 95% (w/w) of the type IX collagen extracted from non-radiolabelled bovine cartilage with 4 M-guanidinium chloride lacked a GAG side chain [11]. Such observations suggest that either (*a*) type IX collagen is synthesized initially in a PG form, but is processed on deposition in the extracellular matrix, or (*b*) two distinct populations of type IX collagen are synthesized which may have different functions in the cartilage matrix. The experiments reported here were designed to test these possibilities.

MATERIALS AND METHODS

Culture and labelling of cartilage

Foetal-bovine epiphysial cartilage was dissected free of adhering tissue, diced into 1–2 mm pieces and washed (three times) with Ham F-12 medium containing Hepes (25 mM), 10% (v/v) foetal-calf serum, ascorbic acid (50 μg/ml), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). The tissue was equilibrated for 1 h at 37 °C in fresh medium and labelled subsequently with either L-[5-^{3}H]proline (20 μCi/ml) or $Na_2{}^{35}SO_4$ (20 μCi/ml) or double-labelled with $Na_2{}^{35}SO_4$ (20 μCi/ml) and [^{14}C]proline (2 μCi/ml), each for 24 h in the presence of β-aminopropionitrile (β-APN; 64 μg/ml).

In pulse–chase experiments, tissue was labelled for 1.5 h with L-[5-^{3}H]proline (20 μCi/ml), with or without the addition of β-APN (64 μg/ml). The tissue was washed extensively with medium and then chased in medium containing non-radioactive proline (14 mM) for various times up to 26 h. Labelled medium was separated from the tissue by centrifugation at 30000 *g* for 1 h and supplemented with the proteinase inhibitors phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM), 6-aminohexanoic acid (25 mM) and EDTA (25 mM).

Salt precipitation of macromolecules secreted into the medium

Proteins secreted into the culture medium were precipitated by addition of $(NH_4)_2SO_4$ to 30% saturation at 4 °C. The precipitate was dissolved in, and dialysed against, 0.5 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing the above proteinase inhibitors and stored at −20 °C before use.

Extraction of labelled tissue

Tissue slices were homogenized in a Polytron homogenizer and extracted sequentially with 10 vol. of 50 mM-Tris/HCl, pH 7.4, containing 1 M-NaCl and 4 M-guanidinium chloride in the presence of proteinase inhibitors as above. The proteins in the guanidinium chloride extracts were separated from the major proteoglycans by CsCl-density-gradient centrifugation, the proteins concentrating as a gel at the top of the gradient [11]. The guanidinium chloride-insoluble residues were washed (three times) with water, suspended in 0.5 M-acetic acid and digested with pepsin (EC 3.4.23.1) as described previously [12].

Chondroitin ABC lyase digestion

Solutions containing proteins from the medium and the 1 M-NaCl and 4 M-guanidinium chloride extracts of the tissue were dialysed against 0.1 M-Tris/HCl, pH 8.0, containing 0.04 M-sodium acetate and the proteinase inhibitors phenylmethanesulphonyl fluoride (0.2 mM), *N*-ethylmaleimide (10 mM), EDTA (2.5 mM) and 6-aminohexanoic acid (0.25 mM) and digested with chondroitin ABC lyase (EC 4.2.2.4) (0.2 unit/ml) for 16 h at 37 °C [13].

Abbreviations used: PG, proteoglycan; GAG, glycosaminoglycan; β-APN, β-aminopropionitrile.

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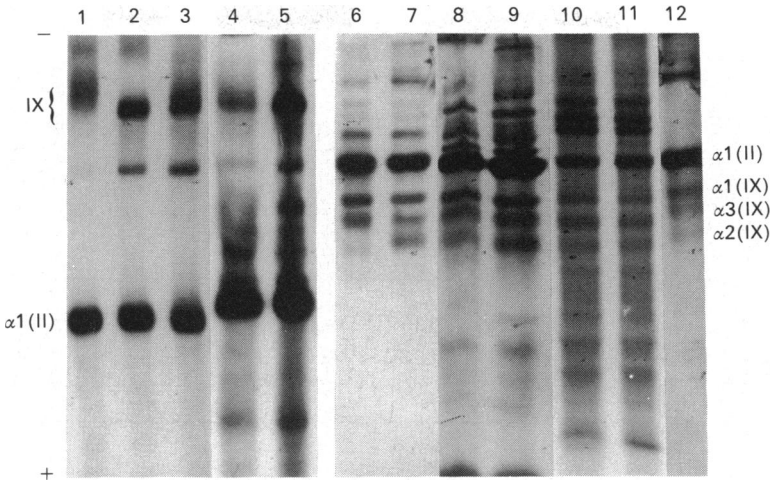

Fig. 1. Fluorogram of newly synthesized [^{3}H]proline-labelled proteins in the medium and tissue extracts of cultured epiphysial cartilage

Medium proteins were precipitated by $(NH_4)_2SO_4$ at 30% saturation and the tissue was extracted sequentially with 1 M-NaCl and 4 M-guanidinium chloride as described in the text. Samples were analysed in the unreduced state on SDS/5%-acrylamide gels (tracks 1–5) and after reduction with 50 mM-dithiothreitol on SDS/8%-acrylamide gels (tracks 6–12). Samples were: 30%-satd.-$(NH_4)_2SO_4$ precipitate (tracks 1 and 2, 6 and 7); 1 M-NaCl extract (tracks 4 and 5, 8 and 9); 4 M-guanidinium chloride extract (tracks 10 and 11); standard [^{3}H]acetic anhydride-labelled types II/IX collagens (tracks 3 and 12). Samples in tracks 2, 5, 7, 9 and 11 were treated with chondroitin ABC lyase.

Immunoprecipitation of type IX collagen

Type IX collagen was immunoprecipitated from solutions of medium proteins or 1 M-NaCl extracts before or after treatment with chondroitin ABC lyase. Immunoprecipitation was carried out by the method of Cooper *et al.* [14], either (*a*) in 0.05 M-Tris/HCl, pH 8.0, containing 0.4 M-NaCl, EDTA (5 mM) and 1% (v/v) Nonidet P40 (NET buffer) or (*b*) in the chondroitin ABC lyase buffer after addition of 1% Nonidet P40. Samples (1 ml) were incubated for 2 h at room temperature with an antiserum (10 μl) that was specific for the COL1 domain of type IX collagen and that showed no cross-reactivity with other native collagens when measured by the direct e.l.i.s.a. technique [11]. Protein A–Sepharose (60 μl of a 1:1 suspension in NET buffer) was added and the mixture incubated for 16 h at 4 °C with end-over-end mixing. The mixtures were centrifuged and the precipitated antigen–antibody–Protein A complexes washed (twice) with NET buffer (1 ml) and (twice) with 0.01 M-Tris/HCl, pH 6.8 (1 ml). The final pellets were then analysed by SDS/PAGE.

Preparation of radiolabelled type IX collagen standard

Foetal bovine cartilage was extracted with 4 M-guanidinium chloride, and the collagens and other proteins were separated from the major proteoglycans by CsCl-density-gradient centrifugation [11]. The protein gel at the top of the gradient was suspended in, and dialysed against, 0.05 M-Tris/HCl, pH 7.4, containing 1 M-NaCl, and the soluble collagens were precipitated sequentially by addition of NaCl to final concentrations of 3.5 M and 4.5 M. The 4.5 M-NaCl precipitate was enriched in intact type IX collagen, but also contained type II collagen [15]. This preparation was then radiolabelled with [^{3}H]acetic anhydride by the method of Gisslow & McBride [16].

SDS/PAGE

Labelled macromolecules were analysed by SDS/PAGE before or after reduction with dithiothreitol (50 mM) [17], followed by fluorography [18]. The newly synthesized type IX collagen was compared with the type IX collagen extracted directly from bovine cartilage and radiolabelled as described above. M_r values quoted are based on collagenous standards of known M_r.

Quantification of type IX collagen

The relative amounts of type IX collagen and its PG and non-PG forms were calculated from (*a*) densitometric scanning of the fluorograms of both total polypeptides in each fraction and the specific type IX collagen immunoprecipitates before and after chondroitin ABC lyase treatment, and (*b*) the total non-diffusible radioactivity in each fraction assessed by scintillation spectrometry. Fractions obtained from tissue labelled with [^{3}H]proline for 24 h in the presence of β-APN were used for the quantification, and under these conditions approx. 90% (w/w) of the newly synthesized type IX collagen was released into the medium and extracted from the tissue before pepsin digestion. The relative amounts calculated for the two forms of type IX collagen are therefore representative of the tissue as a whole.

RESULTS

The electrophoretic profiles of the proteins secreted into the medium and extracted from the tissue after a labelling period of 24 h with [^{3}H]proline are shown in Fig. 1. The major polypeptides in both the medium and 1 M-NaCl extract were the α-chains of types II and IX collagens, identified by reference to a type II/IX collagen standard. The three α-chains of bovine type IX collagen were well separated, with M_r values of 84000, 72000 and 66000 for the α1(IX), α3(IX) and non-glycosylated (GAG-less) α2(IX) chains respectively. The type IX collagen in the medium was present in the PG form, as shown by the absence of the non-glycosylated α2(IX) chain before chondroitin ABC lyase digestion and the appearance of this chain after digestion, when analysed under reducing conditions (Fig. 1; tracks 6 and 7). It should be noted that the glycosylated α2(IX) chain is heterogeneous and migrates as an extremely diffuse band that, when labelled with [^{3}H]proline, is not observed readily, even after immunoprecipitation with a specific antiserum (see Fig. 2*a*; tracks 5 and 7). However, it is easily distinguished when labelled with [^{35}S]sulphate (see Fig. 3), as reported previously [6,9]. The

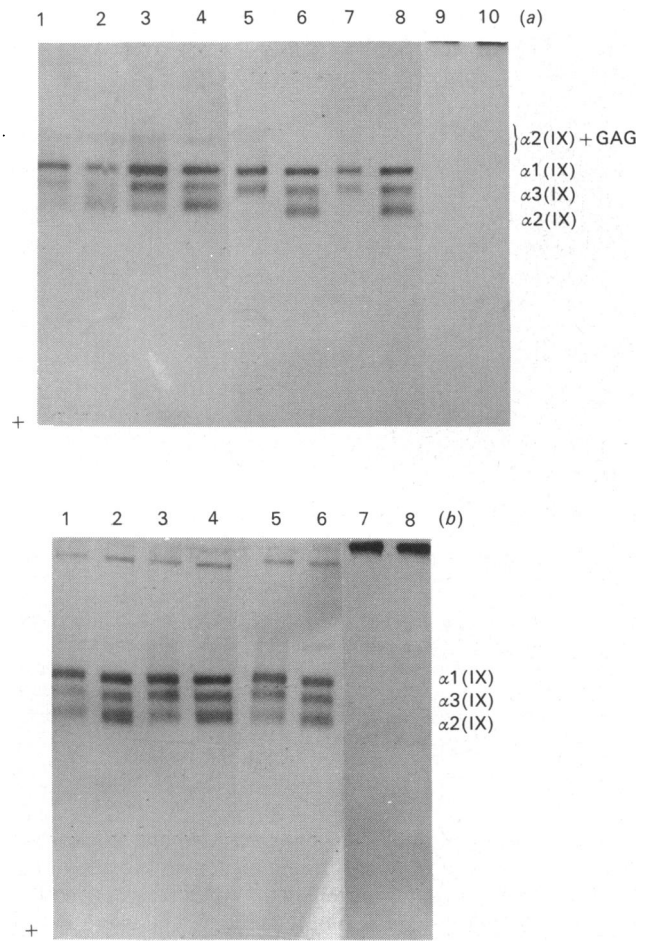

Fig. 2. Fluorogram of type IX collagen immunoprecipitated from the medium and 1 M-NaCl extract of epiphysial cartilage pulsed with [^{3}H]proline and chased for various times

The 30%-satd.-$(NH_4)_2SO_4$ precipitates from media and 1 M-NaCl extracts of tissue were equilibrated in NET buffer and immunoprecipitated with a specific anti-(type IX collagen) serum as described in the text. Immunoprecipitates were analysed on SDS/8%-acrylamide gels after reduction with 50 mM-dithiothreitol, except for tracks 9 and 10 in (*a*) and tracks 7 and 8 in (*b*), which were electrophoresed without reduction. Even-numbered samples were treated with chondroitin ABC lyase. (*a*) Type IX collagen immunoprecipitated from media after 0.5 h (tracks 1 and 2), 1.5 h (tracks 3 and 4), 16 h (tracks 5 and 6) and 26 h (tracks 7–10) chase. (*b*) Type IX collagen immunoprecipitated from 1 M-NaCl extracts after 0.5 h (tracks 1 and 2), 1.5 h (tracks 3 and 4) and 26 h (tracks 5–8) chase.

type IX collagen extracted from the tissue with 1 M-NaCl consisted of a mixture of the PG and non-PG forms, as shown by the presence of the non-glycosylated α2(IX) chain and increased intensity of this chain, before and after enzymic digestion respectively (Fig. 1; tracks 8 and 9). These observations were confirmed by analysing the medium and 1 M-NaCl extract under non-reducing conditions. The type IX collagen in both the medium and tissue fractions migrated as a disulphide-bonded aggregate of M_r approx. 270000. However, the medium form was more diffuse and had a slower mobility than the tissue form before chondroitin ABC lyase digestion (compare tracks 1 and 2 with 4 and 5, Fig. 1). The type IX collagen extracted by 4 M-guanidinium chloride occurred predominantly in the non-PG form, as indicated by the presence of the three α-chains with similar intensities before and after chondroitin ABC lyase treatment (tracks 10 and 11).

In order to explore the possibility that the GAG chain is lost when type IX collagen is deposited in the matrix, pulse–chase experiments were carried out. In addition, to exclude any ambiguity in the analyses, the type IX collagen was immunoprecipitated from both the medium and 1 M-NaCl fractions by a specific anti-(type IX collagen) serum. In the case of the medium, both the PG and non-PG forms were present early in the chase period (Fig. 2*a*; tracks 1–4). However, with time, the proportion of the PG form increased, and after a 26 h chase this form was the major species in the medium (Fig. 2*a*; tracks 7–10) as observed for the 24 h-labelled medium (Fig. 1, tracks 6 and 7). In contrast, the relative proportions of the two forms in the 1 M-NaCl extracts of the tissue did not change throughout the chase period, the ratio of PG to non-PG form being approx. 3:2 (Fig. 2*b*; tracks 1–8). The type IX collagen in the guanidinium chloride extracts could not be specifically immunoprecipitated, since most proteins in these extracts were insoluble in the low-ionic-strength buffers used for immunoprecipitation. However, the intensities of the three α-chains did not change with time of chase (results not shown).

The relative proportions of the type IX collagen present in the medium, NaCl and guanidinium chloride fractions after labelling the tissue for 24 h with [^{3}H]proline in the presence of β-APN were 3:6:1 respectively. Since the medium and guanidinium chloride fractions contained only the PG and non-PG form respectively, and the ratio of PG:non-PG form in the NaCl extract was 3:2, approximately twice as much of the PG form was synthesized compared with the non-PG form. However, the amount of PG and non-PG forms in the tissue was approximately the same.

Fig. 3 shows the type IX collagen immunoprecipitated from the medium and 1 M-NaCl extract of the tissue labelled for 24 h either with [^{35}S]sulphate (tracks 1–5) or with both [^{35}S]sulphate and [^{14}C]proline (tracks 6–12). The profiles confirmed the results obtained by labelling the tissue with [^{3}H]proline, namely that type IX collagen occurs in a PG form in the medium and as a mixture of the PG and non-PG forms in the 1 M-NaCl extract. However, [^{35}S]sulphate-labelled high-M_r aggregates were observed at the top of the gel after reduction in the case of the NaCl extracts (tracks 4 and 11). The sulphate was present as GAG, as shown by its disappearance after digesting the immunoprecipitates with chondroitin ABC lyase (tracks 5 and 12). The weak sulphate-labelled component (*, track 5) presumably indicates the α2(IX) chain with a residual GAG stub attached. [^{35}S]Sulphate-labelled aggregates were also observed in the low-density protein fraction after CsCl-density-gradient centrifugation of the guanidinium chloride extracts. However, their relationship to type IX collagen could not be assessed, since these extracts could not be immunoprecipitated (result not shown).

The immunoprecipitate from the 1 M-NaCl extract contained a component with M_r similar to that of the non-glycosylated α2(IX) chain but which, unlike this chain, was present before reduction and was only revealed after exposing the fluorograms for longer periods of time (Fig. 3, tracks 7 and 8). The relationship of this minor 'α2(IX)-like' component to type IX collagen requires further investigation.

DISCUSSION

The electrophoretic analyses of both the proline- and/or sulphate-labelled cartilage indicate that bovine type IX collagen is secreted into the culture medium predominantly in a PG form, but both the PG and non-PG forms of type IX collagen are present in the tissue. Pulse–chase analyses demonstrated that the

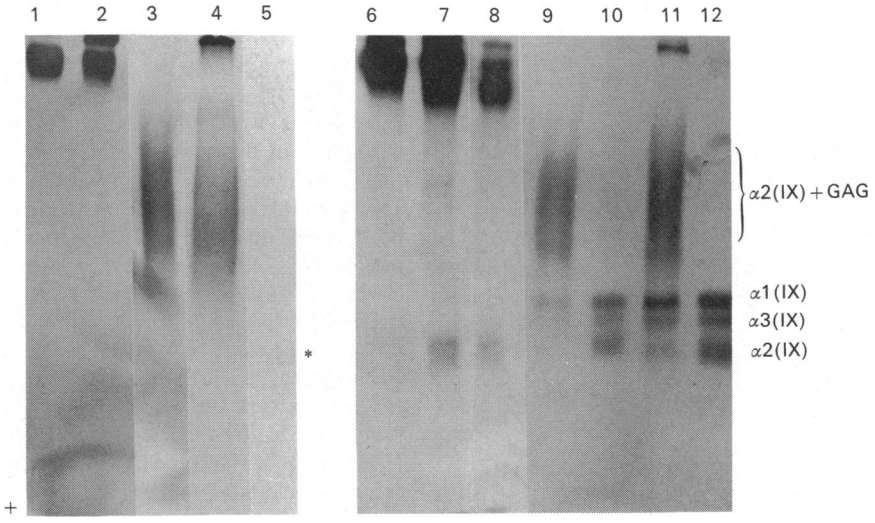

Fig. 3. Fluorogram of type IX collagen immunoprecipitated from the medium and 1 M-NaCl extract of epiphysial cartilage labelled with [^{35}S]sulphate or [^{35}S]sulphate/[^{14}C]proline

The 30%-satd.-$(NH_4)_2SO_4$ precipitate from medium and 1 M-NaCl extract from tissue labelled with $Na_2{}^{35}SO_4$ (tracks 1–5) or $Na_2{}^{35}SO_4$/[^{14}C]proline (tracks 6–12) were equilibrated in NET buffer and immunoprecipitated with a specific anti-(type IX collagen) serum as described in the text. The immunoprecipitates were analysed on SDS/6%-acrylamide gels. Samples were: type IX collagen immunoprecipitated from medium before (tracks 1 and 6) and after (tracks 3, 9 and 10) reduction; type IX collagen immunoprecipitated from 1 M-NaCl extracts before (tracks 2, 7 and 8) and after (tracks 4, 5, 11 and 12) reduction. Samples in tracks 5, 8, 10 and 12 were treated with chondroitin ABC lyase. Tracks 6–8 were exposed for a longer time than were tracks 9–12, to reveal the minor 'α2(IX)-like' component.

proportion of the two forms in the tissue did not change with time of chase, suggesting that the GAG is not gradually removed when type IX collagen is deposited in the matrix. Moreover, analysis of the chase medium indicated a gradual increase with time ion the proportion of the PG form compared with the non-PG form, suggesting a preferential export of the PG form into the medium. The results therefore indicate that the PG and non-PG forms of type IX collagen are synthesized as two discrete populations.

It is obviously important to know whether the two molecular forms of type IX collagen have different functions. In particular, is only one form, or are both forms, bound to the type II collagen fibrils and integrated into the cartilage matrix? Although the collagens extracted from the tissue by both NaCl and guanidinium chloride have not been cross-linked within the matrix, those extracted by guanidinium chloride are believed to be more strongly associated within the matrix and therefore less easily extractable. The type IX collagen extracted by guanidinium chloride was present in a non-PG form, confirming our previous studies on non-radiolabelled cartilage [11], and suggesting that the non-PG form may be preferentially incorporated into the matrix. The selective release of the PG form into the medium would support this hypothesis. However, it is also possible that the two forms of type IX collagen exist in separate extracellular pools and that both become cross-linked within the tissue over longer periods of time. This possibility is difficult to test, since the solubilization of the cross-linked matrix by pepsin digestion results in the cleavage of the NC3 domain and the loss of the GAG chain (if present).

The electron-microscopic observations by Vaughan *et al.* [2] have led to the speculation that the GAG chain of type IX collagen could fit within the 'gap' region of staggered type II collagen monomers. However, there is no direct evidence for this localization, although it is possible that one of the GAG chains detected by the staining method of Scott [19] is bound to type IX collagen. The presence of GAG in the high-M_r aggregates that were specifically immunoprecipitated from the NaCl extracts by the type IX collagen antiserum indicates that the PG form of collagen type IX is capable of forming higher aggregates *in vitro*. However, these aggregates represented a small proportion of the total type IX collagen immunoprecipitated and were evident in extracts of cartilage that had been incubated with β-APN to prevent specific cross-linking. They may therefore merely represent non-specific aggregates of type IX collagen itself, as suggested by Noro *et al.* [9], rather than the specific type II/IX collagen assemblies observed *in vivo* [1,2]. In vitreous humour, the GAG chain is present on the matrix form of type IX collagen, is approximately 10 times larger than that attached to the cartilage collagen [20], and extends away from the type II collagen fibrils [21].

Type IX collagen resembles the small PGs in many respects, particularly the decorin type or PGII that binds to collagen types I and II via the protein core [22]. This ability to bind to collagens and to inhibit collagen fibrillogenesis is independent of the presence of the GAG chain [23]. Similarly, although type IX collagen does not inhibit fibrillogenesis, it decreases the ultimate diameter of the fibres formed, and this effect is also independent of the presence of the GAG chain [24]. It is noteworthy that a proportion of the PGII molecules in tendon and other tissues lacks a GAG chain [25], suggesting that the synthesis of PG and non-PG forms of extracellular-matrix macromolecules may be a common occurrence.

The financial support of the Arthritis and Rheumatism Council is gratefully acknowledged.

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Received 15 May 1991/20 June 1991; accepted 1 July 1991