Identification of the epitope for a monoclonal antibody that blocks platelet aggregation induced by type III collagen

Veronica GLATTAUER, Jerome A. WERKMEISTER, Alan KIRKPATRICK and John A. M. RAMSHAW*

CSIRO, Division of Biomolecular Engineering, 343 Royal Parade, Parkville, Victoria 3052, Australia

A library of eight conformation-dependent monoclonal antibodies that react with distinct epitopes on native human type III collagen has been examined for the ability of these antibodies to inhibit platelet aggregation induced by this collagen. Six of these antibodies had no effects; one, 1E7-D7/Col3, delayed the onset and slowed the rate of platelet aggregation, while another, 2G8-B1/Col3, completely inhibited aggregation. In order to identify the epitope recognized by this inhibitory antibody, a series of peptides that could fold to form triple-helical fragments was examined. Each peptide included six Gly-Xaa-Yaa triplets from

the human type III collagen sequence, where Xaa and Yaa represent the particular amino acids in the sequence, and a C-terminal (Gly-Pro-Hyp)₄ sequence to enhance triple-helical stability. Using these peptides we have identified the epitope as a nine-amino-acid sequence, GLAGAOGLR (where O is the one-letter code for 4-hydroxyproline), starting at position 520 in the human type III collagen helical domain. This sequence is proximal to the site proposed for the interaction of type III collagen with $\alpha_2\beta_1$ -integrin of platelets.

INTRODUCTION

The collagen family of proteins has long been recognized as providing the key structural components of the extracellular matrix, with the interstitial collagens forming the major fibrillar structures. More recently, however, with the wide range of distinct collagen types becoming evident, collagens have been recognized as having more complex functions through a broad range of specific interactions. The interplay between cells and collagens plays an important role in triggering a variety of specific cellular functions. The necessary collagen–cell interactions can occur via specific receptors as well as through cell-adhesion molecules, with the mechanism of interaction between ligand and receptor depending on the cell type. In the case of platelets, the receptor for collagen has been identified as the $\alpha_2\beta_1$ -integrin [1]. The binding domain on type III collagen for this $\alpha_2\beta_1$ -integrin of platelets has been partly defined by study of CNBr fragments [2,3], and a potential domain of interaction has been further examined by peptide models [4].

Previously we have described a range of conformation-dependent monoclonal antibodies (MAbs) to various mammalian collagens [5], including a group of eight antibodies directed against human type III collagen [6]. The epitopes for these eight MAbs have been shown, through mapping to specific CNBr fragments, to be distributed along the helix, and were not restricted to the telopeptide domains. It is possible, therefore, that the epitopes recognized by these MAbs may be in, or at least proximal to, binding domains on the collagen helix, and so capable of blocking particular biological functions.

In the present study we have examined whether any of these eight MAbs leads to changes in platelet aggregation induced by collagen type III. We have found that one of the MAbs led to a slowing of aggregation, while another resulted in the complete inhibition of aggregation. Identification of the epitope recognized by the MAb that blocks aggregation could give additional information on the region of the integrin binding site, although the epitope and the binding site may not necessarily coincide. To

identify this epitope, we have examined an overlapping series of peptides that could fold to form triple-helical fragments. Each peptide included six Gly-Xaa-Yaa triplets from the human type III collagen sequence, where Xaa and Yaa represent the particular amino acids in the sequence, and a C-terminal (Gly-Pro-Hyp)₄ sequence to enhance triple-helical stability. Using these peptides we have demonstrated the location of the epitope recognized by the MAb to type III collagen, and have shown that this epitope is proximal to the site proposed for the collagen–integrin interaction [4].

EXPERIMENTAL

Collagen preparation

Human placental type III collagen was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). It was purified by fractional NaCl precipitation at pH 2.5 and pH 7.4 [7], followed by rapid (NH₄)₂SO₄ precipitation [7], and by precipitation of refolded type III collagen after denaturation in 4 M guanidine hydrochloride [8]. Collagen purity was examined by SDS/PAGE [9] using 5% (w/v) running gels with 3.5% (w/v) stacking gels. To distinguish $\alpha 1$ chains of collagen type III from any $\alpha 1$ chains of collagen type I, an interrupted electrophoresis system was used [10]. Mouse type II collagen was obtained from Elastin Products Company (Owensville, MO, U.S.A.) and was used as supplied.

MAbs

A library of eight MAbs specific to human type III collagen was used; the preparation and characterization of these MAbs has been described previously [6]. MAbs were purified from murine ascites fluid using Protein G–Sepharose (Pharmacia) for IgG-class MAbs and IgM Affinisorb (Windsor Park Laboratories, Teaneck, NJ, U.S.A.) for IgM-class MAbs.

Abbreviations used: MAb, monoclonal antibody; PRP, platelet-rich plasma; O, single-letter code for 4-hydroxyproline (in sequences); Xaa and Yaa, particular amino acids in the collagen sequence; Ac, acetyl.

* To whom correspondence should be addressed.

Platelet assays

For preparation of platelets, whole blood was collected from a healthy donor by venipuncture and added to 0.1 vol. of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood at 160 *g* for 20 min. The top fraction, containing PRP, was collected and the remaining fraction was centrifuged at 3000 *g* for 10 min for preparation of platelet-poor plasma. Platelet counts were in the order of 3.0×10^8 platelets/ml.

Platelet aggregation was carried out using the method of Burns et al. [11]. Aggregation was measured in a Chronolog Model 703 Lumi Aggro-Meter, calibrated so that the platelet-poor plasma gave 10% attenuation and the PRP produced 100% attenuation.

For aggregation assays, human type III collagen fibrils were prepared by dissolving purified collagen (2 mg/ml) in 0.1 M acetic acid at 4 °C, followed by dialysis against 0.02 M Na₂HPO₄ at 4 °C, and incubation at 37 °C for a further 16 h. Fibril formation was confirmed by transmission electron microscopy. MABs were assessed for inhibitory effects on platelet aggregation by first incubating 30 μ l of purified MAB (1 mg/ml in PBS, or dilutions in PBS) with 30 μ l of collagen fibrils (200 μ g/ml) for 45 min at 37 °C. Aggregation was initiated by the addition of 50 μ l of the collagen fibril/MAB mixture to 450 μ l of PRP, which had been preincubated for 5 min at 37 °C. Aggregation was measured by the change in attenuation due to the decrease in turbidity. Controls for platelet function included samples of type III collagen fibrils without antibody, and type I collagen (bovine tendon) fibrils (Helena Laboratories, Beaumont, TX, U.S.A.).

Peptide synthesis

Peptides were synthesized to give the general structure acetyl-(Gly-Xaa-Yaa)₆-(Gly-Pro-Hyp)₄-Gly-Val, where the (Gly-Xaa-Yaa)₆ was a section of six consecutive triplets from human type III collagen [12]. The (Gly-Pro-Hyp)₄ sequence was included to help stabilize triple-helix formation, while the Gly-Val sequence at the C-terminus was present to eliminate possible diketopiperazine formation during synthesis. The peptides are designated by the residue at the N-terminus. For instance, peptide AcT3-505 includes residues 505–522 of type III collagen, while peptide AcT3-508 includes residues 508–525, with numbering based on the helical domain of the sequence of human type III collagen [12]. Ac represents the acetylated form of the peptide. Peptides were synthesized on an Applied Biosystems 430A Synthesizer using the standard FastMoc (Applied Biosystems) method using 9-fluorenylmethoxycarbonyl amino acids and 9-fluorenylmethoxycarbonyl-L-Val-*p*-alkoxybenzyl ester resin (WANG resin). Acetylation was carried out by treatment with acetic anhydride and triethylamine in dimethyl formamide. Side-chain protection was *t*-butyl for Hyp, *t*-butyl ester for Glu and Asp, *t*-butoxycarbonyl for Lys and 2,2,5,7,8-pentamethylchroman-6-sulphonyl for Arg. The purity of all peptides was greater than 95% by reverse-phase HPLC on a Vydac C18 column. Amino acid analysis using a Waters HPLC system with ninhydrin detection confirmed the compositions of the peptides. The preparation of one peptide, AcT3-487, using a standard *t*-butoxycarbonyl N-terminal protection strategy has been described previously [13].

CD spectroscopy

CD spectra were recorded on an Aviv Model 62DS Spectrometer. Peptide solutions (1.0 mg/ml) in 0.15 M NaCl and 20 mM sodium phosphate, pH 7.4, were equilibrated at 4 °C for 48–72 h

prior to analysis. Spectra were collected at 4 °C and 40 °C from 215 to 300 nm.

ELISA

Peptide solutions (1 mg/ml in water) were preincubated at 4 °C for 48 h to allow triple-helix formation prior to binding on to CovaLink NH plates (Nunc) for standard ELISA [14]. For coupling, each peptide was treated at 4 °C with 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide and 0.1 M *N*-hydroxysulphosuccinimide to produce an active ester of the peptide. This was then diluted 1:100 (w/v) with 0.1 M Na₂CO₃/NaHCO₃ buffer, pH 8.6, and reacted with the amino groups of the CovaLink NH plates [15].

Competitive ELISA was performed under non-equilibrium conditions [16]. MAB 2G8-B1/Col3, at a dilution that was not saturating, was incubated for 16 h at 4 °C with preformed triple-helical peptides at various concentrations ranging from 250 to 0.5 μ g/ml. For controls, the same dilution of MAB was incubated with PBS (negative control) or with human type III collagen (positive control). After incubation, an aliquot of each of the MAB/peptide mixtures was assessed for the amount of unbound MAB against an indicator plate of human type III collagen by standard ELISA.

RESULTS

MAB effects on collagen–platelet interactions

The library of eight MABs had been shown previously to interact with epitopes distributed over the full length of type III collagen [6]. They were screened to see which, if any, showed binding to type III collagen fibrils and interfered with the role of this collagen in platelet aggregation. The type III collagen was extensively purified; no type I collagen (which, if present as an impurity, could initiate platelet aggregation) was detected by interrupted SDS/PAGE.

In the absence of MAB pretreatment, the human type III collagen fibrils led to rapid platelet aggregation (Figure 1a). Further, with MAB pretreatment, most (six) of the eight MABs had no effect on platelet aggregation (Figure 1b). However, one MAB, 1E7-D7/Col3, delayed the onset and slowed the rate of platelet aggregation (Figure 1c), while another MAB, 2G8-B1/Col3, completely inhibited aggregation (Figure 1d). Addition of type I collagen fibrils to the PRP/collagen type III/MAB incubation shown in Figure 1(d) rapidly induced normal aggregation (Figure 1e), demonstrating that the inhibition caused by the MAB was specific and did not disrupt platelet integrity in general. The degree of inhibition of platelet aggregation was dependent on the antibody concentration, with concentrations between 500 and 50 μ g/ml showing complete inhibition, while 5 μ g/ml produced a partial effect, with a slower rate of aggregation (results not shown) than in control experiments without antibody.

Identification of the binding region for MAB 2G8-B1/Col3

The MAB 2G8-B1/Col3 had been shown previously to bind to the CB4 region of type III collagen [6]. Comparison of the human [12] and mouse [17] amino acid sequences for this CNBr fragment indicated that there were 15 sequence differences that could provide potential epitopes for the MAB.

Initially three peptides were synthesized that included regions of the sequence where multiple sites of variation occurred close to each other. These peptides were for the regions encompassing

Figure 1 Effects of MAbs on platelet aggregation induced by human type III collagen

(a) Platelet aggregation by human type III collagen fibrils. (b) Lack of inhibition of platelet aggregation by MAb 4F3-C7/Col3, an IgM. (c) Slowed aggregation by type III collagen fibrils after incubation with MAb 1E7-D7/Col3. (d) Complete inhibition of platelet aggregation by type III collagen fibrils after incubation with MAb 2G8-B1/Col3. (e) Continuation of the trace from (d); aggregation is induced by the addition of type I collagen fibrils. Time points of collagen or collagen/MAb additions are indicated by arrows. Time is shown on the horizontal axis, and attenuation is shown on the vertical axis. A decrease in attenuation indicates platelet aggregation.

residues 487–504 (AcT3-487), 517–534 (AcT3-517) and 544–561 (AcT3-544). Equilibrium melting studies on peptide AcT3-487 had previously shown that it formed a stable triple helix with a melting temperature at pH 7.0 of 26 °C [13]. That study had also indicated that addition of an acetyl group to the N-terminus of the peptide increased its melting temperature by about 4 °C. Comparison of the CD spectra at 4 °C and 40 °C for the other two peptides showed the loss on heating of the peak at 224 nm that is characteristic of triple-helical structure, suggesting the presence of triple-helical structures at 4 °C.

Direct ELISA of triple-helical peptides coupled on to CovaLink plates showed that one of these peptides, AcT3-517, which contained residues 517–534, gave a positive reaction with MAb 2G8-B1/Col3, whereas there was no reaction with either of the other two peptides, or when control MAbs were used. These

	Absorbance (405 nm)
AcT3-505: AcGGKG DAGA OGERGPOGLA (GPO) 4GV	0.07
AcT3-508: AcGDAGA OGERGPOGLAGAO (GPO) 4GV	0.08
AcT3-511: AcGAOGERGPOGLAGAOGLR (GPO) 4GV	0.31
AcT3-514: AcGERGPOGLAGAOGLRGGA (GPO) 4GV	0.20
AcT3-517: AcGPOGLAGAOGLRGAGPO (GPO) 4GV	1.22
AcT3-520: AcGLAGAOGLRGAGPOGPE (GPO) 4GV	1.17
AcT3-523: AcGAOGLRGAGPOGPEGGK (GPO) 4GV	0.08

Figure 2 Sequences of seven overlapping peptides

The sequences of seven synthetic peptides used for epitope mapping are shown; O represents the one-letter code for hydroxyproline. The region of sequence identity between the four peptides that gave positive ELISA reactivities (AcT3-511, AcT3-514, AcT3-517 and AcT3-520), presumed to represent the epitope recognized by MAb 2G8-B1/Col3, is indicated. Direct ELISA reactivity values of MAb 2G8-B1/Col3 with peptides coupled on to CovaLink plates are shown. Human type III collagen gave an absorbance of 1.32 units.

peptides had been coupled to the ELISA plate as preformed trimers. If monomer peptides were plated at 20 °C and then held in the presence of excess unbound peptide at 4 °C, a greatly decreased ELISA response was obtained.

A series of peptides was prepared that overlapped each end of AcT3-517, each differing by a translation along the sequence of three amino acids, i.e. one triplet (Figure 2). CD spectroscopy indicated that all peptides contained triple-helical content at 4 °C that was lost on heating. On examination of these peptides by direct ELISA, four gave positive reactivity with MAb 2G8-B1/Col3 (Figure 2), with AcT3-517 and AcT3-520 giving the strongest reactivities, comparable with that of human type III collagen. The differences in ELISA reactivity may be due to variations in chemical coupling caused by Glu side chains in the peptides. Examination of the peptides by a competitive ELISA (Figure 3) confirmed that the same four peptides reacted with MAb 2G8-B1/Col3 and competed effectively against human type III collagen. In this case, less variation was observed between the responses from the individual peptides.

Comparison of the sequences of the four peptides that gave positive ELISA reactions with MAb 2G8-B1/Col3 indicated that a sequence of nine residues, GLAGAOGLR, was common to all sequences (Figure 2), and is thus the probable epitope recognized by the MAb. Examination of the amino acid sequences for other collagens indicated that this sequence is not unique to human type III collagen. It is also present in both mouse [18] and rat (K. Urabe, G. Sarkar and M. E. Bolander, unpublished work; GenBank accession no. L48440) type II collagens, but at a different location (helical domain residues 421–429). Examination of mouse type II collagen by a direct ELISA gave a positive reaction with MAb 2G8-B1/Col3, although the absorbance was only 50 % of that observed with the human type III collagen at comparable concentrations. Competitive ELISA also indicated that the mouse type II collagen reacted with the MAb, but less effectively than in the direct ELISA, requiring four times as much antigen to obtain the same degree of inhibition compared with human type III collagen.

Figure 3 Competitive ELISA for synthetic peptides

Competitive ELISA is shown for MAb 2G8-B1/Col3 using a non-saturating amount of antibody and various concentrations of each peptide. Inhibition of antibody binding was observed with peptides AcT3-511 (○), AcT3-514 (□), AcT3-517 (△) and AcT3-520 (▽), but not with peptides AcT3-505 (●), AcT3-508 (■) or AcT3-523 (▲).

DISCUSSION

For globular proteins, the mapping of conformation-dependent epitopes generally requires detailed tertiary structure information, as polypeptide chain elements from different parts of the structure are normally involved. For antibodies against linear, non-conformation-dependent epitopes, scanning a series of overlapping peptides allows identification of the binding domain. For collagens, because of the rigid, rod-like structure of the molecule formed by the triple helix, a conformation-dependent binding domain is composed of linear peptide sequences. Thus, for collagens, a conformation-dependent epitope can be studied through peptide models, so long as the peptides are capable of refolding into the triple-helical conformation.

Various strategies have been described for synthesizing small, triple-helical collagen peptides. These include the use of a Lys-Lys starting sequence with unprotected ϵ -amino groups to allow synthesis of three linked chains [20,21], and the 'sandwiching' of a collagen sequence, typically of 9–12 amino acids, between (Gly-Pro-Hyp)₃₋₄ sequences, leading to triple-helix formation from single chains [4,22]. In the present study, a modification of this 'sandwich' approach was used in which the N-terminal (Gly-Pro-Hyp)₃₋₄ domain was replaced by additional residues from the collagen sequence, so that a sequence element of sufficient size (18 amino acids) was available for binding studies.

The data from the present study showed that certain of the peptides could bind to the MAb 2G8-B1/Col3, and this allowed the epitope recognized by this MAb to be identified as a nine-residue sequence, GLAGAOGLR (positions 520–528 in the sequence). With the exception of Gly-Pro-Hyp, even the most commonly occurring Gly-Xaa-Yaa (triplet) sequences in collagens are found at only low frequencies, so a specific three-triplet sequence would not be expected to be found in many other collagen sequences. In the present case, the epitope was also present in two rodent type II collagens, but at a different location in the molecule ([18]; K. Urabe, G. Sarkar and M. E. Bolander, unpublished work; GenBank accession no. L48440). The MAb was found to react with mouse type II collagen, supporting the identification of the epitope sequence. However, mouse type II collagen had a lower affinity for the MAb than human

type III collagen, possibly indicating a role for the local structure adjacent to the epitope in determining the affinity.

The epitope recognized by another MAb to type III collagen has previously been proposed [23]. This proposal was based on identification of a nine-amino-acid (three-triplet) repeat within the type III collagen sequence that explained the reaction of this MAb with two separate CNBr fragments, CB4 and CB8 [23]. This proposed epitope (GAOGLRGGA; positions 523–531) partially overlaps the epitope identified in the present study (GLAGAOGLR), and includes the second (GAO) and third (GLR) triplets plus a C-terminal GGA triplet. The epitope recognized by MAb 2G8-B1/Col3 is therefore distinct from that described by Hori and colleagues [23], since it would be expected that, if the epitope included the additional GGA triplet, then MAb 2G8-B1/Col3 should have reacted with CB8 in addition to CB4.

The location of the epitope recognized by MAb 2G8-B1/Col3 is close to the site on type III collagen proposed by Barnes and colleagues as being that recognized by the $\alpha_2\beta_1$ -integrin of platelets [2–4]. The epitope is not necessarily the integrin binding site, since the size of the MAb is such that it would sterically hinder a binding site within the adjacent region of the sequence. The effectiveness of 2G8-B1/Col3 in preventing platelet aggregation induced by type III collagen (Figure 1d) indicates that the off-rate for the antibody from collagen fibrils is probably very low. This may be an effect of this antibody being an IgM, although another IgM directed against human type III collagen showed no inhibition of platelet aggregation (Figure 1b). The slowing down of aggregation caused by MAb 1E7-D7/Col3 (Figure 1c) may be due to this MAb having a higher off-rate from the collagen fibrils; alternatively, its binding site may be further removed from the site of collagen–integrin interaction, such that its steric effects are limited. CNBr fragment mapping for 1E7-D7/Col3 indicated that it recognizes a different region of the sequence (fragment CB8; residues 243–371) [6]. For platelet aggregation, fibrillar collagen is necessary. Thus the effects of this MAb may be due to its epitope being close to the $\alpha_2\beta_1$ -integrin recognition site when the quarter-stagger arrangement of the collagen molecules in a fibril is taken into account.

The use of a peptide model for collagen has allowed the precise determination of the binding site for a conformation-dependent MAb. A wide range of macromolecules have now been described as showing specific, functional binding to collagen [24]. While the domains for the interaction of collagens with these other molecules have sometimes been broadly defined, the molecular details of the specific binding domains on these collagens are generally still poorly understood. The present study using a MAb indicates that peptide models are useful for accurately defining binding domains on collagens, and will be valuable in developing an understanding of the mechanisms of functionally important interactions.

We thank Dr. P. David Toman for sending us the deduced amino acid sequence of mouse type III collagen prior to its publication, Ms. Jacinta F. White for examining type III collagen fibrils by transmission electron microscopy prior to their use in platelet assays, and Professor Barbara Brodsky for helpful discussions.

REFERENCES

- Kunicki, T. J., Nugent, D. J., Staats, S. J., Orzechowski, R. P., Wayner, E. A. and Carter, W. G. (1988) *J. Biol. Chem.* **263**, 4516–4519
- Morton, L. F., Fitzsimmons, C. M., Rauterberg, J. and Barnes, M. J. (1987) *Biochem. J.* **248**, 483–487
- Zijenah, L. S. and Barnes, M. J. (1990) *Thromb. Res.* **59**, 553–566
- Morton, L. F., McCulloch, I. Y. and Barnes, M. J. (1993) *Thromb. Res.* **72**, 367–372
- Glattauer, V., Ramshaw, J. A. M., Tebb, T. A. and Werkmeister, J. A. (1991) *Int. J. Biol. Macromol.* **13**, 140–146

- 6 Werkmeister, J. A. and Ramshaw, J. A. M. (1991) *Biochem. J.* **274**, 895–898
- 7 Trelstad, R. L., Catanese, V. M. and Rubin, D. F. (1976) *Anal. Biochem.* **71**, 114–118
- 8 ChandraRajan, J. (1978) *Biochem. Biophys. Res. Commun.* **83**, 180–186
- 9 Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- 10 Sykes, B., Puddle, B., Francis, M. and Smith, R. (1976) *Biochem. Biophys. Res. Commun.* **72**, 1472–1480
- 11 Burns, G. F., Cosgrove, L., Triglia, T., Beall, J. A., Lopez, A., Werkmeister, J. A., Begley, C. G., Haddad, A. P., d'Apice, A. J. F., Vadas, M. and Cawley, J. C. (1986) *Cell* **45**, 269–280
- 12 Ala-Kokko, L., Kontusaari, S., Baldwin, C. T., Kuivaniemi, H. and Prockop, D. (1989) *Biochem. J.* **260**, 509–516
- 13 Venugopal, M. G., Ramshaw, J. A. M., Braswell, E., Zhu, D. and Brodsky, B. (1994) *Biochemistry* **33**, 7948–7956
- 14 Werkmeister, J. A., Ramshaw, J. A. M. and Ellender, G. (1990) *Eur. J. Biochem.* **187**, 439–443
- 15 Søndergård-Anderson, J., Lauritzen, E., Lind, K. and Holm, A. (1990) *J. Immunol. Methods* **131**, 99–104
- 16 Rennard, S. I., Berg, R., Martin, G. R., Foidart, J. M. and Robey, P. G. (1980) *Anal. Biochem.* **104**, 205–214
- 17 Toman, P. D. and de Crombrughe, B. (1994) *Gene* **147**, 161–168
- 18 Metsäranta, M., Toman, D., de Crombrughe, B. and Vuorio, E. (1991) *J. Biol. Chem.* **266**, 16862–16869
- 19 Reference deleted
- 20 Roth, W. and Heidemann, E. (1980) *Biopolymers* **19**, 1909–1917
- 21 Fields, C. G., Lovdahl, C. M., Miles, A. J., Matthias Hagen, V. L. and Fields, G. B. (1993) *Biopolymers* **33**, 1695–1707
- 22 Li, M.-H., Fan, P., Brodsky, B. and Baum, J. (1993) *Biochemistry* **32**, 7377–7387
- 23 Hori, H., Keene, D. R., Sakai, L. Y., Wirtz, M. K., Bächinger, H. P., Godfrey, M. and Hollister, D. W. (1992) *Mol. Immunol.* **29**, 759–770
- 24 Kadler, K. (1994) *Protein Profile* **5**, 554–555

Received 26 September 1996/14 November 1996; accepted 15 November 1996