ROLE OF POLYMORPHIC Fc RECEPTOR FcγRIIa IN CYTOKINE RELEASE AND ADVERSE EFFECTS OF MURINE IgG1 ANTI-CD3/T CELL RECEPTOR ANTIBODY (WT31)

WIL J.M. TAX, W. P.M. TAMBOER, COR W. M. JACOBS, LEON A. M. FRENKEN, AND ROBERT A. P. KOENE

Department of Medicine, Division of Nephrology, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands

Anti-CD3 monoclonal antibody (mAb) OKT3 is immunosuppressive, but causes severe adverse effects during the first administration ("first-dose reaction"). These adverse effects are presumably caused by cytokine release that results from T-cell activation. In vitro, T-cell activation by anti-CD3 mAb requires interaction with monocyte Fc receptors. The Fc receptor for murine IgG1, FcγRIIa, is polymorphic. In some individuals, murine IgG1 anti-CD3 mAb causes T-cell proliferation and cytokine release in vitro (high responders [HR]), whereas in individuals with the low-responder (LR) phenotype it does not. We have now investigated the role of this FcγRIIa polymorphism in the release of cytokines in vivo and the occurrence of adverse effects after the administration of WT31, a murine IgG1 anti-CD3/T cell receptor mAb. WT31 caused an increase of plasma tumor necrosis factor-α in all four HR patients and none of the five LR patients. In all HR patients except one, plasma γ-interferon and interleukin 6 also increased, and a first-dose response was observed, whereas no cytokine release or adverse effects occurred in any of the LR patients. WT31 caused lymphopenia in all HR and none of the LR patients. FACS analysis demonstrated that in HR patients, after the initial disappearance of CD3+ cells from peripheral blood, modulation of CD3 occurred, whereas in LR patients a high degree of coating of the lymphocytes was observed. Surprisingly, WT31 also induced a marked granulocytopenia, as well as a decrease of thrombocytes, in three of the four HR patients (and in none of the LR patients). These data provide direct clinical evidence that Fc receptor interaction determines the release of cytokines and the occurrence of adverse effects after administration of anti-CD3/T cell receptor mAb. Furthermore, these data suggest that tumor necrosis factor-α by itself is not sufficient to induce the first-dose reaction.

Monoclonal antibodies (mAb*) against the T cell antigen CD3 can be immunosuppressive during transplant rejection episodes, as illustrated by the mAb OKT3. The first administration of OKT3, however, usually evokes severe adverse effects that presumably are related to a massive release of cytokines in vivo. High serum levels of tumor necrosis factor-α (TNFα), γ-interferon (IFN-γ), and, to a lesser extent, interleukin (IL) 2 are observed a few hours after the first injection, presumably resulting from T-cell activation in vivo (1-3). Previous studies have demonstrated that in vitro T-cell activation and proliferation induced by anti-CD3 mAb are dependent on the presence of monocytes bearing Fcγ receptors that bind murine IgG (4, 5). Different human Fcγ receptors are involved in the binding of murine IgG, depending on the isotype of the antibody. Murine (m) IgG2a antibodies such as OKT3 interact with human FcγRI, whereas the Fcγ receptor responsible for binding of mIgG1 is FcγRIIa, an isoform of FcγRII that occurs on monocytes, macrophages, granulocytes, and thrombocytes (6). FcγRIIa is known to be polymorphic: mIgG1 anti-CD3 mAb induces (monocyte-dependent) T-cell proliferation in vitro in only 70% of normal Caucasian individuals (high responders [HR]), but not in low-responder (LR) individuals (4, 5). This genetic polymorphism of FcγRIIa is also reflected in the release of cytokines (IFN-γ and IL-2) observed in vitro when mononuclear cells are incubated with mIgG1 anti-CD3 mAb (7).

Murine IgG1 anti-CD3 mAb have not yet been tested in vivo for their immunosuppressive effect and adverse effects. In LR patients, the adverse effects might be milder or even absent. If at the same time the immunosuppressive effect would be independent of interaction with Fcγ receptors, such an anti-CD3 mAb might provide (at least in LR patients) immunosuppression without serious adverse effects (8). The present study was set up primarily to investigate whether WT31, an mIgG1 mAb directed against the CD3-associated T cell receptor (TCR), induces the release of cytokines in vivo and the cytokine-associated "first-dose reaction" more frequently in those patients whose FcγRIIa can bind mIgG1 (HR) than in LR patients.

MATERIALS AND METHODS

Monoclonal antibody WT31. WT31 is an mIgG1 mAb developed in our laboratory and is directed against the CD3-associated TCR (9, 10). The large-scale production of WT31 and its purification from culture supernatant were performed at the Dutch National Institute of Public Health and Environmental Protection, as described elsewhere (11).

Determination of FcγRIIa phenotype and genotype. To determine whether patients to be treated with WT31 were HR or LR, T-cell proliferation induced by WT31 in vitro was measured (4). The FcγRIIa genotype was also determined by analysis of genomic DNA, essentially as described by Osborne et al. (12). Briefly, DNA was extracted from peripheral blood cells and amplified by polymerase chain reaction. Polymerase chain reaction amplification products were.

1 These studies were supported by the Dutch Kidney Foundation (grant CB6.598) and by a senior fellowship (W. J. M. T.) from the Royal Netherlands Academy of Arts and Sciences.

2 Address correspondence to: W. J. M. Tax, PhD, Department of Medicine, Division of Nephrology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

* Abbreviations: HR, high responder; IFN-γ, γ-interferon; IL, interleukin; LR, low responder; m, murine; mAb, monoclonal antibody; TCR, T cell receptor; TNFα, tumor necrosis factor-α.
were then separated on agarose gel, blotted, and hybridized first with an oligonucleotide specific for the LR allele and then, after stripping, with an oligonucleotide specific for the HR allele. The sequences of the allotype-specific oligonucleotides have been published (12). Polymerase chain reaction products from cell lines U937 (homozygous HR) and K562 (heterozygous) were included as controls.

Treatment with WT31. This study was approved by the institutional ethical committee, and informed consent was obtained from all patients. WT31 was administered by intravenous infusion to nine patients with a rejection episode after kidney transplantation. The diagnosis of rejection was made using clinical criteria, and confirmed by histology of allograft biopsy (except in LR patient 4, for whom no biopsy could be performed). Four patients were HR, and five were LR. All LR patients received 6 mg of WT31 on the first day of treatment. The first HR patient also received an initial dose of 6 mg, but in view of the severe side effects (see below), the initial dose administered to the other three HR patients was 1 mg of WT31. Furthermore, corticosteroids (50 mg of intravenous prednisolone 1 hr before WT31 administration) were given to all HR patients except the first one. The same premedication was given to the first LR patient treated with WT31, but not to the other LR patients.

Monitoring of patients. Monitoring of adverse effects (clinical symptoms included fever, chills, dyspnea, nausea, diarrhea, and headache) was performed at regular intervals after each dose of WT31.

Plasma was obtained from blood samples collected in EDTA tubes before and during the first hours after the first administration of WT31, by immediate centrifugation at 4°C, and aliquots were stored at −70°C until tested. An immunoradiometric assay was used to measure TNFα, IFN-γ, IL-6, and IL-2 (Medgenix Diagnostics, Fleurus, Belgium).

Production of human antibodies against WT31 was measured by analyzing serum samples in an ELISA. Differential cell counts of peripheral blood were made before the first administration of WT31, during the first hours after starting WT31, and then daily. Lymphocyte subsets were determined daily, using direct immunofluorescence with anti-CD3 (anti-Leu-4), anti-CD4 (anti-Leu-3a), anti-CD8 (anti-Leu-2a), and anti-TCR (WT31) mAb labeled with phycoerythrin or fluorescein (Becton Dickinson, Mountain View, CA). Cells were also stained with fluorescein isothiocyanate-labeled anti-mouse Ig to evaluate the amount of murine antibody bound in vivo to the lymphocytes (“coating”). Flow cytometry was performed using a Coulter Epics Elite (Coulter Corp., Hialeah, FL).

Statistica. The hypothesis that WT31 would cause more cytokine release and cytokine-related adverse effects in HR patients was tested statistically by calculating one-tailed P-values in the Fisher exact probability test.

RESULTS

Responder status of WT31 patients. The responder status of patients to be treated with WT31 was initially determined by the T-cell proliferation assay. Individuals with an HR phenotype may be either heterozygous or homozygous genotypically. Recently, a method was described for analysis of the genotypes of both allotype-specific oligonucleotides. This method was applied to DNA obtained from peripheral blood cells from all patients who had been treated with WT31 (except LR patient 3 from whom no cellular material was available). As shown in Figure 1, the results from this genotyping confirmed the responder status determined by the T-cell proliferation assay. Furthermore, all HR patients were found to be heterozygous.

Clinical adverse effects of WT31 in HR patients. Administration of WT31 (6 mg) to the first patient treated with WT31 (HR patient 1) induced a clear-cut first-dose reaction, including high fever and chills, starting approximately 45 min after the start of the infusion. These side effects, and the low numbers of granulocytes and thrombocytes (see below), necessitated the interruption of WT31 treatment in this patient for several days. In view of the observed side effects, the other HR patients received a much lower initial dose (1 mg) of WT31, and prednisolone as premedication. Despite this, all HR patients except HR patient 2 had a first-dose response (although to a lesser extent than the first HR patient). One HR patient (patient 2) had some symptoms of the cytokine-related syndrome, but only on the day after the start of WT31 and not immediately afterward. In complete contrast, none of the five LR patients showed any sign of first-dose reaction, despite the fact that the first dose of WT31 was 6 mg and four of these patients had received no premedication with corticosteroids. Higher doses of WT31 (up to 30 mg/day) were given to two LR patients later during treatment, and these high doses were also tolerated very well.

Effect of WT31 on lymphocytes, granulocytes, and thrombocytes. Administration of WT31 induced an immediate and large decrease of lymphocytes (to values below 150/μl) in all HR patients, as illustrated for HR patient 4 in Figure 2A. Only a slight and transient decrease of lymphocytes occurred in LR patient 2 (maximal percent decrease after the first dose was 36%). In the other LR patients, the percent decrease of lymphocytes was 18% or less, as illustrated for LR patient 1 in Figure 2B.

In three of the HR patients, WT31 also induced a profound granulocytopenia (Fig. 2A). This granulocytopenia necessitated the interruption of WT31 treatment for 1 or several days in these patients. In HR patient 2, the number of granulocytes decreased only marginally. Although there were day-to-day fluctuations in the number of granulocytes, there was no consistent decrease of granulocytes in any of the LR patients, as illustrated for LR patient 1 in Figure 2B.

In HR patient 1, the number of thrombocytes (which was already low at the start of WT31 therapy) decreased further, and remained very low for several days. In the other HR patients, there was a short-lasting and modest decrease of thrombocytes (maximal percent decrease was 24%, 30%, and 14%). No decrease of thrombocytes was observed in LR patients.

Effect of WT31 on rejection episode. In HR patient 1, WT31 treatment had to be interrupted for several days because of

Figure 1. Genotyping of patients treated with WT31. DNA from peripheral blood cells was isolated and amplified by polymerase chain reaction. Southern blots obtained after agarose electrophoresis were then hybridized with oligonucleotides specific for the HR allele (a) or LR allele (b) of the FcγRIIA gene. Lane 1: water; lane 2: U937 (homozygous HR); lane 3: K562 (heterozygous HR); lanes 4–7: HR patients 4, 3, 2, and 1; lanes 8–11: LR patients 2, 1, 4, and 5.
profound granulocytopenia, as mentioned above. For the same reason, also in HR patient 4 only a few (low) doses of WT31 could be given (Fig. 2A). In the two HR patients to whom WT31 could be administered without interruptions, kidney function improved. In HR patient 2, serum creatinine decreased from 307 μmol/L before treatment to 163 μmol/L after the last dose of WT31. In HR patient 3, serum creatinine also decreased during WT31 treatment, as illustrated in Figure 3A. In the first two LR patients, we found no indications for an immunosuppressive effect of WT31, as illustrated for LR patient 1 in Figure 3B. Because of these results, and because the main interest was to study the cytokine-associated adverse effects in relation to the FcyRIIa polymorphism, it was decided to treat the following LR patients after the start of WT31 treatment is indicated. In the HR patient, WT31 appeared to be effective. In the LR patient, serum creatinine continued to rise despite an increased dose of WT31, and antithymocyte globulin (ATG) was then administered, resulting in a decrease of creatinine.

**Figure 2.** Total number of lymphocytes (▲) and granulocytes (■) during WT31 treatment of HR patient 4 (A) and LR patient 1 (B). Cells were counted daily before, during WT31 treatment, and a few hours after each administration of WT31.

In LR patients, there was no decrease in the percentages of CD3+ cells, CD4+ cells, and CD8+ cells increased again. The intensity of the CD3 staining, however, was about threefold lower during these final days of WT31 treatment than before treatment (the intensity of CD4 or CD8 staining did not change).

In LR patients, there was no decrease in the percentages of cells staining with anti-CD3 or anti-CD4 (Fig. 4), or anti-CD8. The intensity of staining with anti-CD4 and anti-CD8 antibodies remained at the same level, and there was only a slight decrease in the intensity of anti-CD3 staining. There was, however, a marked increase in the degree of coating of mononuclear cells from LR patients during WT31 treatment, whereas coating was virtually absent in HR cells (Fig. 5).

**Figure 3.** Serum creatinine during WT31 treatment of HR patient 3 (A) and LR patient 1 (B). On the x axis, the number of days before or after the start of WT31 treatment is indicated. In the HR patient, WT31 appeared to be effective. In the LR patient, serum creatinine continued to rise despite an increased dose of WT31, and antithymocyte globulin (ATG) was then administered, resulting in a decrease of creatinine.

**Cytokine release induced in LR patients.** In plasma samples obtained from HR patient 1 during the first hours after the start of WT31, very high concentrations of circulating cytokines were measured. Peak values were 1750 pg/ml for TNFα, 7600 pg/ml for IL-6, 10.4 IU/ml for IFN-γ, and 2.2 IU/ml for IL-2 (pretreatment values were 66 pg/ml, 16 pg/ml, 0.6 IU/ml, and 0.5 IU/ml, respectively). An increase of TNFα was measured in plasma samples from all HR patients but not in any of the five LR patients (Fig. 6). It is noteworthy that peak levels of TNFα of these HR patients were much lower than the level measured in the first HR patient who received a higher dose of WT31 and no premedication. No increase of IFN-γ was observed in the five LR patients, whereas increased concentrations were measured in all HR patients except the one who showed no immediate first-dose

**Figure 4.** Cells were counted daily before, during WT31 treatment, and a few hours after each administration of WT31. Granulocytes were counted daily before, during WT31 treatment, and a few hours after each administration of WT31.
% CD3- or CD4-pos.

0 20 40 60 80 100

CD3 (LR #2)
CD3 (HR #3)
CD4 (HR #3)
CD4 (LR #2)

Days after first WT31

0 2 4 6 8 10

Figure 4. Percentage of CD3+ or CD4+ lymphocytes during WT31 treatment of HR patient 3 (solid lines) and LR patient 2 (dashed lines): percentage of CD3+ cells in HR patient (○) and LR patient (○); percentage of CD4+ cells in HR patient (■) and LR patient (□). As described in the text, the expression of CD3 on recurring CD3+ lymphocytes of the HR patient was strongly decreased when compared with the pretreatment value.

MFI

1,000

800

600

400

200

0

0 1 2 3 4 5 6 7 8 9

Days after first WT31

LR #1

LR #2

HR #3

HR #2

Figure 5. Coating of lymphocytes in vivo after administration of WT31 to HR patients (solid lines) and LR patients (dashed lines). Mean fluorescence intensity (MFI) in arbitrary units is indicated on the y axis (○, HR patient 2; ■, HR patient 3; ○, LR patient 1; □, LR patient 2).

DISCUSSION

It has previously been suggested that Fc receptor interaction is responsible for the "first-dose effect" of anti-CD3 mAb. This hypothesis was mainly based on the results of experiments in the mouse model, e.g., studies with F(ab')2 anti-CD3 (13) or with anti-CD3 combined with anti-FcR antibodies (14). This study was set up primarily to test the hypothesis that in HR patients WT31 would cause more cytokine release and cytokine-related adverse effects than in LR patients. Our results clearly demonstrate that cytokine release and adverse effects indeed correlate with the presence of the HR allele of FcyRIIa. Increased values of plasma TNF-α were measured in all four HR patients, and in three HR patients there was also an increase of IFN-γ and IL-6, and a clinical first-dose effect. No adverse effects and no cytokine was observed in any of the five LR patients. The complete absence of TNF-α release in LR patients confirms that the antibody preparation is free of endotoxin (known to be a potent inducer of TNF-α [15]).

It is remarkable that one of the HR patients (HR patient 2) showed no immediate side effects of WT31 administration (although some adverse effects, including fever and myalgia, were observed on the second day of treatment). The cause for this discrepancy with the other HR patients is not entirely clear. The number of CD3+ cells in peripheral blood before treatment was similar to that of other HR patients. It may be relevant that, in contrast to the other patients, this patient was treated with WT31 soon after the transplantation (within the first month). At this time he received still high doses of basic immunosuppression, including prednisolone, which may have dampened cytokine release and side effects. In this patient there was an increase of plasma TNF-α, but no increased IFN-γ or IL-6. These findings are reminiscent of those obtained by Chatenoud et al. (16), who observed only
mild or no side effects after administration of BMA031 (an mIgG2b anti-TCR mAb) despite a sharp increase of TNFα but not IFN-γ. Similar results were obtained with an mIgA anti-CD3 mAb: TNFα but not IFN-γ increased, and adverse effects were significantly less than those observed with an mIgG2a of precisely the same specificity that induced both TNFα and IFN-γ (17). Apparently in addition to TNFα, IFN-γ is required to induce the characteristic first-dose reaction. This hypothesis is supported by the protective effect of anti-IFN-γ antibodies on anti-CD3-induced morbidity and mortality in mice (18).

The polymorphism of FcγRIIa was also reflected in the disappearance of lymphocytes from peripheral blood after the first dose of WT31, which occurred in all of the HR patients and none of the LR patients. In LR patients we also found no evidence for a transient lymphopenia during the first hours after administration of the antibody, as was seen with an mIgA anti-CD3 mAb (17). In a recent study it was demonstrated that in vitro mIgA anti-CD3 induced T-cell adhesion to endothelium, whereas for WT31 this was observed only with HR cells and not with LR cells (S. Buysmann, Academic Medical Center, Amsterdam, The Netherlands, personal communication 1996). It is conceivable that in HR patients this increased T-cell adhesion has contributed to the initial
decrease of peripheral T cells. The disappearance from peripheral blood of CD3+ cells caused by WT31 in HR patients only is consistent with published data obtained in patients treated with an mIgG1 anti-CD8 antibody showing that the CD8+ cells disappeared from peripheral blood only in HR patients (19). In humans, the disappearance of lymphocytes from peripheral blood induced by mIgG1 antibodies apparently requires FcR interaction. This contrasts with the findings in mice, where such a disappearance of lymphocytes occurs after administration of F(ab')2 fragments of anti-CD3 or anti-TCR antibodies (13, 20). One should be careful in extrapolating the results obtained in animal studies to the clinical situation. The outcome of treatment with a monoclonal antibody will presumably depend on many factors, including species, the affinity of the antibody, and its specificity.

The disappearance of CD3+ lymphocytes from peripheral blood of HR patients was confirmed by FACS analysis. One should keep in mind that in HR patients only the very small number of T lymphocytes that have not disappeared from the peripheral blood is involved in this FACS analysis. Even among this strongly decreased number of lymphocytes, the percentage of CD3+ cells decreased to approximately 20% or less. Furthermore, the intensity of immunofluorescence per lymphocyte was about fourfold lower than pretreatment values. This decreased CD3 intensity of HR cells can only for a small part be ascribed to coating of the cells with the anti-TCR antibody WT31, since in LR patients the degree of cell coating was much higher, and in these LR patients only a slight decrease of CD3 intensity was observed. We have previously reported that in vitro WT31 can only partly inhibit the binding of anti-CD3 mAbs (9). The most likely explanation of the decreased intensity of CD3 staining in HR patients is modulation of CD3/TCR from the cell surface after in vivo binding of WT31. The antigenic modulation apparently was not complete, since with the anti-Leu-4 antibody we could still detect a low-intensity staining on some T lymphocytes. This pattern (initial disappearance of T cells, followed by a period of CD3 modulation) is similar to that obtained with mIgG2a mAb OKT3 (21) and WT32 (22).

In LR patients, we observed virtually no disappearance of CD3+ cells from peripheral blood, or modulation of CD3. The most striking feature of the FACS analysis in the LR patients was the very high degree of coating of lymphocytes with the WT31 antibody. This finding further underscores that the HR allele of FcyRIIa is required for the disappearance of T cells from peripheral blood induced by mIgG1 mAb. In HR patients, most of the few T lymphocytes that remained present were also coated with WT31, but the degree of coating per lymphocyte was much lower than with LR cells.

A remarkable finding in the present study was the profound and long-lasting granulocytopenia in HR patients after administration of WT31. In a previous study with mIgG2a, anti-CD3 mAb WT32 granulocytopenia was not observed (as a matter of fact, granulocytosis occurred [22]). Immediately after administration of OKT3, a modest decrease (approximately 40%) of granulocytes has been described, but this decrease was transient and granulocyte counts returned to normal values within 1 hr (23). The differential effects on granulocytes of WT31 versus OKT3 and WT32 may be related to the fact that mIgG1 interacts with another human Fc receptor than mIgG2a (FcγRIIa and FcγRI, respectively), and that FcγRIIa but not FcγRI is present on granulocytes. The disappearance of granulocytes may be caused by an increased adhesion to endothelium, resulting from triggering of granulocyte FcγRIIa or perhaps mediated by WT31-induced granulocyte-macrophage colony-stimulating factor (24, 25). An alternative explanation is that in HR patients WT31 activates T cells to kill granulocytes, since anti-CD3/TCR mAb can induce nonspecific cytotoxicity against target cells that express the appropriate Fcγ receptor (26).

WT31 also caused a decrease of thrombocytes in HR patients. Again, such a decrease has not been observed with mIgG2a antibodies OKT3 or WT32. FcγRIIa (but not FcγRI) is present on thrombocytes. In a patient with B cell leukemia who was treated with an mIgG1 anti-idiotypic mAb, a decrease of thrombocytes also occurred, presumably as a result of the binding of immune complexes containing mIgG1 to FcγRIIa on the thrombocytes (27).

Anti-WT31 antibodies were present in serum of some but not all WT31-treated patients. Their presence did not correlate with the FcγRIIa polymorphism, although more patients in the HR group exhibited a humoral immune response to the mAb. This might be related to the fact that most of the HR patients received WT31 doses over a more prolonged period than the LR patients.

It is still uncertain whether interaction with Fc receptors is required for the immunosuppressive effects of anti-CD3 mAb in humans. In the two HR patients to whom WT31 could be administered without interruptions, kidney function improved, whereas in the first two LR patients we found no indications for an immunosuppressive effect of WT31, despite the fact that the WT31 dose was increased to 12 mg or even 30 mg. We real that the number of patients is too small to draw any definitive conclusions from the present data with respect to the immunosuppressive potential of WT31. A tentative conclusion, however, might be that WT31 may not be immunosuppressive in LR patients but it may be immunosuppressive in HR patients. If this is true, it would imply that FcR interaction plays a role not only in the adverse effects but also in the immunosuppressive mechanism.

We recently reviewed the possible clinical significance of the polymorphism of human Fc receptors for immunotherapy (8). The data presented here clearly demonstrate that the FcγRIIa polymorphism defined by in vitro experiments indeed has direct implications for the in vivo use of mIgG1 mAb, and provide direct clinical evidence that the occurrence of adverse effects of anti-CD3/TCR antibodies is correlated with the presence of an Fc receptor that can interact with the mAb. It is hoped that the knowledge obtained from pilot studies such as the present one can be helpful in designing suitably modified antibodies that combine optimal efficacy with minimal toxicity (28).

Acknowledgments. We thank Dr. E.C. Beuvery and co-workers at the Dutch National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands) for the purification of WT31. Louis van de Locht (Department of Hematology, University Hospital Nijmegen) assisted in preparing the oligonucleotides. The skillful assistance of the medical and nursing staff of our transplantation ward is gratefully acknowledged. We also thank C. Glauudemans for technical assistance in some of the initial experiments.
REFERENCES


13. Hirsch R, Bluestone JA, DeNenno L, Greer RE. Anti-CD3 F(ab')2 fragments are immunosuppressive in vivo without evoking either the strong humoral response or morbidity associated with whole mAb. Transplantation 1990; 49: 1117.


Received 10 May 1996.
Accepted 15 August 1996.