Transpl Int (2003) 16: 572–577
DOI 10.1007/s00147-003-0589-z

ORIGINAL ARTICLE

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Histamine-degrading enzymes as cellular markers of acute small bowel allograft rejection

Received: 6 May 2002
Revised: 23 August 2002
Accepted: 28 January 2003
Published online: 29 April 2003

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Abstract Intestinal histamine-degrading enzymes diamine oxidase (DAO) and histamine *N*-methyltransferase (HNMT) activities are relatively constant per individual and bowel segment, and they reflect the functional integrity of the intestinal mucosa. It was, therefore, hypothesised that a decrease in these enzymes could be indicative of acute rejection of an intestinal allograft. Enzymatic activities of DAO and HNMT were determined in mucosal biopsies of isogeneic (Lewis-to-Lewis, $n=48$) and allogeneic (Brown Norway-to-Lewis, $n=48$) heterotopic small bowel transplants in a rat model at various time periods. Allograft recipients were not given any immunosuppression. While no changes in enzyme activities were observed in isografts up to day 8 following transplantation, significantly reduced activities of both enzymes were found in all allografts 6–8 days after transplantation. Activities of both DAO and HNMT exhibited a strong negative correlation with the histological rejection score ($P<0.01$). We can conclude that DAO and HNMT activities in gut mucosa are reliable quantitative markers of acute intestinal allograft rejection in the rat that support histopathological analysis.

Keywords Small bowel transplantation · Allograft rejection · Rats · Biochemical markers · Diamine oxidase · Histamine *N*-methyltransferase · Histamine metabolism

Introduction

Although the clinical results of small bowel transplantation (SBTx) have improved significantly during the past decade, mainly due to the development of new immunosuppressive drugs and better patient management, early and late outcome of SBTx are still unsatisfactory when compared with other organ transplants [6]. Acute rejection is the most common complication of intestinal transplantation and remains the main obstacle to long-term success [16]. Early and reliable detection of mucosal injury is one of the crucial factors in the diagnosis of acute rejection and is usually done by histological examination of biopsy specimens obtained endoscopically. While an increase in serum transaminases, bilirubin and creatinine may indicate acute rejection after liver and kidney transplantation, no reliable biochemical marker has been established that is associated with the extent of the injury of the small bowel and that would reflect the function of the graft [9, 10]. Although a preliminary study reported a decline in serum citrulline concentration with increasing grade of acute cellular rejection in SBTx [17], predictive biochemical parameters are urgently needed for rapid diagnosis and successful treatment of early rejection episodes in SBTx.

Several animal models have been developed to study various aspects of SBTx. The Brown Norway (BN)-to-Lewis transplantation model in the rat is known to be highly allogeneic, with acute rejection occurring within a few days after grafting [26]. Since the heterotopic

transplant technique has been shown to be extremely useful for the assessment of morphological alterations after transplantation, we also decided to use it to determine new biochemical markers of acute rejection and to study their relevance for early diagnosis of small bowel allograft rejection. Acute rejection of such allografts leads to destruction of mucosal cells by immunological and inflammatory processes [8]; therefore, a biochemical marker suitable for the detection of acute rejection should exhibit a rapid change upon initiation of these cellular events.

Diamine oxidase (DAO) and histamine *N*-methyltransferase (HNMT) catalyse alternative pathways for inactivation of the inflammatory mediator, histamine [15]. While HNMT occurs ubiquitously in mammalian tissues, including the gut [2, 19], DAO is found in just a few organs of humans and rats, the intestine being one of the major sites of DAO expression [1]. DAO has been located in differentiated intestinal epithelial cells, where the enzyme is present in soluble form in plasma membrane-associated vesicular structures [5, 23]. The exact location of HNMT within the cell is still unknown.

Impaired histamine degradation resulting from alterations in DAO and HNMT activities is suspected to contribute to pathophysiological changes in various inflammatory and allergic diseases, especially of the gastrointestinal tract. It has been shown that inflammatory bowel diseases lead to a dramatic decrease in DAO activity in the affected region, and measurement of the enzyme released into the circulation after heparinisation has been proposed as a sensitive marker for assessment of the functional integrity of the intestinal mucosa [13, 14, 20]. For small bowel transplantation DAO and HNMT appear to be interesting, not only as mucosal marker enzymes, but also with respect to their role in histamine inactivation. A decrease in the expression and/or activity of these enzymes is accompanied by a reduced histamine degradation capacity that could enhance allograft rejection by histamine-mediated local inflammation. Therefore, we asked whether small bowel allograft rejection is associated with changes in the activities of DAO and HNMT in the graft tissue and whether these changes in enzymatic activities might be useful for early detection of this process.

Materials and methods

Animals

Male Brown Norway (BN) and Lewis rats weighing 200–220 g were used as donors and recipients in this study. They were purchased from Harlan–Sprague Dawley (Indianapolis, Ind., USA). The animals were maintained on a 12-h light/dark cycle and fed on commercially available rat chow and tap water ad libitum, which conforms with the NIH guidelines and the current national laws. The rats received 5% dextrose and normal saline ad libitum overnight before surgery.

Operating procedure

Rats were anaesthetised by isoflurane inhalation via the Med-Vet Anaesthesia Surgical system (VetEquip, Pleasanton, Calif., USA), which is designed for small animals. General anaesthesia was maintained with isoflurane inhalation during the whole operation. The abdomen was shaved and prepared with Betadine. The peritoneal cavity was opened under sterile conditions, and the organs were handled gently, moist Q-tips being used. One-step heterotopic small bowel transplantation was carried out as described previously, under surgical microscopy [18]. Briefly, the jejuno-ileum was removed from the donor animal and transplanted to the recipient. Using microsurgical techniques we anastomosed (10.0 nylon) the graft vessels to the infrarenal aorta and vena cava. We then anastomosed the proximal and distal lumen of the graft to the abdominal wall, using interrupted sutures (3.0 Vicryl). The abdomen was closed with two running sutures (3.0 Vicryl).

Postoperative care

The rats were kept on a warming blanket and put under a heating lamp for the first 24 h after surgery. They usually recovered from anaesthesia within a few hours of the operation. Immediately after surgery, they were given 0.4 mg/kg Buprenex (Reckitt & Coleman Pharmaceuticals, Richmond, Va., USA) subcutaneously for pain control. After the operating procedure, rats had access to regular food and water ad libitum.

Experimental groups

Group 1 ($n=48$): we performed syngeneic heterotopic small bowel transplantation (SBTx) from Lewis donors to Lewis recipients to assess the effect of the transplantation procedure; this group served as control. Six animals were killed for analysis on postoperative days (PODs) 1 to 8.

Group 2 ($n=48$): we performed allogeneic heterotopic SBTx from BN donors to Lewis recipients to investigate the effects of acute rejection on enzyme activities. Six animals were killed and analysed on each postoperative day until POD 8. Grafts were removed for overall inspection and fixation of representative aliquots of each tissue specimen for histopathological assessment or immediate freezing for later biochemical analysis.

Histopathological examination

Tissue specimens of the graft including the mesentery approximately 3 cm in length were fixed in 4% buffered formaldehyde for 2 days and then embedded in paraffin. Three 4-μm serial cross-sections per specimen were stained with haematoxylin–eosin. All samples were examined by a pathologist (P.O.) blind to the grouping. All histopathological abnormalities were recorded, and acute rejection was graded on a scale from 0 to 4, depending on the extent of mucosal injury, the degree of inflammatory infiltration and crypt apoptosis [12]. Grade 0 indicated no rejection; grade 1, incipient acute rejection; grade 2, mild acute rejection; grade 3, moderate acute rejection; grade 4, severe acute rejection.

Biochemical analysis

For determination of DAO and HNMT activities, frozen small bowel samples of 100–300 mg were thawed on ice and then homogenised, in an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) with an S25N-8G probe, at 10,000 rpm

for 30 s in five volumes of 20 mmol/l bis-Tris hydrochloride pH 7.0 containing 1 mmol/l phenylmethanesulphonyl fluoride. The homogenates were cleared by centrifugation for 10 min at 23,000 *g* and the supernatants were used to determine DAO and HNMT activities and protein concentrations. DAO activity was measured in a radiometric procedure with [1,4-^{14}C] putrescine as the substrate, essentially as previously described [22]. Briefly, 30 µl homogenate was incubated in a total volume of 100 µl containing 100 mmol/l sodium phosphate buffer pH 7.2 and 10 nCi [1,4-^{14}C] putrescine hydrochloride (0.45 mmol/l, specific radioactivity 0.222 Ci/mol; Amersham Pharmacia Biotech, Buckinghamshire, UK) for 30 min at 37 °C. The reaction was stopped by addition of 10 µl 10% perchloric acid, the pH was adjusted by addition of 50 µl 0.6 mol/l sodium carbonate pH 12.2, the reaction product Δ_1-[2,5-^{14}C] pyrroline was extracted into toluene containing 0.35% 2.5-diphenyloxazole, and the radioactivity was determined by liquid scintillation analysis. Background activity was measured in assays without homogenate. HNMT activity was measured by transmethylation of histamine by *S*-adenosyl-L-[methyl-^{14}C] methionine. The homogenate was pre-incubated for 15 min on ice with 100 µmol/l aminoguanidine to inactivate DAO activity. We then incubated 10 µl homogenate in a total volume of 100 µl containing 100 mmol/l sodium phosphate buffer pH 7.5, 50 µmol/l histamine, and 10 nCi *S*-adenosyl-L-[methyl-^{14}C] methionine (50 µmol/l, specific radioactivity 2 Ci/mol (Amersham Pharmacia Biotech) for 30 min at 37 °C. The reaction was stopped by addition of 60 µl 500 mmol/l boric acid/1,000 mmol/l sodium hydroxide followed by extraction of the reaction product N^{τ}-[methyl-^{14}C] methylhistamine into toluene/isoamyl alcohol (1:1) containing 0.17% 2.5-diphenyloxazole and determination of the radioactivity by liquid scintillation analysis. Background activity was measured in assays without histamine. We determined the protein concentration of the homogenates by the Bradford method [3], using a commercially available kit (Bio-Rad Laboratories, Munich, Germany). Mean specific enzymatic activities from duplicate assays of each sample were calculated in microunits per mg protein (µU/mg), where 1 µU converts 1 pmol substrate per minute at 37 °C.

Statistical analysis

Statistical analysis was by SPSS for Windows version 10.0 software package (SPSS, Ill.). Differences in enzymatic activities on different PODs within each group of animals were analysed with the Kruskal–Wallis test and the Mann–Whitney U test. We tested differences between the groups of iso-transplant and allo-transplant animals, using a general linear model with the group as fixed effect and the POD as random effect. We analysed correlations between DAO activity, HNMT activity and rejection score, using Spearman correlation statistics. A two-tailed *P* value below 5% was considered to be statistically significant.

Results

General observation

Only animals that survived the immediate postoperative period of 24 h were included in the study, whereas the rats that died during this span were considered to be technical failures and were excluded from analysis. All surviving animals (98%) recovered well from surgery and remained essentially healthy throughout the whole study period. All animals from both groups ate normally, had regular bowel movement, and showed no clinical signs of rejection.

Macroscopic findings

Isogeneic transplants from animals in group 1 had a normal appearance and did not show any gross abnormalities. By contrast, allogeneic grafts from animals in group 2 developed grossly visible abnormalities from PODs 6–8, such as dilatation and thickening of the wall with some peritoneal adhesions. The mesenteries showed moderate shortening when compared with control animals, and mesenteric lymph nodes were enlarged.

Histopathological findings

Histopathological examination revealed distinct abnormalities of the allografts, cellular infiltrates increasing with postoperative time, mucosal injury and crypt apoptosis, especially from PODs 4–8 (Fig. 1). All specimens obtained from PODs 6–8 showed signs of moderate-to-severe acute rejection. The mean grading of acute rejection in these tissue samples was 3.7 to 4 (Fig. 2). In comparison, more than 90% of small bowel isografts did not show signs of acute rejection when analysed histopathologically. Only three isografts were given a rejection score higher than 0 (2, 3, and 4) on PODs 4, 6, and 8 (Fig. 2). Additionally, reperfusion injury was observed in some grafts of both groups (Fig. 3).

Histamine-degrading enzymes

The mean activities of both DAO and HNMT were found to be lower in non-transplanted intestinal control samples from BN rats than those of Lewis rats. Mean ± SD DAO activity was 707 ± 174 µU/mg in Lewis rats ($n = 6$) and 457 ± 189 µU/mg in BN rats ($n = 6$), which was not significantly different (*t*-test, $P = 0.3$). HNMT activity was 348 ± 60 µU/mg in Lewis rats ($n = 6$) and

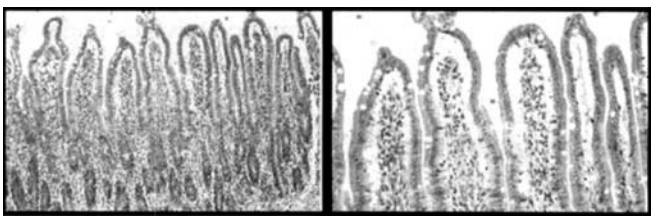

Fig. 1 Characteristic histopathological finding of severe acute allograft rejection in an animal of group 2, analysed on day 8 after allogeneic SBTx. This specimen, classified as grade 4 on the rejection score, shows the typical pathological changes associated with acute allograft rejection, including a marked degree of crypt damage, deep lymphocytic infiltration and abundant apoptotic activity. *Left panel* magnification ×100, *right panel* magnification ×200

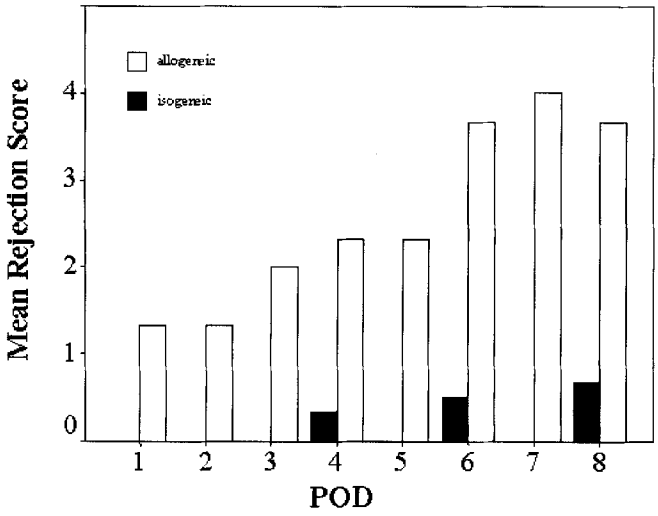

Fig. 2 Histological analysis of graft rejection. Three parallel samples per graft were fixed, stained with haematoxylin–eosin and scored for the extent of rejection on a scale from 0 (no rejection) to 4 (severe rejection). The mean rejection score for the isotransplant (*black bars*, group 1) and allotransplant (*grey bars*, group 2) animals is plotted against the POD. Six animals per group were analysed on each POD

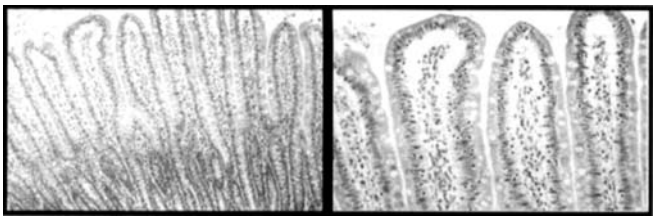

Fig. 3 Histological assessment of an animal from group 1, analysed on POD 8 after isogeneic SBTx. While no signs of acute rejection can be seen (grade 0), a reperfusion injury characterised by minimal changes with the range of alterations that affect the superficial epithelium is apparent in this specimen. *Left panel* magnification ×100, *right panel* magnification ×200

123 ± 56 μU/mg in BN rats ($n = 6$), which was significantly different (t-test, $P < 0.01$). However, these activities when measured in native small bowel samples (DAO = 707 ± 174 μU/mg; HNMT = 348 ± 60 μU/mg) did not significantly differ from those determined in the respective transplanted samples (DAO = 648 ± 280 μU/mg; HNMT = 305 ± 89 μU/mg) analysed on POD 1.

In recipients of a syngeneic graft, no significant changes were observed in the activities of DAO (Fig. 4, upper panel) or HNMT (Fig. 5, upper panel) up to POD 8. In contrast, both DAO and HNMT activities in the allografted tissue of group 2 decreased from PODs 1–8 (Figs. 4 and 5, lower panels). Mean DAO activity (232 μU/mg) from POD 6, and mean HNMT activity (40 μU/mg) from POD 7, were significantly lower than the respective activities that were determined on POD 1 (DAO = 457 μU/mg, HNMT = 123 μU/mg; Mann–Whitney U test, $P < 0.05$). We also confirmed the differing enzymatic activities of DAO and HNMT between isotransplant and the allotransplant animal groups, using a general linear model for statistical evaluation ($P < 0.01$).

Discussion

Small bowel transplantation has become a therapeutic option for patients with short-bowel syndrome or intestinal failure who do not tolerate long-term parenteral nutrition. Early diagnosis of acute rejection is desired, so that early graft loss can be prevented and satisfactory long-term results obtained. Biochemical markers indicating acute allograft rejection are available for kidney and liver transplantation [9, 10], but there are no such markers for the small bowel.

The highly allogeneic strain combination BN-to-Lewis rat has frequently been used in the study of acute allograft rejection and was, therefore, also used in this study. Heterotopic small bowel transplantation in rats is known to produce higher survival rates when compared with orthotopic SBTx, thus posing an ideal model for investigation of the acute rejection process [25].

This study used isogeneic and allogeneic heterotopic small bowel transplants to evaluate the usefulness of the histamine-degrading enzymes, diamine oxidase and histamine *N*-methyltransferase, as biochemical markers for acute small bowel allograft rejection. While no signs of acute rejection were observed macroscopically or histopathologically in most isotransplant animals up to POD 8, the extent of acute rejection markedly increased postoperatively in allotransplant rats, and signs of moderate-to-severe acute allograft rejection were found in all animals after POD 5. Three animals in the syngeneic group revealed histological signs of acute rejection in the absence of an allogeneic stimulus. This phenomenon has been described as occurring randomly in animal models but is not necessarily indicative of graft rejection [7]. Concomitantly, the activities of DAO and HNMT decreased in the allografted tissue after transplantation, reflecting the mucosal injury observed histologically. Moderate-to-severe acute rejection was associated with significantly decreased activities of both enzymes, and enzymatic activities correlated inversely with the rejection score.

Intestinal DAO activity and post-heparin plasma DAO activity have previously been used as sensitive markers for intestinal mucosal damage and mucosal integrity for various disease-associated alterations in animals and humans [4, 13, 14, 20]. Serum DAO activity has also been studied in an intestinal auto-transplantation model in dogs, which showed that DAO activity does not change in the absence of graft rejection [21]. In

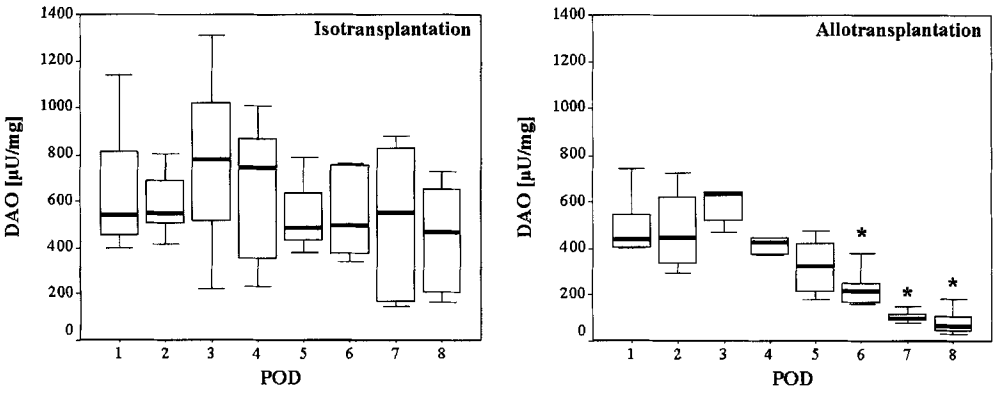

Fig. 4 Box plot of graft DAO activity in isotransplant (*upper panel*) and allotransplant (*lower panel*) animals on the given POD. The *boxes* represent the first and third quartiles, the *vertical lines* the ranges, and a *thick horizontal line* in each box shows the particular median value. Statistically significant differences between POD 1 and subsequent PODs were analysed with the Mann–Whitney U test and are indicated by *asterisks* ($P < 0.05$)

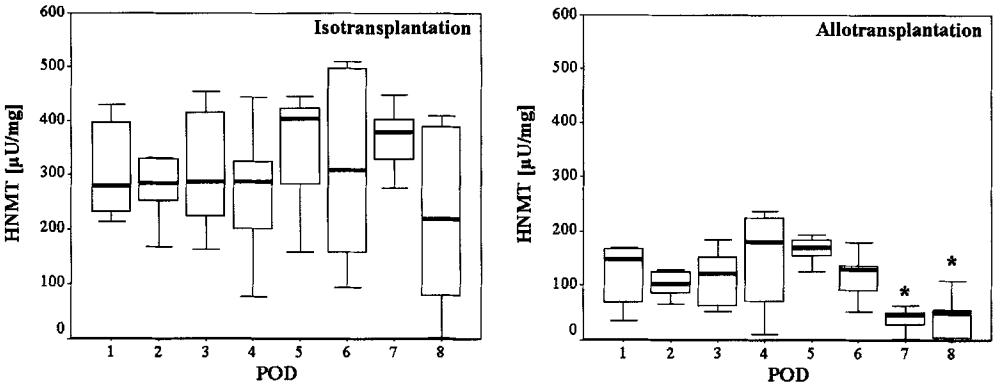

Fig. 5 Box plot of graft HNMT activity in isotransplant (*upper panel*) and allotransplant (*lower panel*) animals on the given POD. The *boxes* represent the first and third quartiles, the *vertical lines* the ranges, and a *thick horizontal line* within each box shows the particular median value. Statistically significant differences between POD 1 and subsequent PODs were analysed with the Mann–Whitney U test and are indicated by *asterisks* ($P < 0.05$)

a rat model of acute intestinal allograft rejection [24] it was found that post-heparin serum DAO was not useful as a marker, especially in the early postoperative period. Since the mechanism of DAO release and the correlation of serum DAO with intestinal DAO are not clear at present, we focused on the tissue enzyme levels that have been shown to be altered by mucosal-tissue damage. Our study demonstrates, for the first time, that acute allograft rejection causes a decrease in DAO activity in the graft, thus emphasising the usefulness of DAO as a biochemical marker of this process. Additionally, we were able to show a corresponding change in the activity of HNMT, catalysing the alternative pathway of histamine inactivation. Although decreased activities of DAO and HNMT generally reflect mucosal damage and are not specific for rejection, they allow assessment of the functional integrity of the graft in transplant patients.

The activities of both enzymes can reliably be determined in very small tissue samples obtained by endoscopic biopsies, by the use of sensitive radiometric assays [11, 22]. These biopsies can be easily performed for multiple samples in a routine setting. Therefore, DAO and HNMT constitute useful new biochemical markers that indicate acute small bowel allograft rejection. Such rejection appears not to be detected earlier by measurement of DAO and HNMT levels than by histopathological assessment, when groups of animals are analysed. This may be due to the individual variation in basal activities. However, if it is considered that DAO and HNMT activities are individually constant in healthy, functional gut tissue, and as there is a strong negative correlation of activities with rejection score, these enzymes will probably allow assessment of changes in the individual follow-up of transplant patients.

In addition to the possible diagnostic value, the fact that DAO and HNMT activities are decreased during acute allograft rejection should also be investigated with respect to changes in histamine metabolism. It is conceivable that a reduced capacity to degrade this inflammatory mediator may also play an important role in the progression of the acute rejection process itself. Decreased inactivation of released histamine may dramatically augment the local inflammatory reaction and thus accelerate the host's attack on the graft. Therefore, our study not only identifies new biochemical markers for acute allograft rejection, but also reveals metabolic changes that might be important for the inflammatory component of the rejection process and thus possibly offer new therapeutic strategies.

Both DAO and HNMT activities show a statistically significant strong inverse correlation with the rejection score (Spearman's correlation, $P < 0.01$). It appears that

these enzymes accurately reflect the extent of cellular damage that accompanies graft rejection in this intestinal transplantation model. We conclude that the histamine-degrading enzymes DAO and HNMT are excellent biochemical markers for the degree of mucosal damage associated with acute small bowel allograft rejection and provide quantitative data on the functional integrity of the graft, thereby supplementing histopathological analysis.

Acknowledgements This work was supported by grant P14923 from the Austrian Science Fund to HGS. The work performed by Monica T. Castanedes is gratefully acknowledged.

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