Naturally Induced Secretions of the Potato Cyst Nematode Co-stimulate the Proliferation of Both Tobacco Leaf Protoplasts and Human Peripheral Blood Mononuclear Cells

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Naturally induced secretions from infective juveniles of the potato cyst nematode *Globodera rostochiensis* co-stimulate the proliferation of tobacco leaf protoplasts in the presence of the synthetic phytohormones α -naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). With the use of a protoplast-based bioassay, a low-molecular-weight peptide(s) (<3 kDa) was shown to be responsible for the observed effect. This mitogenic oligopeptide(s) is functionally dissimilar to auxin and cytokinin and, in addition, it does not change the sensitivity of the protoplasts toward these phytohormones. In combination with the mitogen phytohemagglutinin (PHA), cyst nematode secretions also co-stimulated mitogenesis in human peripheral blood mononuclear cells (PBMC). The stimulation of plant cells isolated from nontarget tissue—these nematodes normally invade the roots of potato plants—suggests the activation of a general signal transduction mechanism(s) by an oligopeptide(s) secreted by the nematode. Whether a similar oligopeptide-induced mechanism underlies human PBMC activation remains to be investigated. Reactivation of the cell cycle is a crucial event in feeding cell formation by cyst nematodes. The secretion of a mitogenic low-molecular-weight peptide(s) by infective juveniles of the potato cyst nematode could contribute to the redifferentiation of plant cells into such a feeding cell.

Cyst nematodes are highly specialized plant parasites that intervene in the developmental program of plant cells. Feeding site formation is induced by pre-parasitic second-stage juveniles (J2), which penetrate the rhizodermis preferentially in the elongation zone and migrate intracellularly through the cortex in search of a cell with high meristematic potential near the vascular bundle (Golinowski et al. 1996). Feeding site

formation is not restricted to plant roots per se; cyst nematodes are able to induce feeding sites in stems and leaves of plants grown in vitro (Sijmons et al. 1991; A. Goverse, *unpublished observations*). The competence of a cell to differentiate into a feeding cell seems to depend on its position (preferably a cell opposite to the protoxylem pole) and on developmental cues (Scheres et al. 1997). Subsequently, this cell redifferentiates into a metabolically highly active transfer cell. In all cyst nematode-plant interactions, feeding cell induction is characterized by similar dramatic cytological changes. Partial cell wall dissolution results in the expansion of the feeding cell within the stele along the xylem. Subsequent fusion of the protoplasts results in a multinucleated cell complex, a syncytium, that can include up to 200 cells (Jones 1981). Studies on the morphogenesis of feeding cells induced by the cyst nematodes *Heterodera glycines*, *H. schachtii*, and *Globodera rostochiensis* (Endo 1986; Golinowski et al. 1996; Sembdner 1963, respectively) suggest that the ontogeny of feeding cells is accomplished by completion of a general developmental program irrespective of the plant organ—root, stem, or leaf—or the host plant.

Feeding cell proliferation coincides with a reactivation of the cell cycle (Gheysen et al. 1997), which is a prerequisite for the development and maintenance of the feeding cell. In *Arabidopsis thaliana*, activity of the cell cycle genes *cdc2aAt* and *cyc1At* was detected within a few hours after feeding cell induction by the beet cyst nematode *H. schachtii*. *cdc2aAt:gus* was strongly expressed inside the feeding cell. Expression of *cyc1At:gus* was weak inside the feeding cell, but strong in cells immediately surrounding the syncytium at the growing edges. Moreover, ³H-thymidine incorporation was observed in dividing cells prior to incorporation into the feeding cell (Niebel et al. 1996). The necessity of cell cycle activation for nematode development was shown by the application of oryzalin (inhibitor of mitosis) and hydroxyurea (inhibitor of DNA synthesis). Both resulted in a complete obstruction of feeding cell development in roots of *Arabidopsis* (De Almeida

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Engler et al. 1999). In addition, DNA endoreduplication was observed in the enlarged nuclei of elongating feeding cells (Endo 1971). The increase in copy number of essential genes could be functionally relevant in the metabolically highly active syncytium. It is assumed that cyst nematodes directly or indirectly induce the reactivation of the cell cycle by initial mitotic stimulation of the precursor cell prior to incorporation of adjacent cells in the growing syncytium (Gheysen et al. 1997). The intriguing question is how the infective nematode initiates these plant responses, thereby forcing a change in cell fate.

Nematode secretions are thought to play a key role in feeding cell development and maintenance (reviewed by Williamson and Hussey 1996). Microscopic observations indicate that stylet secretions of the salivary glands are released during migration through the plant root and injected into the selected root cell by pre-parasitic J2 (Wyss and Zunke 1986). Reduction of secretory granule density in the subventral esophageal glands corresponds with the preparation period during which the nematode stylet is inserted in the parasitized cell and no food ingestion takes place (Wyss 1992). These results suggest that esophageal gland secretions consist of pathogenicity factors, including those involved in feeding cell induction. Recently, the first pathogenicity factors of cyst nematodes were cloned. Four genes encoding endogenous cellulases were isolated from the cyst nematodes *H. glycines* and *G. rostochiensis*. These cellulases were demonstrated to be specifically expressed in the subventral glands of infective juveniles (Smant et al. 1998). Secretions of pre-parasitic J2 of *G. rostochiensis* can be collected upon exposure to potato root diffusate (PRD; Smant et al. 1997). These naturally induced secretions include functional cellulases. The ease with which natural secretions can be collected potentially facilitates the detection of regulatory molecules involved in feeding cell initiation and development.

This paper focuses on the biological relevance of nematode secretions in relation to plant cell proliferation. A tobacco protoplast-based bioassay was used to monitor tobacco leaf protoplast proliferation in response to secretions of infective juveniles of the potato cyst nematode *G. rostochiensis*. In the presence of the phytohormones auxin and cytokinin, cell division activity of the protoplasts was significantly enhanced by secretions. This is the first report describing a mitogenic compound in plant-parasitic nematode secretions. It is demonstrated that the protoplast activating compound(s) is a small peptide(s). Remarkably, nematode secretions also stimulated cell proliferation in human peripheral blood mononuclear cells (PBMC) in the presence of the mitogen phytohemagglutinin (PHA). These results suggest that nematode secretions are able to increase the proliferative response of both plant and mammalian cells.

RESULTS

Tobacco leaf protoplast proliferation assay.

Tobacco mesophyll protoplasts have the potential to divide in vitro (Nagata and Takebe 1970), and protoplast-based assays have been used to study the effect of external stimuli (Takahashi et al. 1989; Delbarre et al. 1994; Matsubayashi and Sakagami 1996). A tobacco leaf protoplast assay (Stickens and Verbelen et al. 1996) was adapted to investigate the effect

of nematode secretions on plant cell division. For each experiment protoplasts were isolated from the successive leaves of a 6-week-old in vitro plant. Protoplast proliferation was determined after 5 days of culturing and division activity was quantified by counting the number of dividing cells relative to the total number of cultured tobacco leaf protoplasts. Despite the rigorous standardization of plant growth and the protoplast isolation procedure, the cell division rate in standard medium supplemented with 1 µg of α-naphthaleneacetic acid (NAA) per ml and 1 µg of 6-benzylaminopurine (BAP) per ml ranged from 30 to 57% between the different protoplast batches (Figs. 1–6). However, provided that a single protoplast batch (protoplasts isolated from a single plant) was used as starting material, no significant differences were observed between replicates within an experiment (at $P > 0.1$; see Tables 1–5). Hence, this assay is reliable and distinctive as long as a single split protoplast batch is used per experiment.

Fig. 1. Dose-responsive stimulatory effect of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* on the proliferative response of cultured tobacco protoplasts in the presence of 1 µg of α-naphthaleneacetic acid (NAA) per ml and 1 µg of 6-benzylaminopurine (BAP) per ml. A gradual, significant increase in cell division rate was observed upon addition of secretions (at $P < 0.05$; see Table 1). As a control, 100 µl of standard pore water (SPW) was added instead of nematode secretions. Data are expressed as the percentage of dividing cells relative to total number of cells. Data presented are from two independent experiments (assay I and II).

Fig. 2. The proliferative response of tobacco leaf protoplasts in the absence (control) or presence of 100 µl of standard pore water (SPW). SPW is normally used for collecting nematode secretions. Protoplasts were cultivated in medium supplemented with 1 µg of 6-benzylaminopurine (BAP) per ml and variable α-naphthaleneacetic acid (NAA) concentrations for 5 days. Proliferation was expressed as percentage of dividing cells relative to total number of cells. Data presented are from one representative experiment out of three with comparable results. For SPW, the mean ± SD of two independent counts are shown.

Specific stimulation of cell division activity of tobacco protoplasts by secretions of *G. rostochiensis*.

Embedded tobacco protoplasts were exposed to increasing quantities of naturally induced secretions from infective juveniles (J2) of *G. rostochiensis* (5, 50, and 100 μ l) in the presence of 1 μ g of NAA per ml and 1 μ g of BAP per ml. Figure 1 shows a gradual, significant increase in the cell division rate upon addition of secretions (at $P < 0.05$; see Table 1). Addition of 100 μ l of secretions (equivalent to the amount produced by 1,000 secreting nematodes) resulted in a significant effect ($P < 0.001$ in all pairwise t tests; data not shown). Addition of 5 and 50 μ l of secretions did not significantly affect the cell division rate.

To test whether standard pore water (SPW)—the solution in which secretions were collected—affected protoplast proliferation, cells were exposed to medium with a fixed BAP concentration (1 μ g/ml) and a variable NAA concentration (0, 0.5, 1, 3, and 7 μ g/ml) in the presence or absence of 100 μ l of

Fig. 3. Heat and Pronase E treatments of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* result in loss of mitogenic activity of secretions in cultured tobacco protoplasts. Within individual protoplast batches no significant difference was observed between the proliferative responses after the addition of 100 μ l of standard pore water (SPW) and heat (secH) or Pronase E (secP) inactivated secretions (at $P > 0.1$). Effect of addition of nematode secretions (sec) on cell division rate is highly different from the control group (at $P < 0.001$; see Tables 2 and 3). Similar significant stimulation of cell division activity was observed for secretions incubated for 3 h at 37°C omitting Pronase E (secW). Data presented are from one representative experiment out of two with comparable results. Data of the treatments are expressed as the mean $\pm$ SD of triplicates.

Fig. 4. A low-molecular-weight (LMW) fraction (<3 kDa) of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* harbors a mitogenic compound(s). Effect of addition of the LMW fraction on cell division rate is highly different from that of the high molecular weight (HMW) fraction as well as from the addition of 100 μ l of standard pore water (SPW; at $P < 0.001$). Data presented are from one representative experiment out of three with comparable results.

SPW. SPW had no detectable effect on the division activity of the protoplasts in these treatments (Fig. 2).

Contribution of microbial contaminants could be excluded, since secretions were obtained from surface-sterilized, pre-parasitic J2. To check whether the antibiotic treatment was adequate, in vitro tomato or potato plants were inoculated with J2. This resulted in normal nematode development without microbial contamination (data not shown). Hence, the observed effect on cell division activity was induced by a compound(s) derived from *G. rostochiensis*.

To exclude the possibility that PRD (used for nematode hatching) was responsible for the observed mitotic stimulation of protoplasts, an excess of 100 μ l of PRD was added to the culture medium. At this concentration, PRD did not affect cell

Fig. 5. Co-stimulatory effect of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* in the presence of 1 μ g of 6-benzylaminopurine (BAP) per ml and variable low α -naphthaleneacetic acid (NAA) concentrations. Cultured tobacco protoplasts do not proliferate in the absence of NAA, but cell division activity was significantly enhanced in the presence of 100 μ l of secretions, compared with the control (100 μ l of standard pore water [SPW], at $P < 0.001$; see Table 4). Data presented are from one representative experiment out of two with comparable results. Data of the additive effect of secretions are expressed as the mean $\pm$ SD of two independent counts.

Fig. 6. Co-stimulatory effect of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* results in significant enhanced cell division rates in the presence of 1 μ g of 6-benzylaminopurine (BAP) per ml and variable high concentrations auxin (3 and 7 μ g of α -naphthaleneacetic acid (NAA) per ml; at $P < 0.001$; see Table 5). Standard pore water (SPW) was used as a control treatment. Data presented are from one representative experiment out of two with comparable results. Data of the additive effect of 100 μ l of secretions are expressed as the mean $\pm$ SD of two independent counts. Data presented in Figures 5 and 6 were obtained with different protoplast batches and, hence, they were analyzed separately.

division activity ($P > 0.1$ in both pairwise t tests; data not shown).

To check whether the observed effect could be attributed to nonspecific nematode compounds, different amounts of culture fluids collected from mixed life stages of the free-living nematode *Caenorhabditis elegans* were tested. Culture fluids from *C. elegans* had an inhibiting effect on protoplast proliferation at all concentrations tested ($P < 0.005$ in all pairwise t tests; data not shown).

These data demonstrate that the cell division activity of tobacco protoplasts is specifically enhanced by secretions of pre-parasitic J2 of *G. rostochiensis*. When protoplasts were exposed to secretions solely, no cell division was observed (data not

Table 1. Deviance table for effect of five different concentrations of potato root diffusate-induced secretions of infective juveniles of *Globodera rostochiensis* on cell division rate as tested in two independent protoplast batches

Source	df ^a	Deviance	Deviance ratio ^b	
Secretions	4	34.80	8.70	* ^c
Batch	1	2.39	2.39	NS
Residual	4	6.60 ^{d,e}		
Total	9	43.79		

^a Degrees of freedom.

^b Deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.

^c * = $0.01 < P < 0.05$; NS = nonsignificant ($P > 0.1$).

^d Residual deviance indicates goodness of fit of the model.

^e Residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, df = 4).

Table 2. Deviance table showing effect of Pronase E on mitogenic activity of secretions of infective juveniles of *Globodera rostochiensis* as tested in two protoplast batches and three independent replicates

Source	df ^a	Deviance	Deviance ratio ^b	
Secretions	2	70.17	35.09	*** ^c
Batch	1	10.01	10.01	**
Replicates	2	0.860	0.43	NS
Residual	12	14.03 ^{d,e}		
Total	17	95.07		

^a Degrees of freedom.

^b Deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.

^c ** = $0.01 < P < 0.001$; *** = $P < 0.001$; NS = nonsignificant ($P > 0.1$).

^d Residual deviance indicates goodness of fit of the model.

^e Residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, df = 12).

Table 3. Deviance table showing effect of heat treatment on mitogenic activity of secretions of infective juveniles of *Globodera rostochiensis* as tested in two protoplast batches and three independent replicates

Source	df ^a	Deviance	Deviance ratio ^b	
Secretions	2	43.51	21.75	*** ^c
Batch	1	5.97	5.97	*
Replicates	2	2.19	1.10	NS
Residual	9	11.81 ^{d,e}		
Total	14	43.79		

^a Degrees of freedom.

^b Deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.

^c * = $0.01 < P < 0.05$; *** = $P < 0.001$; NS = nonsignificant ($P > 0.1$).

^d Residual deviance indicates goodness of fit of the model.

^e Residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, df = 9).

shown). In the secretion concentration range tested, stimulation of protoplast cell division was only observed in the presence of both auxin and cytokinin. Addition of 100 μ l of secretions resulted in a significant increase in cell division rate ($+10 \pm 3\%$ [mean $\pm$ SD]) and this quantity was used for further testing.

Tobacco protoplast cell division is stimulated by an oligopeptide(s) released by infective juveniles of *G. rostochiensis*.

Proteolytic digestion of nematode secretions with the exo-protease Pronase E resulted in the inactivation of secretions (Fig. 3 and Table 2). Pronase E treatment of the secretions resulted in cell division rates that were significantly lower, compared with undigested controls (at $0.05 > P > 0.01$). Incubation of secretions for 3 h at 37°C per se did not affect mitogenic activity of the secretions. The effectiveness of the exo-protease Pronase E (1 μ g/ μ l) treatment was checked by digestion of total J2 homogenate (1 μ g/ μ l) at 37°C for 3 h. Complete digestion of the proteins was observed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining of the gel. Homogenate without Pronase E revealed the expected total protein pattern of J2 (data not shown).

Table 4. Deviance table showing (interactive) effect of secretions of infective juveniles of *Globodera rostochiensis* in combination with four low concentrations of α -naphthaleneacetic acid (NAA) on proliferative response of cultured tobacco protoplasts as tested in two protoplast batches and two independent replicates

Source	df ^a	Deviance	Deviance ratio ^b	
Secretions	1	87.32	87.32	*** ^c
Batch	1	28.39	28.39	***
Auxin	3	82.58	27.53	***
Replicates	1	3.83	3.83	NS
Auxin×Secr	3	9.21	3.07	NS
Residual	18	24.92 ^{d,e}		
Total	27	236.25		

^a Degrees of freedom.

^b Deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.

^c *** = $P < 0.001$; NS = nonsignificant ($P > 0.1$).

^d Residual deviance indicates goodness of fit of the model.

^e Residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, df = 18).

Table 5. Deviance table showing (interactive) effect of secretions of infective juveniles of *Globodera rostochiensis* in combination with three high concentrations of α -naphthaleneacetic acid (NAA) on proliferative response of cultured tobacco protoplasts as tested in three protoplast batches and two independent replicates

Source	df ^a	Deviance	Deviance ratio ^b	
Secretions	1	19.81	19.81	*** ^c
Batch	1	3.99	3.99	NS
Auxin	2	809.96	404.98	***
Replicates	1	3.22	3.22	NS
Auxin×Secr	2	7.49	3.74	NS
Residual	10	20.00 ^{d,e}		
Total	17			

^a Degrees of freedom.

^b Deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.

^c ** = $0.01 < P < 0.001$; *** = $P < 0.001$; NS = nonsignificant ($P > 0.1$).

^d Residual deviance indicates goodness of fit of the model.

^e Residual deviance is just above the chi-square threshold value of 18.3 (at $\alpha = 0.05$, df = 10).

The mitogenic activity of nematode secretions on cultivated tobacco leaf protoplasts was also lost by heat inactivation (Fig. 3 and Table 3). The addition of heat-inactivated nematode secretions resulted in cell division rates that did not differ significantly from those observed for the control protoplast population exposed to 100 μ l of SPW (at $P > 0.1$).

The mitogenic stimulus was demonstrated to be present in the low-molecular-weight (LMW; <3 kDa) fraction of the secretions (Fig. 4). Large differences were found between the cell division rates supplemented with the LMW fraction and the control treatments (at $P < 0.001$ in all pairwise t tests). No significant activity was observed in the high-molecular-weight (HMW; >3 kDa) fraction, which revealed cell division rates similar to those of the control treatments (at $P > 0.05$).

It is concluded that a LMW peptide(s) released by parasitic J2 of *G. rostochiensis* upon exposure to PRD is responsible for the observed enhanced cell division rate in this tobacco protoplast assay.

Secretions of *G. rostochiensis* infective juveniles co-stimulate auxin/cytokinin-induced protoplast proliferation.

Tobacco protoplasts divide in the presence of both NAA and BAP, and dose-response curves (e.g., Fig. 2) were used to obtain information about the nature of the active compound(s) in nematode secretions. Theoretically, nematode secretions could stimulate protoplast proliferation by changing the sensitivity of protoplasts toward auxin and/or cytokinin or by functionally replacing one of these phytohormones. An increased sensitivity of the protoplasts toward NAA should result in the curve in Figure 2 shifting to the left; a reduction of protoplast sensitivity should result in a shift to the right. Similar phenomena would be expected if the sensitivity toward BAP was changed. Alternatively, if secretions could functionally replace NAA, an increase of the protoplast division rate would be observed at below optimal concentrations of NAA (<1 μ g/ml). The largest effect would then be observed in the range of very low NAA concentrations. Increasing the NAA concentration

would lower the relative contribution of the secretions. At above optimal NAA concentrations (>1 μ g/ml), virtually no change in the shape of the curve would be expected. Similar changes could be foreseen when nematode secretions would functionally replace BAP.

Protoplasts were exposed to 100 μ l of secretions in the presence of suboptimal phytohormone concentrations. Addition of secretions resulted in a significant increase in cell division activity when protoplasts were cultured in low auxin concentrations (0, 0.05, 0.1, 0.5, and 1 μ g of NAA per ml) in combination with a fixed cytokinin concentration (1 μ g of BAP per ml) (Fig. 5 and Table 4). In a second assay, protoplast proliferation was also significantly enhanced by secretions in the presence of low cytokinin concentrations (0.01, 0.1, 0.25, 0.5, 0.75, and 1 μ g/ml) and a fixed NAA concentration (1 μ g/ml). This was based on calculated P values for the F tests of the mean deviance ratios (at $P < 0.001$; data not shown). Finally, the effect of secretions was investigated at above optimal auxin concentrations (3 and 7 μ g of NAA per ml versus 1 μ g of BAP per ml). Addition of 100 μ l of secretions again resulted in a significant increase in cell division activity (at $P < 0.001$; Fig. 6 and Table 5).

As shown in Figures 5 and 6, addition of nematode secretions resulted in an increase in protoplast cell division, which was apparently independent of the phytohormone concentration used. This implies that secretions do not functionally replace auxin/cytokinin or change the sensitivity of the protoplasts for these phytohormones. Statistical analysis of these data showed that there is indeed no interaction between the addition of secretions and the effect of either phytohormone on the cell division rate of cultured tobacco protoplasts. Mean deviance ratios for the interactive effect between secretions and, respectively, NAA (Tables 4 and 5) or BAP were not significant (at $P > 0.1$) at both below optimal and above optimal phytohormone concentrations. Hence, it is concluded that secretions co-stimulate tobacco protoplast proliferation in the presence of variable auxin and cytokinin concentrations.

Secretions of *G. rostochiensis* co-stimulate PHA-induced human PBMC proliferation.

To test whether secretions could also stimulate cell division in a mammalian cell system, a proliferation assay was conducted with human PBMC. PBMC are a mixture of lymphocytes including monocytes and T cells and proliferation of these cells can be induced by the addition of various mitogenic compounds or antibodies that activate receptor complexes present at the cellular plasma membranes. Mitogenic stimulation of the cells is routinely determined by measuring 3 H-thymidine incorporation during DNA synthesis.

Co-mitogenic activity of secretions was examined in the presence of the mitogen PHA. Incorporation of 3 H-thymidine was significantly increased after addition of 25 μ l of secretions (equivalent to the amount produced by 250 secreting potato cyst nematodes), compared with the response obtained in the presence of 25 μ l of SPW and 25 μ l of heat-inactivated secretions (Fig. 7). A strong enhancement of cell proliferation of approximately 44% was only detected for cells cultured in the presence of optimal concentrations of the mitogen PHA (25 μ g/ml). No mitogenic activity of secretions was detected in the absence of PHA or in the presence of a suboptimal concentration of PHA (5 μ g/ml). These data suggest that nematode

Fig. 7. Co-stimulatory effect of 25 μ l of potato root diffusate-induced secretions (sec) of infective juveniles of *Globodera rostochiensis* results in a significant increase in 3 H-thymidine incorporation during phytohemagglutinin (PHA)-induced peripheral blood mononuclear cells (PBMC) proliferation, compared with 25 μ l of standard pore water (SPW) or 25 μ l of heat-inactivated secretions (secH). Data presented are from one representative experiment out of two with comparable results. Data of treatments are expressed as mean $\pm$ SD of quadruplicates.

secretions only increase ^3H -thymidine incorporation when the upper limit of PHA stimulation is approached or reached.

This experiment was repeated with monoclonal antibodies (MAbs) raised to the accessory molecules CD2 and CD28 present on the plasma membrane of T cells. Addition of secretions did not result in a significant increase in ^3H -thymidine incorporation, neither at optimal nor suboptimal concentrations of the stimulating anti-CD2 /anti-CD28 MAbs (data not shown).

DISCUSSION

In this paper, evidence is provided that pre-parasitic J2 of the potato cyst nematode *G. rostochiensis* release (an) LMW peptide(s) upon stimulation by PRD. This oligopeptide(s) enhance(s) plant cell proliferation in the presence of the synthetic phytohormones NAA and BAP. It is demonstrated that nematode secretions neither change the sensitivity of the protoplasts toward NAA or BAP nor functionally replace these phytohormones. Hence, it is concluded that nematode secretions co-stimulate the proliferative response of tobacco leaf protoplasts. Since tobacco is a nonhost for *G. rostochiensis*, the stimulation of cell division in tobacco protoplasts by naturally induced secretions of the potato cyst nematode illustrates the activation of a general mechanism in plants. Moreover, nematode secretions co-stimulate mitogenesis in human PBMC in the presence of the mitogen PHA. Purification of the mitogenic compound(s) is in progress to elucidate whether similar activating principles are involved in both systems.

The role of peptide growth factors in animal and plant cell proliferation.

In animal systems, cells release and detect extracellular signaling molecules such as hormones, cytokines, and growth factors. This is presumably the most significant way in which animal cells communicate. Oligopeptides play a major role in cell-cell communication and in this respect insulin, glucagon, interleukins, and epidermal growth factors (EGF) can be named. A remarkable feature of these biologically active peptides is that all are formed by truncation of a larger polypeptide. Dozens of signaling peptides have been identified and the majority are recognized by specific receptors anchored in the plasma membrane of the recipient cell (reviewed by Hancock 1997). Mitogenic peptides play a major role in cell cycle control and re-activation of the cell cycle can be induced by these extracellular signal molecules (Hughes 1992).

Oligopeptides are also involved in communication between filarial nematodes and their host. The presence of *Trichostrongylus colubriformis* in the small intestine of sheep and goats is associated with epithelial renewal. It has been demonstrated that excretory-secretory (ES) products from this nematode are responsible for this cell proliferation. ES products from fourth-stage larvae of *T. colubriformis* stimulated HT29-D4 cell growth in vitro. This effect (partially) disappeared upon heat treatment and trypsin digestion (Hoste et al. 1995). This and other examples (Colina et al. 1990; Robinson and Gustad 1997; Deehan et al. 1998) indicate that the presence of mitogenic peptides in ES products from animal parasitic nematodes may not be uncommon.

In plants only two kinds of mitogenic oligopeptides have been identified so far. Expression of *enod40* in transgenic

roots of *Medicago truncatula* resulted in extensive cortical cell divisions, and bombardment of roots with an *enod40* expression cassette resulted in dedifferentiation and cell division of cortex cells (Charon et al. 1997). A second group are the phytosulfokines. PKS- α and PKS- β (a sulfated pentapeptide and a tetrapeptide, respectively) induce the proliferation of *Asparagus officinalis* mesophyll protoplasts, in cultures, that do normally not respond to any combination of phytohormones (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997). On the surface of rice cells, both high- and low-affinity specific saturable binding sites were demonstrated that were not occupied by auxin or cytokinin. This suggests the activation of a novel signal transduction pathway, by these oligopeptides in the presence of auxin and cytokinin, that is involved in plant cell proliferation (Matsubayashi et al. 1997, 1999).

Animal peptides stimulating the proliferation of plant cells have, to the best of our knowledge, not been reported before. A mitogenic oligopeptide(s) released by infective juveniles of the cyst nematode *G. rostochiensis* upon exposure to PRD could be pathogenesis related. Whether this oligopeptide(s) is co-responsible for syncytium formation remains to be proven.

Nematode secretions co-stimulate plant cell and PBMC proliferation.

In animal systems, it is often observed that combinations of mitogenic growth factors stimulate cell proliferation. Co-stimulation is a term used for growth factors, which solely do not induce cell proliferation but enhance the proliferative response of cells upon mitogenic stimulation (reviewed by Hughes 1992). An example of co-stimulation is the effect of the amphiphilic peptide substance P in PBMC proliferation in the presence of suboptimal concentrations of PHA. It is suggested that the neuropeptide substance P enlarges the proliferative response of PBMC to the mitogen PHA by increasing the intracellular Ca^{2+} concentration of the cells (Kavelaars et al. 1993).

In this paper, we demonstrate that naturally induced secretions from *G. rostochiensis* co-stimulate tobacco protoplast proliferation in the presence of auxin and cytokinin. Statistical analyses did not reveal an interaction between secretions and the concentrations of the phytohormones NAA or BAP on the proliferative response of tobacco protoplasts. This implies that nematode secretions neither change the sensitivity of the protoplasts toward NAA or BAP nor functionally replace these phytohormones. Similarly, nematode secretions co-stimulated PBMC proliferation in the presence of the mitogen PHA only. In both systems, nematode secretions increase the proliferative response of plant and mammalian cells given that the appropriate mitogens are present. As such, co-stimulation properly reflects the observed activity of potato cyst nematode secretions.

Specific co-stimulation of PHA-induced PBMC proliferation by mitogenic compounds of infective juveniles of *G. rostochiensis*.

Secretions of the potato cyst nematode activate human PBMC in the presence of the mitogen PHA. PHA is isolated from the red kidney bean (*Phaseolus vulgaris*) and is a mixture of five tetrameric glycoproteins. This multivalent lectin stimulates the proliferation of PBMC by cross-linking recep-

tors and accessory molecules on the cell surface of T cells and monocytes (reviewed by Geppert 1992). This elicits the production of growth factors—respectively, the interleukins IL-1 and IL-6 by monocytes, and IL-2 by T cells—that finally results in the activation of intracellular signal transduction pathways involved in proliferation of these cells (Kishimoto 1994). Thus, nematode secretions probably stimulate the production of interleukins by the T cells and/or the monocytes.

Specific activation of the T cell population can be achieved with the two stimulating MABs anti-CD2 and anti-CD28, which bind to the corresponding CD2 and CD28 accessory molecules only present at the T cell surface (reviewed by Fletcher and June 1992). Secretions were not able to co-stimulate PBMC proliferation in the presence of anti-CD2/anti-CD28. This result indicates that nematode secretions are not able to induce the production of interleukins in T cells and that the observed proliferative response is the result of interleukin production by the monocytes. Alternatively, nematode secretions stimulate parallel pathways, in T cells, that are solely induced in the presence of PHA. Since a large number of specific agonists and antagonists are available in combination with several mitogens, the use of standard PBMC proliferation assays will greatly facilitate the elucidation of signal transduction mechanism(s) underlying the co-stimulation of PBMC by naturally induced nematode secretions.

Mode of action of mitogenic oligopeptides from infective juveniles of *G. rostochiensis*.

Mitogenic secretions of the infective cyst nematode might be involved in feeding cell development. In potato and tomato roots, differentiated cortex cells opposite to the protoxylem poles are preferred for feeding cell initiation by *G. rostochiensis* (Jones and Northcote 1972; A. Goverse, *unpublished observations*). Induction of feeding cells coincides with the re-activation of the cell cycle by the parasitic nematode (Gheysen et al. 1997). It remains to be proven whether mitogenic peptides released by infective cyst nematodes are (co)responsible for the redifferentiation of these cells. It is remarkable to see that in roots of leguminous plants cortical cells in a similar position are susceptible to *Rhizobium* spp. Nod factors (mitogenic lipo-chitin oligosaccharides). These lipo-chitin oligosaccharides mediate re-entry of the cell cycle during nodule formation (Libbenga and Harkes 1973; Yang et al. 1994).

The mitogenic compound(s) present in cyst nematode secretions co-stimulate cell proliferation at extremely low concentrations. The secretions of a single infective juvenile suffice to increase cell division activity significantly in a pool of 160 PBMC or 200 tobacco leaf protoplasts. This peptide(s) could interact extracellularly with receptors anchored in the plasma membrane. Alternatively, this LMW compound could act intracellularly, bypassing the plasma membrane. The latter was demonstrated for amphiphilic oligopeptides such as the wasp venom peptide mastoparan (Higashijima et al. 1990) and the neuropeptide substance P (Mousli et al. 1990a), which act by direct activation of the G-protein complex (Mousli et al. 1990b). During feeding cell induction, the nematode carefully protrudes its stylet through the cell wall. Hence, nematode secretions may be deposited between the plasma membrane and the cell wall or secretions may be released directly into the cytosol through a perforation in the plasma membrane at

the stylet orifice (Williamson and Hussey 1996). So, from the cyst nematode's biology one cannot deduce whether its secretions act directly on cytosolic compounds or interact with membrane receptors present in tobacco protoplasts.

In summary, we conclude that secretions of infective juveniles of the potato cyst nematode *G. rostochiensis* consist of a mitogenic peptide(s) that is capable of intervening in signal transduction pathways involved in plant and animal cell proliferation. Though by no means proven, mitogenic compound(s) in secretions could be co-responsible for feeding cell development upon nematode infection. The detection of such key intermediates, which might be involved in communication between parasite and host, will contribute to the understanding of feeding cell induction and development by sedentary plant parasitic nematodes. Our findings support the hypothesis that manipulation of plant cell development by cyst nematodes is preceded by initial mitogenic stimulation of the cell. As a next step we will identify this secreted oligopeptide(s) and investigate its role in feeding site induction.

MATERIALS AND METHODS

Nematode secretions.

Secretions were collected from pre-parasitic J2 from the potato cyst nematode *Globodera rostochiensis* Ro1 Mierenbos, basically according to Smant et al. (1997). Thousands of dry cysts were rehydrated in tap water on 100- μ m nylon sieves for 7 days at 20°C. Large numbers of pre-parasitic J2 were obtained by hatching the nematodes in 0.22- μ m-filter, sterilized PRD for 3 days. The nematode suspension was transferred to conical glass tubes and mixed with freshly prepared 70% (wt/vol) sucrose in a 1:1 ratio. A 1-ml layer of tap water was placed onto the (35%) sucrose solution and the J2 were collected by centrifugation at $1,000 \times g$ for 5 min. The J2 were washed three times with tap water by inverting the glass tube.

Pre-parasitic J2 were surface sterilized by incubating the nematodes successively in a cocktail of 0.5% (wt/vol) streptomycin sulfate-penicillin G (20 min), 0.1% (wt/vol) ampicillin-gentamicin (20 min), sterile tap water (5 min), and 0.1% (vol/vol) chlorhexidin-digluconate, followed by three washing steps of 5 min in sterile tap water. Finally, surface-sterilized juveniles were concentrated and suspended in sterile SPW (Schouten and Van der Brugge 1989) supplemented with 15 mM NaCl. Juveniles were placed in sterile glass petri dishes in a final density of approximately 10,000 J2 per ml of SPW and incubated overnight at room temperature. The suspension was transferred into sterile glass tubes to concentrate the juveniles by centrifugation at $1,000 \times g$ for 2 min. Supernatant containing secretions released by the nematodes was removed and stored in 1-ml aliquots at -80°C. The protein content of the supernatant was too low to be quantified by the method of Bradford (1976). Therefore, the concentration was expressed as the amount of secretions produced overnight by the number of pre-parasitic J2 per microliter of SPW.

Different developmental life stages of the free-living nematode *Caenorhabditis elegans* strain Bristol N2 were obtained from a stock culture kindly provided by H. Van Luenen (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Nematodes were collected, surface sterilized, and incubated as described above for *G. rostochiensis*. Culture fluid

was obtained by incubating the nematodes in SPW omitting 15 mM NaCl to circumvent osmotic stress for this nematode species.

Fractionation of secretions.

Secretions obtained from surface-sterilized infective juveniles of *G. rostochiensis* were separated into two molecular weight fractions: proteins with a molecular mass of 0–3 kDa or >3 kDa, respectively. Microcon microcentrator units with a membrane cut-off of 3 kDa (Amicon, Beverly, MA) were rinsed with sterile SPW before adding sterile secretions. LMW products were obtained by centrifugation of the microcentrator at $14,000 \times g$. HMW products were retained on the 3-kDa cut-off membrane and were washed with an excess of SPW. Finally, they were taken up in sterile SPW corresponding to the original sample volume.

Inactivation of secretions.

Inactivation of secretions was achieved by heat treatment or proteolytic digestion. Secretions were heat inactivated by incubation at 100°C for 10 min. Proteolytic digestion was performed by complete digestion of sterile secretions in SPW with 1 µg of the exoprotease Pronase E per µl (Sigma, St. Louis, MO) for 3 h at 37°C. Pronase E was removed by centrifugation at $14,000 \times g$ with a Microcon microcentrator unit with a membrane cut-off of 3 kDa (Amicon, Beverly, MA). As a control, secretions were incubated under the same conditions in the absence of Pronase E.

Proliferation assay with tobacco leaf protoplasts.

For each assay, protoplasts were isolated according to Denecke et al. (1989) from successive leaves of a single 6-week-old plant of *Nicotiana tabacum* cv. Samsun NN. This plant was grown in vitro on Murashige and Skoog culture medium containing 3% sucrose with a regime of 16 h light. Leaves were incubated in TEX enzyme solution 1.2% (wt/vol) cellulase Onozuka R10 (Yakult Honsha, Tokyo) and 0.5% (wt/vol) macerozyme R10 (Yakult Honsha) omitting phytohormones for 20 h at 28°C. Protoplasts were collected over a 80-µm nylon sieve and concentrated in 1 ml of W5 medium by centrifugation at $500 \times g$ for 5 min. To determine the number of protoplasts, the cells were transferred to a Falcon 14-ml polystyrene tube (Becton Dickson Labware, Lincoln Park, NJ) into a final volume of 10 ml of W5 solution. After 30 min, protoplasts were washed twice with W5 solution by centrifugation at $300 \times g$ for 5 min. Protoplasts were resuspended in TEX medium to a final concentration of 2×10^5 protoplasts per ml. Protoplasts were embedded in a so-called liquid-thin-layer culture (Stickens and Verbelen 1996; Potrykus and Shillito 1986). For each treatment, 1 ml of the protoplast suspension was carefully immobilized in 6-cm disposable petri dishes by addition of 1 ml of 1.2% (wt/vol) SeaPlaque agarose (Duchefa, Haarlem, The Netherlands) in TEX medium at 37°C. The thin agar layer was covered with 2 ml of TEX medium supplemented with the synthetic auxin NAA and cytokinin BAP.

Unless indicated otherwise, the final NAA and BAP concentrations were 1 µg/ml (discounting the volume of the solidified culture medium). Aliquots of secretions were added to the TEX-hormone solution. In controls, an equal volume of SPW was added instead of secretions. Petri dishes were

wrapped with Parafilm and incubated for 5 days in the dark at 24°C.

Microscopic observations of cell division rate.

Embedded tobacco protoplasts were observed with an inverted light microscope (Leitz, Wetzlar, Germany). The cell division rate was determined for each treatment by counting the number of dividing cells relative to the total number of cells. Per treatment several spots were randomly selected throughout each petri dish, and all the vital cells present in that particular location were examined ($\times 200$ magnification). Protoplasts were classified either as dividing or nondividing.

Statistical analysis.

To estimate the systematic effects of multiple variables on a counted response variable in the form of a ratio of counts, regression analyses were carried out by the use of generalized linear models (McCullagh and Nelder 1989). The explanatory variables such as replicate, protoplast batch, auxin concentrations, presence/absence of secretions, and plant hormone $\times$ secretion interactions were fitted to the cell division rate by means of logistic regression models with the variance proportional to the binomial variance and a logit-link function. The deviance was used as a measure for goodness of fit for the model. The model was accepted when the residual deviance did not exceed the chi-square test value (McCullagh and Nelder 1989). The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate. *F* tests for the mean deviance ratios were used to assess the treatment effects. Pair-wise differences between treatment means on the logit scale were assessed with *t* tests. All statistical analyses were performed with Genstat 5 version 3.2 (Genstat 5 Committee; Payne et al. 1987).

Proliferation of human PBMC.

Human PBMC were isolated from heparinized blood, obtained from the National Bloodbank (The Netherlands), by centrifugation over Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradients (1.077 g/cm^3) at $1,000 \times g$ for 20 min (Kavelaars et al. 1995). Cells (4×10^4 per well) in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 5% fetal calf serum (FCS) were stimulated to proliferate with 25 µg of PHA (Murex Diagnostics, Chatillon, France) per ml in a final volume of 150 µl. PHA is a component in aqueous extracts of seeds of *Phaseolus* spp. In addition, cells were cultivated in the presence of suboptimal concentrations of PHA (5 µg/ml) or in the absence of PHA. Cells were incubated at 37°C and 5% CO₂ for 3 days. An excess of ³H-thymidine (1 µCi) was added to the cells 16 h before the end of the culture. Cells were harvested by the use of an automated cell harvester (Skatron, Lie, Norway) and incorporation of ³H-thymidine was determined with a scintillation counter. Alternatively, cells were stimulated to proliferate with anti-CD2₁ plus anti-CD2₂ MAb (1:8,000) in combination with anti-CD28 MAb (1:1,000; CLB, Amsterdam). Mitogenic activity of naturally induced nematode secretions of pre-parasitic J2 from *G. rostochiensis* was determined in the presence of above mentioned concentrations of mitogen by including 25 µl of sterile secretions in medium of the cells. Specificity of the response was determined by the addition of

25 µl of SPW and 25 µl of heat-inactivated secretions. Each treatment was done four times in two independent proliferation assays with PBMC from different donors.

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