

Immunomodulating properties of lymphocytes activated by farmer's lung associated bacterium *Thermoactinomyces vulgaris* in vitro

H. J. W. JÄGERROOS

Department of Clinical Microbiology, University of Kuopio, Finland

Thermoactinomyces vulgaris, one of the aetiological agents of farmer's lung, was analysed for its ability to activate non-immune spleen lymphocytes from 10- to 14- week-old mice. The lymphocytes were found to exert both enhancing and suppressing signals after activation, depending on the secondary stimulation used. The enhancing effect is due to interleukin-2 secreted mainly by L3T4+ lymphocytes. The suppressive action is associated with Lyt2+ lymphocytes, which are probably activated by prostaglandins or histamine; but the suppression inducer action of L3T4+ lymphocytes may also be important. Genetic regulation, independent of H-2K and D, was also detected between different inbred strains of mice in response to *T. vulgaris*. The genetic regulation appeared associated with the different responsiveness of Lyt-2 positive lymphocytes between different strains. These results emphasize the importance of nonspecific activation in hypersensitivity disorders induced by microbial antigens.

Key words: lymphocytes; nonspecific activation; *Thermoactinomyces vulgaris*.

Accepted for publication 12 July 1988

Polyclonal lymphocyte proliferation can be induced *in vitro* by a wide range of bacterial preparations. Other immunostimulatory effects include: 1) release of soluble mediators, 2) direct activation of complement, 3) production of antibodies, and 4) activation of mononuclear phagocytes and natural killer cells (3). It has been suggested that some of the *in vitro* actions serve in the first line of defence against bacterial infections *in vivo* (14). Nonspecific immunological activation has also been associated with certain hypersensitivity disorders. One of the widely studied agents with such actions is the Gram negative bacteria lipopolysaccharide (LPS). LPS is associated with human lung disorders (18), and a wide spectrum of immunological responses has been shown after LPS treatment *in vitro*. These include B-lymphocyte transformation and release of interleukin 1 and prostaglandins (6).

Farmer's lung is an allergic alveolitis caused

by inhalation of the products of bacteria and fungi growing in moldy hay. Histologically, the disease is characterized by granulomatous pneumonitis which can lead to interstitial fibrosis. The participation of both humoral and cell-mediated immune response against the antigen is well established (2). In Finland, *Thermoactinomyces vulgaris*, a thermophilic actinomycete, is often associated with the disease (20). In previous studies we have shown that intranasal sensitization of mice with *T. vulgaris* causes a response that, both histologically and immunologically, resembles hypersensitivity pneumonitis (9, 16). In *in vitro* experiments components from *T. vulgaris* were shown to induce immunoactivation. This included both lymphocyte proliferation and macrophage activation with release of interleukin 1 and prostaglandins (7). *In vitro* stimulation with *T. vulgaris* was found to induce functional differentiation of mouse spleen lymphocytes (8).

In local immunization the recruitment of lymphocytes to the lung has been nonspecific (1). Nonspecific activation has also been shown in other circumstances during the response to microbial antigens (11, 12). The inflammatory stimuli provided by several species of bacteria are also well known (3). As there are few studies of the nonspecific immunological activation by etiological agents of farmer's lung disease the inflammatory or regulatory role of such actions is poorly understood. The aim of this study was to investigate further the cellular differentiation and functions of lymphocytes activated with *T. vulgaris*, with emphasis on the characterization of helper- and suppressor-like activity.

MATERIAL AND METHODS

Inbred C57BL/6J (B6), DBA/2N (D2) and Balb/c mice (Zentralinstitut für Versuchstiere, Hannover, FRG) were raised in the Laboratory Animal center of the University of Kuopio. They were given water and food ad libitum. Mice of both sexes aged 10–14 weeks were used.

An extract of *T. vulgaris* was prepared from the mycelial growth (Mycological Reference Laboratory, London, U.K.). *T. vulgaris* was grown on Tryptic soy agar (Difco Laboratories, Michigan, U.S.A.) for 3–5 days at 50°C. After incubation the growth was scraped off the plates with phosphate-buffered saline containing 0.5 % (v/v) formalin. The growth was washed 3 times to remove all media components and resuspended in the same solution. This suspension was then sonicated for 5 min (MSE 1174 Mk2, Crawley Down, Sussex, U.K.) and dialysed extensively against distilled water at 4°C. The dialysate was freeze dried, and the dry weight measured.

The medium used in all experiments was RPMI 1640 (Flow Laboratories, U.K.) supplemented with 5 % foetal calf serum (K.C. Biological, Lenexa, Kansas, U.S.A.), penicillin (200 IU/ml), streptomycin (200 µg/ml), and glutamine (0.3 mg/ml). The incubation atmosphere was 5 % CO₂ and the temperature was +37°C.

Lymphocytes from mouse spleen were prepared as described previously (9) and stimulated with *T. vulgaris* in total volumes of 5–15 ml of culture medium in 25 ml tissue culture bottles (Nunc, Copenhagen, Denmark). The cell concentration was 4×10^6 /ml and the optimal mitogenic concentration of *T. vulgaris* (50 µg/ml) was that indicated in Results. Incubation time was 72 h because preliminary tests have shown this to induce the greatest activity. After incubation, the cells were washed three times with RPMI 1640, and incubated for 1 h in a petri dish with culture medium in a CO₂ chamber. This step was done in order to remove adherent macrophages from the cell population. After this, DNA synthesis was blocked with Mitomycin C treatment by resuspending the stimulated cells to medium containing 50 µg/ml of Mitomycin C (Sigma Chemical Co., Mo, U.S.A.). After 30 min incubation, the cells were washed three times with RPMI 1640 and used as effector cells. ³H-thymidine uptake of the effectors was tested each time. Normal spleen lymphocytes blocked in the same way were used as controls.

Effector cell function was assayed by stimulating spleen lymphocytes, i.e. responder cells in the presence of effector cells. To avoid allogeneically mixed lymphocyte culture, responder and effector cells were always from the same inbred strain of mice. The cells were stimulated by suboptimal concentration of 1 µg/ml of Concanavalin A (ConA, Pharmacia Fine Chemicals, Sweden). In preliminary tests pokeweed mitogen and antigen-induced stimulation were also investigated, but in the experiments presented here only ConA was used. The responder cells were incubated in U-bottom microtiter plates for various lengths of time in a total volume of 250 µl. Total number of responder cells in one well was 2.0×10^5 and the same number of effectors was used in the stimulation. Indomethacin (Dumex, Copenhagen, Denmark), an inhibitor of prostaglandin synthesis, cimetidine (Medica, Finland), an histamine₂ receptor blocker, or cyclosporin A (Sandoz, Basel, Switzerland), an inhibitor of IL2 synthesis, was used in some experiments. Indomethacin and cyclosporin A

were used at a final concentration of 1 µg/ml, while cimetidine was used at a concentration of 10 µg/ml. For the last 6 h of the incubation ³H-thymidine (Amersham, Buckinghamshire, U.K.), was added; the samples were harvested with a semi-automatic harvester, and the acid precipitable radioactivity was measured in a liquid scintillation counter (Ultro-Beta, Wallac, Finland).

For characterization of effector cell phenotypes, spleen lymphocytes stimulated with *T. vulgaris* were studied with flow cytometry. The cells were stained with fluorescein isothiocyanate (FITC) anti-immunoglobulins (Dako, Denmark, Copenhagen) and T-lymphocytes subpopulations were stained with phycoerythrin-conjugated anti L3T4 or anti-Lyt-2 followed by FITC-anti-rat (Becton & Dickinson, CA, U.S.A.). The cells were counted in a FACScan software (Becton & Dickinson, CA, U.S.A.) with the gate set for lymphocytes.

In some experiments certain subpopulations of effector cells were depleted by incubating them with antibody and complement. For this purpose 50 µl of the cell suspension was mixed with 10 µl of anti-Lyt-1 or anti-Lyt-2 (Becton & Dickinson, CA, U.S.A.) or 20 µl of anti-mouse immunoglobulins (Dako, Copenhagen, Denmark) and incubated 60 min at 0°C. The cells were washed, resuspended in 50 µl of guinea pig complement, diluted two-fold, and incubated 45 min at 37°C. The cells were then washed and resuspended in culture medium. The efficacy of the treatment was controlled by immunofluorescent staining of the residual effectors with the above-mentioned sera; the secondary antibody was fluoresceinisothiocyanate- conjugated anti-rat serum. The effectors were counted under a Leitz Orthoplan fluorescence microscope with appropriate filters. The treatment was considered satisfactory if the number of effectors stained with a given serum was reduced to less than 10 % of the original.

RESULTS

In the first set of tests the kinetics of the responder stimulation influenced by the effectors was analysed. As expected from results of previous experiments, effector-induced helper activity was detected. At days 5-9, however,

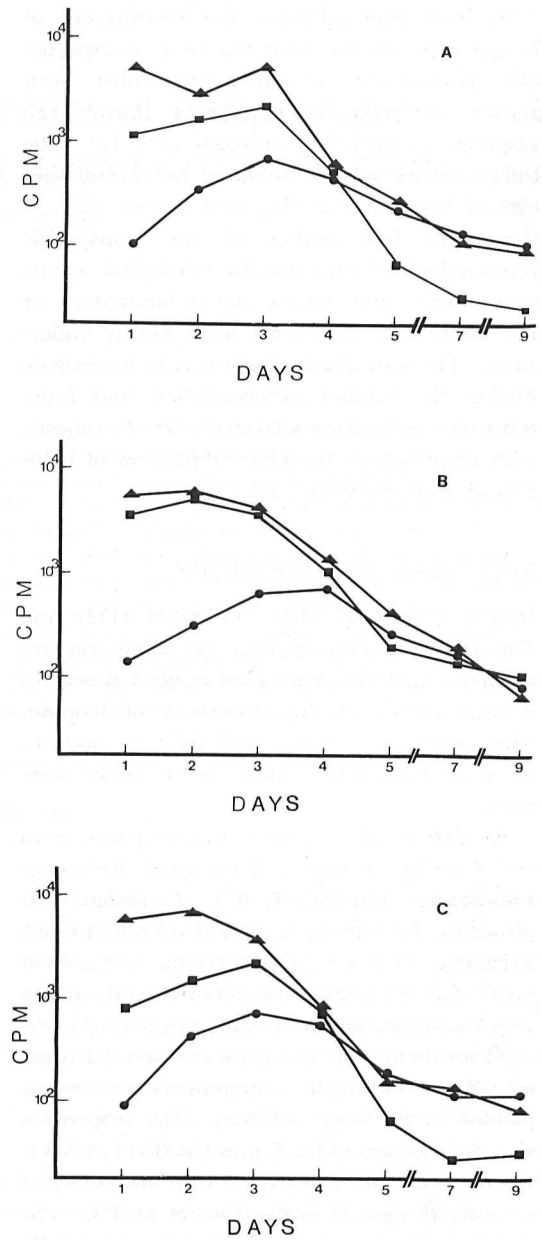


Fig. 1. Responders stimulation curves from Day 1 to Day 9. To the stimulations are added controls (●), effectors (■) or effectors and indomethacin (▲). A = B6 mice, B = D2 mice, C = Balb/c mice. The results are expressed as mean of the duplicates. SD < 15 %

the helper activity changed to suppressor activity in B6 and Balb/c mice. The lymphocytes from D2 mice were an exception. In the

animals helper activity was detected; but at longer responder incubation times the stimulation curves followed those of the controls with no detectable suppressor (Fig. 1). Supernatant solutions from effector cell cultures could induce some helper effect but were devoid of any suppressive effect.

To study the roles of prostaglandin and histamine in this model, indomethacin and cimetidine, respectively, were used. Indomethacin blocks the cyclooxygenase pathway of arachidonic acid metabolism, which leads to the synthesis of prostaglandin, prostacyclin and thromboxane. Cimetidine blocks the histamine₂ receptor; its affinity to histamine₁ receptor is low. In these experiments both indomethacin and cimetidine augmented the helper activity at days 1-4 during responder stimulation in B6 and Balb/c mice. With these agents no changes were seen in D2 stimulation. Typical results at Day 3 are shown in Table 1. Without effectors neither indomethacin nor cimetidine had a detectable effect on the stimulation of responder cells.

With longer incubation time indomethacin,

and to a lesser extent cimetidine, restored the activity. The previously detected difference between inbred strains of mice was also evident at this time. The action of both indomethacin and cimetidine appears to augment responder proliferation during the whole secondary stimulation. It first acts to enhance the helper effect and later to reverse the suppressive effect. Typical results at Day 7 are shown in Table 2.

Interleukin-2 has been shown partly responsible for the helper effect (8). To test this finding, cyclosporin A was used because its best-characterized action is blocking synthesis and release of interleukin-2 (19). It did not, however, affect the development of the suppressor activity. Results from Day 3 and Day 7 are shown in Table 3.

T. vulgaris treatment was found to stimulate preferably T-lymphocytes (Table 4). Both L3T4 + Lyt-2- and L3T4 - Lyt-2 + T-lymphocytes were found to proliferate, but the ratio of these cells changed in favour of L3T4-Lyt-2 + towards the end of the stimulation in Balb/c but not in D2 mice. The percentage of stained cells indicates double-staining of some cell popula-

Table 1

The effect of indomethacin (I) and cimetidine (C) on effector (E) influenced responder (R) stimulation at day 3

	B6	Balb/c	D2
R	2567 ± 354	2679 ± 243	4457 ± 345
R + Co	3111 ± 341	2972 ± 312	5211 ± 686
R + E	12456 ± 1341	12610 ± 1100	44012 ± 3567
R + E + I	61078 ± 4675	53231 ± 1279	43621 ± 4152
R + E + C	43786 ± 6534	39856 ± 3576	45987 ± 3549
R + E + I + C	59671 ± 3498	54243 ± 4719	47681 ± 5673

The results are expressed as mean ± SD. Co = controls

Table 2

The effect of indomethacin (i) and cimetidine (C) on effector (E) influenced responder (R) stimulation at day 7

	B6	Balb/c	D2
R	3300 ± 132	3413 ± 453	2197 ± 234
R + Co	3255 ± 245	3249 ± 354	2637 ± 302
R + E	435 ± 43	567 ± 231	2289 ± 312
R + E + I	2987 ± 312	3397 ± 213	2413 ± 159
R + E + C	2572 ± 238	3019 ± 354	2456 ± 354
R + E + I + C	3014 ± 254	3299 ± 160	2354 ± 457

The results are expressed as mean ± SD. Co = controls

tions, the most likely being L3T4 + Lyt-2 +. The depletion of effector subpopulations indicated that both Lyt-1 + and Lyt-2 + T-lymphocytes act in this model. Depletion of B-lymphocytes (surface membrane Ig+ cells) had no effect, thus indicating their nonreactivity as effectors. Results from depletion experiments with B6 effectors are shown in Table 5.

DISCUSSION

T-lymphocytes stimulated *in vitro* with *T. vulgaris* were investigated in a model similar to that used in assay of human suppressor cells stimulated with ConA (4). Both the enhancing and suppressing actions of effectors can be detected, depending on the incubation time of the responder. Suppressor activity can only be seen when incubation times of responder cells are longer, but down regulation of the helper effect is already seen with shorter incubation times. Suppressive activity induced with related microbes has been shown earlier (10). The suppression found with longer incubation time could be an artefact caused by a change in the kinetic curve of the secondary stimulation or simply by deteriorated viability of the responders. This would mean that the helper effect moves the kinetic curve to the left, which would naturally lead to the impression of suppression because of the proliferative cycle which ends earlier with declining incorporation of thymidine. On the basis of the present findings this would not seem to be the case. Cyclosporin A, which destroys the helper activity, cannot reverse the suppression. Both indomethacin and cimetidine can partly restore the proliferative activity during suppression, indicating true histamine- and prostaglandin-induced suppression.

The coexpression of indomethacin- and cimetidine-sensitive suppression in two strains and the lack of both in the third is interesting. From this study one cannot conclude with certainty that there is a causal relationship between indomethacin and cimetidine action. However, this is strongly suggested by the finding that the combined action of indomethacin and cimetidine is not stronger than that of indomethacin

alone. The association of histamine and prostaglandins is not surprising *per se*. Histamine activity is well known to induce release of prostaglandins in certain circumstances (5), although this association in the generation of suppression is not simple. Histamine can activate suppressor cells via the histamine₂ receptor, and at least the prostaglandins E₁ and E₂ can activate suppressor cells (23). Such activation has been shown to be induced by the change in levels of lymphocyte prostaglandins. The same

Table 3

The effect of cyclosporin A (CyA) on effector (E) influenced responder (R) stimulation in B6 mice

Day	R	R + E	R + E + CyA
3	3567 ± 345	15467 ± 1342	1983 ± 234
7	2541 ± 317	312 ± 64	299 ± 71

The results are expressed as mean ± SD

Table 4

Effector phenotypes of D2 and Balb/c mice

		Day			
		1	3	5	8
L3T4	Balb/c	18	33	29	10
	D2	13	27	48	48
sIg	Balb/c	55	21	23	4
	D2	71	48	25	20
Lyt-2	Balb/c	32	52	61	90
	D2	33	52	47	23

The results are expressed as percentage of positively stained lymphocytes as detected by flow cytometry

Table 5

Responder (R) stimulation influenced by effectors (E) depleted of Lyt-1, sIg or Lyt-2 positive cells in B6 mice

Depleted effectors	Day	
	3	7
None	13584 ± 1601	367 ± 31
Lyt-1	4327 ± 495	611 ± 35
sIg	12564 ± 811	465 ± 84
Lyt-2	9977 ± 775	3217 ± 259

The results are expressed as mean ± SD

prostaglandins can also directly suppress interleukin-2 synthesis (22). Interestingly, suppression of cellular immunity to *Listeria monocytogenes* can be mediated by prostaglandins (13). On the other hand, exogenous interleukin-2 restores the activity to microbial antigens (21). Our earlier results indicate that some of the suppression is mediated via interference of interleukin-2 action (8).

No genetic differences were found in the alveolar macrophage response to *T. vulgaris* (7). In this study there is a variation between inbred strains of mice that is independent of H-2K and H-2D, which corresponds to HLA A, B and C antigens in man. This variation is shown by the differing behaviour of D2 and Balb/c mice which have the same H2K and D haplotypes. On the other hand, B6 and Balb/c mice, which have different H2 haplotypes, behave similarly, i.e. the indomethacin-sensitive suppression is also sensitive to cimetidine. The suppression encountered is not similar to the suppression induced by *T. vulgaris* and mediated by alveolar macrophages because in this model macrophages were removed. As effector supernatants have no suppressive activity, suppression is linked to Lyt-2+ lymphocytes. In addition to the depletion tests there is a difference between effector phenotypes of D2 and Balb/c mice. Relatively few Lyt-2+ lymphocytes are found in D2 compared with Balb/c effectors of which up to 90% are Lyt-2+ at the longer incubation times. Thus these inbred strains of mice show differing activation of lymphocyte subsets by *T. vulgaris*.

Besides LPS there is some data suggesting the importance of nonspecific stimuli in response to microbial and other components. Nonspecific inflammation *in vivo* has been shown to improve the response to mitogens (17). Large numbers of lymphocytes have been shown to undergo polyclonal blast transformation *in vivo* after *Trypanosoma cruzi* infection (11, 12). In allergic alveolitis the cells invading the lungs are mainly T-lymphocytes (15), and in local immunization the recruitment of lymphocytes to the lung was nonspecific (1). All these results emphasize the role of nonspecific immunological activation. Our results indicate

the potential of *T. vulgaris* in such nonspecific activation, but additional studies are needed to further our understanding of its action *in vivo*.

ACKNOWLEDGEMENT

This study was supported by the North-Savo Cultural Fund.

REFERENCES

1. Bice, D. E., Degen, M. A., Harris, D. L. & Muggenburg, B. A.: Recruitment of antibody-forming cells in the lung after local immunization is nonspecific. *Am. Rev. Respir. Dis.* 126, 635-639, 1984.
2. Emanuel, D. A. & Kryda, M. J.: Farmer's lung disease. *Clin. Rev. Allergy* 1, 509-532, 1986.
3. Gialdroni-Grassi, G. & Grassi, C.: Bacterial products as immunomodulating agents. *Int. Arch. Allergy Appl. Immunol.* 76, 119-127, 1985.
4. Goeken, N. E. & Thompson, J. S.: Kinetic characteristics of human suppressor cells. *Transpl. Proc.* XI, 1823-1826, 1979.
5. Goodwin, J. S. & Webb, D. R.: Regulation of the immune response by prostaglandins. *Clin. Immunol. Immunopathol.* 15, 106-122, 1980.
6. Hayari, Y., Kukulansky, T. & Globerson, A.: Regulation of thymocyte proliferative response by macrophage-derived prostaglandin E2 and interleukin 1. *Eur. J. Immunol.* 15, 43-47, 1985.
7. Jägerroos, H. J. W., Nuortio, L. T., Kurup, V. P. & Mäntyjärvi, R. A.: Interactions of *Thermoactinomyces vulgaris* and mouse alveolar macrophages. Loss of mitogenic activity and release of suppressive prostaglandins and interleukin 1. *Scand. J. Immunol.* 24, 381-386, 1986.
8. Jägerroos, H. J. W., Nuortio, L. T. & Mäntyjärvi, R. A.: Polyclonal helper activity induced *in vitro* with *Thermoactinomyces vulgaris* and strain variation in regulation of the activity. *Int. J. Immunopharmacol.* 1988 (in press).
9. Jägerroos, H. J. W., Seppä, A. V. & Mäntyjärvi, R. A.: Pulmonary and immune responses to a *Thermoactinomyces vulgaris* antigen respiratory sensitization in C57BL/6J mice. *Exp. Pathol.* 29, 95-102, 1986.
10. Maier, B. F., Baron, D. P., Bockhorn, H., Drautz, H., Langharig, J. & Bessler, W. G.: Mitogenic and suppressive activity of mycelia from gram-positive bacteria on murine and human lymphocytes. *Immunobiology* 163, 427-435, 1982.
11. Minoprio, P. M., Eisen, H., Forni, L., D'Imperio Lima, M. R., Joskowicz, M. & Coutinho, A.: Polyclonal lymphocyte responses to murine *Trypanosoma cruzi* infection. I. Quantitation of both T- and B-cell responses. *Scand. J. Immunol.* 6, 661-668, 1986.
12. Minoprio, P. M., D'Imperio Lima, M. R., Joskowicz, M. & Coutinho, A.: Polyclonal lymphocyte response to murine *Trypanosoma cruzi* infection. II. Cytotoxic T

- lymphocytes. *Scand. J. Immunol.* 6, 669-680, 1986.
13. Petit, J.-C., Richard, G., Burghoffer, B. & Daguët, G.-L.: Suppression of cellular immunity to *Listeria monocytogenes* by activated macrophages: Mediation by prostaglandins. *Infect. Immunol.* 49, 383-388, 1985.
14. Räsänen, L., Karhumäki, E. & Arvilommi, H.: Bacteria induce lymphokine synthesis polyclonally in human B lymphocytes. *J. Immunology* 121, 418-420, 1978.
15. Semenzato, G., Agostini, C., Zambello, R., et al.: Lung T cells in hypersensitivity pneumonitis: Phenotypic and functional analyses. *J. Immunol.* 137, 1164-1172, 1986.
16. Seppä, A. V., Jägerroos, H. J. W. & Mäntyjärvi, R. A.: Experimental allergic alveolitis in mice induced by *Thermoactinomyces vulgaris*. *Acta. Pathol. Microbiol. Immunol. Scand. Sect. A.* 92, 125-131, 1984.
17. Sheng, Y. A., Bird, J., Florentin, I., Willoughby, D. A. & Giroud, J. P.: *In vivo* effects of an acute nonimmunological inflammation in rats on the lymphoproliferative response to mitogens. *Int. J. Immunopharmacol.* 6, 563-567, 1984.
18. Snella, M.-C. & Rylander, R.: Lung cell reactions after inhalation of bacterial lipopolysaccharides. *Eur. J. Respir. Dis.* 63, 550-555, 1982.
19. Solbach, W., Heeg, K., von Steldern, D. U., Rollinghoff, M. & Wagner, H.: On the partial suppression of IL-2 receptor expression and the prevention of lectin-induced lymphoblast formation by cyclosporine. *A. Clin. Exp. Immunol.* 60, 501-508, 1985.
20. Terho, E. O.: Microbiological and serological studies on farmer's lung disease. Publication of the University of Kuopio. Medicine. Series Original Reports 1, 1978.
21. Vismara, D., Lombardi, G., Piccolella, E. & Colizzi, V.: Dissociation between interleukin-1 and interleukin-2 production in proliferative responses to microbial antigens: Restorative effect of exogenous interleukin-2. *Infect. Immunol.* 49, 298-304, 1985.
22. Walker, C., Kristensen, F., Bettens, F. & deWeck, A. L.: Lymphokine regulation of activated (G1) lymphocyte. I. Prostaglandin E2-induced inhibition of interleukin 2 production. *J. Immunol.* 130, 1770-1773, 1983.
23. Webb, D. R. & Nowowiejski, I.: Mitogen-induced changes in lymphocyte prostaglandin levels: A signal for the induction of suppressor cell activity. *Cell. Immunol.* 41, 72-85, 1978.

Address:

H. J. W. Jägerroos

Dept. of Clinical Microbiology

University of Kuopio

POB 6

SF-70211 Kuopio

Finland

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.