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INHIBITION OF C-MYC EXPRESSION INDUCED BY 4-HYDROXYNONENAL, A PRODUCT OF LIPID PEROXIDATION, IN THE HL-60 HUMAN LEUKEMIC CELL LINE

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4-Hydroxynonenal is a highly reactive aldehyde, produced by cellular lipid peroxidation, able to inhibit cell proliferation "in vitro" and "in vivo". Its concentration in non proliferating cells ranges up to 1 μM, whereas in the highly undifferentiated tumour cells, it is very low or undetectable. We have now demonstrated that micromolar concentrations of 4-hydroxynonenal inhibit c-myc but not N-ras expression in HL-60 human leukemic cells. This inhibitory effect is observed after an incubation of 1 hour with both 1 and 10 μM aldehyde. Moreover, we report that down-regulation of c-myc expression increses when repeated additions of 1 μM 4-hydroxynonenal are performed, to maintain the cells in presence of aldehyde for 7.5 hours. These results indicate that not only the concentration but also the length of exposure to the aldehyde is important in determining the extent of the c-myc expression inhibition and suggest a role of lipid peroxidation products in the control of gene expressions.

4-hydroxynonenal (HNE) is one of the major products of the oxidative breakdown of biological membranes (1). This aldehyde is highly reactive towards thiol compounds and, by this mechanism, inhibits several enzymatic activities at concentrations ranging from 10^{-5} to 10^{-3} M (2). It is well known that these high HNE concentrations inhibit cell proliferation in different cell lines (3, 4). At micromolar non toxic concentrations, HNE induces

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chemiotactic activity in polymorphonuclear leukocytes (5) and stimulates adenylate cyclase (6) and phospholipase C (7) activities. The physiological HNE concentration, in the different tissue types, ranges from 0.2 to 2.8 μM (8) and represent the steady - state amount between the HNE continously produced and that catabolyzed into the cells. Moreover, it has been reported that the lipid peroxidation as well as the concentration of its products are inversely related to the rate of cell proliferation and directly related to the level of cell differentiation (8, 9). HL-60 cells were originally derived from the peripheral blood of a patient with acute myelogenous leukemia (10), like other leukemic cell line (11), they did not show detectable level of lipoperoxidation, even if induced by prooxidant substances (unpublished results). Moreover the exogenous HNE, added to the HL-60 cell culture, disappear from the culture medium within 45-60 minuts (12). We have reported that, when HL-60 cells were repeatedly treated with 1 μM HNE, at regular intervals of 45 min for 7 to 9 hours, their growth was blocked and there was the appearence of a differentiated phenotype in about 50% of the cells (12, 13). Moreover, a single treatment with micromolar HNE concentrations increase globin expression and inhibit c-myc expression in K562 cells (11).

HL-60 cells represent a good model for the study of gene expression and differentiation , in fact they can be induced to differentiate into either monocyte/ macrophage-like cells or granulocytes by several chemical compounds such as the tumour promoting phorbol diester (TPA), 1,25 dihydroxyvitamin D3, dimetylsulfoxide and retinoids (14). The HL-60 genome contains amplified c-myc sequences (15) as well as an activated N-ras allele (16). A multistep mechanism involving both N-ras and c-myc has been proposed as the basis of transformation induced by genomic DNA of HL-60 transfected into NIH3T3 cells (16). C-myc oncogene product is a nuclear protein and its involvement in the control of cell proliferation and differentiation has been extensively demonstrated (17, 18, 19). On the other hand the products of the mammalian ras genes (H-ras, K-ras, N-ras) are located at the inner surface of the cell membrane (20) and bind to GTP or GDP with high affinity (21). Although the p21 ras product may play a role in cell proliferation, in various cell types it is known to induce differentiation (22). A large amount of data demonstrate that c-myc expression decreases during the differentiation process, whereas the levels of ras mRNAs are

reported to increase (23,24), decrease (17, 25) or not change at all (19, 26).

The aim of this study was to investigate the effects of micromolar concentration of HNE , similar to those found in the normal cells, on the expression of these two oncogenes in the HL-60 leukemic cells.

MATERIALS AND METHODS

Cell and culture conditions.

The human HL-60 promyelocytic cell line was cultured at 37 °C in a humidified atmosphere of 5% CO_2- air using a RPMI 1640 medium supplemented with 2mM glutamine, antibiotics and 10% fetal calf serum (FCS) . In all experiments growth rate and cell viability were monitored daily by the trypan blue exclusion test.

HNE treatments.

HNE (kindly provided by Prof. Esterbauer, University of Graz, Austria) was prepared as previously described (27). This aldehyde is a very unstable electrophylic molecule which rapidly reacts with the serum proteins (1). In the presence of fetal calf serum 1 μM HNE disappears from the culture medium in about 45 min (4). In the present paper, HL-60 cells were treated with HNE according to two different experimental procedures:

1) Single treatment : exponentially growing HL-60 cells were resuspended at a concentration of 300,000 cells/ml in RPMI medium without FCS and treated with 1 or 10 μM HNE. After 1 hour, the cells were washed, resuspended in RPMI medium supplemented with 10% fresh FCS and then collected at different times for RNA extraction.

2) Repeated treatments: 1 μM HNE was added to the cell suspension (300,000 cell/ml) at regular intervals of time (45 min) up to 10 treatments. After each treatment aldehyde concentration was monitored by HPLC and the obtained values never exceeded 1 μM (data not shown). After 7.5 hours (45 min. after the last treatment) HNE was no more detectable in the culture medium. The cells were harvested, without washing, at different times for the RNA extraction.

Isolation and Northern blot analysis of RNA.

Total cellular RNA was purified at various times after HNE treatments. Cells (10^8 cells for each time point) were homogenized in 2 ml of lysis solution (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5 % Sarcosyl, 0.1 M 2-mercaptoethanol, pH 7). The homogenates were transferred in "Corex" tubes and 0. 2 M sodium acetate, 2 ml of phenol and 0.4 ml of chloroform-isoamylalcohol mixture (49:1) were added in each tube. The final suspension was cooled in ice for 15 minutes and centrifuged at 10,000 xg for 20 minutes at 4 °C in a Beckman J2-21 centrifuge. The aqueous phase was transferred to a fresh tube, mixed with 2.2 ml of absolute ethanol and then placed at -20 °C overnight to precipitate RNA. Sedimentation at 10.000 xg for 20 min at 4 °C was again performed and the resulting pellet of RNA was dissolved in 0.8 ml of lysis solution and reprecipitated with 1 vol of absolute ethanol at -20 C for 1 hour. After centrifugation at 10,000 xg for 10 minutes, the RNA pellet was resuspended in 4 ml of 75% ethanol, sedimented at 10,000 xg for 10 min at 4 °C, dried under N_2, and dissolved in distilled water. Twenty μg of RNA were fractionated on a 1.5 % agarose-formaldehyde gel and blotted onto a nylon membrane. All filters were hybridized with labeled probes at 65°C in a solution

containing Na_2HPO_4 0.25 M pH 7.2, SDS 5% and EDTA 1 mM pH 8 solution. The filters were washed at 60 °C in Na_2HPO_4 20 mM pH 7.2 , SDS 5% and EDTA 1 mM pH 8 solution and then at 65 °C in an analogous solution in which SDS was 1%. After washing, the filters were exposed to a X-ray film at -80 °C for 48-72 hours.

Autoradiograms were quantified by densitrometrical scanning using a laser densitometer (LKB 2022 ultroscan) and all values were normalised against the relative ethidium bromide stained total RNA.

DNA probes.

In this study we utilized two probes: 1) a 1.4 kb c-myc cDNA Cla I - Eco RI fragment corresponding to the third exon of the gene (28); 2) a N-ras cDNA obtained by a PCR reaction corresponding to the first and second exon of the gene. The DNA probes were labeled with ^{32}P dATP by a multiprime procedure.

Differentiation assay.

The degree of differentiation , 5 days after HNE treatments, was evaluated by counting the number of HL-60 cells phagocytosing the opsonized zymosan as previously described (12).

RESULTS

The growth of HNE treated HL-60 cells was analyzed both after the single treatment with 1 or 10 μM HNE and after repeated treatments with 1 μM HNE (Fig. 1). Cell proliferation was not significanly inhibited by a single dose of 1 μM HNE, whereas 10 μM HNE blocked cell growth for 24 hours. In the following days, treated cells resumed proliferation at the same rate as the control cells. In contrast, when cells were given 10 consecutive treatments with 1 μM HNE, the growth was inhibited for 5 days.

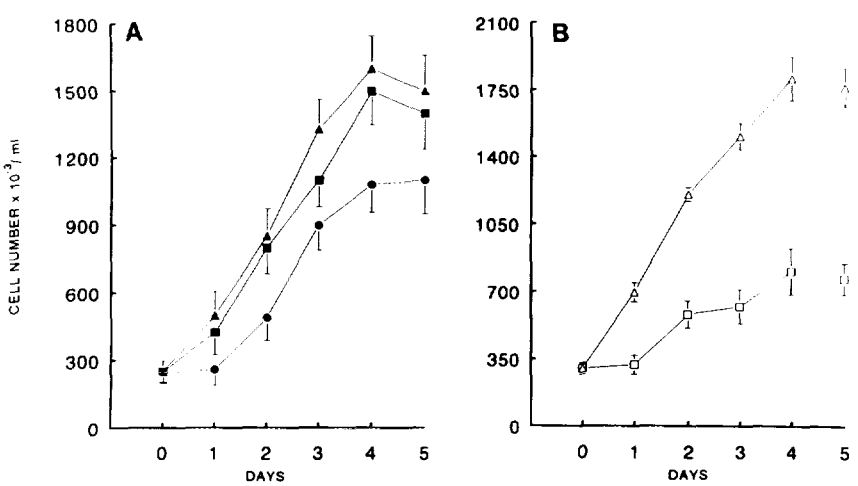
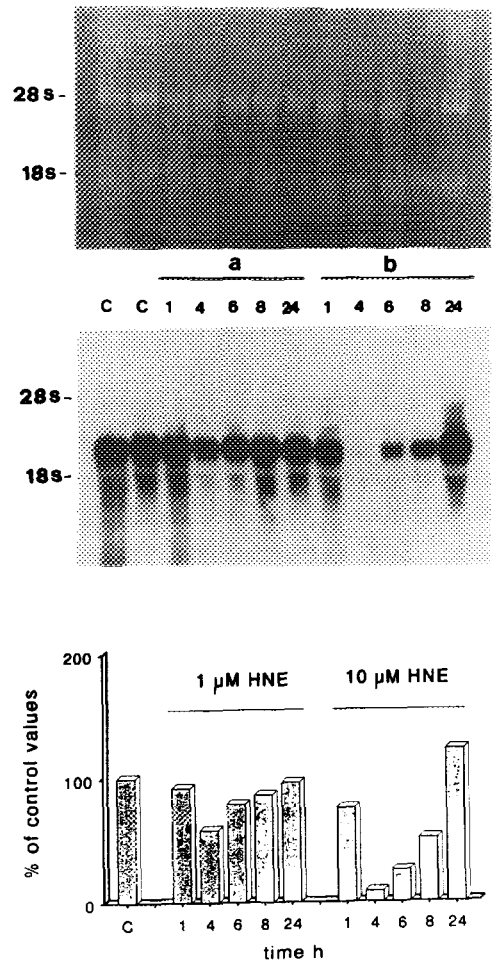


Figure 1. Growth of HL-60 cells; A: cells treated for 1 hour with HNE, ▲ controls, ▩ 1 μM HNE, ● 10 μM HNE; B: cells treated for 10 times, at intervals of 45 minutes with 1 μM HNE △ controls , □ treated cells. Values are the mean + S.D. of 6 separate experiments.



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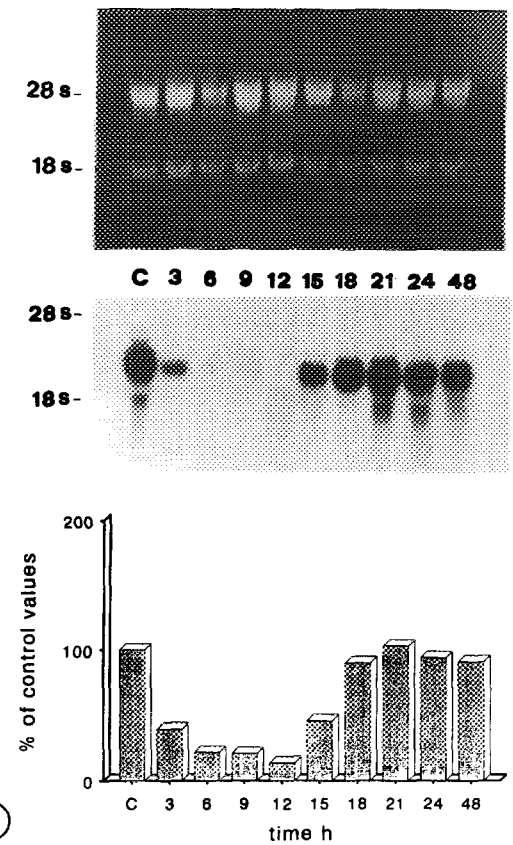


(3)

Figure 2. c-Myc expression in HL-60 cells treated for 1 hour with HNE; a: hours after treatment with 1 μM HNE; b: hours after treatment with 10 μM HNE. Bar graphs are obtained by the values of densitometric scanning of the autoradiographs and are expressed as percentage of the controls.

Figure 3. c-Myc expression in HL-60 cells treated 10 times with 1 μM HNE at intervals of 45 minutes. Numbers are the hours from the beginning of the treatments. Bar graphs are obtained by the values of densitometric scanning of the autoradioraphs and are expressed as percentage of the controls.

Phagocytosis capability, 5 days after the repeated treatments was present in 40% of the cells, while at this day after a single treatment (with 1 or 10 μM HNE), the number of cells phagocytosing the opsinized zymosan was simalar to that found in the control cells (about 5-8%).

The pattern of c-myc expression after a single treatment with 10 or 1 μM HNE is shown in Fig.2. c-Myc expression was inhibited

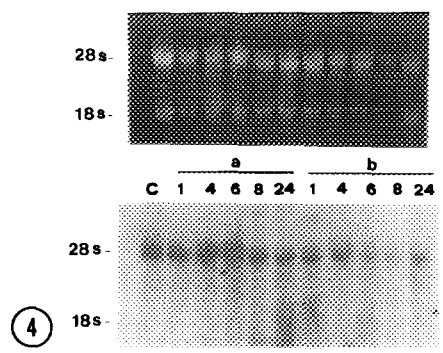
4 hours after treatments with both concentrations. As measured by densitometric scanning of the autoradiographs, 4 hours after a single treatment with 1 μM HNE , c-myc mRNA decreased by approximately 35% with respect to control value. At 8 hour the inhibition was only of 20% and c-myc expression was restored to the control level within 24 hours. The highest HNE concentration (10 μM), caused a reduction of c-myc expression of 92 % after 4 hours and the inhibition was still considerable (50%) after 8 hours. Also in this case, however, the level of c-myc expression was completely restored by 24 hours.

The pattern of c-myc expression after repeated treatments with 1 μM HNE is shown in fig. 3. During the treatments (3 and 9 hours) c-myc RNA level was strongly depressed (from 60% to 70%) and remained inhibited (86%) after 12 hours. At 15 hours , c-myc expression was still reduced by 50% and returned to the control values at 21-24 hours.

By contrast,N-ras expression,studied both after single and repeated treatments, showed no significant variation (Fig.4 and 5).

DISCUSSION

The present results shown that HNE treatment causes down regulation of c-myc transcript levels. c-Myc expression rapidly decreases even after the single treatment with HNE. The extent as well as the duration of the inhibition is concentration dependent. An analogous result has been previously obtained in



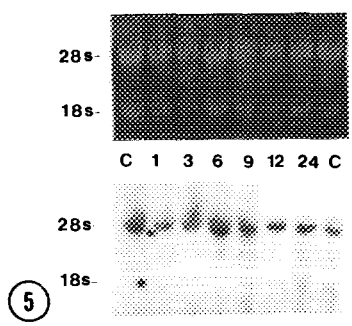


Figure 4. N-ras expression in HL-60 cells treated for 1 hour with HNE; a: 1 μM HNE; b: 10 μM HNE. Numbers are the hours after the treatment.

Figure 5. N-ras expression in HL-60 cells treated 10 times with 1 μM HNE. Numbers are the hours from the beginning of treatments.

K562 cells treated with HNE (11). c-Myc expression is related to the cell proliferative state and in same cases it does parallel the loss of clonogenic capacity and acquisition of differentiated phenotypes (29-31). In our study, 1 hour of incubation with 1 or 10 μM HNE was not sufficient to induce a differentiated phenotype in HL-60 cells. However, 10 μM HNE caused a transient block of the cell growth. Therefore, the down regulation of c-myc expression by HNE treatment seems to be associated with an inhibition of cell proliferation rather than with the induction of differentiation.

Repeated treatments with 1 μM HNE cause an inhibition of the c-myc expression stronger than a single tratment with both 1 and 10 μM HNE. In fact, 10 treatments with 1 μM HNE (7.5 hours of incubation in presence of the aldehyde) provoked an 86% inhibition of c-myc expression which persisted after 12 hour. This result indicates that the extent of the effect is also related to the duration of exposition to the aldehyde.

As previously demonstrated by us in an extensive way (12), repeated treatments with 1μM HNE are able to induce a differentiated phenotype in approximately 40% of HL-60 cells after 5 days. This has been confermed in the present study by testing the phagocytosing capability of the HL-60 cells treated for 10 times with 1 μM HNE. It has been proposed that the down regulation of c-myc is an early event in the metabolic pathway leading to differentiation (32). However, most of the differentiation inducers caused a reduction of c-myc expression for several days, whereas the HNE effect on this parameter was reversible. This may be due to the fact that these inducers are stable in the culture medium up to the end of the experiments, whereas, the instability of HNE causes its disappearence from the cell suspension 7.5 hours after the beginning of repeated treatments. It has been reported that when an inducer is removed from the culture medium, c-myc mRNA reappears after a few hours [30]. Thus, the reversibility of the c-myc down regulation after HNE treatments may be due to the short period of real exposure of the cells to this aldheyde.

We were also interested in studying the expression of the proto-oncogene N-ras in addition to c-myc, because both these oncogenes seems involved in myelomonocytic differentiation (17-23). HNE does not modify the level of N-ras mRNA both after single or repeated treatments. Other reports indicated that little, if any, decrease in the level of N-ras mRNA was observed

in HL-60 cells induced to differentiate with DMSO, HMBA, 1,25-Dihydroxy vitamin D3 and TPA (17, 19, 23). HL-60 cellular differentiation would therefore appear to involve primarily alterations in c-myc expression rather than in that of c-ras genes (19). Therefore, HNE seems to act on c-myc and N-ras expression in HL-60 cell line, like the majority of inducers of myeloid differentiation. In addition the fact that this aldehyde don't modify N-ras mRNA level demonstrates that the inhibition of c-myc expression is not the result of the decrease of total RNA synthesis but is a specific HNE-mediated event.

It is noteworthy that a strong inhibitory effect on c-myc expression was exerted by 1 μM HNE when this low concentration, similar to those found in the normal cells, was maintained in the culture medium for a few hours. This suggest that the HNE, when restored in the neoplastic cells, may contribute to the recovery of their proliferative control.

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REFERENCES

1. Esterbauer, H. (1982) In Free radicals, lipid peroxidation and cancer (McBrien D.C.H. and Slater T.F. Eds), pp. 101-128 Academic Press, London.
2. Esterbauer, H., Zollner, H., and Schaur, R.J.(1988) ISI Atlas Sci. 1, 311-317.
3. Hauptlorentz, S., Esterbauer, H., Moll, W., Pumpel, R., Shauestein, E. and Puschendorf, B. (1985) Biochem. Pharmacol. 34, 3803-3809.
4. Barrera, G., Biasi, F., Fazio, V.M., Paradisi, L., and Dianzani, M.U. (1991) In Chemical Carcinogenesis 2 (Columbano A. et al. Eds.), pp. 337-342. Plenum Press, New York.
5. Curzio, M., Esterbauer, H., Di Mauro, C., Cecchini, G. and Dianzani, M.U. (1986) Biol. Chem. Hoppe-Seyler 367, 321-329.
6. Paradisi L., Panagini, C., Parola, M., Barrera G. and Dianzani. M.U. (1985) Chem. Biol. Interact. 53, 209-217.
7. Rossi M.A., F. Fidale, A. Garramone, Esterbauer, H. and Dianzani M.U. (1990) Biochem. Pharmacol. 39, 1715-1719.
8. Esterbauer, H. Zollner, H. and Schaur, R.J. (1990) In Membrane Lipid Oxidation (C. Vigo Delfrey C. Ed.), Vol.1, pp. 240-268. CRC Press Inc., Boca Raton, FL.
9. Dianzani, M.U., Poli, G., Canuto R.A., Rossi, M.A., Biocca, M.E., Biasi, F., Cecchini, G., Muzio, G., Ferro M., and Esterbauer H. (1986) Toxicol. Pathol. 14, 404-410.
10. Collins, S.J., Gallo R.C. and Gallagher, R.E. (1977) Nature 270, 347-349.

11. Fazio, V.M., Barrera, G., Martinotti, S., Farace, M.G., Giglioni, B., Frati, L., Manzari, V., and Dianzani, M.U. (1992). Cancer. Res. 52, 4866-4871.
12. Barrera, G., Di Mauro, C., Muraca, R., Ferrero, D., Cavalli, G., Fazio, V.M., Paradisi, L., and Dianzani, M.U. (1991) Exp. Cell. Res. 197, 148-152, 1991.
13. Fazio, V.M., Rinaldi, M., Ciafrè, S., Barrera, G .and Farace, M.G. (1993) in Molec. Aspects Med. Vol 14, pp. 217-228, Pergamon Press, London.
14. Hozumi, M. (1983) Adv. Cancer Res. 38, 121-169.
15. Collins, S.J. and Groudine, M. (1982) Nature 298, 679-681.
16. Murray, M.J., Cunningham, J.M., Parada, L.F., Dautry, F., Lebowitz, P. and Weinberg, R.(1983) Cell 33, 749-757.
17. Imaizumi,M.and Breitman,T.R.(1988) Cancer Res. 48, 6733-6738.
18. Grosso, L.E. and Pitot, H.C. (1984) Biochem. Biophys. Res. Commun. 119, 473-480.
19. Watanabe, T., Sariband, E., Mitchell, T. and Kufe, D. (1985) Biochem. Biophys. Res. Commun. 3, 999-1005.
20. Polakis,P.G., Rubinfeld, B., Evans,T., and McCormick,F.(1991) Proc. Natl. Acad. Sci.USA, 88, 239-243.
21. Barbacid, M. (1987) Ann. Rev. Biochem. 656, 779-827.
22. Haubruck, H. and McCormick, F.(1991) Biochim. Biophys. Acta, 1072, 215-229.
23. Studzinski,G.P.and Brelvi,Z.S.(1987) Lab.Invest. 56, 499-504.
24. Yamashiro,S., Takayama, K., Shiku, H. and Furukawa,K. (1993) Leuk. Res. 17, 129-136.
25. Filmus, J. and Buick, R.N. (1985) Cancer Res. 45, 822-825.
26. Sariband,E., Mitchell,T. and Kufe,D.(1985) Nature 316, 64-66.
27. Di Mauro, C., Cavalli,G., Amprimo, M.C., Paradisi, L., Scano, G., Curzio,M.and Dianzani, M.U.(1990) Cell Biochem.Funct. 8, 147-155.
28. Dalla Favera,R., Martinotti,S., Gallo,R., Erikson, J., Croce, C.M.(1983) Science 219, 963-967.
29. Szabo, G. Jr., Szekely, L., Schablik, M., Klein, G., Sumegi, J. and Szabo, G. (1991) Exp. Cell Res. 193, 420-424.
30. Reitsma, P.H., Rothberg, P.G., Astrin S.M., Trial, J., Barshavit, Z., Hall A., Teitelbalum S.L. and Kahan A.J. (1983) Nature 306, 492-306.
31. Morimoto, S., Shiraishi, T., Fukuo, K., Koh, E., Kitano, S., Yaasuda, O., Tamatani, M. and Ogihara, T (1992) Biochem. Int. 28, 313-321.
32. Yen, A. and Forbes, M.E.(1990) Cancer Res. 50, 1411-1420.