Carcinogenesis vol.17 no.9 pp.2069–2072, 1996

SHORT COMMUNICATION

Medroxyprogesterone acetate accelerates the development and increases the incidence of mouse mammary tumors induced by dimethylbenzanthracene

C.Marcelo Aldaz[1], Qiao Yin Liao, Michael LaBate and Dennis A.Johnston

The University of Texas M.D. Anderson Cancer Center, Department of Carcinogenesis, Science Park-Research Division, Smithville, Texas 78957, USA

[1]To whom correspondence should be addressed

Chemical induction of mammary tumors in mice requires usually a long latency period and is often complicated by high non-mammary tumor related mortality. Classically hormone stimulation has been used as the means to increase tumor incidence. The synthetic progestin medroxyprogesterone acetate (MPA) was postulated by some authors to increase mammary tumor incidence in various rodent models. However, controversy exists regarding the role of MPA in experimental and human carcinogenesis. In our study we tested the use of a protocol of combined MPA- and dimethylbenz[*a*]anthracene (DMBA) treatment for the obtention of mammary tumors with a short latency and with a lower toxicity than the classical multiple dose DMBA protocol. MPA was very effective in accelerating the development and increasing the incidence of mammary tumors induced by DMBA in CD2F1 mice. MPA by itself did not produce any mammary tumors. The mean latency for tumor development from the end of carcinogen treatment was 99 ± 51 days in the group that received a combination of MPA and four DMBA doses. This group showed significantly earlier mammary tumor incidence ($P < 0.0001$) and higher tumor numbers than the groups that received only DMBA. Mammary tumors were also analyzed for effects on the mutation rate affecting the Ha-*ras* and Ki-*ras* genes. Our data is consistent with MPA probably increasing the number of target cells at risk for mutation by the chemical carcinogen DMBA and possibly promoting the faster development of tumors.

Rodent models of chemically-induced mammary carcinogenesis have been used over the years in order to gain insight into the biology of breast cancer. However, marked differences exist between rats and mice in their susceptibility to mammary tumor development after chemical carcinogen treatment. It is known that in general, laboratory mice are much more resistant to chemical induction of mammary tumors when compared with rats. For instance in rats a single dose of 5–10 mg/100 g b.w. dimethylbenz[*a*]anthracene (DMBA*), without any exogenous hormonal manipulation, is sufficient to induce a high incidence of mammary tumors (1); while mice require a regimen of multiple applications of comparable doses of DMBA to obtain a relatively modest tumor response with a long tumor latency, high morbidity and mortality non-mammary tumor related (2–4). Rat mammary tumors are largely hormone dependent for growth, while most mouse mammary tumors once developed become hormone independent. Partially, for these reasons the rat mammary tumor models are much more widely used than the mice models. In our laboratory we have been using rat mammary tumor models to elucidate the role of critical genetic and chromosomal events in mammary carcinogenesis (5,6). However an obvious problem is the relative paucity of information at the genetic level in the rat (7). Because of more extensive knowledge of the mouse genome, it would be expedient to use mouse models for comparison with findings in human breast cancer.

One of the goals of the studies reported here, was to develop a simple and reliable protocol for the chemical induction of mouse mammary tumors with relatively short latency and high tumor incidence. It is very well documented that hormonal stimulation is a requisite for obtaining a high mammary tumor incidence in the mouse (2–4,8). Exogenous hormone administration and pituitary isografts have been classically used (8,9). Recently, Pazos *et al.* reported that multiple *N*-methyl-*N*-nitrosourea (NMU) doses in combination with medroxyprogesterone acetate treatment (MPA) were very effective in the induction of mouse mammary adenocarcinomas in BALB/c mice (10). Based in part on these observations and to clarify some of the controversy on the role of MPA in carcinogenesis, in our study we tested the use of a protocol of combined MPA–DMBA treatment for the obtention of mammary tumors with a short latency and with a lower toxicity than the classical multiple dose DMBA protocol. In order to obtain some information on the molecular mechanisms of carcinogenesis, tumors induced with this protocol were also analyzed for the incidence of mutations in Ha-*ras* and Ki-*ras* genes.

Virgin female CD2F1 (Balb/C×DBA/2) mice purchased from the National Cancer Institute (Fredericksburg, MD) were randomized and separated into four groups, Groups 1–4 (housed 10/cage) of ~40 mice each for the different treatment protocols (Figure 1) and one control group 5 (mPA only) of 20 mice. At 6 weeks of age, animals from Groups 3, 4 and 5 received a subcutaneous implant in the interscapular area of two compressed pellets, standard release no binder, of medroxyprogesterone acetate, 20 mg each (Hormone Pellet Press, Kansas City, KS). DMBA (Sigma) at a concentration of 1 mg/dose, was administered intragastrically dissolved in 0.1 ml cottonseed oil at the time points indicated in Figure 1. Animals were monitored weekly by palpation for mammary tumor development.

Mice were euthanized when palpable mammary tumors reached ~1.5 cm in diameter. A part of tumor tissue was frozen for DNA analysis and a part was fixed in formalin, embedded in paraffin and processed for standard hematoxylin–eosin staining. Tumor histologies were classified as previously described (2).

For the statistical analysis of tumor incidence curves and tumor latency, time 0 was considered as the time when

*Abbreviations: MPA, medroxyprogesterone acetate; DMBA, dimethylbenz[*a*]anthracene; NMU, *N*-methyl-*N*-nitrosourea; SSCP, single strand conformation polymorphism.

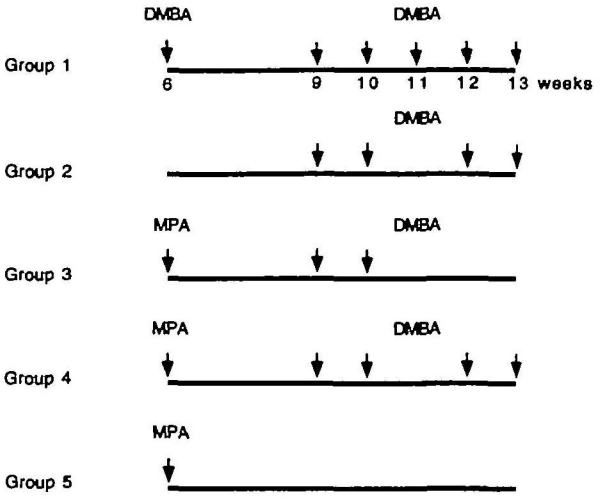

Fig. 1. Mammary carcinogenesis treatment groups in CD2F1 mice.

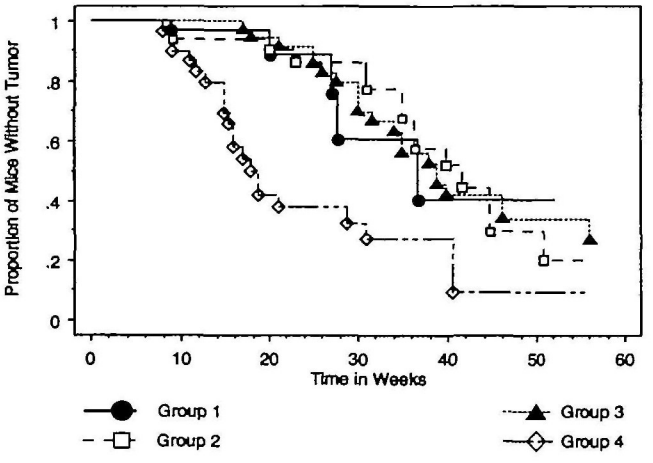

Fig. 2. Cumulative proportion of mice without mammary tumors, using Kaplan–Meier survival statistics. Treatment groups as indicated in Figure 1. Group 4 showed a significantly earlier ($P < 0.0001$) mammary tumor incidence than groups 1, 2 and 3 Group 5, with zero tumor incidence was not plotted.

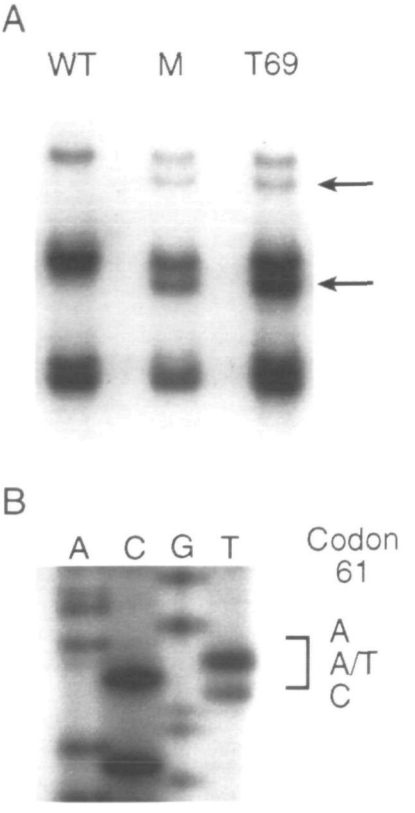

Fig. 3. DMBA-induced Ha-*ras* mutations in mouse mammary tumors. Tumor DNA from exon 2 (containing codon 61) of the Ha-*ras* gene was amplified as described in Materials and methods. (**A**) The ^{32}P-labeled PCR products were run on an SSCP gel containing MDE acrylamide. Known wild type (WT) and mutant (M) DNAs are shown with sample T69. In comparison with the WT DNA, two additional/shifted bands (indicated by arrows) can be seen in the M and T69 lanes. The lower, more intense band was excised and reamplified as described in Materials and methods and subjected to cycle sequencing (**B**) A typical A→T transversion in codon 61 (5′-CAA-3′) can be seen.

treatment ended in all groups at week 13 of age. The time to tumorigenesis was noted for each animal. Causes of morbidity and mortality other than mammary tumors were considered as censored observations. The time to tumorigenesis was analyzed using Kaplan–Meier survival statistics with Gehan–Breslow significance test (Statistica: CSS, Tulsa, OK). Tumor latencies, unadjusted for censoring, were also compared by using the Student's *t*-test. Animals were observed for a total of 15 months.

Ten randomly selected tumors from group 4 and ten from the DMBA only groups (1 and 2) were analyzed for mutations in codons 12/13 and 61 of the Ha-*ras* and Ki-*ras* genes. Genomic DNA (0.2 μg) was added to a 10 μl reaction containing 0.5 mM of each dNTP, 6 pmol of each primer, 0.6 U of Taq polymerase and 5 μCi [α-^{32}P]dCTP in amplification buffer (50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$). Ha-*ras* amplifications were performed using primers previously reported (11). Primers used for Ha-*ras* exon 1 amplification included 5′-GCTAAGTGTGCTTCTCATTGGC-3′ and 5′-CTCTATAGTGGGATCATACT-3′. Primers for Ha-*ras* exon 2, included 5′-GACTCCTACCGGAAACAGGT-3′ and 5′-TGATGGATGTCCTCGAAGGA-3′. Primers for amplification of exons 1 and 2 of Ki-*ras* were selected based on published sequence (12). They included 5′-GTAAGGCCTGC-TGAAAATG-3′ and 5′-GGGTCGTACTCATCCACAA-3′ (Ki-*ras* exon 1); 5′-GACTCCTACAGGAAACAAGT-3′ and 5′-GGTGAATATCTTCAAATGAT-3′ (Ki-*ras* exon 2). After an initial denaturation step at 95°C for 2 min, 40 cycles were performed at 95°C for 30 s and either 51°C (Ha-*ras* exon 1 and Ki-*ras* exon 2), 55°C (Ki-*ras* exon1) or 60°C (Ha-*ras* exon2) for 30 s, and 72°C for 1 min for all four exons.

Single strand conformation polymorphism (SSCP) gels were run in order to screen potential point mutations (13). Two different gel systems were used to screen for mutations. In gel system A, gels containing 6% polyacrylamide (acrylamide:bis, 19:1), 10% glycerol and 1×TBE (1×TBE = 90 mM Tris-borate, 2 mM EDTA) were electrophoresed in 0.5×TBE at 4 W for 14 h at room temperature and were used to resolve [α-^{32}P]dCTP-labeled PCR products from exon 1 (including codons 12/13) of both the Ha-*ras* and Ki-*ras* genes. In gel system B, gels contained 0.5×MDE acrylamide (AT Biochem) and 0.6×TBE and were electrophoresed in 0.6×TBE at 4 W for 14 h at room temperature. They were used to resolve mutations in exon 2 (including codon 61). Aliquots of the PCR reactions were diluted 1:5 in 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanole. Samples were heated at 95°C for 5 min and cooled on ice. Typically, 5 μl of this mixture were electrophoresed. Gels

Table I. Influence of MPA treatment on formation of DMBA induced mammary tumors in CD2F1 mice

Group	No. of mice	Total mammary tumors (MT)	Mice with MT	MT/mouse	Latency for MT development (days) $\bar{X}$ (± SD)[a]	Non-MT related deaths[c]
1 (DMBA×6)	32	8	5/32 (16%)	0.25	152 ± 75	26 (81%)
2 (DMBA×4)	35	24	15/35 (43%)	0.69	218 ± 72	18 (51%)
3 (MPA-DMBA×2)	36	28	21/36 (58%)	0.78	210 ± 65	11 (31%)
4 (MPA-DMBA×4)	30	35	21/30 (70%)	1.17	99 ± 51[b]	8 (27%)
5 MPA	20	0	0/20 (0%)	0.0	–	0.0

[a]Latency for tumor development from the end of all treatments at week 13 (time 0) unadjusted for censoring.
[b]Statistical significance versus Group 4 = $P < 0.1$, $P < 0.001$ and $P < 0.001$ comparison with Groups 1,2 and 3 respectively.
[c]Includes deaths due to other neoplasias. these were: Group 1 = 3 mice, Group 2 = 4 mice, Group 3 = 4 mice and Group 4 = 5 mice.

Table II. Mammary tumors histopathological classification

Group	Total tumors	Type B adenocarcinomas[a]	Type A adenocarcinomas	Adenosquamous carcinomas[b]	Myoepitheal adenocarcinomas
1	8	5 (63%)	1 (12%)	1 (12%)	1 (12%)
2	24	15 (63%)	5 (21%)	3 (13%)	1 (4%)
3	28	16 (57%)	8 (29%)	3 (11%)	1 (4%)
4	35	25 (71%)	2 (6%)	6 (17%)	2 (6%)

[a]Includes well and poorly differentiated carcinomas.
[b]Includes tumors with >10% of focal squamous metaplasia.

were dried on blotting paper, and exposed to X-ray film. Gel bands from samples that were shifted relative to those from wild type DNA were excised from the dried gel, eluted reamplified and sequenced as previously described (14).

In Group 1 of the tumor experiment (Figure 1), we followed the conventional multiple DMBA dose treatment (2,4), six 1 mg doses per mouse. We observed that this protocol was extremely inefficient in the generation of mammary tumors, see Table I. This was due to the very high morbidity and mortality during the course of the experiment for causes other than mammary tumors. In Group 2, where DMBA was given four times, morbidity and mortality were much lower. As a result, mammary tumor incidence was higher, although considerably less than the tumor incidence obtained in the MPA–DMBA treated groups. The combination of MPA with four DMBA doses (Group 4) was the most efficient protocol inducing tumors with the shortest latency and lowest non-mammary tumor mortality (Figure 1) in CD2F1 mice.

Group 4 showed a significantly earlier mammary tumor incidence curve ($P < 0.0001$) than Groups 1, 2 and 3 (Figure 2). The first tumor arose at 42 days, counted from the time that all treatments ended at week 13. Fifty percent of the mice had tumors by ~18 weeks from that time point. This protocol also showed, the highest tumor incidence since 21 of 30 (70%) mice developed mammary tumors, and tumor multiplicity was also the highest with 1.17 mammary tumors/mice (Table I). Most of the mammary tumors induced by the MPA–DMBA protocols were Type B adenocarcinomas (2), i.e. with no squamous metaplasia, Table II. Interestingly however, a high proportion of the tumors generated in Group 3, (MPA–DMBA×2) were of the tubular type (Type A). The most important conclusion from our study is that MPA accelerated the development and increased the incidence of the DMBA induced mammary tumors.

Depot medroxyprogesterone acetate is widely used in humans as a contraceptive, in the treatment of breast cancer, endometriosis, and other pathological conditions (15). Controversy surrounds its use and potential influence on the risk for developing breast cancer (16). Recently, it was concluded that there is an increase in developing breast cancer only in recent or current MPA users, with the highest risk seen when used 5 years or less prior to the development of breast neoplasia (17). These authors further speculated that one of the possible explanations would be that depot MPA may accelerate the development of preexisting tumors (17).

The results of our studies are analogous to previous observations of Pazos *et al.* (10) who demonstrated that MPA in combination with the direct alkylating agent nitrosomethylurea was effective in the induction of mammary tumors in Balb/C mice, in our experiment, however, we used a much lower dose of MPA. Several years ago Lanari and coworkers (18) reported that MPA depot alone at very high doses, 40 mg every 2 months during a year, was sufficient to induce mammary tumors in Balb/C mice after a very long latency period. In our experiment, however, MPA by itself at the dose of 40 mg MPA once in s.c. pellet implants, did not induce any mammary tumors during the observation period of 15 months. This indicates that in this system, MPA fits the operational definition of a tumor promoter, since it was not, by itself, carcinogenic but enhanced the tumor response and promoted the tumor growth of the mammary neoplasias induced by DMBA. Nevertheless, we cannot rule out that MPA could be acting as a co-carcinogenic agent and that the dose utilized in our study is sub-threshold for the generation of tumors by itself but still sufficient to observe a synergistic effect with other carcinogens. Russo and Russo (15) have shown that MPA at a single 5 mg dose, equivalent to the human pharmacologic dose, was sufficient to induce a very significant cell proliferative response in the rat mammary terminal end buds, inhibiting differentiation. These authors showed that pretreatment with MPA also increases the tumor response in the DMBA rat model (15,19). On the other hand, other investigators have reported that

pretreatment with MPA inhibits the DMBA tumor response in rats (20). Controversies such as this one, using the same experimental system (19,20), exemplifies the need for further studies to better define and clarify the role of MPA in mammary carcinogenesis.

In order to analyze the level of involvement of some known putative molecular mechanisms of chemical carcinogenesis we also analyzed the occurrence of mutations in the Ha-*ras* and Ki-*ras* proto-oncogenes in the mammary adenocarcinomas induced by DMBA alone and compared these with mutations found in those tumors induced by MPA–DMBA. It was of interest for us to explore whether the MPA treatment affected the rate and/or type of mutations affecting this oncogene family. Ha-*ras* codon 61st mutations have been reported previously in DMBA induced mouse mammary tumors. However, in those studies tumors were generated from hyperplastic (preneoplastic) mammary outgrowth lines (21,22), no information is available on the true frequency of Ha-*ras* mutations in 'primary' DMBA induced mouse mammary tumors. Mutations affecting the Ki-*ras* gene (codon 12) have been reported in NMU induced mouse mammary tumors (9). In our analysis we found that three tumors out of ten analyzed (30%) from the MPA–DMBA group 4 showed activating mutations in codon 61st of the Ha-*ras* gene versus only one tumor out of ten (10%) analyzed in the DMBA group had a mutation. On sequencing these, all four samples showed a CAA→CTA transversion (Gln→Leu) in codon 61 (Figure 3). No other mutations were observed in either codons 12 or 13 of Ha-*ras* or in the Ki-*ras* gene codons 12, 13 and 61. We can conclude that MPA pretreatment did not generate any novel mutations in Ha-*ras* or Ki-ras, however it appeared to increase the rate of Ha-*ras* codon 61st mutations generated by DMBA.

Although our original objective was to develop an efficient mouse mammary chemical carcinogenesis protocol, it is relevant that our findings add weight to previous reports that postulated that MPA can promote the development of mammary tumors both in rats and mice (10,19). As suggested by Russo and Russo (15), it is possible that MPA could act by generating a proliferative response in a specific compartment of the mammary gland, thus increasing the number of target cells at risk for mutation by the carcinogen treatment. Still much has to be learned to fully understand the effect of this potential tumor promoter on the mammary gland.

Acknowledgements

Supported by US Public Health Service grant CA59967 to C.M.A. and NCI CA16672 from the National Institutes of Health. We are grateful to Aaron Chen for technical assistance and to Michelle Gardiner for secretarial assistance.

References

1. Welsch,C.W. (1985) Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. *Cancer Res.*, **45**, 3415–3443.
2. Medina,D. (1982) Mammary Tumors. In Homburger,F. (ed), *The Mouse in Biomedical Research*. Academic Press, New York, vol. IV, pp. 373–396.
3. Medina,D. (1974) Mammary tumorigenesis in chemical carcinogen-treated mice. I. Incidence in BALB/c and C57BL mice. *J. Natl Inst.*, **53**, 213–221.
4. Medina,D., Butel,J.S , Socher,S.H. and Miller,F.L. (1980) Mammary tumorigenesis in 7,12-dimethybenzanthracene-treated C57BL×DBA/2f F_1 mice. *Cancer Res.*, **40**, 368–373.
5. Aldaz,C.M., Chen,A., Gollahon,L.S., Russo,J. and Zappler,K. (1992) Nonrandom abnormalities involving chromosome 1 and *Harvey-ras-1* alleles in rat mammary tumor progression. *Cancer Res.*, **52**, 4791–4798.
6. Gollahon,L.S., Chen,A. and Aldaz,C.M. (1995) Loss of heterozygosity at chromosome 1q loci in rat mammary tumors. *Mol. Carcinogen.*, **12**, 7–13.
7. Levan,G., Szpirer,J., Szpirer,C., Klinga,K., Hanson,C. and Islam,M. (1991) The gene map of the Norway rat (*Rattus norvegicus*) and comparative mapping with mouse and man. *Genomics*, **10**, 699–718.
8. Medina,D. (1974) Mammary tumorigenesis in chemical carcinogen-treated mice. II. Dependence on hormone stimulation for tumorigenesis *J. Natl Cancer Inst.*, **53**, 223–226.
9. Guzman,R.C., Osborn,R.C , Swanson,S M., Sakthivel,R., Hwang,S.-I., Miyamoto,S. and Nandi,S. (1992) Incidence of *c*-Ki-*ras* activation in *N*-methyl-*N*-nitrosourea-induced mammary carcinomas in pituitary-isografted mice. *Cancer Res.*, **52**, 5732–5737.
10. Pazos,P., Lanari,C., Meiss,R., Charreau,E.H. and Pasqualini,C.D. (1991) Mammary carcinogenesis induced by *N*-methyl-*N*-nitrosourea (*MNU*) and medroxyprogesterone acetate (*MPA*) in *BALB/c* mice. *Breast Cancer Res. Treatment*, **20**, 133–138.
11. DiGiovanni,J., Beltrán,L., Rupp,A., Harvey,R.G. and Gill,R.D. (1993) Further analysis of *c*-Ha-*ras* mutations in papillomas initiated by several polycyclic aromatic hydrocarbons and papillomas from uninitiated, promoter-treated skin in SENCAR mice. *Mol. Carcinogen.*, **8**, 272–279.
12. George,D.L , Scott,A.F., Trusko,S., Glick,B., Ford,E. and Dorney,D J. (1985) Structure and expression of amplified c-Ki-*ras* gene sequences in Y1 mouse adrenal tumor cells *EMBO J.*, **4**, 1199–1203
13. Orita,M., Suzuki,Y., Sekiya,T. and Hayashi,K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphism using the polymerase chain reaction. *Genomics*, **5**, 874–879.
14. Brenner,A.J. and Aldaz,C.M. (1995) Chromosome 9p allelic loss and p16/CDKN2 mutational analysis in uncultured breast tumors. Evidence of p16 inactivation in immortal breast epithelial lines. *Cancer Res* , **55**, 2892–2895.
15. Russo,I.H. and Russo,J. (1991) Progestagens and mammary gland development: Differentiation versus carcinogenesis. *Acta Endocrinol.* (Copenhagan), **125**, 7–12.
16 Chilvers,C. (1994) Breast cancer and depot-medroxyprogesterone acetate: A review. *Contraception*, **49**, 211–222.
17. Skegg,D.C.G., Noonan,E.A., Paul,C., Spears, G.F.S., Meirik,O. and Thomas,D.B. (1995) Depot medroxyprogesterone acetate and breast cancer *JAMA*, **273**, 799–804.
18. Lanari,C., Molinolo,A.A. and Pasqualini,C.D. (1986) Induction of mammary adenocarcinomas by medroxyprogesterone acetate in BALB/c female mice. *Cancer Lett.*, **33**, 215–223.
19. Russo,I.H. and Russo,J. (1988) Hormone prevention of mammary carcinogenesis: A new approach in anticancer research. *Anticancer Res.*, **8**, 1247–1264.
20. Labrie,F., Li,S , Bélanger,A., Côté,J., Mérand,Y. and Lepage,M. (1993) Controlled release low dose medroxyprogesterone acetate (MPA) inhibits the development of mammary tumors induced tumors induced by dimethylbenz[*a*]anthracene in the rat. *Breast Cancer Res. Treatment*, **26**, 253–265.
21 Dandekar,S., Sukumar,S., Zarbl,H., Young,L J.T. and Cardiff,R.D. (1986) Specific activation of the cellular Harvey-*ras* oncogene in dimethylbenzanthracene-induced mouse mammary tumors. *Molec Cell. Biol.*, **6**, 4104–4108.
22. Kumar,R., Medina,D. and Sukumar,S. (1990) Activation of Ha-*ras* oncogenes in preneoplastic mouse mammary tissues. *Oncogene*, **5**, 1271–1277.

Received on March 14, 1996; revised on June 3, 1996; accepted on June 10, 1996