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Antagonism of Aminoglutethimide and Danazol in the Suppression of Serum Free Oestradiol in Breast Cancer Patients

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Abstract—*The response rate of advanced postmenopausal breast cancer patients to treatment with aminoglutethimide (AG) + danazol was significantly worse than that with AG alone. The suppression of serum oestradiol levels by AG + danazol was similar to that by AG alone, but the combination treatment also suppressed sex hormone binding globulin (SHBG) levels and increased the % free oestradiol, whilst these parameters were unaffected by AG alone. The degree of suppression by AG + danazol of free oestradiol concentrations was less than the suppression of total oestradiol level and in some patients the concentration of the free fraction was increased above pretreatment levels. These effects on the presumed biologically active unbound fraction of oestradiol may explain the poor clinical response rate to AG + danazol.*

INTRODUCTION

AMINOGLUTETHIMIDE and danazol are two of a number of agents which have been used in the medical endocrine treatment of advanced breast cancer. Although aminoglutethimide was initially used in breast cancer because of its supposed effects on adrenal steroidogenesis [1], its mode of action is now thought to be by suppression of plasma oestrogen levels resulting from its inhibition of the aromatase enzyme system [2-5]. The mechanism of action of danazol in breast cancer is unknown.

In postmenopausal patients (who were not selected on the basis of oestrogen receptor status) the objective response rate to aminoglutethimide was 32% (mean of 19 studies [6]) and to danazol was 17% [7]. In an attempt to improve on the individual response rates the two drugs were combined and compared in a randomized trial against aminoglutethimide alone in the treatment of postmenopausal advanced breast cancer patients. Contrary to expectations, the response rate to the combination (13%) was significantly worse than to aminoglutethimide alone (33%) [8]. We report here endocrine studies which were undertaken in an attempt to explain the poor response to the combination.

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MATERIALS AND METHODS

Clotted blood samples were collected from postmenopausal patients with advanced, histologically proven breast cancer, who were part of a randomized trial (the full details of which have been published separately [8]) of treatment with aminoglutethimide (AG), either with or without danazol, at the Peter MacCallum Hospital, Melbourne. All patients received 250 mg AG three or four times daily according to tolerance and cortisone acetate 25 mg in the morning and 12.5 mg at night. In addition, patients randomized to AG plus danazol received the latter in a dose of 200 mg three times daily. The patients had received no other endocrine treatment for at least 1 month before starting therapy. Samples were obtained before and during treatment from 15 patients on AG alone and 12 patients on AG + danazol. Serum was separated and was transported frozen to London for analysis.

Sex hormone binding globulin (SHBG) binding capacity was measured by the method of Iqbal

and Johnson [9]. This method was found to be unaffected by the presence in serum of 10^{-6} M danazol, 10^{-5} M 2-hydroxymethylethisterone or 3×10^{-7} M ethisterone (the two major circulating metabolites of danazol). These concentrations are higher than the respective peak concentrations found *in vivo* [personal communication, Dr R. S. Andrews, Sterling Winthrop Research]. The percentage of oestradiol unbound to protein (i.e. the % free) was measured by centrifugal ultrafiltration-dialysis according to our previously described modifications [10] of the method of Hammond *et al.* [11].

Total unconjugated oestradiol was measured by radioimmunoassay using the [^{125}I]oestradiol kit from EIR (Zurich, Switzerland), after organic extraction and chromatographic purification of the sample. Approximately 1000 cpm of [2,4,6,7-^{3}H]oestradiol (94 Ci/mmol, Amersham International) were added as a recovery control to 1 ml of serum, which was then extracted with ether, which had been freshly purified through alumina (B.D.H.). The dried extract was applied to a Sephadex LH20 column (80 mm length × 5 mm diameter) and oestradiol was eluted after passage of between 4 and 6 ml dichloromethane:methanol, 95:5 (analytical grade solvents). It was established that danazol, ethisterone and 2-hydroxymethylethisterone did not co-elute with oestradiol. After solvent evaporation the residue was reconstituted in 0.5 ml of assay buffer and 2 × 0.1 ml were taken for radioimmunoassay and 0.2 ml for recovery estimation. Mean recovery was 69% and assay sensitivity was 0.4 fmol/tube. The mean solvent blank was 3.9 ± 2.0 (S.D., $n = 7$) pmol/l. This was not subtracted from the result. The within and between assay c.v.s were 8.6 and 12.3% respectively at a serum concentration of 60 pmol/l. The concentration of free oestradiol was calculated from the total concentration and the % free oestradiol.

For all analyses all samples from the same patient were run in the same batch. Statistical analyses were performed by paired and unpaired *t* tests.

RESULTS

Total oestradiol concentration (Fig. 1a)

There was a fall in mean oestradiol levels after 1-2 months on both treatments of about 50% compared to pretreatment levels ($P < 0.05$). There was no further significant change in oestradiol levels with either treatment during the next 2 months. There was no significant difference in oestradiol levels between the two treatment regimens at any time point.

SHBG binding capacity (Fig. 1b)

There was no significant difference in mean SHBG binding capacity between patients in the two groups before treatment, and there was no significant change in the levels in the AG-treated patients during treatment. However, there was a marked fall in the SHBG binding capacity of patients treated with AG + danazol during the first 2 months of treatment ($P < 0.001$). During the next 2 months on treatment there was an apparent further fall in binding capacity, but this was not statistically significant. The mean fall in SHBG binding capacity was 74% after 1-2 months and 83% after 3-4 months.

Percentage free oestradiol

This was measured before treatment and after 1-2 months of treatment in five patients on AG and in eight patients on AG + danazol. The mean values for patients on AG before and during treatment were not significantly different [1.33 ± 0.08% (S.E.M.) and 1.34 ± 0.06%, respectively]. The eight patients on AG + danazol all showed an increase in % free oestradiol during the first 2 months on therapy: pretreatment mean, 1.13 ± 0.08%; on-treatment mean, 1.88 ± 0.06, $P < 0.001$. The mean increase as a percentage of pretreatment value in these patients was 72 ± 12, whilst the mean percentage fall in SHBG binding capacity in these eight patients was 79 ± 5.

Concentration of free oestradiol

For six patients treated with AG + danazol sufficient sample before and after 1-2 months of treatment was available for analysis for both % free oestradiol and total concentration of oestradiol. Concentration of free oestradiol was thus calculated for these samples and is shown in Table 1. For each patient the on-treatment concentration of both total and free oestradiol is

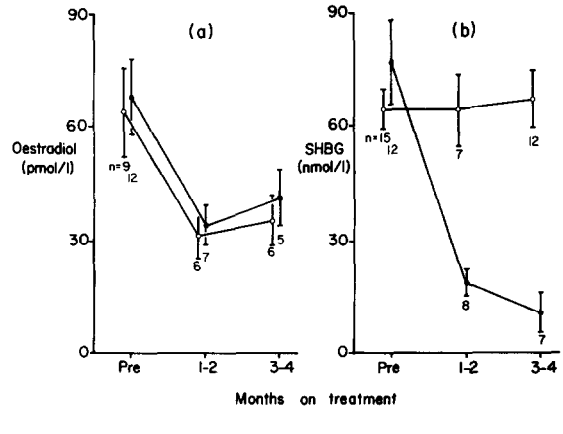

Fig. 1. The effects of AG (○) and AG + danazol (●) on the mean (± S.E.M.) serum levels of (a) oestradiol and (b) SHBG binding capacity in postmenopausal patients with advanced breast cancer.

Table 1. Clinical response (assessed according to standard UICC criteria) and concentration of free oestradiol before and after 1-2 months *treatment with aminoglutethimide plus danazol (AG + D) or AG alone*

Patient	Concentration of free oestradiol (pmol/l) Pretreatment	On-treatment	Clinical response
AG + D:			
A	1.53	0.55	R
B	0.27	0.23	PD
C	0.60	0.58	SD
D	0.50	0.57	SD
E	0.57	0.92	PD
F	0.50	0.76	PD
AG only:			
G	1.54	0.21	R
H	0.59	0.28	PD
I	0.69	0.48	R

R: response; SD: stable disease; PD: progressive disease. Patients are identified by the same letters as in Fig. 2.

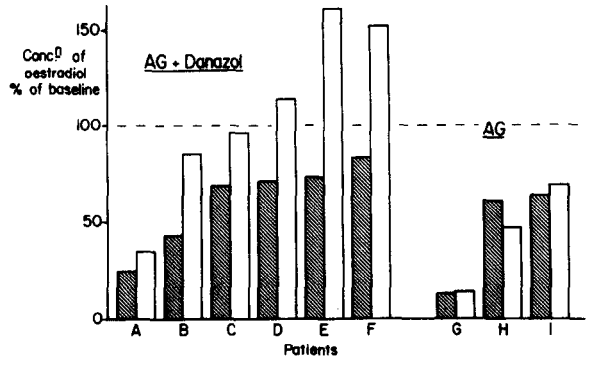

Fig. 2. The effects of AG and AG + danazol on the concentration of total (hatched bars) and free (open bars) serum oestradiol in individual postmenopausal patients with advanced breast cancer. Levels after 1-2 months *treatment are expressed as a percentage of the pretreatment value.*

shown in Fig. 2 as a percentage of the pretreatment concentration. In each patient the on-treatment total concentration of oestradiol was found to be suppressed, although the degree of suppression is variable. The suppression of free oestradiol, however, was less for each patient than the suppression of total oestradiol, and in 3/6 patients the on-treatment concentration of free oestradiol was higher than pretreatment levels.

Analysis of samples from three patients treated with AG alone showed similarly consistent suppression of total oestradiol concentrations, but in this case there was little disparity between the suppression of total and free oestradiol concentrations.

The concentration of free oestradiol for the nine patients prior to treatment ranged from 0.3 to 1.5 pmol/l. The clinical response of these patients is shown in Table 1.

DISCUSSION

A number of recent investigators have found that % free oestradiol is higher in breast cancer patients than in matched controls [12-14]. In addition, % free oestradiol is higher in British women than in Japanese women (who have a lower incidence of breast cancer) [15] and, importantly, is higher in normal women who on follow-up had developed breast cancer than in those who had not [16]. The current study supports the importance of free (and possibly albumin-bound) rather than total oestradiol to the progression of breast cancer.

It is generally accepted that oestrogen deprivation is the major mechanism by which both medical and surgical endocrine treatment of breast cancer is effective [17]. AG achieves this in postmenopausal patients by suppression of oestrogen synthesis by the peripheral aromatase enzyme system [2-5]. Although danazol is marketed as an antigonadotrophin and is commonly used in the treatment of endometriosis and benign breast disease, its mode of action is unclear. Interaction with progesterone and oestrogen receptors, and inhibition of adrenal and gonadal steroidogenesis are possible mechanisms [18]. More recently we have suggested that the therapeutic action of danazol in endometriosis may depend on its androgenic activity [19]. Danazol markedly increases the % free testosterone by suppression of SHBG levels and by competition for SHBG binding sites [20]. The mechanism by which danazol is effective in causing regression in some breast cancer patients is equally unclear but in postmenopausal patients it seems most unlikely that it is by suppression of gonadotrophin release. It is notable that the

objective response rate in the only study reported for danazol in breast cancer was 17% [17] and this is similar to the response rate for three androgen preparations (testosterone propionate, fluoxymesterone and either dihydroxymethyltestosterone or dihydromethyltestosterone propionate), which were 14, 14 and 18% respectively when tested in at least 300 patients each [21].

There is no indication that danazol is an aromatase inhibitor and it was therefore hoped that its combination with AG might increase the response rates above that found with the individual drugs. However, the trial of AG vs AG + danazol found a significantly lower response rate for the combination: objective response rate in assessable patients 33% (21/64) vs 13% (5/38) ($P < 0.05$). In addition, the median duration of response was 15 months for AG vs 11 months for AG + danazol. Oestrogen receptor data were unavailable on these patients but there was no difference between the groups in their response to previous endocrine treatment or in disease free interval, numbers of sites of involvement, age or site of involvement, which suggests that the patients in the AG + danazol group were not inherently more endocrine-resistant.

From the results obtained it is also clear that there was little difference between the two groups in the degree of suppression of total oestradiol, which was similar to that found in other studies on aminoglutethimide conducted by this laboratory [22, 23]. The difference in response cannot therefore be explained by a difference in suppression of total oestradiol levels.

The effects of the combined treatment on SHBG, however, may explain the difference in response. It is now widely accepted that only that portion of a steroid which is unbound to protein (i.e. free) is able to cross the plasma membrane and interact with cytoplasmic receptors. There is an inverse relationship between SHBG binding capacity and the fraction of oestradiol which circulates unbound to protein [10, 24]. Thus the suppression of SHBG binding capacity by danazol, which has been shown here to also occur in combination with AG, would be expected to cause an increase in % free oestradiol, and this has also been confirmed in this study. We have shown that as well as suppressing SHBG binding capacity, danazol and its metabolites ethisterone and 2 hydroxymethylethisterone increase % free testosterone by competition for SHBG binding sites (data to be published elsewhere), and this is likely also to contribute to the increase in % free oestradiol. Overall the effect of this increase in % free oestradiol in all patients treated with AG + danazol was to counteract the suppressive effect of AG on the concentration of the free, biologically active fraction of oestradiol. In some patients this resulted in these effects of AG being totally nullified. Thus the marked suppression of SHBG binding capacity may be responsible for the poor efficacy of the combined therapy. The number of patients in whom suppression of free oestradiol concentration was measured was too small to make a meaningful correlation with clinical response, but it is interesting to note that of the six patients on AG + danazol, only the patient with the greatest % suppression (patient A) showed an objective response.

A fall of SHBG binding capacity will cause an increase in % free testosterone as well as % free oestradiol. This effect might also be detrimental to a regimen aimed at oestrogen deprivation since this could increase substrate concentration for the aromatase enzyme. However, the observation that suppression of total oestradiol concentration was unaffected by the inclusion of danazol in the regimen indicates that this effect is probably unimportant in terms of overall oestrogenicity. It has been shown previously that danazol does not affect the total oestradiol concentration in postmenopausal women [25].

Although it is widely accepted that it is the free fraction of oestradiol that is biologically active, there is much contentious discussion as to whether the albumin-bound fraction is biologically available, because of its very rapid dissociation rate, and should therefore be considered in terms of tissue stimulation [26–28]. The albumin-bound fraction was not examined in this study. However, the ratio of free oestradiol to that bound to albumin is constant as long as albumin concentration remains unchanged [29], and this indicates that the suggestion that danazol is detrimental to the combined regimen because of its effects on oestrogen binding is valid whether the biologically important fraction is the free or the albumin-bound plus the free.

The only circumstance in which combined endocrine treatment has been shown to give an improved response rate above single-agent endocrine therapy was in the use of AG + danazol + tamoxifen [30]. At first sight it is difficult to reconcile that result with the poor efficacy of AG + danazol and the observation that tamoxifen + AG has no benefit in terms of response rate above tamoxifen alone [31]. We have suggested [32] that the latter observation may be due to the oestrogenic activity of tamoxifen being potentiated by serum oestrogen suppression by AG. The combined endocrine effects of danazol, AG and tamoxifen are difficult to predict, but one effect of tamoxifen, which we have shown to be maintained in the presence of AG [32], is to increase SHBG binding capacity. In ten patients

on treatment with tamoxifen + AG + danazol we found the SHBG binding capacity to be reduced from 62.1 ± 5.5 nmol/l before to 34.7 ± 10.6 nmol/l after 3 months treatment [unpublished results, samples kindly provided by Drs R. C. Coombes and T. J. Powles]. Examination of the relationship between % free oestradiol and SHBG binding capacity indicates that such a reduction in SHBG binding capacity would only increase % free oestradiol from about 1.1 to 1.3 because of the logarithmic nature of the relationship [10], much less than that with AG + danazol without tamoxifen.

In conclusion, it is clear that the overall endocrine and clinical effects of combination endocrine therapy may be predictable only with a full understanding both of the endocrine effects and of the mechanism of action of the individual drugs. It would seem that it is inappropriate to combine danazol with drug regimens which are designed at suppression of circulating oestrogen levels, because of its effect on protein binding and tissue availability of oestradiol in serum.

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