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Original Paper

Immunoneutralisation of Gonadotrophin Releasing Hormone: a Potential Treatment for Oestrogen-dependent Breast Cancer

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The aim of this study was to assess the therapeutic potential of active immunisation with GnRH-glycys-PPD in a hormone-dependent experimental model. Mammary tumours were induced in female rats using dimethylbenzanthracene (DMBA) and the effects of GnRH immunoneutralisation on tumour development were evaluated. High titres of anti-GnRH IgG correlated with a decrease in oestrogen levels and subsequent tumour suppression. A comparison of immunised and non-immunised animals showed that when GnRH-specific IgG levels were at a maximum titre (80–100 μg/ml), nearly 10% of the GnRH-glycys-PPD treated animals showed mammary masses, compared with all the non-treated animals at the same stage in the study. When the antibody levels fell, tumour regrowth was observed, but to a level below that observed in the non-treated animals. Following further treatment with the analogue, the tumours regressed again, showing their retention of hormone dependency. This is consistent with other endocrine manipulations in the treatment of breast cancer; the advantages of immunisation with GnRH-glycys lies in its non-toxicity and reduction in side-effects, which were mainly adjuvant-induced. © **1997 Elsevier Science Ltd.**

Key words: antibodies, antineoplastic, experimental mammary neoplasms, gonad atrophy, gonadotrophin releasing hormone analogue, hormone-dependent, immunotherapy, serum oestrogen

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INTRODUCTION

GONADOTROPHIN RELEASING hormone (GnRH or LHRH) forms an essential link between the central nervous system and the anterior pituitary gland in the control of fertility. GnRH is released by the hypothalamus, travels to the pituitary gland via a portal system, where it stimulates the release of luteinising hormone and follicle stimulating hormone into the general circulation. These in turn regulate steroidogenesis and gametogenesis in both males and females [1–3]. In a previous paper [4], we described the effects of immunisation with an analogue of GnRH, GnRH-glycys, conjugated to PPD (purified protein derivative of tuberculin) [5], on the female reproductive system. A high antibody response was evoked which correlated well with low serum oestradiol levels. This led us to propose that, potentially, the conjugated analogue could be used therapeutically against hormone-dependent breast malignancies, as well as other oestrogen-sensitive disorders—polycystic ovary syndrome, premenstrual syndrome and endometriosis, which are currently treated with unconjugated GnRH analogues. Endocrine manipulation by immunisation overcomes the disadvantages associated with direct GnRH analogue administration, namely, administrations of large, toxic and expensive drug doses. The use of small quantities of a non-toxic synthetic peptide makes this a more viable and economic therapy. The advantage of using GnRH-glycys as opposed to GnRH is that the analogue has the same conformational preference in solution as the native hormone, but is sufficiently altered in composition to prevent any potential autoimmune reactions [5].

One extensively used animal model makes use of the fact that multiple mammary adenocarcinomas can be induced in female Sprague–Dawley rats by the single administration of the polycyclic hydrocarbon DMBA (7,12-dimethylbenz(*a*)anthracene) [6]. These tumours are considered to be hormone dependent as they regress fol-

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lowing ovariectomy [7], or after the administration of various steroids [6, 8] and antihormones [7, 9]. The model has also proved useful for the evaluation of drugs considered to have antitumour properties [10, 11].

The aim of this study was to assess the effect of immunisation with GnRH-glycys in a hormone-dependent mammary tumour model.

MATERIALS AND METHODS

Treatment schedule (Table 1)

Female Sprague–Dawley rats, aged 16 weeks old (originating from Harlan Olac Ltd, Oxfordshire, U.K.) were divided into three groups of 20, in study week 0. All the rats in Group III were given a subcutaneous injection of 0.1 ml BCG (Evans Biological Ltd, U.K.) equivalent to half the standard human dose, in study week 2. The day after BCG-priming, tumour induction was carried out in Groups II and III (20 mg/ml DMBA in peanut oil, by gavage) and Group I was left untreated. The immunogen, GnRH-glycys-PPD, was prepared as detailed previously (GnRH-glycys was designed by Proteus Molecular Design Ltd, and synthesised by Peninsula Laboratories Inc., U.S.A.), with sulphomaleimido benzoic succinimide as the linker agent. In study weeks 6, 8, 10 and 12, animals in Group III were immunised once a week with 0.2 ml GnRH-glycys-PPD [12] adsorbed on to Imject Alum (Pierce Chemicals, U.K.), intraperitoneally, with the equivalent of 50 μg GnRH/rat. In the second stage of the study, the Group III animals received a booster injection in study week 25 and Group II were immunised in study weeks 25, 27, 29 and 31, having previously been injected in week 21 with BCG (Table 1).

Assessment of the physiological effects of immunisation

During the 46 week study, body weights were measured (food consumption was not monitored) and injection sites were palpated on a weekly basis. In study week 23 (interim kill) and at the end of the study (terminal kill), autopsies were performed and target tissues were fixed in 10% (v/v) formalin in saline for histological slide preparation (BS&S Ltd, Edinburgh, U.K.). The tissues were weighed and statistical analysis performed on the data.

Table 1. Treatment schedule used in the study

Study week	Group I ($n = 20$)	Group II ($n = 20$)	Group III ($n = 20$)
2	–	–	BCG
2	–	DMBA	DMBA
6	–	–	1st immunisation
8	–	–	2nd immunisation
10	–	–	3rd immunisation
12	–	–	4th immunisation
21	–	BCG	–
23	interim kill	interim kill	interim kill
25	–	1st immunisation	5th immunisation
27	–	2nd immunisation	–
29	–	3rd immunisation	–
31	–	4th immunisation	–
45	terminal kill	terminal kill	terminal kill

Female Sprague–Dawley rats, at the age of 16 weeks old (study week 0), were randomised into three groups of 20. Half the animals were sacrificed in study week 23 (interim kill) and the rest in study week 45 (terminal kill)

Serum antibody determination

Serum antibody levels were determined by ELISA using GnRH-glycys-BSA coated plates, as detailed previously [4, 12]. Tail bleeds were carried out at weekly intervals and for a month following immunisation. The mean of duplicate results minus background readings was calculated in micrograms of GnRH specific IgG/ml from a standard curve. The levels of IgG produced were assessed, as well as the IgG subclass and IgM levels, with peroxidase-labelled rabbit anti-rat IgGl, IgG2a, IgG2b, IgG2c or IgM instead of γ chain-specific IgG.

Determination of oestrogenaemia

Estimation of serum oestradiol levels was carried out at the interim kill stage, using a direct competitive RIA (radioimmuno-assay) kit (Sorin Biomedica, Rome, Italy). The reproductive cycle of the animals was assessed from duplicate vaginal smears, taken weekly on two consecutive days. The stage in the reproductive cycle was determined by the relative cellularity of the smear and the proportion of cornified epithelial cells, leukocytes and mucus present [13].

Assessment of tumour progression

On a weekly basis, the animals were palpated for any abnormal masses and their position noted for postmortem identification. Three diameter measurements were taken with calipers when the masses reached a 3 cm length. Gross masses were excised from sacrificed animals at the interim kill (IK) and terminal kill (TK) stages and from animals sacrificed for humane reasons (termed HIK or HTK, depending on whether the animals were sacrificed prior to the interim or terminal kill stages). It was not possible to assess the tissues from animals which had died spontaneously, as they were markedly autolysed. The masses were cut into 1 mm^3 sections and stored in 10% (v/v) formalin in saline until sectioned and stained for histology (BS&S Ltd, U.K.). The slides were evaluated microscopically, the tissue of origin was identified and any mammary tumours were classified.

Control group

Prior to carrying out this study, a group of 20 female rats, 16 weeks old, were treated with DMBA in order to give an indication of the length of time for tumour progression and life expectancy. The animals were palpated weekly and a note was made of any abnormal mammary masses. However, no other evaluations were carried out.

Statistics

Comparisons were calculated using the Mann–Whitney test.

RESULTS

Antibody titres to GnRH

No IgM antibody response was detectable, following immunisation and for the duration of the study. No IgG antibody response was detectable after the first administration of analogue, but after the second injection the mean concentration of IgG was raised to 33.3 ± 1.2 μg/ml in Group II (after the second injection) and 25.2 ± 0.2 /ml in Group

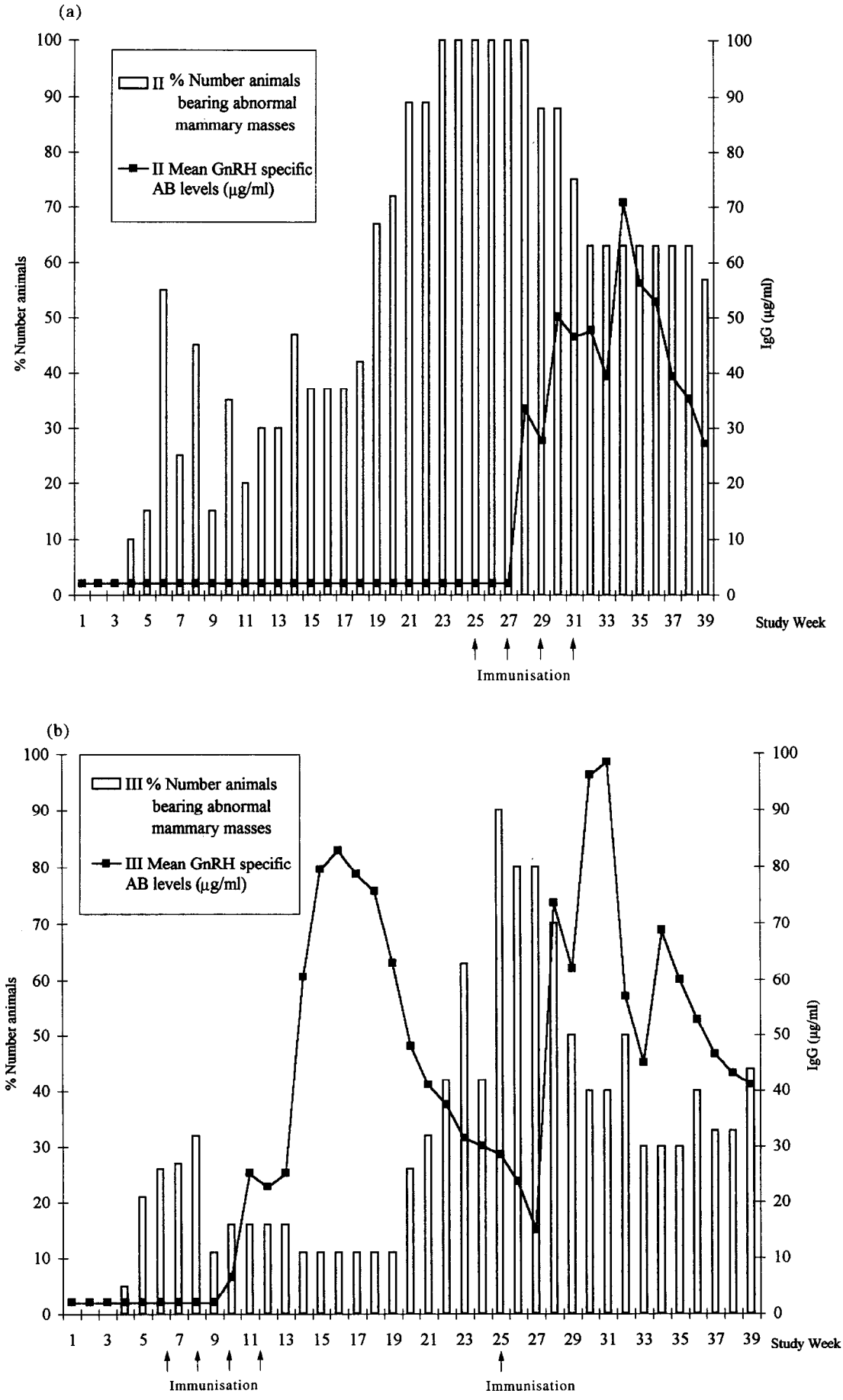

Figure 1. IgG response to immunisation with GnRH-glycys-PPD (equivalent to 50 μg GnRH per injection) in female Sprague–Dawley rats. The percentage of animals bearing mammary masses is also indicated. Immunisations were administered in (a) Group II— study weeks 25, 27, 29, 31; (b) Group III—study weeks 6, 8, 10, 12, 25.

III after the third injection (Figure 1(a), (b)). After each subsequent administration the concentrations increased to 50.1 ± 3.1 and 76.1 ± 2.5 μg/ml in Group II and 60 ± 0.95 μg/ml in Group III. In Group III (Figure 1(b)) the IgG levels peaked at 82.8 ± 4.1 μg/ml before gradually decreasing to below this level of antibody concentration, 30 μg/ml. This concentration was maintained for 4 weeks, until a further booster injection caused the antibody level to rise again. This fifth injection resulted in the mean IgG concentration rising 5 weeks after drug administration, to 98.4 ± 4.2 μg/ml. An examination the IgG subclasses raised showed that mainly IgG1 and IgG2a antibodies were induced.

Serum oestradiol (E_2) levels and vaginal cytology

At the interim kill stage, non-immunised animals showed E_2 levels of 18.6 ± 5.6 pmol/l compared with levels of 4.4 ± 6.0 pmol/l in the Group III animals. This reduction of E_2 levels was significant ($P > 0.025$) and a direct result of immunisation. Vaginal cytology taken during the study revealed that immunisation with GnRH-glycys-PPD caused ovarian failure in both immunised groups, a month following the fourth injection and this lasted for the duration of the study. In general, this took the form of permanent dioestrous.

Palpation assessment

Abnormal masses began to develop approximately two weeks following induction with DMBA. In Group II, all the animals bore abnormal masses by study week 23. However, following the second injection with GnRH-glycys-PPD, a gradual decrease in animals bearing abnormal masses was observed, to around 60% for the duration of the study (Figure 1(a)). In the Group III animals, the percentage of animals with abnormal masses increased steadily until after the second injection with GnRH-glycys-PPD, when the number of animals bearing abnormal masses decreased to 10–20% (Figure 1(b)), as opposed to double that in the non-immunised animals. In the treated animals, cessation of GnRH-glycys-PPD resulted in an increase in the size and number of growths (as determined by palpation). However, the majority of these masses regressed again following a fifth injection in study week 25 (Figure 1(b)). A comparison of the IgG response and development of abnormal masses showed that, while antibody levels remained above 50 μg/ml, the numbers of animals bearing abnormal masses decreased, and vice versa. This was true in animals where immunisation was commenced immediately following tumour induction, as well as in those receiving GnRH-glycys 6 months after tumour induction.

Figure 2 shows the incidence of single and multiple masses occurring in Groups II and III during the first part of the study. In addition to the increasing number of animals showing abnormal growths in Group II, there was also a greater incidence of multiple masses. Immunisation with GnRH-glycys-PPD suppressed the number of animals with abnormal growths, but as the antibody levels fell there was an increase in this number and incidence of multiple masses. In the second part of the study, following immunisation in both Groups II and III, the number of animals bearing abnormal masses decreased together with the incidence of multiple growths (Figure 3).

In the DMBA basic control group, run prior to this study, the numbers of animals bearing abnormal mammary masses and incidence of multiple growths was similar to that observed in Group II prior to immunisation (data not shown). However, beyond 24 weeks, there was a rapid increase in the number of spontaneous and humane deaths and all the animals had multiple growths. This group only survived a further 9 weeks.

Tumour pathology (Table 2)

Figure 4 shows cross-sections through typical malignant and benign compound mammary tumours, although there was no clear boundary between the two types of tumour. Generally the tumours consisted predominantly of ductal structures with variable amounts of stroma and secretory alveoli. Those classified as malignant showed local invasion, a high mitotic rate, large nucleoli, nuclear or cellular pleomorphism and epithelia of several cell thickness, while many of the tumours classed as benign contained areas of cellular atypia. One other type of benign tumour, which is known to occur spontaneously in Sprague–Dawley rats, contains a predominantly fibrous stroma and in this study was grouped separately as fibroadenomas.

In Group II (tumour induction in study week 2 and GnRH-glycys-PPD in study weeks 25, 27, 29, 31), compound mammary tumours occurred in 11 animals, being present in multiple sites in 6 and occurring in a local lymph node in one. Of these 11, 2 animals had benign, 7 malignant and 2 a combination of tumours. Four of the 5 premature humane decedents had malignant tumours. At the interim stage (IK and HIK, $n = 9$), in Group II, 6 animals showed tumours (4 malignant, 1 benign and 1 mixed). Of these, 3 animals showed tumours at multiple sites. By the terminal stage (TK and HTK, $n = 10$), 7 animals developed tumours (3 malignant, 1 benign, 2 spontaneous fibroadenomas and 1 mixed). Of these, 3 animals showed tumours at multiple sites. Although the incidence and type of tumour were similar, the size of the tumours, by palpation, decreased following treatment with GnRH-glycys-PPD.

In the Group III animals (tumour induction in study week 2 and GnRH-glycys-PPD in study weeks 6, 8, 10, 12, 25), the number of compound mammary tumours induced was reduced and multiple sites were involved in only one animal. Furthermore, there was an increase in the number of animals with benign tumours occurring relative to those with malignant tumours. Compound mammary tumours occurred in a total of 5 animals, of which 3 had benign, 1 had a malignant and 1 had both types. At the IK stage ($n = 9$), 1 animal had a compound malignant tumour and 1 a fibroadenoma, but no animals had to be sacrificed prematurely. No TK animals ($n = 5$) had tumours, whereas at the HTK stage ($n = 4$), 3 animals showed benign tumours (one together with a fibroadenoma) and 1 animal a combined tumour. In Group I, only one animal showed a spontaneous fibroadenoma. Table 2 summarises these findings.

Spontaneous premature decedents

In Group II, one animal died spontaneously in week 20. No tissues were preservable and so histopathological assessment was not made. Therefore, the cause of death was unknown. For two weeks preceding death, this animal had a small, singular, abnormal mass (as determined by palpation). In Group III, 2 animals died spontaneously, one in

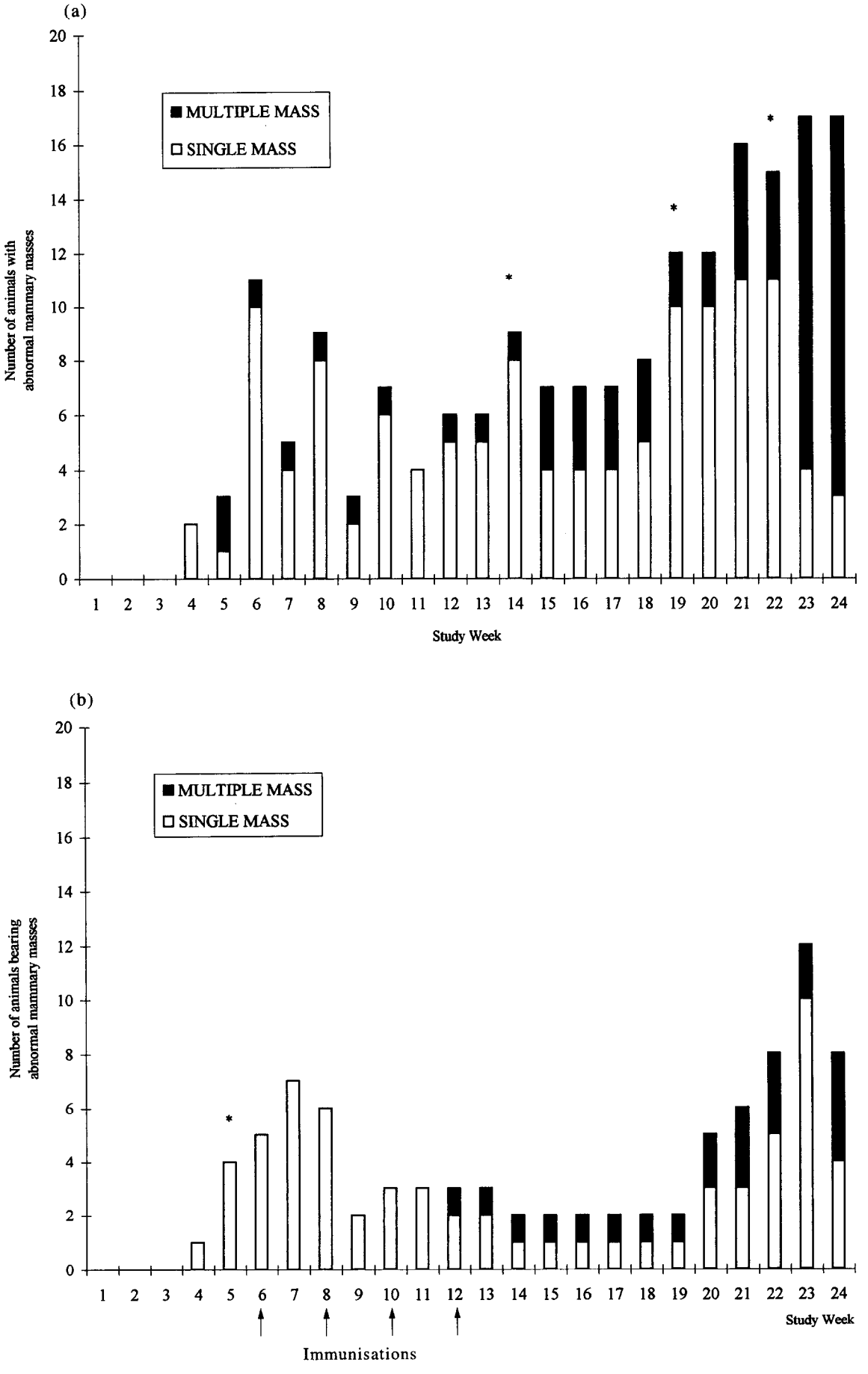

Figure 2. Number of animals bearing abnormal mammary masses, as determined by palpation, during study weeks 0–24. (a) Group II—these animals were non-immunised during this period of time; (b) Group III—immunisations were administered in study weeks 6, 8, 10, 12. *Indicates premature deaths.

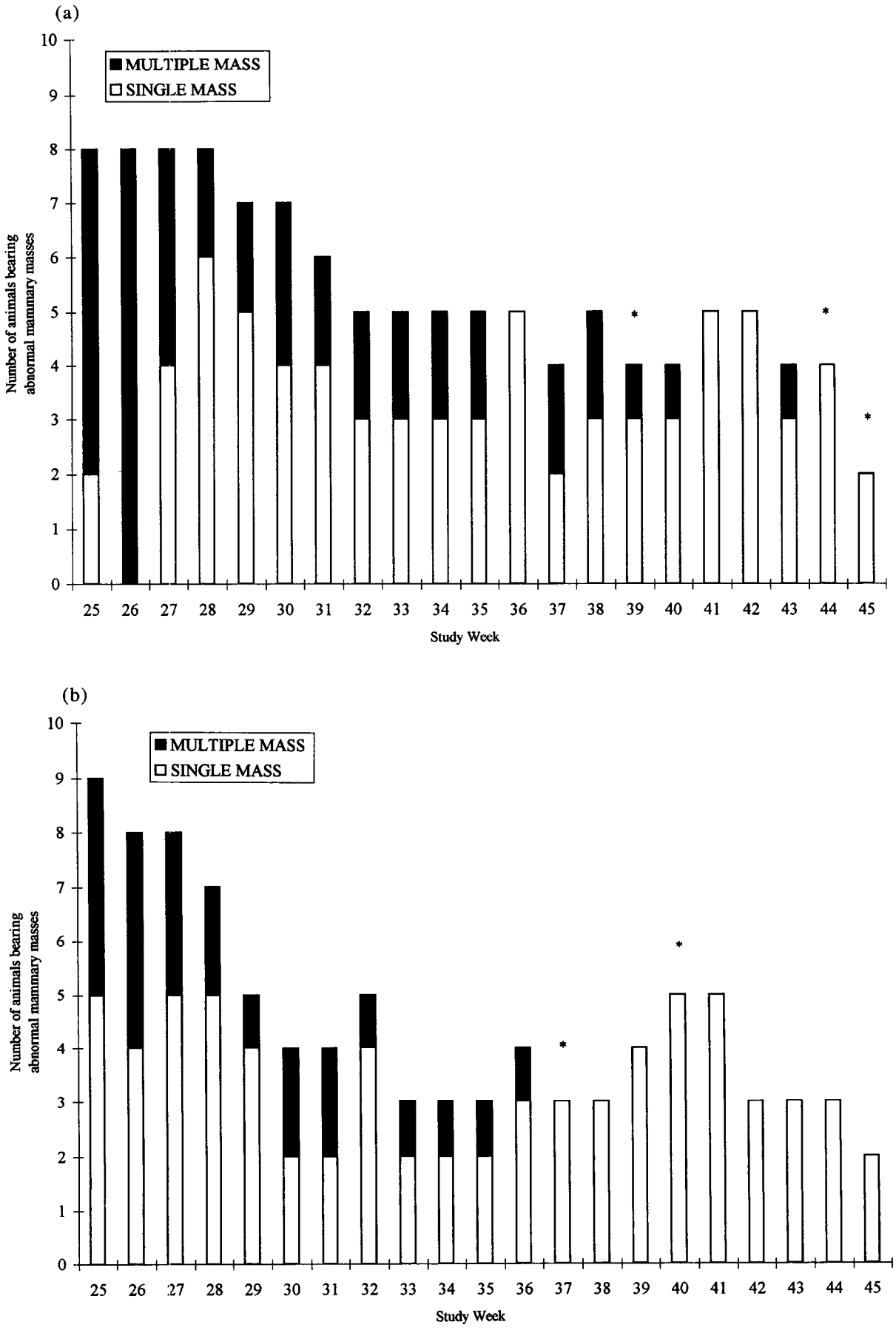

Figure 3. Number of animals in (a) Group II and (b) Group III bearing abnormal mammary masses, as determined by palpation, during study weeks 25–45. *Indicates premature precedents.

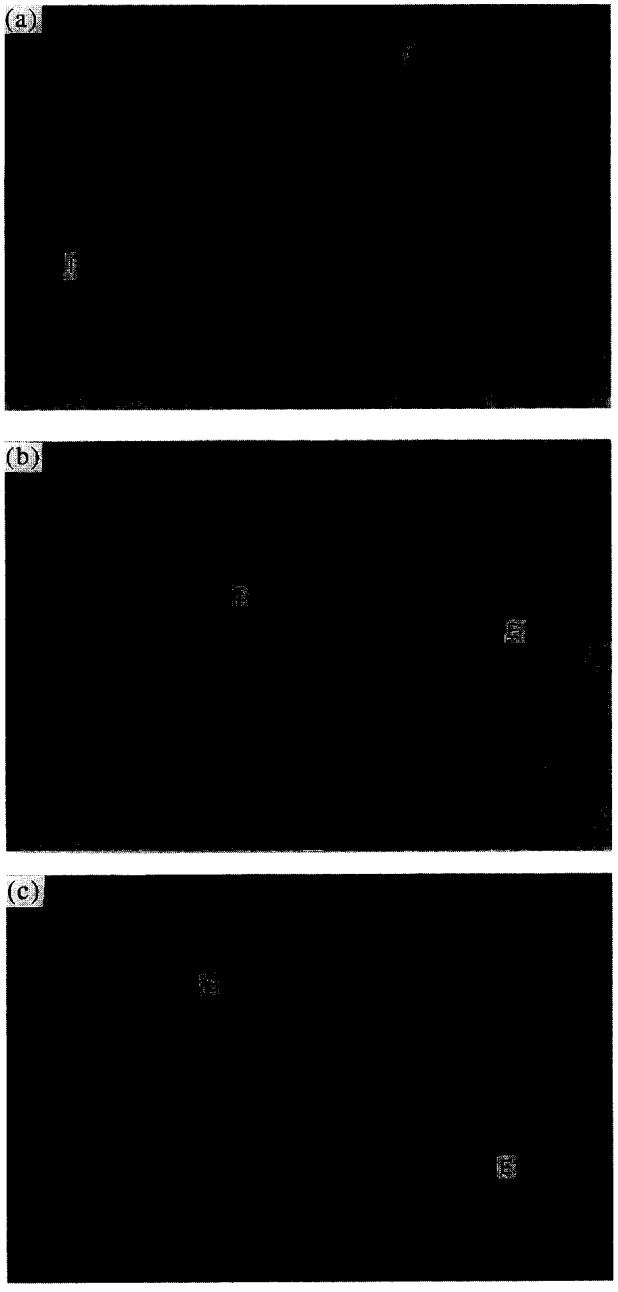

Figure 4. (a) DMBA-induced compound malignant mammary tumour obtained from a female Sprague–Dawley rat, prior to treatment with GnRH-glycys-PPD (Group II, interim kill, study week 23). This section also shows intravascular tumour cell growth (I) and proliferating epithelium (P). (b) DMBA-induced compound benign mammary tumour, obtained from a female Sprague–Dawley rat following immunisation with GnRH-glycys-PPD (Group III, terminal kill, study week 45). The tumour shows single epithelium lined tubules (E). (c) A simple spontaneous fibroadenoma obtained from an untreated female Sprague–Dawley rat, showing single epithelium lined tubules (E).

week 5 and the other in week 38. Again, the tissues were markedly autolysed and the cause of death not known. The animal which died in week 5 did not have any palpable masses prior to death, but the animal which died in week 38 was observed to have a small, singular, abnormal mass during study weeks 21–27.

Physiological effects of immunisation (Table 3)

Histopathology of the autopsied Group II ovaries ($n = 9$), prior to immunisation, showed evidence of ovulation (presence of corpora lutea) in all the animals, but after GnRH-glycys-PPD treatment this was found in only 1 animal ($n = 8$), The Group III animals showed evidence of depressed ovarian activity in the early part of the study (4 out of 9 animals with corpora lutea) and, as in Group II, this was found in only 1 animal ($n = 9$) by the end of the study. This was reflected in the decrease in ovarian weight from the interim to terminal autopsies (Table 3). In Group II, the combined ovarian weight at interim autopsy was 126.3 ± 35.0 mg and at terminal autopsy was 24.3 ± 33.1 mg. The weights for the Group III animals at interim and terminal autopsy were 66.8 ± 38.4 mg and 18.0 ± 16.4 mg, respectively. In comparison, the Group I animals showed combined ovarian weights of 130.5 ± 10.3 mg (interim autopsy) and 129.6 ± 10.9 mg (terminal autopsy). The animals in Group II (terminal autopsy) and the animals in Group III (interim and terminal autopsy) showed a significant reduction ($P < 0.01$) in ovarian weight compared with the equivalent Group I animals.

The effect of immunisation on the uterine weight is also shown in Table 3. There was no difference in weight from the interim to terminal autopsies in the Group II animals. The Group III animals showed a significant decrease ($P < 0.01$) in weight at terminal autopsy (0.172 ± 0.01 g) compared with the Group I animals at terminal autopsy (0.654 ± 0.1 g) and the Group III animals at interim autopsy (0.659 ± 0.22 g). In addition, histopathology revealed atrophy of the uterine and cervico-vaginal tissue of the Group III animals by the end of the study in comparison with Groups I and II. Atrophy of the uterus consisted of a reduction of intercellular matrix and condensation of nuclei; endometrial glands were tubular or slightly coiled and contained a hyperchromatic cuboidal or columnar lining. In extreme cases the uterus was contracted in size as described in a previous publication [4].

The vagina and cervix showed changes in epithelium, following GnRH-glycys-PPD treatment. In Group II (at TK), the epithelium tended to be stratified or keratinised, whereas the Group III animals (at IK and TK) showed mainly keratinisation and in extreme cases the epithelium became low stratified as shown in a previous study [4]. Only one animal in Group III at IK showed similar effects. These changes were unlike those seen in ageing animals, where there are increased amounts of uterine intersitium and cystic endometrial hyperplasia, but closely resembled those seen in juvenile rats.

A significant effect of immunisation was an increase in body weight ($P < 0.025$) after the fourth GnRH-glycys-PPD injection. In Group III the increase in body weight was maintained for approximately three months before returning to normal (compared with Group I); however, the fifth injection resulted in an increase to the previously attained level (Figure 5).

Correlation between tumour presence and gonadal atrophy

None of the animals showing extreme gonadal atrophy (low stratified epithelia of the vagina, contracted uterus) bore compound mammary tumours (Table 4). The animals in Group II with tumours showed stratified or keratinised epithelia of the vagina and only 1 out of five of those treated

Table 2. A summary of tumour pathology findings

Treatment group	Number of animals bearing Malignant tumours	Benign tumours	Combined tumours*	No tumours
Group I				
IK (week 23, $n = 10$)	0	0	0	10
TK (week 45, $n = 10$)	0	1FA	0	9
Group II†				
IK (week 23, $n = 7$)	2	1	1 (MS, 3B, 1M)	3
HIK (before IK, $n = 2$)	2 (MS[1])	0	0	0
HTK (before TK, $n = 3$)	2 (MS[2])	1FA	0	0
TK (week 45, $n = 7$)	1	1, 1FA	1 (MS, 3B, 1M)	3
Group III‡				
IK (week 23, $n = 9$)	1	1FA	0	7
HTK (before TK, $n = 4$)	0	3 (1MS[3])	1 (MS, 1B, 5M)	0
TK (week 45, $n = 5$)	0	0	0	5

[1]One tumour with 3 and the other with 4 sites; [2]one tumour with 2 and the other with 3 sites; [3]one tumour with 2 sites—1 benign and 1 FA. *Combined tumours: the number of benign (B) and malignant (M) sites are shown. †One animal died spontaneously and the tumour was not examined histopathologically. ‡Two animals died spontaneously and the tumours were not examined histopathologically. FA, fibroadenoma; IK, interim kill; HIK, humane interim kill; HTK, humane terminal kill; MS, multiple sites; TK, terminal kill.

with GnRH- glycys-PPD showed ovarian activity (presence of corpora lutea). All of the animals in Group III bearing tumours showed keratinised epithelia and only two of these showed evidence of ovarian activity.

DISCUSSION

The aim of this investigation was to determine the potential therapeutic value of an analogue of GnRH in a hormone-dependent disorder. In previous studies [4, 10, 14], we found that GnRH-glycys conjugated to PPD evoked a marked antibody response in both male and female rats. Effective immunisation resulted in the suppression of gonadal function, a total depression of serum sex steroid levels and there was a good correlation with the antibody levels attained. No IgM response was detectable and the major IgG subclasses consisted mainly of IgG1 and IgG2a. Although the precise mechanism by which gonadal function was disrupted remains unknown [15], it has been suggested from data produced in clinical trials that the induction of a particular subclass may play an important role in the efficacy of treatment [16]. This may be achieved by changing the adjuvants and carrier proteins used in conjunction with GnRH-glycys [17].

In this study, the primary effect of GnRH neutralisation was to substantially reduce serum oestradiol concentrations. Unlike in the male studies, where testosterone levels were totally suppressed, in this investigation a basal level for oestradiol remained. Oestrogens are produced by other tissues (e.g. skin, adipose, adrenals) which are not necessarily under the control of GnRH [18] and this could explain the basal level. The effect of oestrogen depletion on the ovaries of animals receiving the analogue concurrently with tumour induction (Group III) was seen at the interim kill stage, where the combined ovarian weight was half that of untreated animals. At the terminal kill stage, the weight was 7-fold lower than the normal weight. However, in animals bearing established tumours (Group II), immunisation caused the ovarian weight to fall to approximately a fifth of the normal weight (at terminal autopsy), which gives us some indication of the length of time for atrophy of the ovary to occur. With regard to the uterine weight, there was no significant weight difference in the Group II animals, from interim to terminal autopsy. Whereas the effect in the Group III animals was only seen at terminal autopsy. In addition, histology showed that the atrophic effects on the uterus (contracted) and cervix/vagina (low stratification of

Table 3. Effect of immunisation with GnRH-glycys-PPD on gonadal organ and overall body weights

Treatment group	Mean body weight (g) ± S.D.	Combined ovarian weight (mg) ± S.D.	Uterine weight (g) ± S.D.
Group I			
IK ($n = 10$)	271.0 ± 14.0	130.5 ± 10.3	0.664 ± 0.18
TK ($n = 10$)	303.2 ± 24.9	129.6 ± 10.9	0.654 ± 0.10
Group II			
IK ($n = 9$)	277.5 ± 11.9	126.3 ± 35.0	0.660 ± 0.21
TK ($n = 5$)	*318.0 ± 19.9	†24.3 ± 33.1	0.625 ± 0.42
Group III			
IK ($n = 9$)	*305.6 ± 28.2	†66.8 ± 38.4	0.659 ± 0.22
TK ($n = 5$)	*336.0 ± 24.5	†18.0 ± 16.4	†0.172 ± 0.01

The effect of immunisation of female Sprague–Dawley rats with GnRH-glycys-PPD in gonadal organ weights and body weights were compared. These measurements were only taken at interim (IK) (study week 23) and terminal (TK) (study week 45) autopsies. Group I animals were untreated, Group II animals were immunised in study weeks 25, 27, 29, 31 and Group III animals were immunised in study weeks 6, 8, 10, 12, 25. *Denotes a significant increase ($P < 0.025$) in weight after immunisation compared with the appropriate Group I animals. †Denotes a significant decrease ($P < 0.01$) in weight, compared with the appropriate Group I animals.

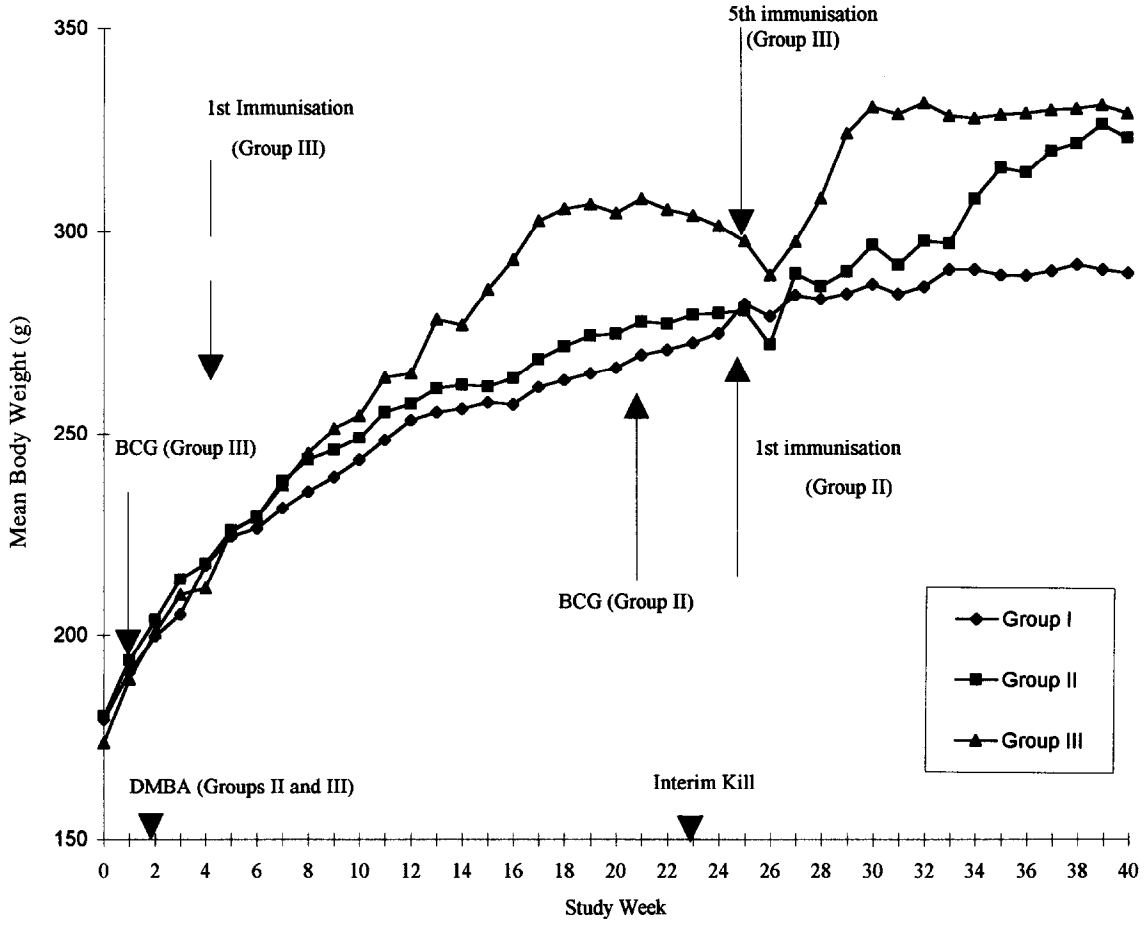

Figure 5. Body weight development in female Sprague–Dawley rats.

Table 4. Correlation between tumour presence and gonadal atrophy

Animal no. (Group)	Time of sacrifice (study week)	No. of tumours and tumour type	Ovarian activity and characterisation of cervico-vaginal epithelium
21 (II)	HIK (week 16)	3 M	corpora lutea, stratified epithelium
22 (II)	HTK (week 43)	2 M	no luteal tissue, stratified epithelium
23 (II)	HTK (week 36)	3 M	corpora lutea, stratified epithelium
27 (II)	TK (week 45)	3 B, 1 M	no luteal tissue, stratified epithelium
28 (II)	HTK (week 41)	1 FA	no luteal tissue, keratinised epithelium
30 (II)	IK (week 23)	1 B	corpora lutea, keratinised epithelium
33 (II)	TK (week 45)	1 M	no luteal tissue, keratinised epithelium
34 (II)	TK (week 45)	1 B	no luteal tissue, keratinised epithelium
35 (II)	IK (week 23)	3 B, 1 M	corpora lutea, stratified epithelium
36 (II)	IK (week 23)	1 M	corpora lutea, stratified epithelium
37 (II)	HIK (week 12)	4 M	corpora lutea, keratinised epithelium
39 (II)	IK (week 23)	1 M	corpora lutea, keratinised epithelium
40 (II)	TK (week 45)	1 FA	no luteal tissue, keratinised epithelium
41 (III)	IK (week 23)	1 FA	Corpora lutea, keratinised epithelium
46 (III)	HTK (week 43)	1 B	no luteal tissue, keratinised epithelium
52 (III)	HTK (week 43)	1 B	no luteal tissue, keratinised epithelium
55 (III)	HTK (week 30)	1 B, 1 FA	no luteal tissue, keratinised epithelium
56 (III)	HTK (week 34)	5 M, 1 B	no luteal tissue, keratinised epithelium
58 (III)	IK (week 23)	1 M	corpora lutea, keratinised epithelium

The effect of immunisation of female Sprague–Dawley rats with GnRH-glycys-PPD in tumour type—malignant (M), benign (B) or fibrous (FA), and gonadal histology. Evidence of ovulation was indicated by the presence of corpora lutea and atrophy of the cervico-vaginal epithelium was determined by low stratification. These assessments were made at interim kill (study week 23, IK), terminal kill (study week 45, TK) and for humanely killed animals (HIK or HTK, depending on whether killed prior to IK or TK).

the epithelium) were, in the main, only seen at the terminal kill stage. It is possible that, in time, gonadal atrophy in the Group II animals would also show similar long-term effects of immunisation as seen in the Group III animals. These results were consistent with those observed in other female animal studies [10, 19]. Details of changes to the gonadal tissues have been published previously [4]. Histology of the gonads showed the effects of analogue administration to be consistent with those seen as a result of oestrogenaemia. However, the effects more closely resembled those seen in juvenile animals as opposed to aged animals.

In addition to rendering the animals infertile (as assessed by non-cycling indicated in vaginal cytology), the effect of lowering oestrogen concentrations caused regression of abnormal mammary growths. The relationship between high antibody concentrations and low oestrogen levels meant that the number of animals bearing abnormal growths was lowest when the antibody response peaked. However, as the antibody levels fell, an increase in incidence of mammary masses was observed. This is consistent with other endocrine manipulations in the treatment of breast cancer. One theory is that renewed growth following oestrogen deprivation is likely to result from adaptive changes within the tumour—for example, an increase in oestrogen-resistant cell clones [18], but in our study, this was not the case. Tumours developing after treatment were shown to retain their hormone dependency as they regressed following further immunisation (Group III). Histologically, there was a spectrum of morphological 'malignancy' and the borderline between benign and malignant was not distinct. Many of the tumours classed as benign contained areas of cell atypia, indicative of previous malignancy or a potential for future malignancy. However, the incidence of growths was low in the Group III animals and by the end of the study none of the surviving animals showed any compound mammary tumours. In addition, during periods of high GnRH-specific antibody levels, the occurrence of multiple tumours was low. Although the experimental group sizes were not large, there is an indication that the mammary tumours induced by DMBA, although heterogeneous in nature, were hormone dependent as they regressed in size and number a few weeks following immunisation. In the animals bearing established tumours (Group II), tumour incidence and histological classification were similar pre- and postimmunisation. Nevertheless, in both groups, the size and incidence of multiple tumours, by palpation, was seen to decrease following immunisation and it is likely that, as in the Group III animals, with further boosting and time, the incidence and nature of the tumours would be altered. However, since Group II more closely represents the clinical situation, with hindsight, it would have been better to have prolonged the terminal autopsy. Furthermore, it is interesting to note that none of the animals showing extreme gonadal atrophy (which is dependent on the time factor) showed compound mammary tumours. In the clinical situation, the development of a vaccine which can effectively lower serum oestradiol levels has many potential clinical applications in hormone-dependent disorders, but the complications caused by gonadal atrophy and oestrogenaemia would have to be evaluated on an individual basis.

In the model used in this study, the effect of oestrogen deprivation compared favourably with other GnRH-related therapies [10, 20, 21]. The data produced show that GnRH- glycys is non-toxic and a comparison with a DMBA control group showed that life expectancy was increased. Any adverse effects were induced by the use of an adjuvant [4]; this took the form of inflammatory changes noted around the liver, spleen, lymph nodes and in the peritoneal cavity [4, 14]. However, problems associated with aluminium hydroxide could be eliminated in the near future with advances in adjuvant formulations and we are currently investigating the suitability of novel adjuvants and alternative carrier proteins in conjunction with GnRH-glycys. One non-adjuvant induced effect was seen in an increase in body weight which took place after the final injection, when the antibody levels peaked. This problem could be alleviated by anti-androgenic administration and was not seen as a major drawback. In addition, any symptoms of oestrogenaemia and gonadal atrophy could be relieved by hormone replacement therapy and topically applied drugs. In conclusion, this study has given an indication of the antitumour potential of immunisation with GnRH-glycys and further studies are being carried out to formulate an effective therapeutic for hormone-dependent cancers.

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