phosphorylation sites of whole myosin and those of the isolated light chains, the data are presented in an alternative form in the inset of Fig. 2. It could be inferred from Fig. 2 that the phosphorylation sites of whole myosin fall into two classes, the first 50 percent being phosphorylated relatively easily and the second 50 percent phosphorylated with more difficulty. When the light chains are removed from the myosin molecule, only one class of sites is apparent. This behavior can be explained by assuming cooperative interaction of the myosin heads, and it is suggested that phosphorylation of the first head hinders that of the second head. This would generate a sequential or ordered phosphorylation process.

Alternatively, it might be argued that the nonlinearity of the phosphorylation reaction is due to progressive reduction of available substrate. This possibility cannot be eliminated since the Michaelis constant, K_m, for the myosin and myosin light chain kinase is not known under our assay conditions. However, we think this explanation is unlikely because a similar phosphorylation profile is obtained at two different myosin concentrations (0.4 and 1.9 μM with respect to the 20,000-dalton light chain) and because phosphorylation of the isolated light chains (at 0.5 and 1.0 μM) showed a markedly different dependence on kinase concentration.

These results imply that the two heads of the smooth muscle myosin molecule do not act independently. This cooperative behavior of smooth muscle myosin has apparently not been noted previously, possibly because of the use of more complex protein systems and different assay conditions (*7*) and because the prephosphorylation levels of myosin are often not taken into account.

In these studies we used the simplest assay system that was available with our current level of expertise. Although this approach has the advantage that it allowed a less ambiguous interpretation of the effects of phosphorylation than was possible previously, it has the disadvantage that the system does not fully represent the native situation. The regulatory mechanism must be more complex than our experiments indicate, and the cooperative behavior of myosin remains to be investigated when additional components, such as tropomyosin, are added.

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A Conjugate of α-Amanitin and Monoclonal Immunoglobulin G to Thy 1.2 Antigen Is Selectively Toxic to T Lymphoma Cells

Abstract. *A covalent conjugate of an α-amanitin azo derivative and a monoclonal immunoglobulin G to the Thy 1.2 antigen on murine T lymphocytes was synthesized. The conjugate was 375- to 750-fold more inhibitory to murine T lymphoma S49.1 cells than the unconjugated derivative. At 0.7 × 10^{-7} to 1.5 × 10^{-7}M and at 4 × 10^{-7}M amanitin equivalents, the conjugate inhibited protein synthesis in S49.1 cells by 50 percent and 80 to 96 percent, respectively. At these concentrations, mutant Thy 1–deficient S49 cells and other murine lymphomas lacking Thy 1 altogether or carrying Thy 1.1 antigens were unaffected. This result demonstrates the potential for targeting amanitin to specific cell types.*

Conjugates of toxins and proteins have been constructed by coupling diphtheria toxin, ricin, the toxin A chains, gelonin, and amanitin to antibodies, hormones, or concanavalin A; and the targeting potentials of the conjugates have been evaluated with mammalian cells (*1–6*). Conjugates in which a monoclonal antibody directed against a colorectal carcinoma are linked either to a diphtheria A chain or to a ricin A chain are cell-specific and inhibit protein synthesis by the carcinoma cells at a median effective dose (ED_{50}) of $10^{-9}M$ (*5*). An ED_{50} of 10^{-9} to $10^{-11}M$ has been demonstrated with an epidermal growth factor coupled to a ricin A chain (*2*), with a monoclonal antibody to Thy 1.2, a differentiation antigen on murine T cells, coupled to a ricin A chain (*1*), and with a monoclonal antibody to Thy 1.2 coupled to ricin (*6*). However, in several studies, the conjugates of ricin, diphtheria toxin, or the toxin A chains with antibodies or hormones were of relatively low potency or completely nontoxic, even when the receptor-binding activity was retained (*1*, *3*). Although it is not known to what extent this decreased toxicity of the conjugates is due to the lysosomal degradation of the toxins before they reach intracellular targets, such degradation may be significant in inactivating many of the chimeric toxins.

α-Amanitin, a potent and relatively specific inhibitor of RNA polymerase II, is a bicyclic octapeptide (*7*, *8*). Although lactoperoxidase inactivates α-amanitin in vitro (*9*), the relative stability of amanitin observed in studies of whole animals (*10*) indicates that it is not readily affected by lysosomal inactivation. Conjugates of amanitin with monoclonal antibodies of varying specificities would therefore be useful in comparisons with conjugates of diphtheria toxin, ricin, or their respective A chains for the study of cellular uptake and processing of the conjugates. We now report that a conjugate of an α-amanitin azo derivative and a monoclonal immunoglobulin G (IgG) to Thy 1.2 (anti-Thy 1.2) is a selective toxic agent directed by the antibody to a murine T lymphoma S49.1. The conjugate is 133 to 277 times more toxic against S49.1 cells than native α-amanitin and 375 to

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750 times more toxic than the azo derivative. With this differential in toxicity and the targeting potential of the monoclonal antibody carrier, the conjugate of amanitin and anti-Thy 1.2 may be useful as a model system for amanitin targeting to specific cell types in vivo as well as in vitro.

An α-amanitin–anti-Thy 1.2 IgG conjugate was prepared under aseptic conditions by a modified carbodiimide method that provides minimal intra- and intermolecular IgG cross-linking and minimal IgG precipitation. A free carboxyl group was first introduced into α-amanitin with *p*-aminobenzoyl-*N*-glycylglycine (*11*). The resulting derivative, α-amanitinyl-azobenzoyl-*N*-glycylglycine (ABGG), was then covalently conjugated to reactive amino groups of IgG by using *N*-ethyl-*N'*(dimethylaminopropyl)carbodiimide (EDC) (*12*). The conjugates of ABGG with nonimmune IgG and of ABGG with anti-Thy 1.2 IgG had molar ratios of 3.6 to 6.3 mole of ABGG per mole of IgG. The conjugates contained less than 10 percent IgG cross-linking, as indicated by 8 percent sodium dodecylsulfate–polyacrylamide gel electrophoresis (data not shown) (*13*). The potency of the ABGG–anti-Thy 1.2 IgG conjugate with respect to inhibition of calf thymus RNA polymerase II was reduced by a factor of 46 when compared to free α-amanitin and by a factor of 19 when compared to ABGG (Table 1). The specificity of the conjugated antibody toward Thy 1.2 was retained, as indicated by ^{51}Cr release microcytotoxic tests on S49.1 cells, which carry the Thy 1.2 antigen on the cell surface, and C1498 cells, which lack Thy 1.2 (*14*). Fifty percent lysis of S49.1 cells with guinea pig complement required 0.05 μg of anti-Thy 1.2 IgG alone or 0.07 μg IgG equivalent of the ABGG–anti-Thy 1.2 IgG conjugate. Up to 1 μg IgG equivalent of the conjugate was not cytolytic against C1498 cells.

The cytotoxicity of α-amanitin conjugates was examined by exposing the S49.1 cells to the toxic agents continuously for 48 hours and determining the amino acid incorporation into cells. With 48 hours of continuous exposure, the cytotoxicity of α-amanitin conjugated to anti-Thy 1.2 IgG increased 47-fold compared to α-amanitin alone and 131-fold compared to ABGG alone (Table 1). With the ABGG–nonimmune IgG conjugate up to 1 μ*M* of α-amanitin equivalent or anti-Thy 1.2 IgG alone (10 μg/ml), the amino acid incorporation of S49.1 cells was reduced only by 20 percent. When 0.4 μ*M* ABGG was used simultaneously

Table 1. Concentrations of toxic agents required for 50 percent inhibition of calf thymus RNA polymerase II activity or protein synthesis in S49.1 cells. Conditions for the assay of RNA polymerase II were similar to those described in (*7*). The concentration of [^{3}H]-uridine 5′-triphosphate (2 Ci/mmole, Schwarz/Mann) was 16 μ*M*. Protein synthesis was determined as described for the 1-hour exposure in the legend to Fig. 1. For 48-hour exposure, 20 μl of toxin diluted in 0.1*M* sodium phosphate, *p*H 8, or 20 μl of 0.1*M* sodium phosphate, *p*H 8, as control were added to 200 μl of cells in 12 by 75 mm tubes, at 1×10^5 cells per milliliter. After 48 hours of incubation at 37°C with 5 percent CO_2, 100 μl of cells were collected and treated as described in the legend to Fig. 1; N.D., not determined.

Inhibitors	Concentration for RNA polymerase II inhibition (*M*)	Concentration for protein synthesis inhibition	
		1-hour exposure (*M*)	48-hour continuous exposure (*M*)
α-Amanitin	2.5×10^{-9}	2.0×10^{-5}	1.8×10^{-6}
ABGG	6.0×10^{-9}	5.4×10^{-5}	5.0×10^{-6}
ABGG–anti-Thy 1.2 IgG	1.1×10^{-7}	7.2×10^{-8}	3.8×10^{-8}
ABGG–nonimmune IgG	N.D.	$> 1 \times 10^{-6}$	$>1 \times 10^{-6}$

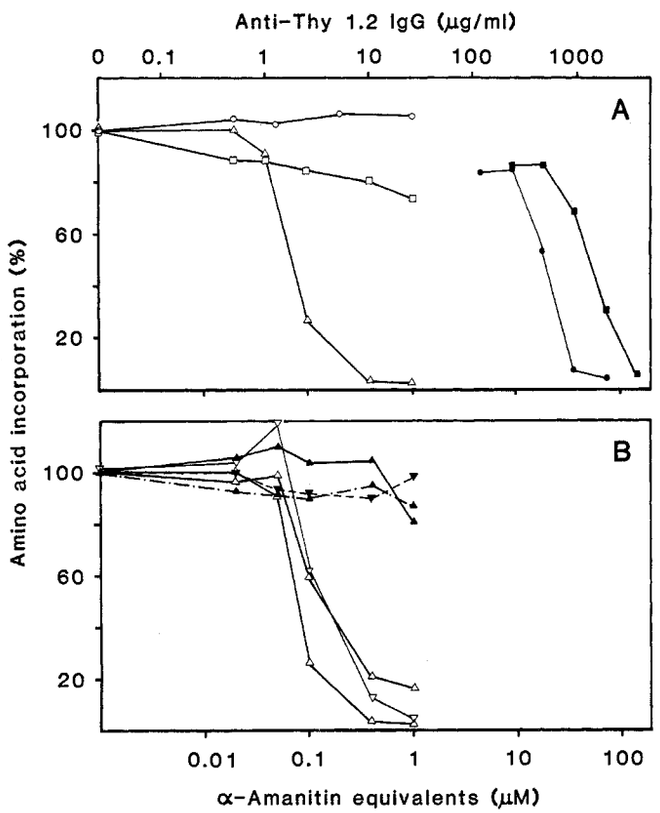

Fig. 1. Inhibition of protein synthesis, expressed as the percentage of U-^{14}C–labeled amino acids incorporated by untreated cells (*18*). (A) S49.1 cells exposed for 1 hour to (●) α-amanitin, (■) ABGG, (□) anti-Thy 1.2 IgG, (○) ABGG–nonimmune IgG, or (△) ABGG–anti-Thy 1.2 IgG. (B) S49.1 cells (△ and ▽) exposed for 1 hour to two different preparations of α-amanitin–anti-Thy 1.2 IgG. One preparation was used for (▲) S49 (Thy 1–deficient) cells, (▲–·–▲) C1498 cells, and (▼– – –▼) BW5147 cells. Each point represents an average of triplicate samples. To 10 μl of cells in each of 24 Falcon flat-bottom wells at 4×10^5 cells per milliliter were added 10 μl of toxins diluted in 0.1*M* sodium phosphate, *p*H 8, or 10 μl of 0.1*M* sodium phosphate, *p*H 8, as controls. After 1 hour of incubation at 37°C, 1 ml of warm growth medium was added, and the cells were incubated for 71 hours longer. At 30 hours before the end of the incubation period, 20μl of U-^{14}C–labeled amino acid mixture (Amersham, specific activity > 50 mCi per milligram atom of carbon) was added to the cells to a final concentration of 1 μCi/ml. Finally, 0.7 ml of cells were collected from each well into 1 ml of cold phosphate-buffered saline, poured over a GF/C filter, washed, and counted (*13*). Cytotoxicities of α-amanitin and ABGG against S49 (Thy 1–deficient) cells were similar or within onefold of those observed for S49.1 cells (data not shown) (*13*).

with, but not coupled to, anti-Thy 1.2 IgG (10 μg/ml), the reduction of amino acid incorporation was also 20 percent, an indication that there was no synergistic effect between ABGG and anti-Thy 1.2 IgG.

The increase in the cytotoxicity of α-amanitin when it was conjugated to anti-Thy 1.2 IgG was even more pronounced when S49.1 cells were exposed to the agents for 1 hour and then diluted 50-fold with fresh medium (Fig. 1 and Table 1). As indicated by the cytotoxic levels, the 1-hour exposure seems to be sufficient to allow nearly as much binding and uptake of ABGG–anti-Thy 1.2 IgG as the 48-hour continuous exposure. The cytotoxicity of ABGG–anti-Thy 1.2 IgG was 133 to 277 times greater than that of α-amanitin alone and 375 to 750 times greater than that of ABGG alone (*15*). The enhancement of cytotoxicity of the conjugated α-amanitin is even more pronounced if the further 46-fold decrease in RNA polymerase II inhibition with the conjugated α-amanitin is taken into account. However, this decrease in RNA polymerase II inhibition may be a property manifested only in cell-free systems, since intracellular lysosomal degradation of the conjugate may release a derivative more inhibitory than the intact conjugate.

The inhibition of amino acid incorporation was cell type–specific and correlated with the presence of the Thy 1.2 antigen. α-Amanitin–anti-Thy 1.2 IgG, at 0.4 μM α-amanitin equivalent, inhibited protein synthesis in S49.1 cells by 80 to 96 percent without affecting protein synthesis in the mutant, Thy 1–deficient S49 cells and the lymphoma C1498, which lacks Thy 1 antigens altogether, or the T lymphoma BW5147, which carries Thy 1.1 instead of Thy 1.2 antigen (Fig. 1). The ABGG–nonimmune mouse IgG conjugate, at the same concentration range used for the ABGG–anti-Thy 1.2 IgG conjugate, was not toxic to S49.1 cells. Anti-Thy 1.2 IgG alone at 25 μg/ml reduced the amino acid incorporation in S49.1 cells by only 28 percent.

Although there may be marked differences among various cells and cell antigens in promoting entry of bound antibody, α-amanitin-anti-Thy 1.2 IgG seems to be more effective than diphtheria toxin or ricin conjugates in the delivery of toxins to intracellular target molecules. In comparison with diphtheria toxin, which could within 1 day inactivate the cell's supply of elongation factor II with only one molecule of toxin (*16*), α-amanitin is much less potent, having an inhibition constant (K_i) against calf thymus RNA polymerase II of $3 \times 10^{-9} M$ (*7*). However, when conjugated to anti-Thy 1.2, α-amanitin inhibited protein synthesis by 50 percent in S49.1 cells at $0.7 \times 10^{-7} M$ to $1.5 \times 10^{-7} M$ α-amanitin equivalents. The difference in α-amanitin concentration between the values for K_i and 50 percent inhibition of protein synthesis is only 30- to 60-fold. It may be that the stability of α-amanitin contributes to the relative effectiveness of the conjugate in the delivery of toxins to intracellular target molecules.

The 375- to 750-fold enhancement of ED_{50} in vitro when the amanitin azo derivative is conjugated to monoclonal anti-Thy 1.2 makes this conjugate particularly attractive for cell-specific targeting in vivo. A 135-fold difference in toxicity was seen between the concentrations of free ABGG and conjugated α-amanitin that are required to inhibit protein synthesis in S49.1 cells by 50 percent and 80 to 96 percent, respectively. The conjugates of the A chains of diphtheria toxin or ricin and a colorectal carcinoma monoclonal antibody show about 100-fold enhancement of ED_{50} in vitro over the respective A chains alone (*5*). With lactose present to block the lectin binding activity of ricin, the anti-Thy 1.2 IgG–ricin conjugate is 700 times more toxic to T leukemic EL4 cells that contain the Thy 1.2 surface antigen than to cells lacking it (*6*). However, the concentrations of the above conjugates that are required to inhibit protein synthesis or cell viability by 80 to 98 percent of the target cells are not much lower than the ED_{50} of the A chains alone or ricin plus lactose. A recently reported conjugate of monoclonal anti-Thy 1.2 IgM and ricin A chain has an ED_{50} against WEHI-7 cells (a mouse leukemia expressing Thy 1.2) that is 2000- to 6000-fold less than that of the ricin A chain alone (*1*). The concentration of the conjugate required to kill 80 to 90 percent of the WEHI-7 cells is about 100-fold less than the ED_{50} of the ricin A chain. While a rigorous comparison of amanitin and other toxins requires the conjugation of toxins to identical antibodies and inhibition of the same cells, it is apparent that amanitin may be targeted to specific cell types with a selective toxicity at least comparable to that of other toxins. Furthermore, the nonspecific toxicity of the amanitin moiety might be further reduced by employing amanitin derivatives—for example, amanullin—which are less toxic to cells or animals while retaining potent inhibition of RNA polymerase II (*8*).

One possible drawback of the anti-Thy 1 system in vivo is the anticipated reaction of the anti-Thy 1 antibody with normal T cells of the host. However, Bernstein *et al.* (*17*) treated a mouse leukemia in vivo with monoclonal anti-Thy 1.1 and did not observe any obvious side effects or generalized toxicity.

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12. For the preparation of ABGG-IgG conjugate, a freshly prepared aqueous solution of EDC (Eastman) was added to an aqueous solution of ABGG in sodium phosphate buffer, *p*H 5. The final concentrations of EDC, ABGG, and sodium phosphate were 45.3, 2.28, and 12.5 mM, respectively. After 2 minutes of incubation at room temperature, the reaction mixture was added to mouse nonimmune IgG (Sigma) or mouse monoclonal anti-Thy 1.2 IgG (No. 1330, Becton, Dickinson) that had been previously dialyzed against 0.256M sodium phosphate buffer, *p*H 8. The final concentrations for EDC, ABGG, IgG, and sodium phosphate were 10, 0.5, 0.005, and 200 mM, respectively. The mixture was incubated at room temperature overnight and then dialyzed extensively against 0.1M sodium phosphate, *p*H 8. The extent of the conjugation of ABGG to IgG was determined by A_{278} and A_{395}. The A_{395} measured the azo moiety at 14,000 $M^{-1} \cdot cm^{-1}$. A mole of ABGG was equal to a mole of α-amanitin.
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Gonadotropin-Releasing Hormone Receptor Binding and Pituitary Responsiveness in Estradiol-Primed Monkeys

Abstract. *Anterior pituitary tissue from ovariectomized cynomolgus macaques* (Macaca fascicularis) *contains a single class of high-affinity receptor for gonadotropin-releasing hormone (GnRH). Exogenous estradiol causes a twofold increase in the tissue concentration of GnRH receptor within 36 hours without affecting receptor affinity. Release of luteinizing hormone in response to exogenous GnRH is initially suppressed by estradiol, but pituitary responsiveness is restored within 36 hours of introduction of estradiol. The pituitary tissue concentration of GnRH receptor is positively correlated with estradiol-induced release of luteinizing hormone only during the phase of potentiated response, an indication that although the augmentation of responsiveness by estradiol may reflect an increased GnRH receptor concentration, the suppression of the luteinizing hormone response by estradiol probably reflects estradiol actions at loci other than the pituitary GnRH receptor.*

In primates, ovulation is the climax of the interplay between ovarian, neural, and adenohypophyseal factors (*1*). At midcycle the schema of functional changes in the hypothalamus and anterior pituitary leading to ovulation is keyed to increased secretion of estradiol (E_2) from the developing Graafian follicle (*1, 2*). Indeed, administration of E_2 to ovariectomized monkeys or to monkeys or women in the early follicular phase of the reproductive cycle induces changes in the hypothalamic-hypophyseal axis that are similar to those obligatory for ovulation in the normal cycle (*1, 3*). Elevation of serum E_2 to midcycle levels (300 to 600 pg/ml), either during the normal cycle or by the introduction of exogenous E_2, augments pituitary responsiveness and induces a marked discharge of gonadotropins (*1, 3, 4*).

The activity of the anterior pituitary gland is regulated by the hypothalamus. Therefore, the impact of E_2 on adenohypophyseal function may reflect E_2 action at either one or both of these loci. However, the recent work of Knobil and coworkers (*2*) suggests that E_2 inputs directed at anterior pituitary loci are sufficient to explain the controlling influence of E_2, provided exogenous gonadotropin-releasing hormone (GnRH) is supplied. Although the cellular and subcellular modifications induced by the estrogenic influence have not been precisely defined, Park *et al.* (*5*) reported that when mice were treated with E_2 in vivo, homogenates of their anterior pituitaries showed increased binding of GnRH. Furthermore, recent reports indicate that the GnRH receptor concentration reaches a maximum in the rodent pituitary during the early afternoon of proestrus (*6, 7*). Perhaps not coincidentally, pituitary responsiveness is also greatest at this time (*8*). In an effort to determine the subcellular changes that underlie E_2 modulation of pituitary responsiveness in primates, we have studied the binding characteristics of the GnRH receptor in anterior pituitary tissue of ovariectomized cynomolgus macaques (*Macaca fascicularis*) at various intervals after the introduction of E_2.

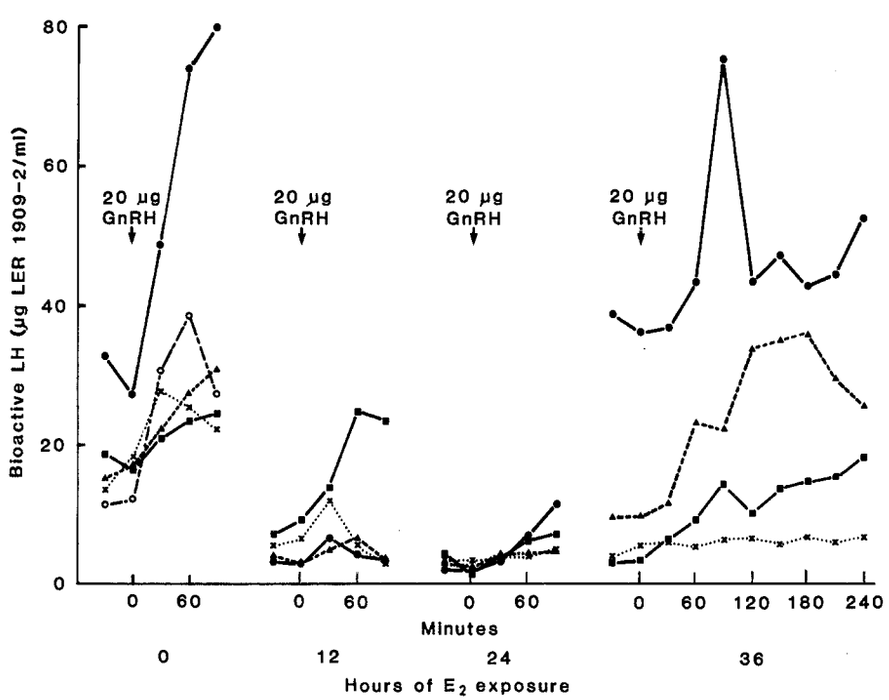

Fig. 1. The effect of hours of exposure to estradiol (E_2) on the magnitude of luteinizing hormone (*LH*) release in response to 20 μg of gonadotropin-releasing hormone (*GnRH*).

Twenty-five monkeys were used in this study, each ovariectomized at least 3 weeks prior to initiation of the experiments. In the first experiment the short-term effect of E_2 on pituitary responsiveness was determined. Serum concentrations of E_2 were increased to midcycle values by subcutaneous insertion of two 3-cm Silastic capsules containing crystalline E_2 (*9*). The concentration of E_2 in serum not subjected to chromatography was determined by radioimmunoassay (*10*). Preimplantation concentrations of serum E_2 (21 ± 8 pg/ml) were increased to 450 ± 30 pg/ml within 6 hours of implantation and were maintained above 400 pg/ml for the duration of E_2 treatment. Animals were challenged with GnRH (20 μg intravenously) at 0, 12, 24, or 36 hours after the insertion of E_2 capsules. Samples of blood were collected at 6-hour intervals until the GnRH challenge and at 30-minute intervals thereafter. The concentrations of luteinizing hormone (LH) in serum were quantified by bioassay with a mouse interstitial cell testosterone (MICT) system (*11*) and the units of LH were expressed in terms of a rhesus macaque standard (LER 1909–2) with a biopotency of 0.003 NIH-S1 units. The magnitude of bioactive LH released in response to challenge with 20 μg of GnRH varied as a function of the length of exposure to E_2 (Fig. 1). Pituitary responsiveness (defined experimentally as the magnitude

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