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Carrier-induced suppression of the antibody response to a 'self' hapten

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SUMMARY

Immunization of male rats and monkeys with gonadotropin-releasing hormone (GnRH) conjugated to a carrier results in a dramatic atrophy of the prostate. GnRH, linked to either diphtheria toxoid or tetanus toxoid as carrier, is now being evaluated for its use in the immunotherapy of hormone-dependent prostatic enlargement in men. This report deals with the phenomenon of carrier-induced, epitope-specific regulation in the GnRH-carrier system. In experiments designed to assess the influence of the carrier on antibody responses to the 'self' hapten GnRH, we show that preimmunization with carriers diphtheria toxoid and tetanus toxoid results in a strain-dependent inhibition of anti-GnRH responses in mice. Results of adoptive transfer experiments indicate that T cells from carrier-presensitized mice are responsible for suppression of anti-haptenic antibodies and that T cells from conjugate-immunized mice, on the other hand, can actually help overcome hyporesponsiveness.

INTRODUCTION

A gonadotropin-releasing hormone (GnRH)-based vaccine is currently being explored for its potential use in the 'immunosurgery' of the prostate in men with carcinoma of the prostate and benign prostatic hypertrophy. This vaccine is based on observations that immunization with GnRH linked to a carrier such as diphtheria toxoid (DT) or tetanus toxoid (TT) results in a dramatic atrophy of the prostate in rats and in monkeys.[1,2] GnRH is an evolutionarily conserved 'self' peptide and a hapten and has therefore to be linked to a carrier in order to elicit antibodies to GnRH. The carrier presumably generates a helper T-cell response that 'helps' GnRH-specific B cells.

While Phase I human clinical trials are currently underway to examine the efficacy of this vaccine, we are also engaged in investigating immunogenetic aspects of antibody responses to this vaccine. Specifically we have been investigating the regulation of anti-hapten (GnRH) responses by the carrier (DT and TT). In this report we show that presensitization with DT and/or TT can induce hyporesponsiveness to GnRH. This phenomenon of carrier-induced, epitope-specific regulation initially described by Herzenberg *et al.*[3-5] and subsequently demonstrated and extended by others[6-11] refers to the observation that presensitization with a given carrier often results in the inhibition of antibody responses to a hapten, when subsequent immunization is done with the hapten linked to the same carrier. This phenomenon has been confirmed for several different haptens and for several different carriers (such as TT, keyhole limpet haemocyanin, β galactosidase, etc.). These studies have confirmed that preimmunization with a carrier often results in an inhibitory effect on the production of antibodies to new epitopes or ligands linked to the same protein.

Previous studies relating to this phenomenon have been done with several different haptens[3,4,6,8] and the conclusion that emerged from these studies is that epitope-specific suppression is not a generalized phenomenon; presensitization with a given carrier does not result in the suppression of responses to all haptens. In some cases an actual enhancement, rather than suppression, resulted from carrier presensitization.[8] Furthermore, carrier-induced suppression is strain-dependent and not all strains of mice are susceptible to hapten-specific suppression when tested with a given hapten–carrier conjugate. It is therefore quite important that every hapten-carrier conjugate vaccine be individually studied from this perspective.

In this report we deal with a 'self' hapten, GnRH, and an as yet untested carrier, DT. We show that presensitization with both DT and TT separately can induce inhibitory effects on anti-GnRH responses. We also show that this is a H-2 strain-dependent phenomenon. Adoptive transfer experiments designed to identify the cells involved in mediating these effects suggest that T cells are responsible for both inducing suppression and for helping overcome suppression.

Abbreviations: AU, Absorbance units; DT, diphtheria toxoid; GnRH, gonadotropin-releasing hormone; TT, tetanus toxoid.

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MATERIAL AND METHODS

Peptide synthesis and conjugation

A modified GnRH decapeptide containing D-lysine (instead of L-glycine) at position six, attached to a linker ε amino caproic

acid, was synthesized by the solid phase method using benzylhydrylamine resin as the solid support.[12] All experiments described in this paper were performed with a single batch of peptide, which was over 90% pure.

The purified peptide was conjugated to DT by the glutaraldehyde method.[13] Briefly, 10 mg of GnRH in 1·5 ml of 0·1 M phosphate buffer, pH 7·0, was added to 7 mg of DT (15 ml, 0·1 M phosphate buffer, pH 7). Fifty-eight microlitres of glutaraldehyde (Sigma Chemical Co., St Louis, MO) in 11 ml of phosphate buffer was cooled and added gradually to the above mixture. The reaction was carried out for 20 h at 4° and then stopped by dialysis against phosphate-buffered saline (PBS).

The procedure for conjugating GnRH to TT was the same as above with the exception that 10·25 mg TT was substituted for DT.

The degree of conjugation of the peptide to the carrier proteins was estimated by amino acid analysis, taking advantage of the presence of the unusual amino acid, ε amino caproic acid, which is present only in the peptide and not in the protein.[14]

Immunization

BALB/c (H-2^d), C57BL/6 (H-2^b), C3H/He (H-2^k), FVB (H-2^q) and SJL (H-2^s) (bred in our animal house) were presensitized once by an intramuscular injection of TT or DT (100 μg) in alum. These and control mice (that received an equivalent volume of alum alone) were immunized 30 and 60 days later with the appropriate GnRH–carrier conjugate (10 μg GnRH) in alum. Sera were collected 7 days after the final immunization and then analysed for anti-GnRH and anti-carrier antibodies.

Assays for anti-GnRH antibodies

Anti-GnRH antibody titres were measured by radioimmunoassay and expressed as antigen binding capacity (Abc). All individual sera were titrated simultaneously by the dilution method, using the same batch of the radioactive tracer. The assay protocol consisted of mixing 50 μl each of normal horse serum (diluted 2·5 times in assay buffer, 50 mM PBS with 0·1% bovine serum albumin), diluted antisera, ^{125}I-GnRH and assay buffer. After an incubation of 18 hr at 4°, the antibody-bound fraction was separated by the method of Jeffcoate *et al.*[15] Antigen binding capacity (expressed in ng/ml) was calculated at a point at which proportionality between the dilution of the antiserum and ^{125}I-GnRH binding was obtained.

Assays for anti-carrier antibodies

Anti-carrier (anti-DT or anti-TT) antibodies were detected by ELISA. Briefly, 1 μg of protein in 100 μl PBS (50 mM, pH 7·4) was coated onto each well of Nunc (Roskilde, Denmark) ELISA plates. This was followed by incubation with 100 μl of diluted antisera and subsequently with 100 μl of anti-mouse immunoglobulins (polyclonal goat anti-mouse Ig) conjugated to horseradish peroxidase. Each incubation lasted for 1 hr at 37° and was followed by three washes, of 5 min duration each, with the washing buffer (PBS, pH 7·4, containing 0·2% Tween 20). Colour was developed by adding 50 μl of orthophenylene diamine as the substrate. The reaction was stopped after 20 min with 50 μl of 5 N sulphuric acid and the absorbance measured at 490 nm.

Cell separation and adoptive transfer

Fifteen days after the second injection of GnRH–TT, lymphocytes were obtained from the spleens of presensitized as well as non-presensitized mice. Enriched T-cell populations were obtained by passing splenocytes through nylon wool.[16] 50×10^6 purified T cells or non-T cells were injected intravenously into recipient mice which received an intramuscular injection of GnRH–TT at the same time. Seven days after this injection, mice were bled and assayed for antibodies to GnRH.

RESULTS

Effect of DT presensitization

Presensitization with DT clearly affected anti-GnRH antibody responses in three of the five strains of mice tested (Table 1). C57BL/6, C3H/He and SJL mice showed significantly lower anti-GnRH responses if they were presensitized; for instance, C3H/He mice presensitized with DT manifested an eightfold lower anti-GnRH response than if they received alum, instead of DT, during presensitization. DT presensitization did not result in the inhibition of anti-GnRH responses in FVB and BALB/c mice. In fact, presensitization of FVB mice resulted in a twofold higher anti-GnRH response.

Anti-DT responses, on the other hand, appeared to be completely unaffected by DT presensitization; anti-DT titres are the same in both experimental and control animals (Table 1).

Effect of TT presensitization

TT presensitization resulted in decreased anti-GnRH titres in three of the four strains studied. There was a 50-fold decrease in anti-GnRH titres in C57BL/6 mice, while BALB/c and C3H/He mice also showed statistically significant depressions in antibody titres (Table 2). The extent of suppression varied from strain to strain. SJL mice were not susceptible to TT-induced hyporesponsiveness.

TT-induced anti-GnRH suppression lasted for at least 120 days from the time of presensitization. On day 120 the anti-

Table 1. Effect of DT presensitization on the antibody response to GnRH and DT

Mouse strain	Presensitization	Antibody response: anti-GnRH (ng/ml)	Antibody response: anti-DT (AU × 100)
BALB/c	DT	8±1·2	15±1·1
BALB/c	Saline	12±0·7	20±2·0
C57BL/6	DT	3±0·4	20±1·8
C57BL/6	Saline	10±1·1	29±2·2
C3H/He	DT	1·5±0·2	26±3·1
C3H/He	Saline	12±1·9	30±3·2
SJL	DT	3·5±0·7	50±4·0
SJL	Saline	9·5±0·9	60±3·9
FVB	DT	43±2·5	40±3·5
FVB	Saline	20±1·5	42±2·4

Table 2. Effect of TT presensitization on the antibody response to GnRH and TT

Mouse strain	Presensitization	Antibody response: Anti-GnRH (ng/ml)	Antibody response: Anti-TT (AU × 100)
BALB/c	TT	0·7±0·05	10±1·2
BALB/c	Saline	4·4±0·4	12±1·5
C57BL/6	TT	0·2±0·04	8±1·0
C57BL/6	Saline	11·8±1·6	13±1·3
C3H/He	TT	2·0±0·6	6±0·9
C3H/He	Saline	4·9±0·9	8±1·3
SJL	TT	7·5±1·3	15±2·0
SJL	Saline	4·3±1·3	17±3·3

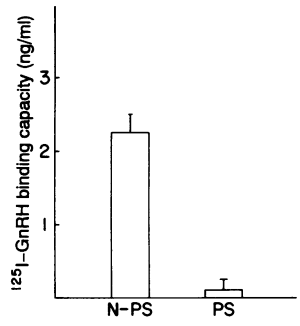

Figure 1. Anti-GnRH antibody response in TT presensitized (PS) and non-presensitized (N-PS) C57BL/6 mice measured on day 120 after TT presensitization. Values are expressed as mean±SE of 10 mice per group.

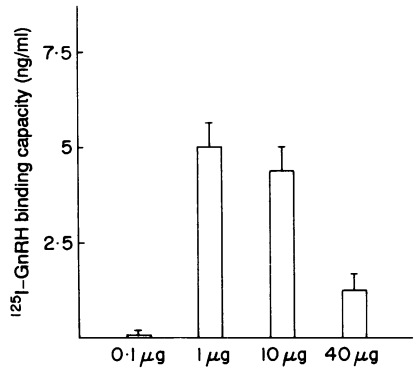

Figure 2. Anti-GnRH antibody response in C57BL/6 mice as a function of the dose of GnRH-DT conjugate injected. Response was measured after two injections of the GnRH-DT conjugate. Values are expressed as mean±SE of 10 mice per group.

GnRH antibody response in C57BL/6 mice was 10-fold lower in presensitized than in non-presensitized (but immunized) mice (Fig. 1).

There was no significant variation in the titre of anti-TT antibodies between the presensitized mice and their respective controls (Table 2).

Antigen dosage and anti-GnRH responses

The antibody response to GnRH could be suppressed even without presensitization if the dose of the conjugate was increased. Figure 2 shows the anti-GnRH antibody response generated in C57BL/6 mice injected with different doses (0·1, 1, 10 and 40 μg GnRH per mouse) of GnRH–DT conjugate. A dose of 1 μg/mouse seemed to be the optimal dose for anti-GnRH antibody generation in C57BL/6 mice. Anti-GnRH titres were significantly suppressed if the dose of the conjugate was increased to 40 μg per mouse. Anti-DT antibody titres were not significantly different in the groups receiving different doses of the conjugate (unpublished observations).

Anti-GnRH response after cell transfer

In order to identify the type of cells mediating this epitope-specific regulation, cells were adoptively transferred from presensitized mice to non-presensitized mice. The protocol called for the transfer of T cells and non-T cells from TT-presensitized C57BL/6 mice and from naive mice into non-presensitized (but GnRH–TT-immunized) mice. The transfer of T cells from TT-presensitized mice resulted in a twofold statistically significant inhibition of anti-GnRH antibodies, while the transfer of non-T cells from TT-presensitized mice and the transfer of T cells from naive mice did not affect anti-GnRH responses (Fig. 3).

In a reverse experiment, cells were transferred from non-presensitized mice to TT-presensitized mice, in an effort to reverse the inhibition of anti-GnRH responses. When T cells were transferred from GnRH–TT-immunized mice, there was a dramatic elevation in anti-GnRH titres, while the transfer of T cells from naive mice had no such effect (Fig. 4). The transfer of non-T cells, from naive or from immunized mice, had no effect whatsoever on anti-GnRH titres.

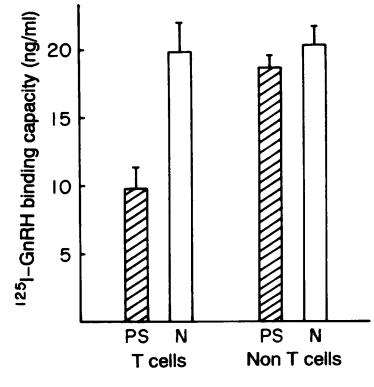

Figure 3. Suppression of anti-GnRH response in non-presensitized conjugate-immunized C57BL/6 mice after transfer of T cells from presensitized (PS) or naive (N) mice.

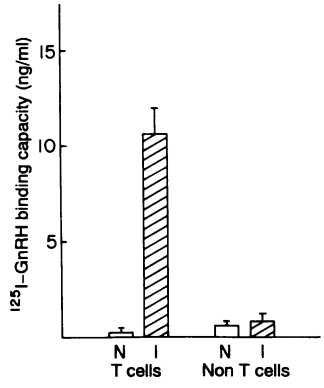

Figure 4. Anti-GnRH-antibody response in TT presensitized C57BL/6 mice after transfer of naive (N) or immune (I) T cells and non-T cells from non-presensitized mice.

DISCUSSION

The GnRH vaccine is intended for use in humans, where the aim is for a universal response; thus, an important prerequisite is the investigation of immunogenetic influences on anti-GnRH antibody titres. We felt it essential to study the effects of carrier-presensitization on the antibody response to the hapten, GnRH. Experiments described in this report were designed to investigate whether epitope-specific suppression could compromise the use of DT or TT as a carrier in vaccines containing GnRH as the hapten.

A number of laboratories have demonstrated epitope-specific suppression in different hapten–carrier systems.[3,11] Our experiments in five strains of mice (H-2^d, H-2^b, H-2^k, H-2^s and H-2^q) showed a strain-dependent suppression of anti-GnRH responses. Presensitization with TT resulted in suppression of anti-GnRH titres in BALB/c, C57BL/6 and C3H/He mice, whereas presensitization with DT suppressed the response in C57BL/6, C3H/He and SJL mice. However, we also found that presensitization with DT did not suppress anti-GnRH responses in BALB/c and FVB mice. Instead, anti-GnRH titres in FVB were actually elevated after preimmunization with DT. This is in accordance with the observations of Lise *et al.*[8] who have shown that responses to some haptens are suppressed, responses to some others are unaffected and responses to yet others are actually enhanced as a result of preimmunization.

We also observed that carrier presensitization was not the only cause of suppression of anti-haptenic responses. Figure 2 shows the suppression of the anti-GnRH response in C57BL/6 mice upon increasing the dose of GnRH–DT conjugate to 40 μg. This would suggest that an excess of carrier might result in 'clonal dominance' of carrier-specific B cells, leading to a paucity of help for some determinants, of which the attached hapten might be one.[7]

Our data clearly indicate that the generation of epitope-specific suppression is not a universal phenomenon; it is strain-dependent and carrier-dependent. For example, TT suppresses anti-GnRH response in BALB/c mice whereas DT does not, and DT suppresses anti-GnRH in SJL mice whereas TT does not. This could be a reflection of H-2-related dominance of a given carrier protein in a given strain. The results of the adoptive transfer experiments described in this report indicate that T cells from TT-preimmunized mice are at least partially responsible for carrier-induced suppression. It is interesting, in this context, that T cells from immunized (as opposed to pre-sensitized) mice can actually 'lift' suppression in presensitized mice. We are currently engaged in characterizing the T cells mediating these effects.

If, as has been suggested previously,[3,4] suppressor T cells are indeed the primary mediators of carrier-induced regulation, one may have to consider the existence of putative suppressor epitopes on TT and DT. Presumably, these suppressor epitopes could be MHC restricted, which might explain the H-2 strain-dependent regulation described here and elsewhere.[5] We are now exploring the nature of T-cell epitopes on DT and TT from this perspective.

Etlinger *et al.*[10] have described a strategy for circumventing TT-induced suppression; the use of a helper T epitope from TT as a carrier, instead of the whole molecule, enables a 'bypass' of this effect. We are currently investigating the possibility of using synthetic 'universal' T-helper epitopes as carriers, instead of TT and DT, to circumvent carrier-induced, epitope-specific regulation.

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REFERENCES

1. Jayashankar R., Chaudhuri M.K., Singh O., Alam A. & Talwar G.P. (1989) Semisynthetic anti-LHRH vaccine causing atrophy of the prostate. *The Prostate*, **14,** 3.
2. Giri D.K., Chaudhuri M.K., Jayashankar R., Neelaram G.S., Jayaram S. & Talwar G.P. (1990) Histopathological changes in reproductive organs of male Wistar rats following active immunization with LHRH. *Exp. Molec. Pathol.* **52,** 54.
3. Herzenberg L.A. & Tokuhisa T. (1982) Epitope specific regulation. I. Carrier-specific induction of suppression for IgG anti-hapten antibody responses. *J. Exp. Med.* **155,** 1730.
4. Herzenberg L.A., Tokuhisa T., Parks D.R. & Herzenberg L.A. (1982) Epitope-specific regulation. II. A bistable, Igh-restricted regulatory mechanism central to immunologic memory. *J. Exp. Med.* **155,** 1741.
5. Herzenberg L.A., Tokuhisa T. & Hayakawa K. (1983) Epitope-specific regulation. *Ann. Rev. Immunol.* **1,** 609.
6. Schutze M.P., Leclerc C., Jolivet M., Audibert F. & Chedid L. (1985) Carrier induced epitopic suppression, a major issue for synthetic vaccines. *J. Immunol.* **135,** 2319.
7. Schutze M.P., Deriaud G., Przewlocki G. & Leclerc C. (1989) Carrier-induced epitopic suppression is initiated through clonal dominance. *J. Immunol.* **142,** 2635.
8. Lise L.D., Mazier D., Jolivet M., Audibert F., Chedid L. & Schlesinger D. (1987) Enhanced epitopic response to a synthetic human malarial peptide by preimmunization with tetanus toxoid carrier. *Infec. Immun.* **55,** 2658.
9. Etlinger, H.M., Felix A.M., Gillessen D., Heimer E.P., Just M., Pink J.R.L., Sinigaglia F., Takacs B., Trzeciak A. & Matile H. (1988) Assessment in humans of a synthetic peptide based vaccine against the sporozoite stage of the human malarial parasite Plasmodium falciparum. *J. Immunol.* **140,** 626.
10. Etlinger H.M., Gillessen D., Lahm H.-W., Matile H., Schonfeld H.-J. & Trzeciak A. (1990) Use of prior vaccinations for the development of new vaccines. *Science*, **249,** 423.
11. John D.D., Wasserman S.S., Torres J.R., Cortesia M.J., Murillo J., Losonsky G.A., Herrington D.A., Sturche D. & Levine

M.M. (1989) Effect of priming with carrier on response to a conjugate vaccine. *Lancet*, **1**, 1415.

12. STEWARD J.M. & YOUNG J.D. (1984) *Solid Phase Synthesis*. Pearce Chemical Co., IL.
13. AVRAMEAS S. (1969) Coupling of enzymes to proteins with glutaraldehyde. *Immunochem.* **6**, 43.
14. SAD S., TALWAR G.P. & RAGHUPATHY R. (1991) Influence of the genetic background and carrier protein on the antibody response to GnRH. *J. Reprod. Immunol.* **19**, 197.
15. JEFFCOATE S.L., FRASER H.M., HOLLAND D.T. & GUNN A. (1974) Radioimmunoassay of LHRH in serum from man, sheep and rat. *Acta. Endocrinol. (Copenh.)*, **75**, 625.
16. JULIUS M.H., SIMPSON E. & HERZENBERG L.A. (1974) A rapid method for isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**, 645.