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Human HER-2/neu protein immunization circumvents tolerance to rat neu: a vaccine strategy for 'self' tumour antigens

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SUMMARY

Many newly defined tumour antigens are 'self' proteins. Immunizing cancer patients against these antigens may be difficult due to tolerance. The HER-2/neu oncogenic protein is such a 'self' tumour antigen. Rat neu is homologous with human HER-2/neu and provides a model system for studying vaccination strategies. Rats are tolerant to rat neu. Vaccination with this 'self' protein elicits no detectable immune response. The current studies evaluated whether tolerance to rat neu can be circumvented by immunizing with the highly homologous foreign human HER-2/neu protein. Rats were immunized with human HER-2/neu intracellular domain (hICD) protein that is 92% homologous to rat neu ICD. Animals immunized with hICD developed significant antibody and T-cell responses that were specific for both human HER-2/neu and rat neu. Neu-specific antibodies were present in titres of greater than 1:200 000. Analysis of the specificity of the antibody response using synthetic peptides demonstrated substantial reactivity to an epitope with 100% homology between rat and human protein. Significant T-cell responses (stimulation index > 10) to hICD and rat neu protein (stimulation index > 4) were detected. The T cells also responded to both human and rat ICD. The results imply that immunization with foreign proteins, which are highly homologous to 'self' tumour antigens, may be an effective vaccine strategy for 'self' tumour antigens.

INTRODUCTION

A new generation of tumour antigens has been defined: self proteins.[1,2] 'Self' proteins expressed by melanoma cells as melanocyte differentiation antigens, such as gp100, MAGE, and MART-1, have been found to be immunogenic in humans who have melanoma.[3–5] 'Self' proteins known to be involved in malignant transformation, such as HER-2/neu and c-myc, have also been found to stimulate an immune response in patients whose cancers express those proteins.[6,7] Mutated cancer-specific proteins, such as p53 and ras, have segments that are unique and thus cancer-specific. However, recent studies suggest that the immunity directed against mutated cancer-specific proteins is often targeted against the non-mutated or 'self' epitopes.[8,9]

Despite the presence of a detectable immune response to 'self' tumour antigens in some patients, immunological tolerance exists and represents a barrier to effectively vaccinating against tumour antigens. One model system in which immunization to a 'self' tumour antigen has been evaluated is immunity to neu in rats. Rat neu is a homologue for human HER-2/neu, an overexpressed oncogenic growth factor receptor. HER-2/neu is a proposed target for therapeutic cancer vaccines in humans. It is overexpressed on a wide variety of adenocarcinomas[10–12] and is associated with a poor prognosis in several subsets of patients.[13,14] Rats are tolerant to rat neu.[15,16] Rats vaccinated with either purified rat neu protein or rat neu extracellular domain (ECD) expressed by vaccinia virus do not develop rat neu-specific immunity.[15,16] Tolerance can be circumvented in the rat, however, by immunization with peptides derived from the rat neu protein sequence.[16] This observation demonstrates cancer vaccines directed against 'self' tumour antigens can be formulated to be effective in generating immunity, but the use of peptides may be problematic as they are thought of as weak immunogens and human leucocyte antigen (HLA) restriction may limit usefulness. An ideal vaccine strategy targeting a 'self' tumour antigen would be one in which vigorous immunity could be elicited with one vaccine formulation for all patients.

The current study evaluated whether immunization with a foreign protein which 'mimics' the protein structure of a 'self' tumour antigen represents an effective strategy.[17] Immunizing cancer patients with foreign proteins which are highly homologous to 'self' human tumour antigens may allow the generation of cross-reactive tumour-specific immunity. Tumour-specific immunity elicited in this fashion, may be of sufficient

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Abbreviations: ECD, extracellular domain of the HER-2/neu oncogenic protein; hICD, human HER-2 neu ICD protein; ICD, intercellular domain of the human HER-2 neu oncogenic protein.

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magnitude to impart an anti-tumour effect. Studies presented here evaluate immunity to rat neu protein as a surrogate for studying immunity to human HER-2/neu protein. Results showed that immunizing rats with human HER-2/neu intercellular domain (ICD), using a variety of different adjuvants, results in the generation of high titre rat neu-specific antibodies and substantial rat neu-specific T-cell immunity. Antibody epitope mapping with synthetic peptides revealed responses to a peptide with 100% homology between rat and human protein. T-cell responses to both rat neu and human HER-2/neu were detected. Given that T-cells normally recognize peptides, the data implies that the T-cell responses are also directed to a common epitope shared between human and rat ICD proteins.

MATERIALS AND METHODS

Animals

Rats used in this study were Fischer strain 344 (CDF (F-344)/CrlBR) (Charles River Laboratories, Portage, MI). Animals were maintained at the University of Washington Animal facilities under specific pathogen-free conditions and routinely used for experimental studies between 3 and 4 months of age. Pathological evaluation of rat tissues was performed by Dr D. Liggitt, University of Washington, Department of Comparative Medicine.

Neu proteins

Rat neu protein was purified using immunoaffinity column purification techniques. Briefly, a lysate preparation of a rat neu overexpressing cell line, DHFRG8 (American Type Culture Collection, Rockville, MD), was incubated overnight at 4° on a prepared immunoaffinity Affigel-10 column (BioRad, Hercules, CA). To generate the lysate preparation 20×10^7 cells were used.[16] The Affigel-10 was coupled to a rat neu-specific antibody, 7.16.4 (kindly supplied by Dr Mark Greene). After incubation with lysate, the column was washed three times, twice with phosphate-buffered saline (PBS) and once with 1 M NaCl. The rat neu protein was eluted with a buffer: pH 2·5, 0·05 M glycine, 0·15 M NaCl, and 0·1% Triton-X and the eluent was immediately brought back to neutral pH with 1 M Tris–HCl. Pooled protein fractions were dialysed against PBS. After dialysis, the protein was concentrated by centrifugation (Centricon-100, Amicon, Beverly, MA). The rat neu protein was sterile filtered (Nalgene, Rochester NY). Protein purity was verified by both protein staining and Western blot.[16] Purified protein was quantified (Bio-Rad Protein Kit). Recombinant human and rat ICD proteins were kindly provided by Dr Kenneth Grabstein (Corixa Corp., Seattle, WA).

Immunization

Rats were immunized recombinant human HER-2/neu intracellular domain protein (hICD) (50 μg), or immunoaffinity column-purified rat neu protein (50 μg). Proteins were administered with either complete Freund's adjuvant (CFA; Sigma ImmunoChemicals, St. Louis, MO) or murine granulocyte–macrophage colony-stimulating factor (GM-CSF) 5 μg (Immunex Corp., Seattle, WA) as adjuvants. Control groups received adjuvant alone. Inoculations with GM-CSF were given intradermally (i.d.) and inoculations with CFA were administered subcutaneously (s.q.). Animals underwent two immunizations each 14–16 days apart. Eighteen to 20 days after the second immunization animals were assessed for immunological response. Sera, spleens and draining lymph nodes were harvested from immunized animals. Experiments included four animals/experimental group. Data shown here were derived from two separate immunization experiments for each group performed more than 2 months apart.

Cell lines

Two cell lines were used as a source of neu proteins. SKBR3, a human breast cancer cell line that is a marked overexpressor of HER-2/neu (American Type Culture Collection) was maintained in culture in 10% fetal bovine serum (FBS) (Gemini Bioproducts, Inc., Calabasas, CA) and RPMI. DHFRG8, an NIH/3T3 cell line cotransfected with c-neu-p and pSV2-DHFR (American Type Culture Collection) was used as a source of non-transforming rat neu protein. This cell line was maintained in 10% FBS and Dulbecco's modified Eagle's minimal medium with 4·5 g/l glucose. DHFRG8 cells were passaged through the same medium supplemented with 0·3 μM methotrexate at every third passage to maintain the neu transfectant.

Enzyme-linked immunosorbent assay (ELISA) for rat neu and human HER-2/neu-specific antibody responses

Ninety-six well Immulon 4 plates (Baxter SP, Redmond, WA: Dynatech Laboratories) were incubated overnight at 4° with either a rat neu-specific antibody (c-neu-4, Oncogene Science) or a human HER-2/neu-specific antibody (520-C9, a kind gift of Dr David Ring) at a concentration of 10 μg antibody per ml in carbonate buffer. After incubation, all wells were blocked with PBS and 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO), 100 μl/well for 4 hr at room temperature. The plate was washed with PBS/0·5% Tween and protein was added. Rows of wells were coated with alternating PBS/1%BSA and DHFR-G8 lysate (rat neu) or SKBR3 lysate (human HER-2/neu) (10^8 cells/20 ml PBS), 50 μl/well, overnight at 4°. After washing, the plate was incubated with rat sera at the varying dilutions in PBS/1% BSA and incubated for 1 hr at room temperature. Sheep anti-rat immunoglobulin horseradish peroxidase (HRP) was added to the wells at a 1:5000 dilution in PBS/1%BSA and incubated for 45 min at room temperature (Amersham Co.). Following the final wash, TMB (Kirkegaard and Perry Laboratories, Gaithersburg, MD) developing reagent was added. The optical density (OD) was read at 450 nm. The OD of each serum dilution was calculated as the OD of the neu-coated wells minus the OD of the PBS/1%BSA-coated wells.

Antigen specificity was confirmed by analysing experimental sera for antibody responses to ovalbumin in an ELISA. In these analyses, plates were incubated overnight at 4° with purified ovalbumin protein at 10 μg/ml concentration in carbonate buffer alternating with rows of buffer alone. Antibody evaluation the proceeded as described above.

ELISA for peptide epitope analysis

Ninety-six-well Immulon 4 plates (Dynatech Laboratories) were incubated overnight at 4° with 50 μl of a neu peptide solution at a concentration of 10 μg/ml diluted in PBS alternating with rows of PBS/1%BSA. The peptides constructed were 15–18 amino acids in length and were derived from the amino acid sequence of the rat neu protein. Some peptides were located in areas of 100% homology between rat neu and

human HER-2/neu. The peptide-coated plate was incubated with rat sera diluted 1:50 and 1:100 for 1 hr at room temperature. Sheep anti-rat HRP was added to the wells at a 1:5000 dilution in PBS/1%BSA and incubated for 45 min at room temperature. Following the final wash, the TMB developing reagent was added. The OD was read at 450 nm. The OD of each serum dilution was calculated as the OD of the peptide-coated wells minus the OD of the PBS/1%BSA-coated wells.

T-cell proliferation assays

For analysis of neu protein-specific responses, immune spleen cells were harvested by mechanical disruption and passage through wire mesh and washed. Two hundred thousand spleen or 1×10^5 lymph node cells/well were plated into 96-well round bottom microtitre plates (Corning, Coring, NY) with six replicates per experimental group. The media used consisted of EHAA 120 (Biofluids) with L-glutamine, penicillin/streptomycin, 2-mercaptoethanol, and 5% FBS. In initial experiments, cells were incubated with 1 μg/ml of the various proteins. Subsequent experiments evaluated increasing concentrations of experimental proteins, recombinant human HER-2/neu ICD and recombinant rat neu ICD, ranging from 0·5 to 2·0 μg/ml. After 4 days, wells were pulsed with 1 μCi of [^{3}H]thymidine for 6–8 hr and counted. Data are expressed as a stimulation index (SI) which is defined as the mean of the experimental wells divided by the mean of the control wells (no antigen). Ovalbumin was used as a negative control antigen for proliferation in all assays at a 1 μg/ml concentration.

RESULTS

Rats immunized with hICD develop high titre human and rat neu-specific antibodies

Previous studies demonstrated that rats, immunized with rat neu protein, do not develop immune responses to rat neu.[15,16] Animals are presumed tolerant to this 'self' protein. For the current study rats were given a priming immunization and a boost immunization with hICD with either GM-CSF or CFA as an adjuvant. All rats immunized with hICD developed significant antibody responses specific for human HER-2/neu protein, with titres greater than 1:200 000 (Fig. 1a). By marked contrast, rats immunized with rat neu protein did not develop human neu-specific antibodies. Ovalbumin was used as a negative control protein. No sera tested was positive for antibodies to ova (data not shown).

Human HER-2/neu ICD is 92% homologous to rat neu ICD at the amino acid level. Analysis was performed to discern whether the human HER-2/neu-specific antibodies were cross-reactive with rat neu. Rats immunized with hICD with either GM-CSF or CFA as an adjuvant had high titre antibody responses specific for rat neu (Fig. 1b). The magnitude of the rat neu-specific antibody responses was nearly identical to that of the human HER-2/neu-specific response.

Human HER-2/neu and rat neu-specific antibodies, generated by immunizing with hICD, are specific for an intracellular domain epitope with 100% homology between rat and human neu. Epitope mapping was done with a series of synthetic peptides ($n = 16$) derived from the amino acid sequence of the rat neu protein. Both intracellular and extracellular peptides were included. Eight of the peptides were derived from regions of the rat neu protein that were 100% homologous with human HER-2/neu protein. The dominant response detected was to an ICD peptide epitope, p932–946 (Fig. 2). The amino acid sequence of this peptide is identical between rat and human.

Immunization of rats with hICD elicits detectable T-cell responses specific for both human and rat neu protein

The conditions for circumventing T-cell tolerance may be more stringent than those needed to break B-cell tolerance. The key for a successful cancer vaccine targeting a 'self' tumour antigen is the ability to generate significant T-cell immunity. T-cell proliferative responses were evaluated in rats immunized with hICD plus either GM-CSF or CFA. T-cell responses to hICD protein were detected from lymph nodes draining the inoculation site (Fig. 3a) and spleen (Fig. 3b). T-cell responses to rat neu protein were also detected, although at a lower magnitude than the hICD response. There was no evidence of response to an irrelevant protein, ovalbumin. Animals immunized with rat neu protein with adjuvants or adjuvants alone did not have a detectable T-cell response to either hICD or rat neu protein.

Rats immunized with hICD developed significant proliferative responses to both human and rat ICD protein in a dose-dependent fashion (Fig. 4). The magnitude of the T-cell immune responses directed against rat or human neu protein was similar in rats immunized with hICD plus CFA at the greatest concentration of antigen tested (2·0 μg). The magnitude of the T-cell response against rat was less than the response to human in rats immunized with hICD plus GM-CSF at all concentrations. However, the possibility exists that the responses would become more equivalent with additional boosting.

Biopsies of skin, liver, lung, gastrointestinal tract, kidney and heart were obtained from immunized animals and evaluated for histopathological evidence of autoimmunity. There was no evidence of autoimmune pathology in these tissues which express basal levels of rat neu protein (data not shown).

DISCUSSION

The HER-2/neu oncogenic protein is an overexpressed 'self' protein implicated in the pathogenesis of many human malignancies. The HER-2/neu protein has been proposed as a target for immunotherapy, including cancer vaccines and monoclonal antibody treatment.[18] Some patients with HER-2/neu-expressing tumours have been found to have pre-existent immunity directed against the protein.[19–21] Investigations in animal models demonstrate that a vigorous immune response directed against HER-2/neu can be beneficial in tumour eradication or protection.[22] Neu transgenic mice treated with a murine HER-2/neu-specific antibody were protected from the development of neu-expressing mammary cancers. In addition, established tumour regressed with increasing doses of neu-specific antibody in animals with breast cancer.[22] Human clinical trials of infusion of HER-2/neu-specific antibodies resulted in regression of tumour in some patients with advanced stage breast cancer.[23] Thus, inducing immunity in patients who have no pre-existing HER-2/neu-specific immune response, or boosting immunity in patients with an existent HER-2/neu-specific immune response is likely to have

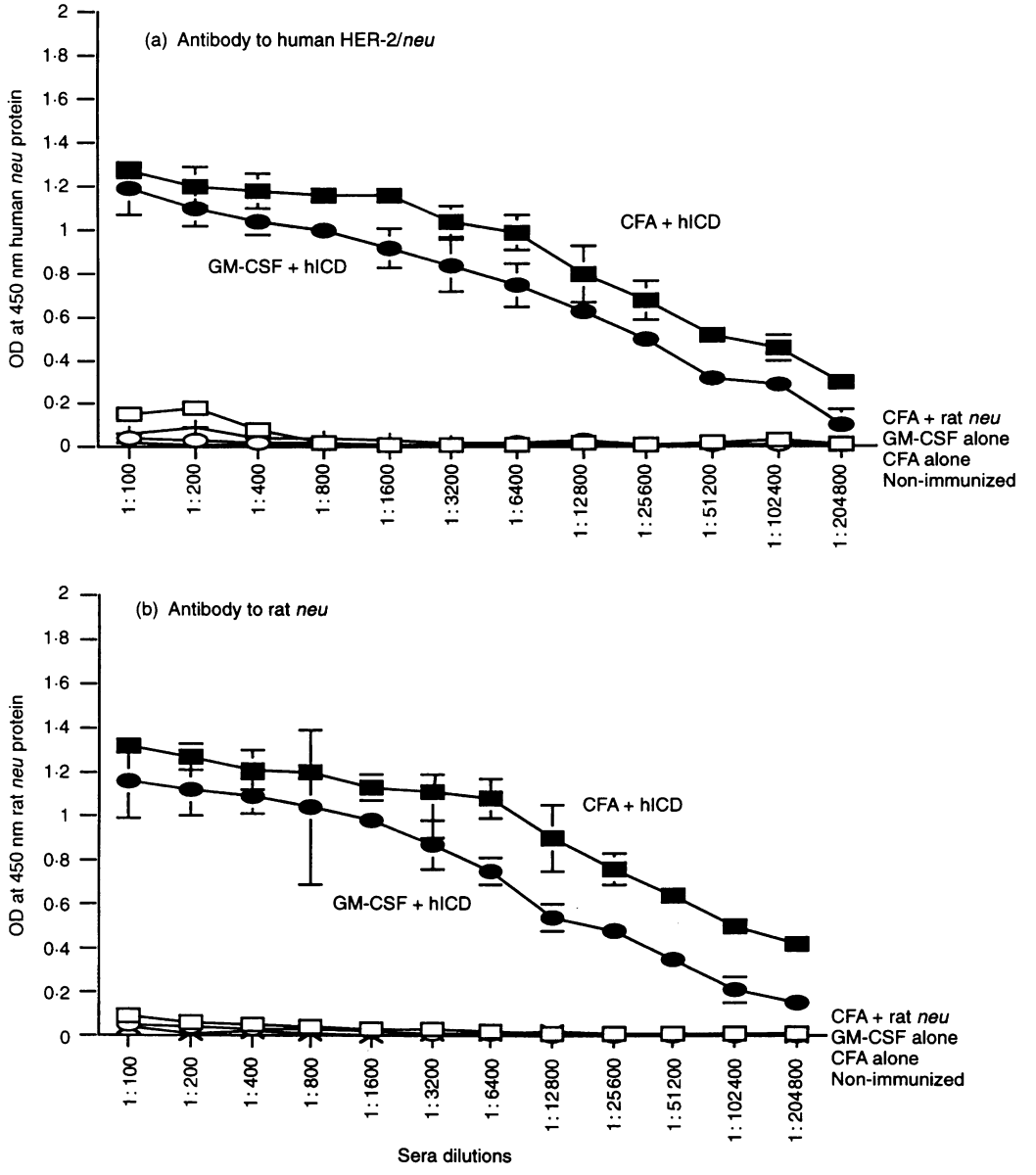

Figure 1. Rats immunized with hICD develop high titre human and rat neu-specific antibodies. This figure represents data collected from two separate experiments; eight experimental animals. The greatest interassay standard deviation, at the most concentrated control sera dilution, was 0·12 OD. Three control animals that were not immunized are shown as an example of a naive rat response to human HER-2/neu and rat neu proteins. (a) Human HER-2/neu-specific antibody responses were determined by ELISA. Results are depicted as the mean and standard deviation of the antibody response of each experimental group at each sera dilution. (b) Rat neu-specific antibody responses were determined by ELISA. Results are depicted as the mean and standard deviation of the antibody response of each experimental group at each sera dilution.

therapeutic benefit. A major current therapeutic question is: how best to immunize patients to this 'self' tumour antigen to elicit vigorous HER-2/neu specific immunity?

Responses to HER-2/neu protein are detectable in only a minority of patients with HER-2/neu-positive cancers. Eliciting a vigorous immune response to the HER-2/neu protein in such patients may be difficult due to immunological tolerance directed against this 'self' protein. The rat offers a good model for the evaluation of immunological strategies directed against the neu protein. Rats have been shown to be tolerant to rat neu.[15,16] Recent studies demonstrate that tolerance can be circumvented by immunizing rats with peptides derived from the rat neu protein.[16] We are currently addressing the question of the efficacy of peptide-based vaccines in human vaccine trials.

While effective, peptide immunization may be difficult to apply to an outbred human population with disparate major histocompatibility complex (MHC) backgrounds. Despite the efficacy of peptide vaccination in the rat model, an ideal vaccine strategy targeting a 'self' tumour antigen would be one in which the vaccine formulation would be effective in all patients, such as by use of protein with multiple potential

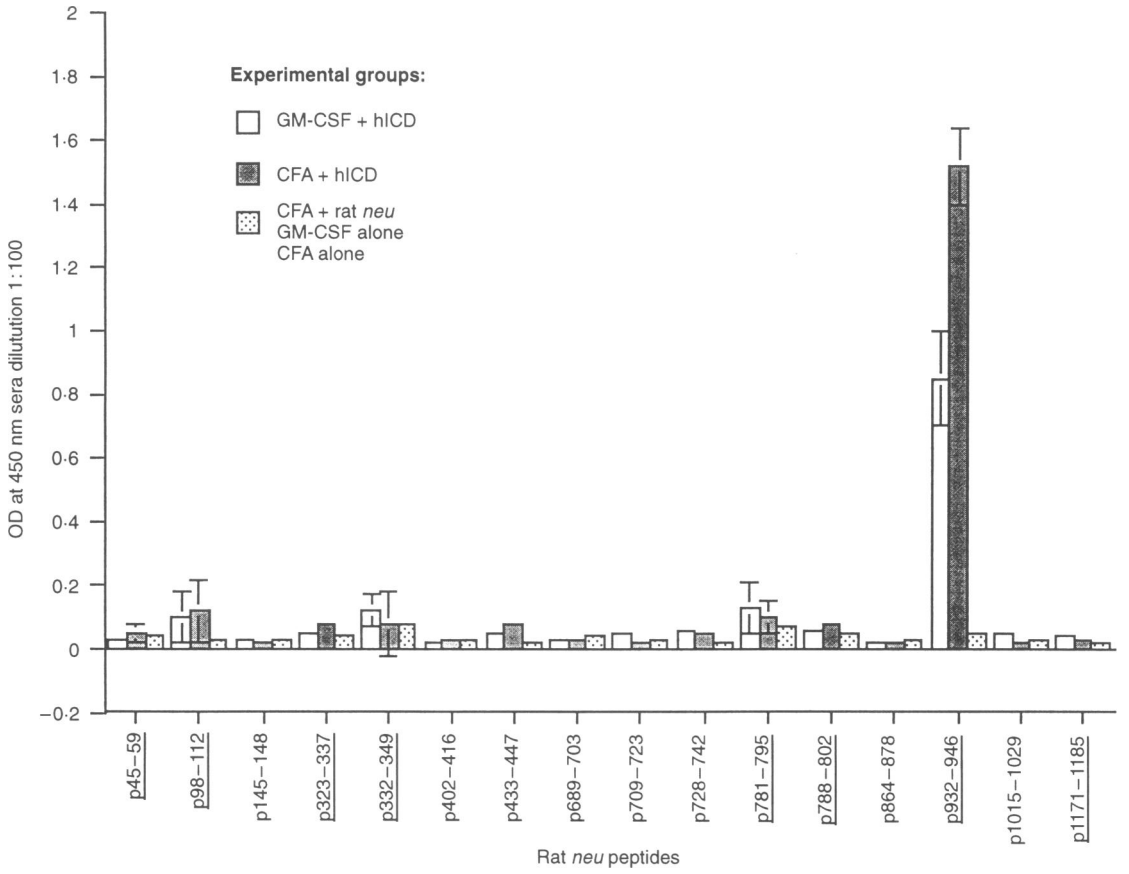

Figure 2. Human HER-2 and rat neu-specific antibodies, generated by immunizing with hICD, are specific for an intracellular domain epitope with 100% homology between rat and human neu. Sera derived from animals in each experimental group were evaluated in ELISA for antibody response to 16 peptides derived from the amino acid sequence of the rat neu protein structure. Eight of the 16 peptides were derived from sections of the rat neu protein that were 100% homologous with human neu. These peptides are underlined. This figure represents data collected from two separate experiments with eight experimental animals in each group. Results are depicted as the mean and standard deviation of the antibody response of each experimental group at a sera dilution of 1:100.

epitopes, rather than peptide with a single epitope appropriate for only a small family of MHC molecules. Vaccination with molecular mimic proteins has been an effective strategy in generating immunity to 'self' particularly in the development of anti-fertility vaccines. In particular, vaccinating animals with purified zona pellucida protein derived from a different species can result in the generation of high titre 'self' zona pellucida antibodies that render the animal infertile.[24,25] The studies described herein addressed whether a similar strategy could be applied to 'self' tumour antigens for the purpose of developing human cancer vaccines.

Rats immunized with human ICD developed antibody titres greater than 1:200 000 specific for hICD. Of note, the human neu-specific antibodies were cross-reactive with rat neu protein. A similarly vigorous antibody response to 'self' neu protein could be detected. By contrast, as previously reported, rats immunized with rat neu protein did not develop antibody responses.[15,16] An expected result might have been vigorous antibody response to human protein with a detectable, but substantially lower titre antibody response to rat neu protein. This scenario would occur if immunity was directed primarily to foreign epitopes in the human HER-2/neu protein. Evaluation of the antibody response, using peptide epitopes derived from the rat neu protein sequence, demonstrated a substantial antibody response directed to an epitope with 100% homology between rat and human neu protein.

Vaccine strategies that elicit significant numbers of 'self'-reactive B cells may lead to the generation of 'self'-reactive T cells. The presence of B cells specific for 'self' protein can result in the circumvention of T-cell tolerance to the same protein.[26] Antibody to 'self' protein can bind to and serve to concentrate the 'self' protein in antigen-presenting cells and in this fashion, stimulate T-cells specific for 'self' in addition to foreign protein epitopes. Accordingly, rats immunized with hICD developed T-cell responses specific for both rat and human neu proteins. Rats immunized with rat neu protein did not generate rat-specific T-cell immunity indicating tolerance to neu was present at the level of the T-cell. Although the magnitude of the T-cell response was greater for the foreign protein, the response to self protein was substantial.

There are several possible explanations for the differences noted between the magnitude of the T-cell responses to hICD as compared to rat neu protein. First, a portion of responding T-cell population might recognize heterologous epitopes only and be specific for ICD only. Another possibility is that the ECD portion of the protein, present in the intact rat neu, has

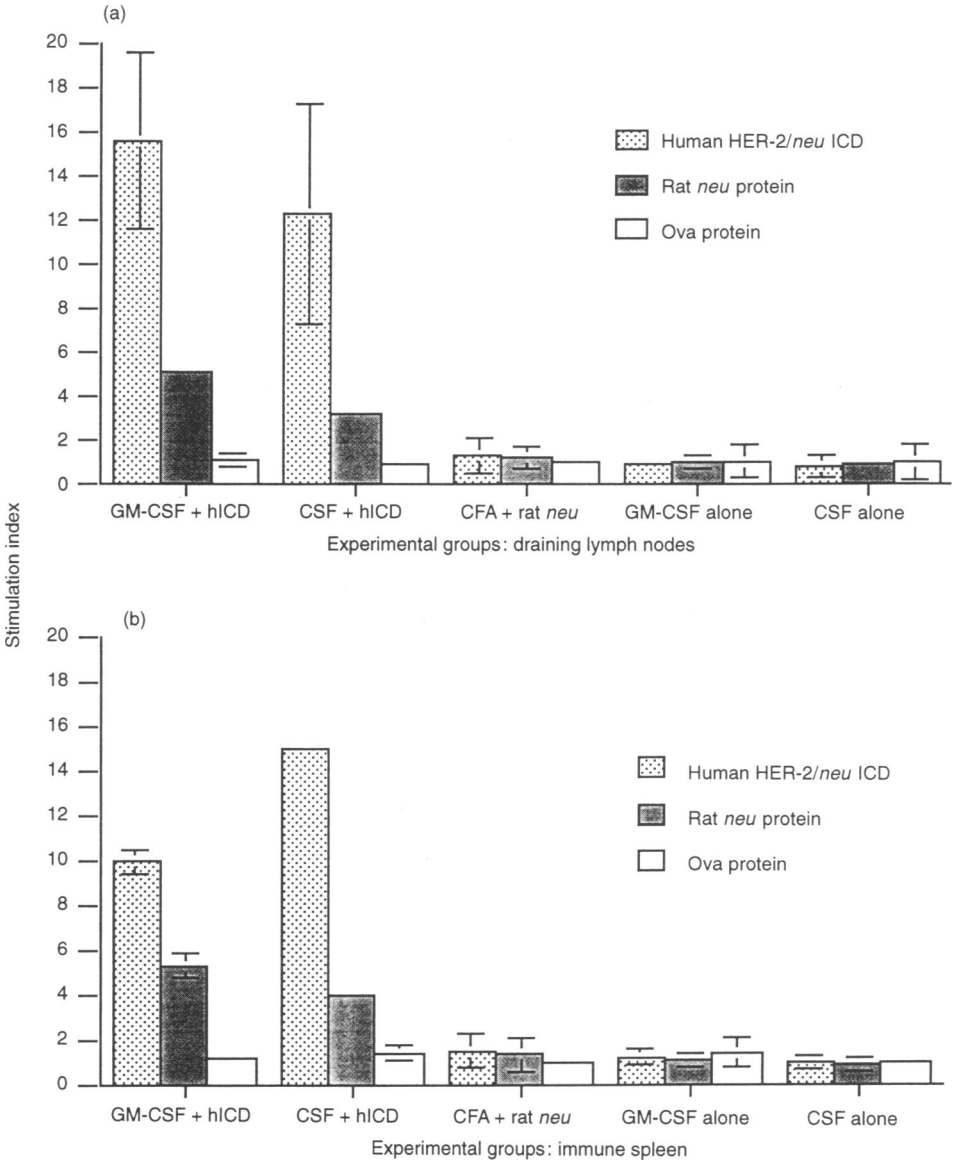

Figure 3. Immunization of rats with hICD elicits detectable T-cell responses specific for both human and rat neu protein. (a) T cells (1×10^5) derived from draining lymph nodes of experimental rats were incubated with 1 μg/ml of recombinant hICD, purified rat neu protein or ovalbumin as an irrelevant control protein. Proliferative responses were assayed after 4 days of culture in six-well replicates. The data are expressed as a stimulation index which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. (b) T cells derived from spleens of immunized rats were assayed in the same fashion as the lymph node cells. Data are the mean and standard deviation of four animals in each experimental group.

a suppressive effect. There may be a difference in the ability to generate immunity to the ICD or the ECD portion of the neu protein. Theoretically, tolerance is most stringent for the self proteins most available to the immune system. By virtue of a normal intracellular location, the ICD is more likely to be sequestered from the immune system and, therefore, less likely to have elicited a prior stringent tolerizing response. The ECD is located extracellularly, is shed and may be more available for prior immune recognition. Thus, the ECD would be more likely to elicit a tolerizing response. It is possible that tolerance to the ECD exists and that presentation of ECD epitopes results in a blunting of the ICD-specific response. Finally, populations of responding cells may have differing affinities for recognition of human neu versus rat neu peptide epitopes. Higher concentrations of rat ICD are required to achieve a similar stimulation index seen at lower concentrations of hICD (Fig. 4).

The field of tumour immunology has been stimulated by observations of existent immune reactivity to 'self' proteins in some patients. However, in the majority of patients, the reactivity is non-existent or of low magnitude. Investigations in many tumour systems in animal models show that a vigorous immune response is required for tumour eradication.[27] Cancer vaccines attempting to boost or generate immunity *in vivo* to 'self' tumour antigens must deal with the role tolerance plays in hampering the immune response. The current studies give

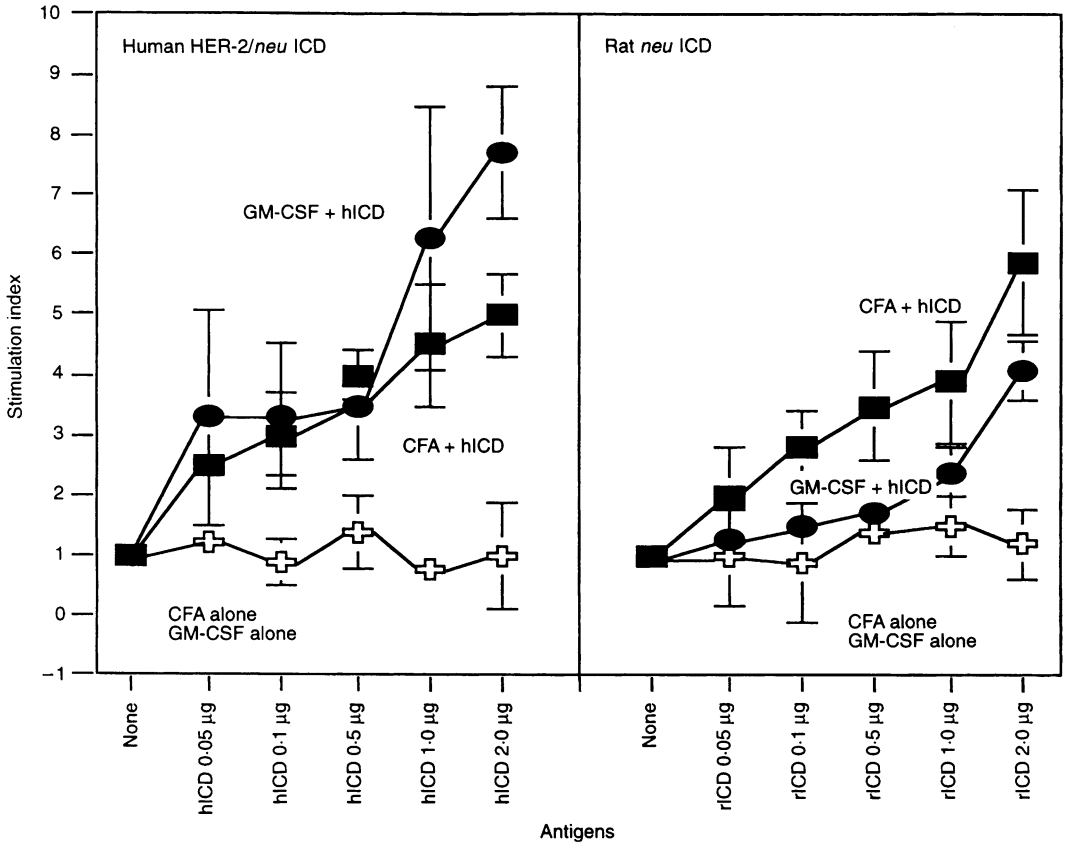

Figure 4. Rats immunized with hICD developed proliferative responses to both human and rat ICD protein in a dose-dependent fashion. T cells (2×10^5) derived from spleens of immunized rats were incubated with increasing concentrations of recombinant hICD or recombinant rat ICD neu proteins. Proliferative responses were assayed after 4 days of culture in six-well replicates. The data are expressed in terms of a stimulation index (SI) which is the mean of the experimental wells divided by the mean of the control (no antigen) wells. None of the animals tested had an SI to ovalbumin greater than 1·5 (data not shown). Data are expressed as the mean and standard deviation of four animals in each experimental group. These data represent a separate experiment from animals immunized in Fig. 3.

credence to the concept that immunizing patients with foreign proteins, which are highly homologous to 'self' tumour antigens, may result in an effective vaccine strategy for the treatment of human malignancy.

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