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SELECTIVE ENHANCEMENT OF TUMOUR NECROSIS FACTOR ACTIVITY: MAPPING REGIONS WITH MONOCLONAL ANTIBODIES

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Abstract: A significant body of evidence now exists to show that under certain conditions, antibodies of particular specificity can dramatically enhance hormonal activity *in vivo*. It has been proposed that such enhancement can originate from the selective masking "active regions" on the hormone. Mapping of the enhancing antigenic site on TNF is reviewed here.

Enhancement of the biological activity of a hormone or immunological mediator *in vivo* or *in vitro*, by site-directed antisera, has been known to occur by a number of possible mechanisms. Historically, these have included increased receptor affinity through antibody bivalency, Fc-region mediated targeting, antibody-induced conformational effects in the antigen and protection of the mediator from proteases resulting in a more optimal delivery (reviewed in reference 1). The recognition that most mediators have both low and high affinity binding sites and, as is the case for TNF, structurally distinct receptors with particular tissue distribution and specificity, has furthermore led to the demonstration that it is possible to selectively target a mediator to a particular receptor subset with a monoclonal antibody (MAb)[2]. Thus, by appropriate selection the MAb it is possible to generate a mediator-MAb complex which binds to some receptors but not others. We describe here such a MAb to TNF (MAb 32), which permits binding of the cytokine to tumour cells but which prevents its interaction with endothelial cells. A peptide sequence region corresponding to the site recognized by MAb 32 has been identified; polyclonal antisera of restricted specificity prepared to a peptide related to the epitope for MAb 32, demonstrate similar properties to MAb 32. The potential benefits of this approach lie in the possibility of selectively targeting highly toxic mediators with consequent improvement in therapeutic utility. We have used epitope mapping techniques combined with multiple peptide synthesis strategies to map the sequence regions recognised by the enhancing anti-tumour necrosis factor antibody (MAb 32). A 'human' version of the antibody is currently being evaluated for its use in reducing cachexia associated with cancer and AIDS without the concomitant blocking of the tumour regressive and antiviral activities of the cytokine.

TNF is produced by activated macrophages in response to infection and during malignancy. In mouse tumour models, systemic administration of TNF results in haemorrhagic necrosis of tumours while *in vivo* it has demonstrable

cytostatic and cytolytic activity on tumour cells[3,4]. However, infusion of recombinant TNF into cancer patients during phase I and II clinical trials has demonstrated little efficacy in terms of tumour regression[5,6]. Indeed, significant toxicity has been observed following TNF treatment, the most serious manifestations being pulmonary failure and coagulopathies. Less serious side-effects include chills, fever, malaise, headache, myalgia and nausea. The systemic toxicity associated with the administration of TNF is believed to be, at least in part, a consequence of its interaction with the endothelium[7,8].

Analysis of an extensive panel of anti-TNF MAbs[9] has enabled the identification of MAb (MAb 32) which enhances the tumour regressive activity of human TNF in mouse syngeneic solid tumour models (WEHI-164 and Meth A sarcoma). Up to a 10-fold enhancement of TNF activity has been observed with concomitant reduction of TNF toxicity, as measured by the ability of MAb 32 to inhibit the TNF induced expression of procoagulant activity on cultured endothelial cells[2]. These *in vivo* findings have recently also been extended to a human breast tumour (MDA 231) subcutanously implanted into nude mice (Fig. 1).

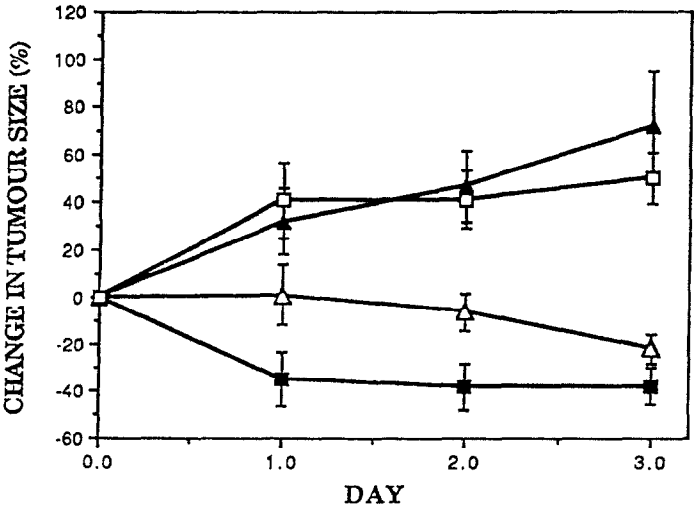

Fig. 1 Effect of MAb 32 on TNF-induced regression of human MDA breast tumours in nude mice. ▲-▲, Saline; Δ-Δ, TNF (10μg);■ - ■, TNF (10μg) + MAb 32 (50μg); □ - □, TNF (10μg) + MAb 47 (50μg).

In these experiments mice were injected with 5 x 10^5 MDA 231. Upon appearance of tumours (approximately 10 mm in diameter), the mice were injected i.p. on 4 consecutive days with recombinant human TNF 1m(10μg) and MAb 32 (50 μg as ascitic globulin fraction prepared by sodium sulphate precipitation), mixed 60 minutes prior to administration. Control groups received injections of one of the following: PBS alone, MAb 32 alone (data not shown), inhibitory anti-TNF MAb 47 in complex with TNF or

an arbitrary control MAb against bovine growth hormone in the presence of TNF (data not shown). MAb 32 was found to significantly enhance the TNF-induced regression of this human breast tumour. The enhancement of TNF activity in this manner is believed to be due to the ability of MAb 32 to selectively 'restrict' the receptor specificity of TNF[10]; that is the resulting complex can bind to tumour tissue but not endothelium [2]. Similarly, univalent Fab' fragments of MAb 32 also retain the ability to enhance TNF induced tumour regression. Thus, the enhancement phenomenon is not the result of antibody bivalency or Fc-region mediated uptake of the TNF complex[2].

In view of the ability of MAb 32 to selectively inhibit TNF binding to endothelial cells but not tumour cells[2] localisation of the antibody's epitope was undertaken in order to relate to function. Preliminary antibody-antibody competition assays indicated that the binding site recognized by MAb 32 overlapped with that for other inhibitory anti-TNF MAbs; however, unlike other antibodies, MAb 32 was unique in its ability to selectively inhibit receptor binding. Synthetic peptides derived from the human TNF sequence were synthesized concurrently on solid-phase pins in the conventional 96 well microtray format[11]. The peptides, synthesized both as 7-mers and 10-mers, overlapped by a single amino acid residue and were mixed with either purified MAb 32 or MAb 32 which had been precomplexed with TNF. The results obtained from the analysis of MAb binding to 10-mers are shown in Fig. 2. These indicate that the regions 1-10, 40-49 and 105-138 may be involved in MAb binding to intact human TNF. However, examination of the three dimensional structure of TNF[12] indicates that only regions 1-10, 40-42, 44-46, 107-114 and 127-130 are in exposed loop structures whereas other residues lie within ß pleated sheet structures Residues 1-10, 40-42, 44-46 and 127-130 are situated at the base of the TNF trimer while residues 107-114 are at the 'apex' end of the molecule. Binding of MAb 32 with region 107-114 was not observed using 7-mer peptides.

The use of multiple peptide synthesis in functional or antigenic screening, in systems such as those requiring the preparation of the peptides on the microtitre-pins, has several advantages. Firstly, overlapping short peptide sequences can be prepare covering the whole molecule (ie for TNF some 150 peptides would be required). Secondly, in more sophisticated analyses a complete replacement set of peptides can be made, in which all possible amino acids are substituted in turn at every position within the epitope. Thus, having identified a functional or antigenic site of interest, the optimal residue substitutions can be made and tested in the screening system permitting the identification of the critical amino acids involved in a particular antibody-antigen interaction. A typical commercially available kit for screening produced the data shown in Fig. 2. The use of short peptides produced either by the

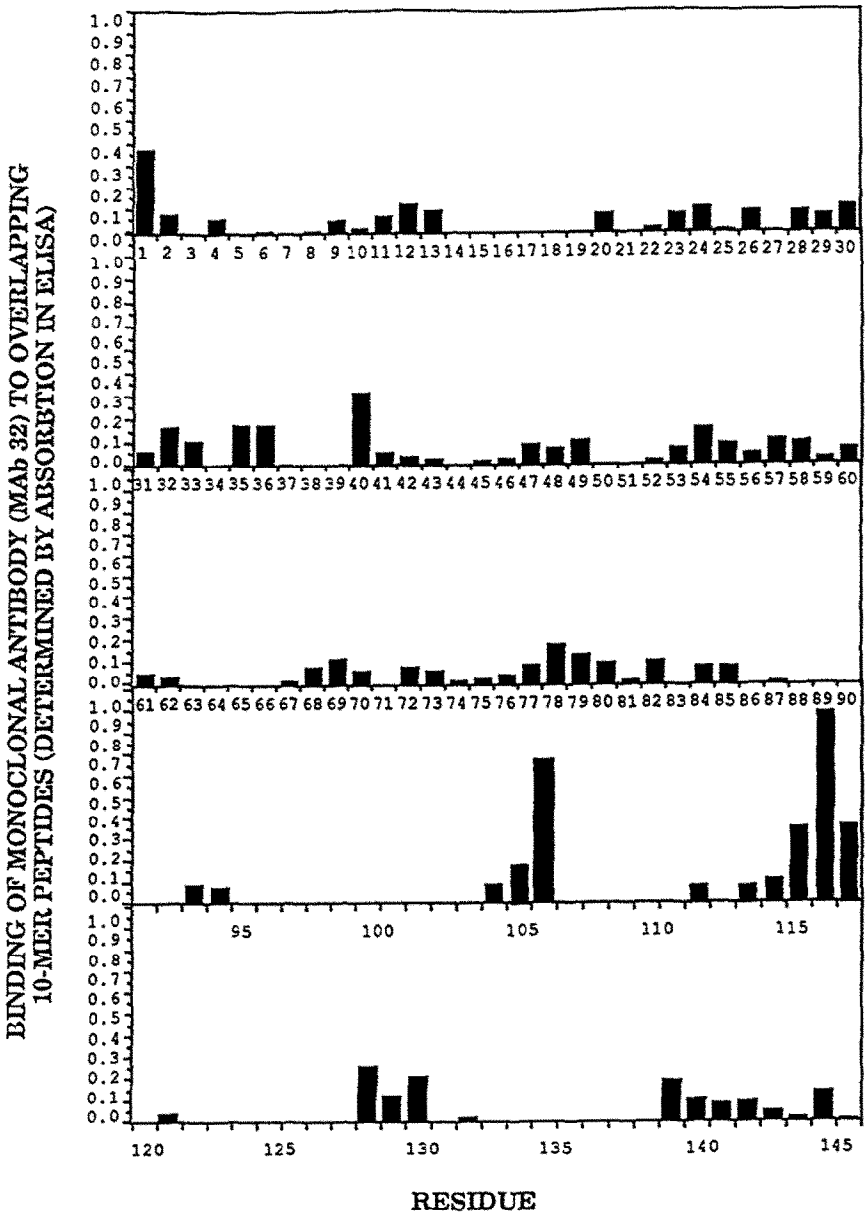

Fig. 2 Binding of Mab 32 to TNF peptides immobilized on pins.

Geysen methodology, described above, or the T-Bag method[13] have made it possible to screen large numbers of peptides, however, a high degree of purity has not been required with these techniques. The graded binding around the antigenic site (see Fig.2 regions 105-107 and 115-119) determined by the former method also reduces the risk of a spurious cross reaction.

More recently, peptide libraries have been constructed using recombinant DNA technology which allows the display of hexapeptides on filamentous phage particles[14,15]. Using a library comprised of 3 x 10^8 recombinants peptides, binding to a MAb against ß-endorphin was identified by successive rounds of selection or panning[14,16]; such a library comprises a complete set of all possible hexapeptides. Similarly, a 15-residue peptide library has been constructed and expressed on the surface of phage comprising 2 x 10^7 recombinants[17] Phage libraries have also been used to express antibody variable domains and have been used to screen for particular antibody specificities[18,19]. These have the advantage of circumventing the necessity of hybridoma technology for the production of MAbs. More recently, the identification of an antigenic determinant recognised by a MAb was reported using mixtures of synthetic peptides prepared by standard t-Boc chemistry and tested in liquid phase[20]. A synthetic pentapeptide library constructed on beads which can then be used to screen for antibodies or receptors has also been reported[21].

While peptides provide useful probes for the elucidation of antibody binding sites, one of the major constraints associated with this approach may be their inability to fold sufficiently in isolation from the parent molecule. In addition, the antigenic repertoire of protein molecules resides in structures which originate from both short linear sequences (sequential epitopes) and from the close proximity of discontinuous sequence regions (conformational epitopes).

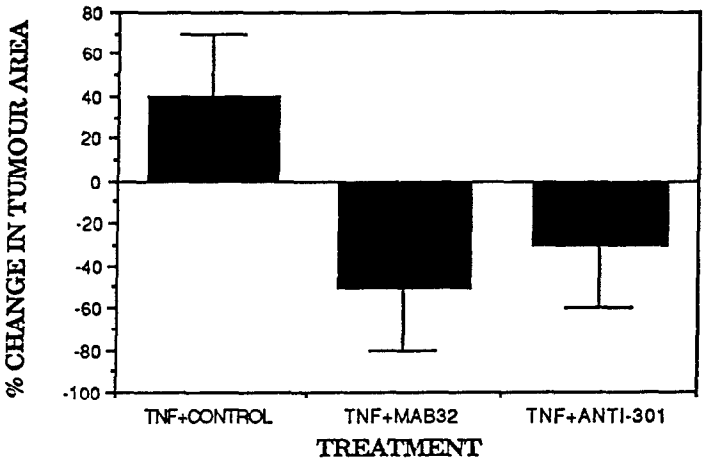

Fig. 3 Effect of anti-peptide antiserum on TNF activity in tumour bearing mice.

Various predictive indices are available which may facilitate recognition of antigenic sites on a particular protein in the absence of crystallographic data. These include hydrophilicity, segmental or atomic mobility determination and secondary

structure information. In the example of anti-TNF MAb 32, we have employed the use of a predictive computer program which placed major emphasis on protein loop regions as antigenic sites after determining the secondary structure propensity of individual resisues[22]. TNF peptides ranging in length from 15 to 25 mers were predicted as epitope sites and synthezed. These peptides were emulsified in Freund's complete adjuvant, antisera raised in sheep and the antiserum used in antibody-antibody competition assays as previously described[1]. Results from these assays indicated that antibody to the first 18 N-terminal amino acids of human TNF competed for binding of radiolabelled TNF with MAB 32 to the same degree as the homologous MAb 32. Furthermore, antiserum to this region enhanced tumour regression in a similar manner to that of MAb 32 (Fig.3).

Monoclonal antibody 32 binds to a region of TNF which appears to be involved in binding to endothelial cell receptors (see reference 2); complexes of TNF and MAb 32 bind to tumour cell receptors and demonstrate enhanced tumour regressive activity. These observations lead us to believe that it is possible to selectively target TNF to tumour cells by preventing or reducing its interaction with the endothelium.

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