Cancer Immunol Immunother (1999) 48:214–217

SYMPOSIUM SERIES

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Prerequisites for the Immunotherapy of Cancer

Accepted: 4 March 1999

Abstract Tumours express proteins not commonly found in normal cells, or over-express certain proteins. These may in some cases serve as target antigens for immunological attack. It is therefore essential to improve our understanding of the nature of these target epitopes and the cells which recognize them, in order to develop immunotherapy as a realistic treatment for cancer. A small group of around 40 investigators recently came together at the Heinrich Fabri Institute of the University of Tübingen to discuss the identification of human tumour antigens and the exploitation of this knowledge for effective immunotherapy.

Characterisation of tumour antigens

A major concern of the tumour immunotherapist must be to select specific target antigens by means of which the tumour may be recognized and controlled by the immune system in the absence of detrimental immunologically-mediated effects on the host. Since 1990 many potentially suitable target antigens have been identified either by molecular genetic or biochemical methods. Most of these are presented by MHC class I molecules to cytotoxic T lymphocytes (CTL), and many of them are melanoma antigens. It is still necessary to invest effort in identifying novel target antigens, especially in tumours other than melanoma. In this regard, Y. de Vries (Nijmegen) outlined the identification of the G250 renal carcinoma antigen, initially identified by a monoclonal antibody (mAb) and present in 90% of primary kidney tumours. Peptides corresponding to sequences in the molecule predicted to bind MHC class I molecules were synthesized and shown to be immunogenic for CTL. One peptide stimulated CTL which killed target cells transfected with the gene. Further studies are required to elucidate whether this antigen may be useful in the treatment of renal cancer. Target antigens detected by antibodies in vivo can also be identified by the so-called SEREX technique. R. Rees (Nottingham) employed SEREX in prostate cancer to identify a number of sequences, of which 5 are novel. Y. de Vries described the SSX-2 cancer/testis (C/T) antigen, which was also identified in this way. It is expressed by 50% of melanomas but also by several other tumours and might be useful for immunotherapy. Three HLA-A2-binding peptides from this molecule were identified, two of which were shown to be capable of sensitizing CTL in vitro. One peptide stimulated CTL which were able to kill HLA-A2+, SSX-2+ melanoma cells.

Another "classical" way to identify novel tumour antigens was explored by F. Jotereau (Nantes) using tumour-infiltrating lymphocytes (TIL) to select targets represented in a cDNA tumour expression libary. She reported the identification of a novel HLA-B13-restricted melanoma antigen using this approach. However, most presentations were more concerned with the identification of novel epitopes derived from known tumour-associated genes than with finding completely

This meeting was organized under the aegis of the European Cancer Research Consortium EUCAPS and took place at the Heinrich Fabri Institute, Blaubeuren, Germany, 4th–7th February 1999

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novel proteins. Thus, Jotereau also reported that by transfecting COS cells with the genes for tyrosinase, gp100, MAGE 3 or MAGE 6 and screening these with TIL clones, she had been able to identify novel HLA-B35-restricted epitopes of these molecules. R. Kiessling (Stockholm) reported on a modified screening system for identifying novel HLA-A2-restricted epitopes of the recently-identified melanoma antigen overexpressed in 70–80% of melanomas, the "melanocortin 1 receptor" (MC1R), using A2-transgenic mice. The mice were immunized against the whole protein and then their T cells stimulated by peptides selected by A2-binding motif prediction; in this way, at least three HLA-A2-restricted epitopes were identified. These seemed to be quite immunodominant for TIL; about half of TIL lines examined recognized at least one of the MC1R peptides. The utilisation of HLA-A2-transgenic mice for epitope screening was also presented by C. Gambacorti-Passerini (Milan). He identified epitopes derived from the NPM/ALK fusion protein by motif prediction, T2-binding and then immunogenicity testing in the transgenic mice. The use of K562 cells transfected with HLA-A2, coupled with the application of HLA-A2 – bcr/abl peptide tetrameric complexes (P. Travers, London) may enable the detection and isolation of T cells specific for naturally processed bcr/abl fusion protein epitopes, which is otherwise very difficult (A. Dodi, London). Peptide modification by amino acid substitution may be able to enhance the immune response also against the original, unmodified, peptide, as reported by Jotereau, and shown by many others. It may be possible to elute peptides from the MHC molecules of the cell surface without killing the cells by performing "acid wash extraction" (AWE). O. Tsitsilonis (Athens) reported AWE fractions of ovarian carcinoma cell lines which sensitized T2 cells for lysis by tumour-specific CTL. Identification of these peptides is currently in progress.

A method for determining whether predicted peptides were in fact expressed by tumours, even without recourse to the use of specific T cells as indicators of expression, was described by H.-G. Rammensee (Tübingen). Synthetic peptides corresponding to the predicted HLA-binding sequences of candidate tumour-specific target molecules were first used to establish mass-spectrometry peaks. Then, HLA eluates from tumour cells were simply run under the same conditions and these predetermined peaks were sought. If present, these were sequenced to ensure that they were not by chance of the same mass but different sequence. In this way, new epitopes from CEA, p53 and an RNA-helicase were identified in tumour cells, including freshly excised colon carcinoma cells.

Very few MHC class II-restricted tumour antigens have been identified thus far, although it is thought that CD4 cells are equally or even more important than CD8 cells in tumour rejection. Gambacorti-Passerini confirmed the class II-restricted immunogenicity of bcr/abl peptides in normal donors but was unable to show this in CML patients, as previously observed by others. The generation of CML-specific T cells using other approaches has also proven very difficult (Dodi). In another system, G. Gaudernack (Oslo) described their attempts to stimulate CD4+ T cells in vitro with ras position 61 mutants in a class II-restricted fashion, following their success with position 12 mutants. However, in several different experimental approaches using different types of antigen presenting cells (APC), they were completely unsuccessful. Nonetheless, position 61 mutant peptides were used to vaccinate melanoma patients, and in vivo responses to the peptides could then be observed (see section "Clinical Applications"). Thus, our in vitro sensitization techniques may still require refinement before they accurately reflect immunogenicity as naturally experienced in vivo. Other approaches to identify MHC class II-restricted epitopes include precipitation of the class II molecules from tumour cell lysates, their elution, HPLC purification and sequencing. The techniques available for sequencing in this rapidly changing, high technological application, were summarized by C. Creaser (Nottingham). Application of elution and sequencing to the identification of HLA-DR-restricted epitopes from melanoma cells was described by G. Pawelec (Tübingen). Two of these, present as a wild-type grp78 sequence and as a possibly mutated (one amino acid-substituted) form were each shown to be specifically immunogenic to T cells in vitro (M. Adibzadeh, Tübingen). Another, derived from an unmutated gp100 molecule, was shown to stimulate T cells which were also able to recognize the native tumour cells from which it was derived but not B cells from the same patient (S. Heinzel, Tübingen). HLA-DQ-bound peptides from the same melanoma cells were examined by T. Halder (Oslo) and several sequences derived from a number of molecules were identified. Two separate sequences from the same molecule, melanoma-associated chondroitin sulphate proteoglycan (MCSP) may be candidates for tumour-specific targets and are currently being tested. MCSP is perhaps a particularly interesting candidate because it is expressed by malignant melanoma cells from >90% of samples examined, but is not expressed by normal melanocytes. Preliminary data with one of these peptides suggest that it is indeed immunogenic for T cells (Adibzadeh).

Generation of APC

Accumulating knowledge of tumour antigens must be matched by information on how the antigens should be best presented in stimulatory form to T cells in vitro and in vivo. Currently, attention is focussed on dendritic cells (DC) as APC in both situations. G. Adema (Nijmegen) discussed optimisation of DC as APC in terms of origin of the cells (monocytes or bone marrow?), cytokine protocols and stability of the maturation stage and matching phenotype, as well as best source of antigen to use for presentation. Molecular analysis of DC using

DDRT-PCR and random sequencing of DC cDNA libraries revealed some new molecular markers including a chemokine, DC-CK1, preferentially attracting naive B cells as well as T cells. Other aspects of DC biology were presented by R. Offringa (Leiden) concerning the effects of dexamethasone (DEX) on human DC. DEX blocks maturation of DC and IL 12 production, but does not block IL 10 production, thus possibly enhancing Th2 responses at the expense of Th1 responses. The development of protocols for the in vitro sensitization of T cells to tumour antigens presented by DC was discussed by E. Celis (Rochester). These protocols were developed for generating T cells for use in adoptive immunotherapy, and employed methods involving a semi-limiting dilution approach to generate T cell cultures and supplementation of the medium with IL 10 as well as IL 7, in order to enhance the production of CTL. In this way, CTL specific for epitopes of her2/neu, p53 and CEA have been developed. The importance of IL 10 in maintaining the specificity of the cells derived in this system, even at the expense of the numbers of T cells obtained, was emphasised. ''Natural'' antigen from whole tumour cells may also offer a good source of all potentially relevant targets for immunotherapy, especially if present in the form of apoptotic bodies, readily taken up by immature DC.

Clinical applications

Adoptive immunotherapy with TIL has been practised for many years with limited success. One reason for this may be derived from the experience of Jotereau, who monitored the frequency of tumour-reactive T cells in TIL populations which had been stimulated in vitro with autologous tumour cells and expanded with IL 2. Using sensitive methods for detecting activated T cells (intracytoplasmic cytokine staining) and antigen-specific T cell receptors (soluble MHC/peptide tetramer complexes), she found that tumour-specific CTL were very infrequent in the TIL populations (0–14%). Many TIL-derived T cell clones (TCC) respond poorly to autologous tumour and secrete little or no IL 2. Use of transfectants showed that this was not due to lack of costimulation. However, increasing the antigen density did restore reactivity to many clones. This suggests that the majority of these TCC have low affinity antigen receptors. It may therefore be beneficial to employ the MHC tetramers to enrich the T cells with the highest avidity receptors for the tumour antigen and use only these to treat the patient.

Active immunotherapy with tumour-specific peptide vaccines is also being intensively tested in current clinical trials. E. Jäger (Frankfurt) described their experience with the use of melanoma peptides together with GM-CSF to activate endogenous DC. The injection sites were monitored for the presence of CD4 and CD8 cells, and cytokines. Both CD4 and CD8 cells can be detected, and IL 2, IFN-γ and TNF-α, but not IL 4 or IL 10. One patient of 12 in the MAGE 1/3 vaccination trial with a baseline CTL activity prior to vaccination showed clinical responses coupled with an increase in CTL activity. In the Melan A/tyrosinase trial, clinical effects were a little more pronounced, with two complete responders, two partial responders and 11 stable disease, out of 26 patients entered. In preparation for a newer trial exploring spontaneous immune responses against the C/T antigen NY-ESO-1, 4 of 6 patients showed NY-ESO-1-specific CD4 and CD8 responses, indicating that the antigen could be recognised in a class I- and II-restricted fashion by patients in vivo. Gaudernack described the first trials (done together with L. Braathen in Berne) using the ras position 61 mutant peptide. Although there was no evidence of immunogenicity of this peptide in vitro (see above), peptide-pulsed DC were injected intra-dermally together with GM-CSF into the legs of melanoma patients. There was evidence of DTH reactivity and development of class II-restricted T cell responses to several different position 61 mutant peptides. M. Gjertsen (Oslo) went on to describe results of clinical trials using ras position 12 mutants in pancreas carcinoma. Altogether 43 mostly inoperable patients were given repetitive injections of peptide together with GM-CSF at the same site. DTH not present before vaccination developed in 22 of the 43 patients, but only after the fourth vaccination. T cell proliferation against various mutant peptides but not against wild-type ras peptide was seen in vitro. Thus, 55% of the patients could be classified as immunological responders; this group of patients survived significantly longer than the non-responders (although non-responders still survived longer than historical non-vaccinated controls). The long-term survivors (some at >500 days thus far) are continued on an intermittent re-vaccination protocol. Given the aggressive nature of pancreatic carcinoma, these data are very encouraging.

Tumour escape from the immune response

It is becoming increasingly clear that an important factor in determining the outcome of immunotherapy is tumour ''escape'' from the immune response. In addition, as pointed out by Offringa, peptide-based immunotherapy may even have tolerogenic effects if the peptide leaks out of the depot and becomes systemically available to unactivated DC elsewhere. This effect can be avoided in animal models by using activating mAb against DC-expressed CD40; their use also potentiates the effect of functional, non-tolerogenic peptide immunisation regimes. Therapeutic approaches to enhance immune responses against tumours by engineering stimulatory cytokines and costimulatory ligands to replace those missing from the tumour cells have also been extensively tested over the years. Along these lines, F. Farzaneh (London) described a novel approach in which mAb were chemically linked to the surface of tumour cells. For example, mAb to CD28 on the tumour

cells ligates the major costimulatory receptor CD28 on the T cells, avoiding the coligation of the inhibitory receptor CD152 which can occur if tumour cells are engineered to express CD80 or CD86 natural ligands. Multiple mAb can be linked to each tumour cell; an animal model using P815 with CD3, CD5 and CD28 mAb on the surface shows a significant improvement in survival compared to P815 alone (70% compared to 30% at 80 days).

Effective immunotherapy selects for tumour cells which no longer express the target antigens, most often by downregulating the HLA restriction molecules. This has been clearly observed in gp100+ melanoma, and as presented here by Jäger, in Melan-A+ tumours as well. In some cases, however, it is the tumour antigens themselves, rather than the HLA molecules, which are downregulated. A novel possible mechanism for this may be the phosphorylation of the antigenic peptide itself, which can result in lack of presentation by HLA (M. Andersen, Copenhagen). In vitro, it is observed that tumour cell variants may support the growth of CTL clones in a heterogeneous fashion, with CD39+ clones being the least able to maintain T cells (A. Kirkin, Copenhagen). A similar effect in vivo might also influence the efficacy of the anti-tumour response. The T cells may be affected by the cytokine environment in a way which influences their growth, as suggested by Y. Sun (Mannheim). He reported that loss of CD30 expression irreversibly caused by exposure to high levels of IFN-γ on certain T cells inhibited their growth. This implies that the immunoenhancing effects of IFN-γ may be tempered by growth suppressive effects at least on some anti-tumour clones. Finally, it was argued by Kiessling that tumour-specific T cells may also be negatively affected by the tumour cells via production of reactive oxygen species by activated macrophages. This results in the downregulation of the zeta chain of the receptor signalling complex in both T cells and NK cells, and their eventual apoptosis. It might be possible to prevent this by using histamine to block macrophage peroxide production, and clinical trials are ongoing to explore this approach.

Conclusions

Cancer immunotherapy can be successful but often fails because of tumour escape. Intensive monitoring of each treated patient to identify and intervene in each instance of escape will be required but will be expensive. One major hurdle to successful application of immunotherapy to cancer will therefore be cost. If economic and regulatory problems can be overcome, routine immunotherapy will more rapidly become a reality.

Acknowledgements This conference was organised under the aegis of the EU cancer research project EUCAPS (Contract BMH4-CT98-3508). We are very grateful to Merck KGaA, Roche Diagnostics, Dianova and Schleicher & Schuell for additional support.