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Chemically defined, cell-free cancer vaccines: use of tumor antigen-derived peptides or polyepitope proteins for vaccination

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Summary

With the advent of gene therapy there has been a revival of immunotherapy of cancer. Preclinical studies with the so called tumor vaccines – syngeneic, irradiated tumor cells secreting cytokines – are at present entering clinical trials and hold much promise for efficacious treatment, maybe even cures, of cancer. However, autologous whole cell vaccines which are cytokine gene-modified are expensive and difficult to standardize. In addition, autologous tumor cell cultures, and hence tumor vaccines, cannot be prepared for all patients. For these reasons we have investigated alternative schemes for vaccination against cancer. We have developed vaccines on the basis of tumor antigen-derived peptides using a method, we called "transloading", to transfer peptides efficiently into cells. Such vaccines are chemically well defined and inexpensive to produce. We show in a preclinical mouse model system for mastocytoma, and to a somewhat lesser extent for melanoma, that peptide vaccines give excellent protection against tumor take, provided tumor antigen peptides are injected subcutaneously in conjunction with polycations like polyarginine. Immunohistological investigations on thin sections show that three days after injection, the vaccine bolus is heavily infiltrated by antigen presenting cells which take up peptides. We posit that such antigen presenting cells then migrate into draining lymph nodes where they activate naive T cells to become anti-tumor cytotoxic lymphocytes (CTL). The consequences of strong dependency of peptide sequence on the HLA-type of patients and possible remedies are discussed. One avenue to be tested in mouse models is the injection of mixtures of peptides covering a multiplicity of MHC-specific peptides. Another is the production of recombinant proteins as vaccines whose application is considerably less dependent on HLA-types of patients to be treated. In addition, such proteins would be expected to contain both class I and class II restricted peptides. Alternatively, artificial proteins incorporating many CTL epitopes of known tumor antigens, including members of the MAGE family, thus yielding a SUPERMAGE vaccine with utility for several human HLA-types could be designed. The prophylactic application of peptide/polyepitope vaccines can be envisaged.

I. Tumor-specific and tumor-associated antigens

After the great successes of vaccination against bacterial and viral infections, physicians and scientists have attempted to harness the exquisite specificity, sophistication and power of immunology to combat cancer, but the results hitherto have been rather disappointing and have led to a rather pessimistic assessment of immunotherapy of cancer. Indeed, at certain periods the postulate emerged that cancers were not

immunogenic. Recent molecular and immunological investigations, however, have revealed the existence of tumor antigens in several tumor types (Boon et al., 1994; Boon and van der Bruggen, 1996; Rosenberg, 1997). This has led to the conclusion, that most, and perhaps all tumors are indeed immunogenic, but that the response of the immune system to tumor antigens is inadequate and in need of enhancement if immunotherapy of cancer is to be attempted.

Peptides derived from tumor antigens, mostly presented by cancer cells in the MHC class I context (Boon et al., 1994; Boon and van der Bruggen, 1996; Robbins and Kawakami, 1996; Rosenberg, 1997) can be classified as having been derived from tumor specific (TSAs) or tumor associated antigens (TAAs). They have been identified mostly by means of CTL clones obtained from cancer patients and by gene cloning or by peptide elution followed by tandem mass spectrometry analysis (Boon et al., 1994; Slingluff et al., 1994; Wang and Rosenberg, 1996). As shown recently by Kinzler and Vogelstein's lab, cancerous transformation leads to extensive reprogramming of the cancer cell in that as many as 500 mRNA types are either up or down regulated (Zhang et al., 1997). As a consequence, cancer cells can express tumor associated antigens which are unconnected with the gene expression programme of the parental cell type from which the cancer cell arose.

Thus it is known for melanoma, that malignant cells express, amongst other TSA and TAA, the MAGE, BAGE and GAGE family of genes which are normally expressed in male germline cells and placenta (Boon and van der Bruggen, 1996) and thus are clearly self-proteins. Because germ line cells are immunologically privileged and do not express MHC surface receptors, clonal selection presumably cannot act to eliminate MAGE, BAGE and GAGE specific CTLs under normal circumstances. The frequent appearance of these related genes in tumors different from melanoma such as head and neck cancer, lung cancer or bladder carcinoma may be a consequence of random demethylation of genes in cancer cells and activation of their promoters by demethylation, as has been shown for the MAGE-1 promoter (De Smet et al., 1996). Another interesting example of TAAs in melanoma cells are the so called differentiation antigens tyrosinase, tyrosinase related proteins 1 and 2 (TRP-1, TRP-2) and other proteins normally encountered during melanocyte differentiation (see **Table I** and Boon et al., 1997; Rosenberg, 1997). Interestingly, the immune system treats these proteins, dysregulated and expressed in the "wrong" context of tumor cells, as foreign antigens. Despite potential earlier clonal selection by the presumed interaction of the immune system with melanocytes, cytotoxic T cells which recognise peptides derived from these proteins are generated and hence are reactive against melanoma cells.

The encoded proteins of oncoviral genes, mutated oncogenes and tumor suppressor genes or mutated cellular genes, are tumor specific (**Table I**). Examples for the first are the oncoproteins of the Epstein-Barr virus (Rickinson and Moss, 1997) or E6 and E7 of HPV16 (Tindle, 1996), for the second and third the mutated Ras or p53 proteins (Disis and Cheever, 1996) and for the last are connexin (Mandelboim et al., 1994; Mandelboim et al., 1995), Her 2/neu (Disis and Cheever, 1997), MUC 1 (Finn et al., 1995), CDK4 (Wolfel et al., 1995) and ß-catenin (Robbins et al., 1996). Being tumor specific, any of these have the potential of eliciting anti-tumor immunity when used as a vaccine.

II. TSA and TAA peptide vaccines

Previous attempts at creating tumor vaccines have concentrated on the stimulation of the immune response by cytokines. The great advantage of using cytokine-secreting autologous tumor cells as vaccines is that in this strategy, unidentified and unknown TSAs and TAAs are presented to the immune system in a milieu of high local cytokine concentration (Pardoll, 1995; Zatloukal et al., 1995). The most widely used cytokine is IL-2, the level of which is highly critical for the outcome of the vaccination as indicated in preclinical studies using the M3 melanoma model (**Figure 1a** and Schmidt et al., 1995). We further determined cytokine optima in the B16/F10 model where the number of metastases were scored. The IL-2 optimum for B16/F10 appears to be higher (3,300 units IL-2/vaccine; **Figure 1b**) as compared to 1,500 units IL-2/vaccine for the M3 model (**Figure 1a** and Schmidt et al., 1995). As shown previously for the M3 system, GM-CSF shows no dosis maximum, but both in the M3 and B16/F10 model, protection increases with cytokine dosage (**Figure 1c**). In the case of IFN-γ it appears that a dose of 165 ng/vaccine yields best protection (**Figure 1c**).

It has been the experience of different groups that despite the great potential that autologous cancer vaccines have, the procedure is cumbersome and expensive, and of limited utility since tissue cultures can only be prepared in roughly 2/3 of the cases for malignant melanoma (E. Wagner, Vienna, unpubl. results). These circumstances call for alternative strategies.

Syngeneic dendritic cells pulsed with tumor antigen peptides matched to the MHC type of the mice strains have repeatedly been shown to protect animals against tumor take (Celluzzi et al., 1996; Mayordomo et al., 1996; Mayordomo et al., 1995; Paglia et al., 1996; Porgador et al., 1996). This approach has also been used in a clinical setting: lymphoma patients were treated with dendritic cells pulsed with autologous idiotype protein (Hsu et al., 1996; Hsu et al., 1997). The results of these studies are promising.

In a seminal investigation, Boon and coworkers vaccinated fifteen terminally-ill melanoma patients with a HLA-A1 matched immunogenic MAGE-3 peptide and detected clinical benefits in five of them in that some tumors regressed (Marchand et al., 1995). This result is even more remarkable, because no adjuvants of any kind were used. Curiously, despite these clinical effects, no

tigens	*MHC*	*Tumor where Ag was initially identified*	*Epitope*	*Ref.*
ıtigens expressed in many tumor types; widely shared				
\GE-1	HLA-A1	melanoma	EADPTGHSY	(Traversari et al., 1992)
	HLA-Cw16	melanoma	SAYGEPRKL	(van der Bruggen et al., 1994)
\GE-3	HLA-A1	melanoma	EVDPIGHLY	(Gaugler et al., 1994)
	HLA-A2	melanoma	FLWGPRALV	(van der Bruggen et al., 1994)
\GE	HLA-Cw16	melanoma	AARAVFLAL	(Boel et al., 1995)
\GE-1	HLA-Cw6	melanoma	YRPRPRRY	(Van den Eynde et al., 1995)
tAME	HLA-A24	melanoma	LYVDSLFFL	(Ikeda et al., 1997)
·acetylglusoaminyl- ransferase-V	HLA-A2	melanoma	VLPDVFIRC	(Guilloux et al., 1996)
.5	HLA-A24	melanoma	AYGLDFYIL	(Robbins et al., 1995)
.fferentiation antigens expressed in melanocyte lineage cells				
\RT-1/Melan-A	HLA-A2	melanoma	AAGIGILTV	(Kawakami et al., 1994)
	HLA-A2	melanoma	ILTVILGVL	(Castelli et al., 1995; Wolfel et al., 1994)
)loo/pmel17	HLA-A2	melanoma	KTWGQYWQV	(Kawakami et al., 1995)
	HLA-A2	melanoma	ITDQVPFSV	(Kawakami et al., 1995)
	HLA-A2	melanoma	YLEPGPVTA	(Cox et al., 1994)
	HLA-A2	melanoma	LLDGTATLRL	(Kawakami et al., 1994)
	HLA-A2	melanoma	VLYRYGSFSV	(Kawakami et al., 1995)
	HLA-A24	melanoma	VYFFLPDHL	(Robbins et al., 1997)
/rosinase	HLA-A2	melanoma	MLLAVLYCL	(Wolfel et al., 1994)
	HLA-A2	melanoma	YMNGTMSQV	(Wolfel et al., 1994)
	HLA-B44	melanoma	SEIWRDIDF	(Brichard et al., 1996)
	HLA-A24	melanoma	AFLPWHRLF	(Kang et al., 1995)
	HLA-DR4	melanoma	QNILLSNAPLGPQFP	(Topalian et al., 1996; Topalian et al., 1994)
	HLA-DR4	melanoma	SYLQDSDPDSFQD	(Topalian et al., 1996; Topalian et al., 1994)
TP-1/gp75	HLA-A31	melanoma	MSLQRQFLR	(Wang et al., 1996)
TP-2	HLA-A31	melanoma	LLPGGRPYR	(Wang et al., 1996)
mor specific, mutated antigens				
·Catenin	HLA-A24	melanoma	SYLDSGIHF	(Robbins et al., 1996)
)K4	HLA-A2	melanoma	ACDPHSGHFV	(Wolfel et al., 1995)
JM-1	HLA-B44	melanoma	EEKLIVVLF	(Coulie et al., 1995)
\SP-8	HLA-B*3503	head and neck cancer	FPSDSWCYF	(Mandruzzato et al., 1997)
)nmelanoma antigens				
ER-2/neu	HLA-A2	breast/ovarian	KIFGSLAFL	(Fisk et al., 1995)
	HLA-A2	breast/ovarian	ELVSEFSRM	(Fisk et al., 1995; Ioannides et al., 1993)
	HLA-A2	breast/ovarian	IISAVVGIL	(Linehan et al., 1995; Peoples et al., 1995)
JC-1	Non-MHC ?	breast/ovarian/pancreas	PDTRPAPGSTAPPAHGVTSA	(Finn et al., 1995)
uman papillomavirus 5, E 7	HLA-A2	cervical carcinoma	YMLDLQPETT	(Alexander et al., 1996; Ressing et al., 1996)
arcinoembryonic ntigen (CEA)	HLA-A2	epithelial cancers	YLSGANLNL	(Ras et al., 1997; Tsang et al., 1995)

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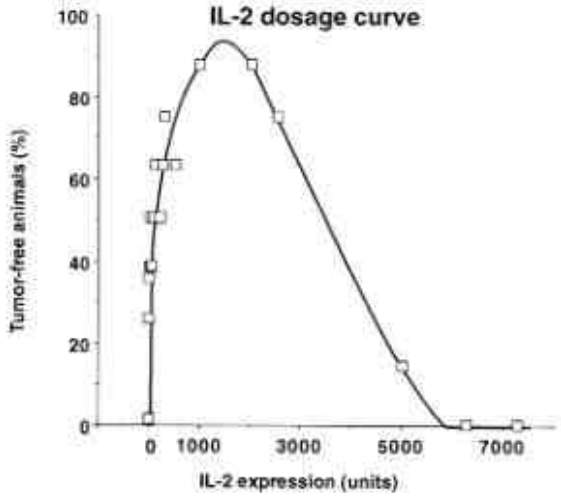
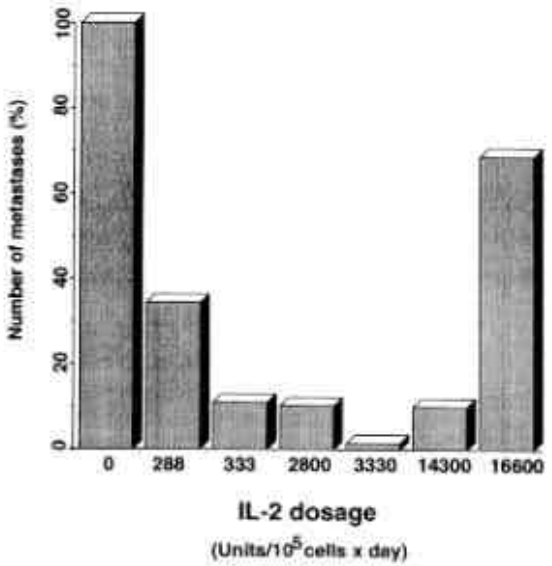


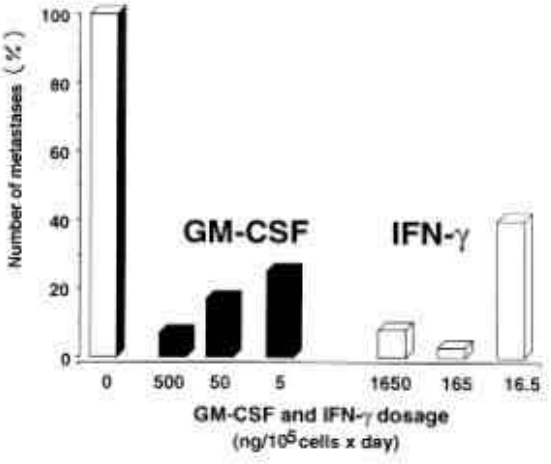
ble I: (previous page) Human tumor specific and tumor associated antigens recognized by T cells known to date. After Boon et al., 1997; Robbins and Kawakami, 1996; Rosenberg, 1997; Wang et al., 1995 with adaptions.

Figure 1: Cytokine dosage effects in experimental murine melanoma systems.

Figure 1a: IL-2 dosage curve as determined in DBA/2 mice (8-10 animals/group) with prophylactic treatment against M-3 melanoma tumor challenge (Schmidt et al., 1995). Mice were vaccinated twice at weekly intervals (10^5 irradiated cells/vaccination/animal) and challenged one week after the second vaccination (3 x 10^5 viable M-3 melanoma cells). Reproduction of a previously published Figure (Schmidt et al., 1995).



IL-2 (**Figure 1b**), GM-CSF and IFN-γ (**Figure 1c**) dosage effects in the treatment of experimental B16-F10 lung metastases in C57BL/6J mice (eight animals/group). Animals were vaccinated three times (10^5 irradiated cells/vaccination/animal). Lung metastases were established by intraveneous injection of 10^4 B16-F10 cells three days before the first vaccination. After 28 days lung metastases were scored and the number obtained for all eight animals of the control group receiving mock-transfected B16-F10 was set to 100 % (n=215).



peptide specific CTLs could be detected in vaccinated patients. However, as reported at this conference, no clinical effects were detected in a repeat experiment by the Parmiani group (Milano).

Following in the footsteps of Boon et al. (Marchand et al., 1995) we have carried out a preclinical study in mice for the efficacious vaccination against tumor take in both the murine M3 melanoma and the P815 mastocytoma model system using small TAA-peptides matched to the MHC-type of the experimental animals (Schmidt et al., 1997a). Since no known tumor antigens are available for the M3 system, it was ascertained by means of RT-PCR that M3 cells expressed tyrosinase and tyrosinase related protein-1 (TRP-1) which are known as TAAs in human melanomas. Much progress has been made in establishing the binding rules for small peptides to MHC class I molecules (Rammensee et al., 1995), therefore, potential tumor antigen peptides can be predicted up to a certain extent by computer analysis. Six potential H-2K^d binding peptides are found in tyrosinase (**Table II**, see also (Schmidt et al., 1997a) and four in TRP-1 (**Table II**). Two each of these two classes of peptides were arbitrarily selected (**Table II**) and combined for subcutaneous injection into mice. In the case of the P815 system it was known that a "self"-peptide SYFPEITHI derived from the JAK1 tyrosine kinase protein occupied an unusually high proportion of 5% of all MHC class I molecules on P815 cancer cells (Harpur et al., 1993; Rammensee et al., 1995) and this peptide was therefore judged to represent a useful target for immunological rejection.

Immunohistological investigations of the vaccination

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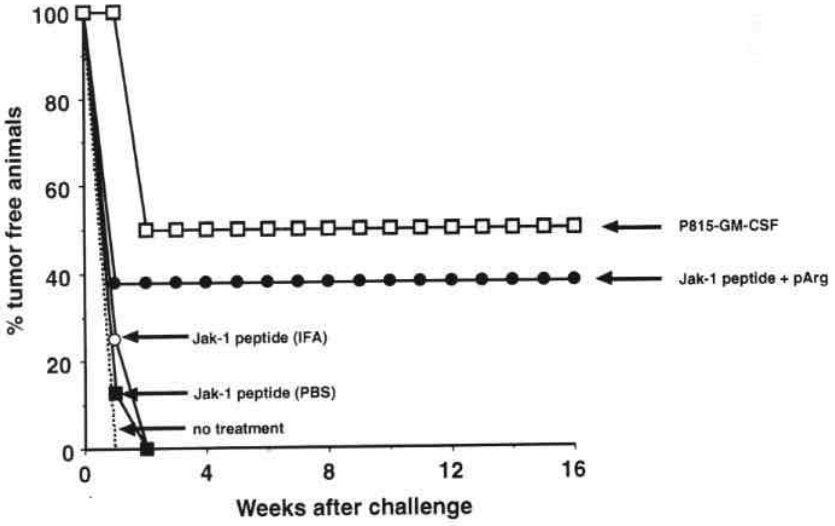
Table II

Protein	Peptides identified (?Y??????L/I)	Peptides selected
tyrosinase	HYYVSRDTL,YYVSRDTL, SYMVPFIPL, VYPEANAPI, LYRNGDFFI, PYLEQASRI	YYVSRDTL, PYLEQASRI
TRP-1	AYISLFLML, YYSVKKTFL, KYDPAVRSL, RYAEDYEEL	RYAEDYEEL, YYSVKKTFL

Table II: Putative peptide antigens used in the M3 melanoma model. The sequences of mouse tyrosinase and tyrosinase related potein 1 (TRP-1) were searched for peptides fulfilling criteria necessary for binding to H-2Kd molecules (?Y??????L/I) (Rammensee et al., 1995).



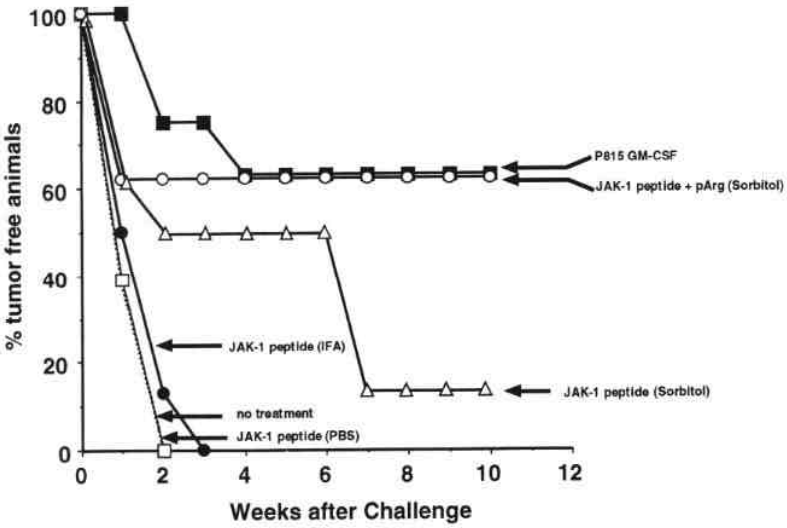


Figure 2: Peptide immunization prevents tumor growth.

Figure 2a: P815 mastocytoma model. DBA/2 mice were immunized with the JAK-1 kinase derived peptide SYFPEITHI. Mice (8 animals per group) were vaccinated three times at weekly intervals and challenged one week after the last immunisation as described in (Schmidt et al., 1997a). Tumor cells stably transfected with a GM-CSF gene (P815 GM-CSF) served as gold standard for vaccine efficiency. Peptides were injected subcutaneously in PBS, emulsified in incomplete Freund's adjuvant (IFA) or as a mixture of peptide and polyarginine (pArg).

Figure 2b: Model as in Figure 2a using the novel sorbitol containing injection medium.

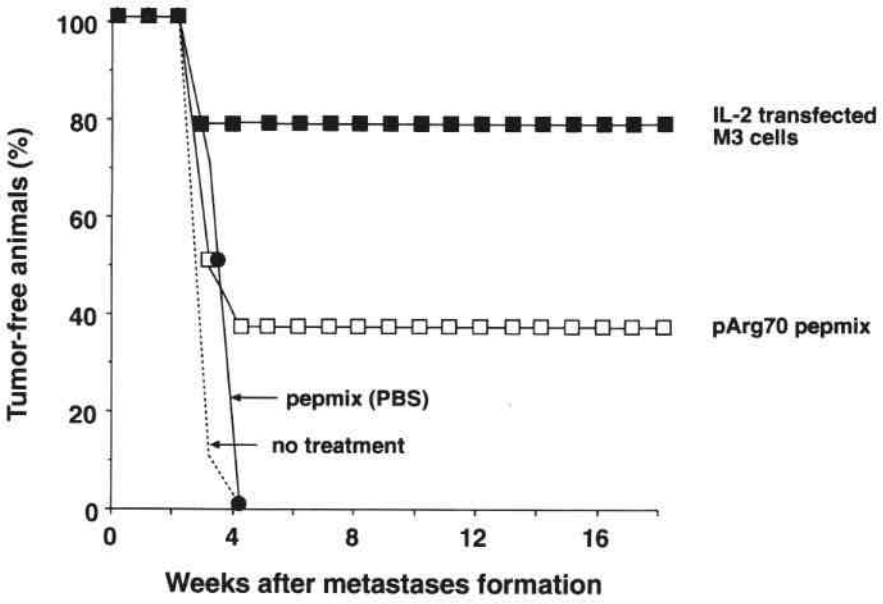


Figure 2c: M3 melanoma model. Experimental metastases (10^4 parental M3 cells) were set 5 days before the first vaccination. Animals were vaccinated subcutaneously 3 times at weekly intervals with a mixture of tyrosinase and TRP-1 derived peptides (Table II). A cellular vaccine consisting of IL-2 gene transfected, irradiated M3 was used to assess the relative efficiency of the peptide vaccine.

site (Bannerji et al., 1994; Maass et al., 1995; Schmidt et al., 1997b) as well as complementary studies of others (Huang et al., 1994) have identified antigen presenting cells (APCs), rather than T cells, to play a pivotal role in initiating the cascade leading to the generation of tumor specific T cells. Our choice of the subcutaneous route for injection of the peptides was the logical consequence of the above cytological investigations in that it was our intention to elicit a cellular response based on APCs rather than a humoral immune reaction. APCs are present in skin at high frequency, with Langerhans constituting as much as 3-4% of the cell mass (Stingl, 1990). We surmised that these APCs could be engaged and thus initiate the immunological events which would lead to the production of anti-peptide (hence anti-tumor) CTLs capable of attacking the tumor cell challenge or pre-existing experimental metastases placed counterlaterally on the back of the mouse.

A trivial finding is that in physiological saline solutions tumor antigen peptides are often quite insoluble and are possibly inactivated by precipitation in the injection medium. This can be circumvented by dissolving the peptide at low ionic strength and making the solution isotonic by addition of sorbitol (unpublished result). The JAK 1 peptide by itself as well as peptide solutions to which incomplete Feund's adjuvant (IFA) had been added did not elicit any protection against tumor take in the P815 test system (**Fig 2a**, see also Schmidt et al., 1997a). When peptide was dissolved in the novel sorbitol medium tumors developed somewhat slower, however, only administration of peptide in conjunction with polyarginine did result in protection levels comparable to a cellular vaccine secreting GM-CSF (**Figure 2b**). We hypothesize that the failure of peptides to elicit protection against tumor take may be caused by a lack of peptide uptake into APCs. Such a hypothesis is supported by the finding that at least *in vitro* APC do not take up small peptides efficiently, but do so, if polycations such as polylysine and polyarginine are included in the peptide medium (Buschle et al., 1997). Such an enhancement of peptide uptake can also be demonstrated to occur *in vivo*: Sectioning and immuno-staining the injection site after application of a fluorescently labelled small peptide clearly reveals importation of the small peptides into APCs when co-applied with polycations, but not, when these compounds are eliminated (**Figure 3** and unpubl. results).

As shown in **Figure 2b**, the JAK 1 peptide administered in the sorbitol medium together with polyarginine was as good as a GM-CSF secreting cellular vaccine and better than peptide injected in isotonic saline (**Figure 2a**). Hence, under appropriate conditions, the potency of peptide vaccines can equal that of a cytokine secreting cellular vaccine in the P 815 model system. The M3 melanoma yields similar answers, although the first generation results show the peptides from tyrosinase and tyrosinase related protein to be less potent (**Figure 2c** and Schmidt et al., 1997a). The less efficient protection against tumor take seen in the M3 system may be explained by a poor immunodominance of the peptides arbitrarily selected for this experiment. Experiments with

the novel sorbitol medium are in progress.

When comparing the peptide vaccines with cytokine secreting cellular vaccines, which have become the gold standard of immunotherapy of cancer, it may be concluded from the P815 experiments that peptide vaccines can be equally efficacious. The situation with the M3 experiment can only be judged once we have identified potent, immunodominant tumor antigen peptides. Also, as demonstrated by Boon's group with a tumor antigen peptide (Marchand et al., 1995), humans may be less refractory to peptide vaccination so that even the incomplete protection seen in the M3 system may already be relevant for the human situation. However, the success of peptide vaccines will always depend on a cogent selection of peptides.

From the above we wish to conclude:

l. Peptide vaccines can be made to work as efficiently as cytokine secreting cellular vaccines.

2. By contrast with cellular vaccines, peptide vaccines are chemically defined and can be easily standardised.

3. They are inexpensive and are available for vaccination without delay and have a long shelf-life.

4. Although today's focus is on developing vaccines to cure cancer or at least minimal residual disease of cancer, the availability of peptide vaccines has the potential to vaccinate prophylacticly to prevent the occurrence of cancer.

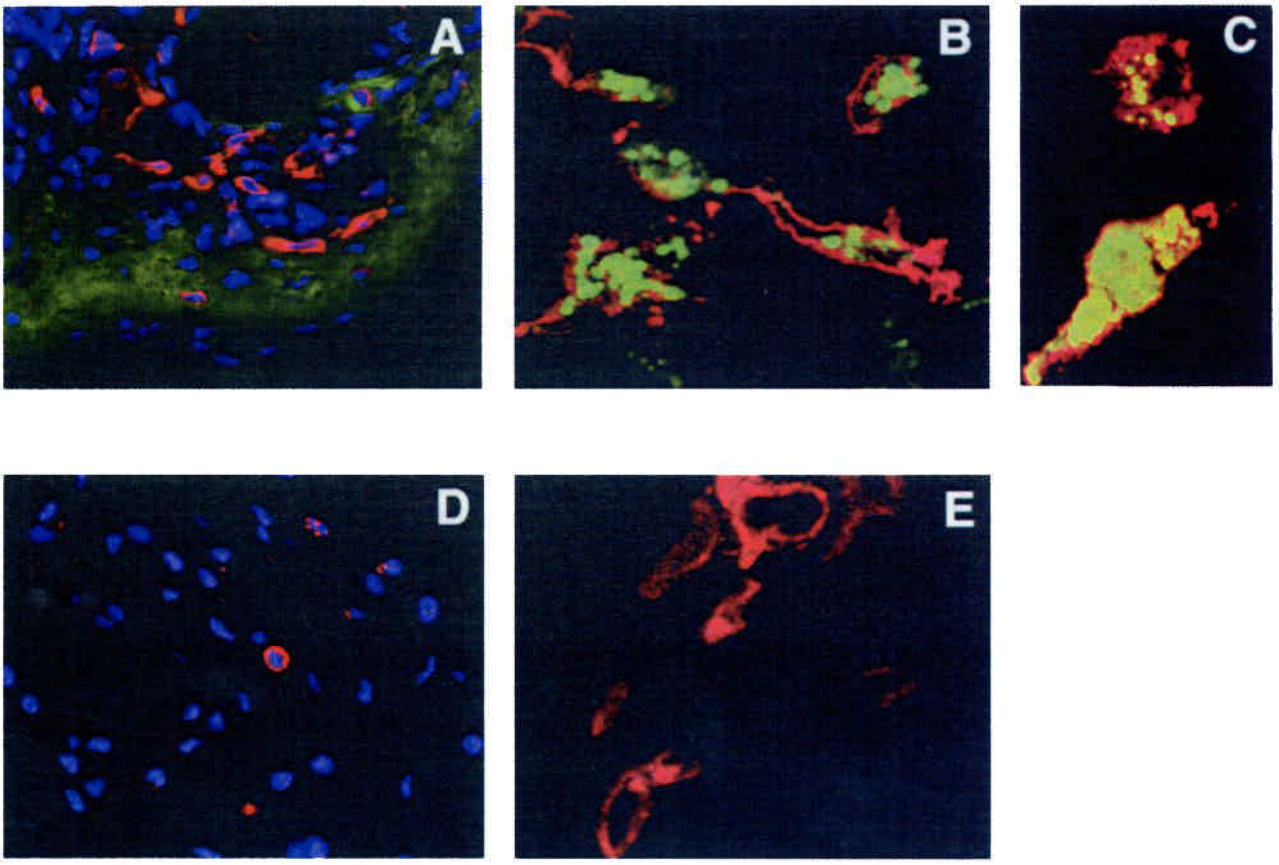


Figure 3: Immunohistological analysis of the injection site after application of peptide vaccines in the presence (**A,B,C**) or absence (**D, E**) of polyarginine.

Fluorescence labeled peptides were injected subcutaneously alone (D,E) or as a mixture of peptide with polyarginine (A,B,C). **A, D**: Fluorescence photomicrographs of injection sites 3 days after vaccination. Cryosections were stained with an antibody recognizing MHC class II followed by detection with a Texas Red tagged secondary antibody. Nuclei were counterstained with 4,6-diamidino-phenylindole (DAPI). **B, C, E**: Confocal microscopy of injection sites 7 days after vaccination. Sections were processed as above except that DAPI was omitted.

III. Cancer peptide vaccines and HLA heterogeneity

We are encouraged by these many positive aspects of peptide vaccines, but a discussion of this field would be incomplete without considering the downside and possible remedies thereof. The single most important impediment for the wide application of such vaccines is the dependence of the TSA and TAA peptide sequence on the HLA-type of the patient. Another type of heterogeneity one has to content with is the apparently diverse appearance of TAAs even within one class of tumors such as melanoma. Another issue which impinges on practicality of these vaccines, is whether different types of cancer share common antigens. The situation here is in flux and no final assessment is as yet possible. Each cancer type may ultimately require its own vaccine composition.

A possible solution to some of these problems, for instance TSA and TAA heterogeneity within one tumor type, is to work with peptide mixtures where it is assumed that mismatched peptides are simply inert. The same approach may be useful when dealing with HLA-heterogeneity although here the complexities are immense and vexing. One positive aspect is that certain HLA-types occur more frequently than others, as is the case for HLA-A2 which in Caucasians is encountered in nearly half of the population. It can be calculated that by combining HLA-A2 with other relatively frequent HLA-types like A1, A3 and A24 approximately 80% of the Caucasian population will be covered on statistical grounds. This proportion can be increased by adding rarer types, but with diminishing returns and, in practice, 100% will never be attained.

TSAs and TAAs, as is the case for cellular proteins, are degraded in the cytoplasm by the proteasome and the resulting peptides transported into the endoplasmatic internal space by the transporter associated with antigen processing (TAP) (York and Rock, 1996). Association of the peptides binding tightly to the groove of the MHC class I molecule occurs in small cellular vesicles which then fuse with the cell membrane, thus exposing the peptide-charged MHC molecule to the extracellular environment. The consequence of such a MHC dependent selection of peptides is that HLA-dependence in peptide vaccines can be largely circumvented by using the entire TSA or TAA for vaccination. The task then becomes that of developing efficient procedures to import the unprocessed (recombinant) TSAs and TAAs into APCs. We have ascertained that the polycations also enhance the entry of large proteins into APCs (unpublished results) and hence this approach should be applicable to protein vaccination. One major advantage of such protein vaccines is that class I as well as MHC class II restricted epitopes are contained within the sequence of a given protein and thus can be presented following entry into APCs (Germain and Margulies, 1993; Kovacsovics Bankowski and Rock, 1995; Watts, 1997; York and Rock, 1996).

IV. Polyepitope proteins as vaccines

Melief et al. (Melief et al., 1996) have recently suggested a general utility of polyepitope proteins to vaccinate against cancer. We wish to point out that when a TAA protein is degraded and some of its peptides ”presented”, much of the protein sequence is dead-weight because peptides derived from most of the TAA sequences will not fit a given MHC molecule. Now, it has been demonstrated (An and Whitton, 1997; Thomson et al., 1996; Thomson et al., 1995; Whitton et al., 1993) that polyepitope proteins consisting entirely of contiguous short CTL epitopes are correctly processed by the immune system to yield CTLs against each of the epitopes. Assuming that this is a general principle we have constructed a gene encoding an artificial protein, “SUPERMAGE”, which consists entirely of known short peptides derived from known melanoma TAA and TSA capable of generating CTLs in different HLA contexts (**Figure 4**). Thus, it would be expected on the basis of the artificial SUPERMAGE sequence that such a protein could be used as a melanoma vaccine, in an HLA setting of A1, A2, A24, A31, B44, and Cw6. Thus a majority of melanoma patients, could be vaccinated with this antigen. It is not surprising that at this stage HLA-A2 peptides are over-represented in the SUPERMAGE protein, reflecting the present day state of the art in CTL analysis. The corresponding gene sequence shown in **Figure 4** contains restriction sites within the coding sequence for easy modification of the protein sequence, as new epitopes become known. Large quantities of the SUPERMAGE protein could rather easily be produced using standard recombinant technologies including expression of SUPERMAGE in insect cells.

Our investigations of the injection sites following peptide vaccination revealed that invasion of the bolus of injected peptides requires several days (**Figure 3a, b** and **c**). By contrast, peptides injected without adjuvants rapidly diffuse from the injection site and are undetectable at time points beyond 24 hours (**Figure 3d, e** and unpublished results). At day 7 numerous APCs are loaded with flourescence labelled peptides, but only when polyarginine is coinjected (Figures 3 b, c and e). Possibly, application of a larger polyepitope protein rather than peptide mixtures may enhance the efficiency of the vaccine by displaying decreased diffusion rates at least when injected subcutaneously. These ideas are now being tested in murine model systems. However, before such strategies including the generation of SUPERMAGE and related proteins of different kinds can be routinely implemented in man it will be necessary to establish the potency of individual epitopes (e.g. in form of peptides) and to determine what minimal number of each epitope in particular is necessary to elicit clinical benefits in different HLA contexts.

A. DNA sequence

ggaattcgccgcc**ATG**GAAGCTGACCCGACAGGTCACAGTTACGAGGTAGATCCCATCGGGCATTTGTACTTTTTGTG
GGGACCCAGGGCTTTGGTATACCGTCCCCGACCGCGACGTTACGTTCTCCCAGATGTGTTCATACGTTGTGCG
TATGGCTTAGATTTTTACATATTAGCAGCCGGGATTGGAATCTTGACGGTAATCCTGACAGTAATCCTCGGTGT
TTTAAAAACTTGGGGCCAATATTGGCAGGTAATCACTGACCAAGTTCCCTTTAGTGTTTACCTAGAACCGGGG
CCCGTAACCGCGCTACTGGATGGTACGGCGACGCTACGGTTGGTACTCTATCGCTATGGAAGTTTTAGCGTAG
TCTACTTCTTTCTACCAGACCACCTAGTCGACATGTTACTCGCTGTACTGTATTGTTTGTATATGAATGGCACT
ATGTCACAAGTGAGTGAAATCTGGAGAGATATTGACTTTGCCTTCCTTCCCTGGCATAGACTATTCATGAGTTT
ACAAAGGCAGTTCCTGCGCCTATTGCCGGGTGGGAGACCCTATCGTtaatctagatt

B. Amino acid sequence

EADPTGHSY **EVDPIGHLY** FLWGPRALV **YRPRPRRY** VLPDVFIRC **AYGLDFYIL** AAGIGILTV
ILTVILGVL KTWGQYWQV **ITDQVPFSV** YLEPGPVTA **LLDGTATLRL** VLYRYGSFSV
VYFFLPDHL-*VD*-MLLAVLYCL YMNGTMSQV **SEIWRDIDF** AFLPWHRLF **MSLQRQFLR**
LLPGGRPYR-**Stop**

C

peptide	protein	MHC
EADPTGHSY	MAGE-1	HLA-A1
EVDPIGHLY	MAGE-3	HLA-A1
FLWGPRALV	MAGE-3	HLA-A2
YRPRPRRY	GAGE-1	HLA-Cw6
VLPDVFIRC	N-acetylglucosaminyl-transferase-V	HLA-A2
AYGLDFYIL	p15	HLA-A24
AAGIGILTV	Mart-1/Melan A	HLA-A2
ILTVILGVL	Mart-1/Melan A	HLA-A2
KTWGQYWQV	gploo/pmell7	HLA-A2
ITDQVPFSV	gploo/pmell7	HLA-A2
YLEPGPVTA	gploo/pmell7	HLA-A2
LLDGTATLRL	gploo/pmell7	HLA-A2
VLYRYGSFSV	gploo/pmell7	HLA-A2
VYFFLPDHL	gploo/pmell7	HLA-A24
MLLAVLYCL	tyrosinase	HLA-A2
YMNGTMSQV	tyrosinase	HLA-A2
SEIWRDIDF	tyrosinase	HLA-B44
AFLPWHRLF	tyrosinase	HLA-A24
MSLQRQFLR	TRP-1	HLA-A31
LLPGGRPYR	TRP-2	HLA-A31

Figure 4: Artificial, polyepitopic SUPERMAGE antigen for the treatment of human melanoma of the HLA haplotypes A1, A2, A24, A31, B44 and Cw6.

A) DNA sequence of the SUPERMAGE polypeptide. 5' Eco R I and 3' Xba I sequences were added for subcloning (underlined). A Sal I site was included for later modification of the molecule (underlined). The ATG start codon (bold) is preceeded by the sequence gccgcc to allowing efficient translation (Kozak, 1984). **B**) Amino acid sequence of the SUPERMAGE antigen (single letter amino acid code). **C**) Peptides used in the SUPERMAGE antigen. For references see Table II.

V. A schedule for curing of cancers ?

Looking far ahead and assuming that both cellular and at least one form of peptide/protein vaccination can be shown to be efficacious in patients, the following scheme shown in **Figure 5** may ultimately apply. For immunotherapy of cancer, tumors would be resected or, if indicated, patients would undergo chemotherapy or radiation therapy in order to remove the cancerous tissue. In this way cancer would be reduced to a minimal residual disease. Blood cells or excised tumor tissue could be used to determine the HLA-type of the patient and the tumor tissue would be screened for any known TSA and TAA by RT-PCR or immunological techniques. If the appropriate peptides or multiepitope proteins are available, patients would be vaccinated with these defined vaccines. Because of the HLA heterogeneity, in practice probably around 80% of the patients (see above) can be anticipated to qualify for such a protocol, if the vaccine is tailored to the four most frequent HLA-types. Alternatively, patients could be treated with recombinant TSA, TAA proteins. If neither of the above are available, either because of a rare HLA genotype or lack of known antigens, the patients would proceed to treatment with either autologous or allogeneic cellular tumor vaccines, if either of these can be shown to be effective in the ongoing clinical trials. If there is no reoccurrence of metastases after peptide/protein vaccination, no further steps would be required. If there is

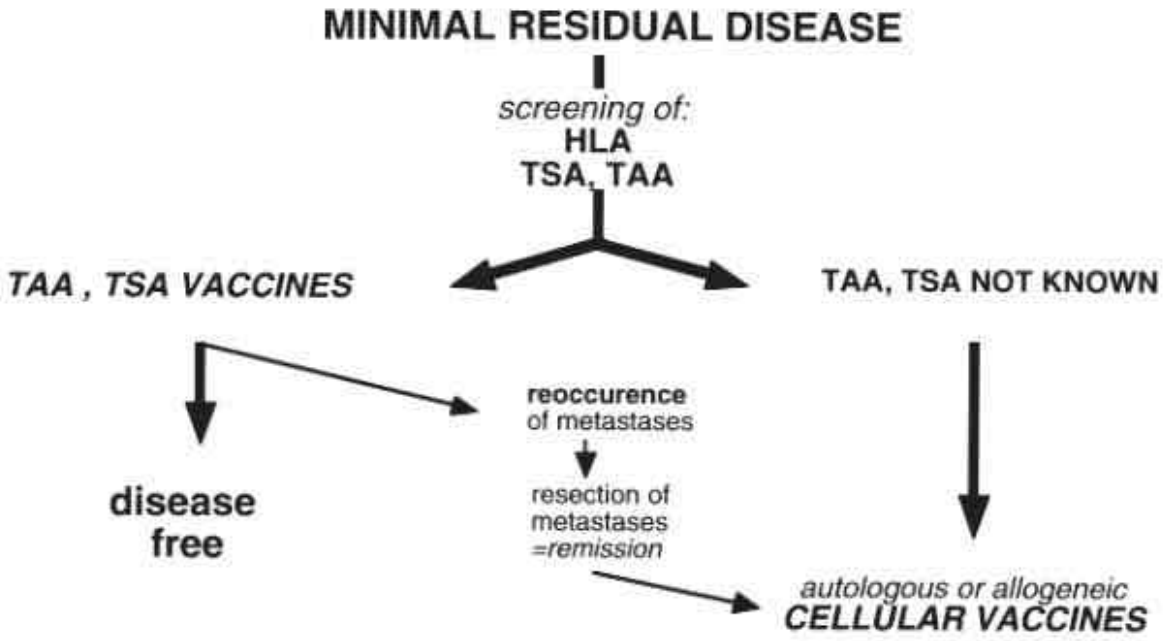


Figure 5: Potential treatment schedule for human cancers

reocurrence of tumor metastases, either because progression of tumors renders the vaccine ineffectual or the peptide epitopes prove insufficiently immunogenic, the patients would undergo remission-inducing conventional therapy followed by treatment with cellular vaccines at a later stage. It is hoped that by applying the above scheme at least select patient populations will benefit from immunotherapy of cancer.

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