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DIRECCIÓN DE NUEVAS CREACIONES

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Abogado(a)/Señor(a)
SILVANA MARIA LÓPEZ DÍAZ

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Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/JP2008/000290				
Fecha de Presentación Internacional	21/02/2008				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No. 13-101095-00000-0000	19/abril/2013
Reivindicaciones:	Radicado No. 13-101095-00000-0000	19/abril/2013
	Radicado No. 13-101095-00003-0000	15/Octubre/2013
Dibujos:	Radicado No. 13-101095-00000-0000	19/abril/2013
Secuencias:	Radicado No. 13-101095-00000-0000	19/abril/2013
Prioridad:	60/902,949	21/Febrero/2007

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Presentación de solicitud Fraccionaria (Art 36 D 486)

Se acepta para examen la solicitud fraccionaria presentada e identificada como 13-101095, porque no implica ampliación de la materia contenida en la solicitud parental 09-102283.

4. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. Para la presente solicitud se estudió el capítulo reivindicatorio completo (13-101095-00003-0000).

Título de la solicitud: “Vacunas peptídicas para cánceres que expresan antígenos asociados con tumores”.

El problema planteado en esta solicitud consiste en la necesidad de identificar péptidos inmunogénicos derivados de genes cuya expresión esté desregulada en tumores y que sean útiles en el desarrollo de drogas anticancerosas.

Para resolver este problema técnico la solicitud en estudio proporciona composiciones farmacéuticas e inmunogénicas que comprenden entre 0.1mg y 10mg de un péptido seleccionado del grupo que consiste de i) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO:110, 111, 387, 112, 394, 395, 133, 135, 137 o 426; y ii) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO: 114, 116, 117, 121, 227, 228, 233, 254, 271, 272 o 288; y un portador.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 14/00, A61K 38/00, A61P 35/00, C07K 11/00, C12N 5/10, G01N 33/15, G01N 33/50.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

En primer lugar, se recuerda a la solicitante que la forma correcta de reclamar una composición farmacéutica o una composición inmunogénica (vacuna) es a partir del ingrediente activo, los excipientes y/o coadyuvantes y las proporciones de los mismos. De modo que como están redactadas las reivindicaciones 1 y 2 no se trata de composiciones verdaderas.

Además no existe sustento en la descripción para la realización de estas composiciones que demuestren su actividad o efecto puesto que la descripción sólo se refiere a la identificación de los péptidos epítopes y a su posterior comprobación experimental para demostrar su capacidad de inducción en células T in vitro, pero no existe evidencia de estudios clínicos de dichas composiciones. Se recuerda a la solicitante que las reivindicaciones deben estar enteramente sustentadas en la descripción.

Por otra parte las reivindicaciones no son claras pues mencionan que la composición comprende un péptido seleccionado del grupo que consiste de i) y ii), y en cada literal se proveen varias secuencias, de modo que no se entiende si debe seleccionarse un péptido de i) y un péptido de ii) para que la composición tenga dos péptidos, o si debe seleccionarse un péptido de ambos grupos en conjunto, como lo da a entender el encabezado. Se sugiere hacer las correcciones y aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 4 grupos inventivos diferentes, correspondientes a péptidos de 4 genes diferentes.

Gen	Péptido SEQ ID. NO.
HIG2	110, 111, 112, 114, 116, 117, 121
INHBB	133, 135, 137, 426
TTK	227, 228, 233, 254
URLC	271, 272, 288

Por lo tanto, se entiende que los grupos inventivos están definidos por las siguientes características:

Grupo inventivo 1: Composiciones que comprenden entre 0.1 mg y 10 mg de un péptido seleccionado del grupo que consiste de SEQ ID NO: 110, 111, 112, 114, 116, 117 o 121 y un portador farmacéuticamente aceptable.

Grupo inventivo 2: Composiciones que comprenden entre 0.1 mg y 10 mg de un péptido seleccionado del grupo que consiste de SEQ ID NO: 133, 135, 137 o 426 y un portador farmacéuticamente aceptable.

Grupo inventivo 3: Composiciones que comprenden entre 0.1 mg y 10 mg de un péptido seleccionado del grupo que consiste de SEQ ID NO: 227, 228, 233 o 254 y un portador farmacéuticamente aceptable.

Grupo inventivo 4: Composiciones que comprenden entre 0.1 mg y 10 mg de un péptido seleccionado del grupo que consiste de SEQ ID NO: 271, 272 o 288 y un portador farmacéuticamente aceptable.

El solicitante puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 21 de febrero de 2007, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Stevanovic 2002	"Identification of tumour- associated T-cell epitopes for Vaccine development"	Julio/2002
D2	Parker et al. 1994	"HLA Peptide Binding Predictions"	1994

El documento D1¹ divulga la identificación de péptidos epítopes con características mejoradas para tratamientos anticancerígenos que pueden ser empleados en la elaboración de vacunas (Resumen).

¹ Stevanovic S. 2002. Identification of tumour-associated T-cell epitopes for vaccine development. Nat Rev Cancer 2(7):514-20.

El documento D2² enseña la herramienta BIMAS, la cual es un sitio web para la predicción de péptidos que se unen a moléculas del complejo mayor de histocompatibilidad (http://www.bimas.cit.nih.gov/cgi-bin/molbio/ken_parker_comboform).

8. Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en “Una composición farmacéutica que comprende entre 0.1mg y 10mg de un péptido seleccionado del grupo que consiste de i) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO:110, 111, 387, 112, 394, 395, 133, 135, 137 o 426; y ii) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO: 114, 116, 117, 121, 227, 228, 233, 254, 271, 272 o 288; y un portador”.

La reivindicación 2 consiste en “Una vacuna que comprende entre 0.1mg y 10mg de un péptido seleccionado del grupo que consiste de i) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO:110, 111, 387, 112, 394, 395, 133, 135, 137 o 426; y ii) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO: 114, 116, 117, 121, 227, 228, 233, 254, 271, 272 o 288; y un portador farmacéuticamente aceptable”.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Composición que comprende	Composición inmunogénica (Pág. 6; tabla 2) que comprende	
entre 0.1mg y 10mg de un péptido seleccionado del grupo que consiste de i) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO:110, 111, 387, 112, 394, 395, 133, 135, 137 o 426; y ii) un péptido sintetizado que consiste de las secuencias de aminoácidos SEQ ID NO: 114, 116, 117, 121, 227, 228, 233, 254, 271, 272 o 288;	Cantidad efectiva de un péptido epítipo TAA (Pág.6; tabla 2)	Péptidos predichos con BIMAS a partir de las secuencias de las proteínas anotadas en Genbank para los genes HIG2 (No. NM_013332), NHBB (No. NM_002193), TTK (No. NM_003318) y URLC (No. NM_017527)
y un portador farmacéuticamente aceptable	Con un adjuvante o portador (Pág. 6 tabla 2)	

El documento D1 se considera el estado de la técnica cercano al objeto reclamado en las reivindicaciones 1 y 2 porque divulga composiciones inmunogénicas basadas en epítopes peptídicos derivados de antígenos asociados a tumores (TAA), las cuales pueden comprender un solo péptido o varios péptidos de antígenos tumorales (Pág. 1) y portadores o adjuvantes apropiados (Pág. 6; tabla 2) y son potencialmente efectivas para el tratamiento de diferentes tipos de cánceres. Dichos péptidos pueden ser identificados con el uso de herramientas bioinformáticas de predicción de epítopes (como BIMAS) a partir de una secuencia de un antígeno tumoral, seguido de ensayos in vitro para la verificación de su actividad en la inducción de células T (Pág. 4, Figura 1).

La diferencia entre la invención, definida en la reivindicación 1 y las composiciones del documento D1 consiste en los péptidos específicos seleccionados para los genes HIG2,

2 Parker, K.C., Bednarek, M.A., Coligan, J.E., 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152, 163.
HLA Peptide Binding Predictions: Recurso en línea: http://www.bimas.cit.nih.gov/cgi-bin/molbio/ken_parker_comboform.

NHBB, TTK y URLC.

El efecto que logra la selección de péptidos de los genes HIG2, NHBB, TTK y URLC para la construcción de composiciones inmunogénicas es que dichas composiciones se hacen específicas para actuar en cánceres relacionados con los antígenos HIG2, NHBB, TTK y URLC.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar las composiciones conocidas en el documento D1 para conseguir composiciones específicas para los genes HIG2, NHBB, TTK y URLC”.

Sin embargo, ya era conocido que los genes HIG2, NHBB, TTK y URLC tenían una expresión desregulada en tumores y sus secuencias estaban plenamente identificadas, como se menciona en la memoria descriptiva (folios 8 – 9). En ese sentido, para una persona medianamente versada en la materia habría sido previsible llevar a cabo el proceso de identificación de péptidos de antígenos tumorales divulgado en el documento D1, a partir del uso de herramientas bioinformáticas como BIMAS y su subsecuente verificación y validación in vitro. De esta manera, por medio de la herramienta BIMAS (D2) se habría introducido la secuencia de aminoácidos de cada una de las proteínas de interés, es decir, HIG2, NHBB, TTK y URLC y los péptidos predichos con el programa se habrían llevado a verificación experimental, como lo sugiere el documento D1. La verificación experimental de los epítopes predichos permite reducir el número de péptidos candidatos potenciales (Schirle et al. 2001)³. Para ejemplificarlo, se muestra a continuación la tabla de resultados arrojados por BIMAS al realizar la búsqueda para la proteína HIG2 (los péptidos correspondientes a las secuencias reclamadas en la presente solicitud están sombreados).

Scoring Results			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	8	YLLGVLTLL	836.253
2	13	VLTLISIFV	650.311
3	15	TLLSIFVRV	488.951
4	4	VLNLYLLGV	271.948
5	9	LLGVLTLL	83.527
6	22	RVMSLEGL	31.957
7	6	NLYLLGVVL	28.027
8	16	LLSIFVRVM	6.053
9	11	GVVLTLLSI	2.100
10	44	SQLANTEPT	1.370
11	38	TSWTTRSQL	1.157
12	29	GLLESPSPG	1.130
13	30	LLLESPSPGT	0.811
14	47	ANTEPTKGL	0.572
15	5	LNLYLLGVV	0.350
16	54	GLPDHPSRS	0.306
17	1	MKHVLNLYL	0.277
18	33	SPSPGTSWT	0.268

3 Markus Schirle, Toni Weinschenk, Stefan Stevanovic. 2001. Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens Journal of Immunological Methods 257: 1–16.

19	23	VMESLEGLL	0.218
20	2	KHVLNLYLL	0.119
21	18	SIFVRVMES	0.087
22	12	VVLTLLSIF	0.039
23	19	IFVRVMESL	0.028
24	41	TTRSQLANT	0.018
25	7	LYLLGVVLT	0.015

En consecuencia, una persona mediamante versada en la materia llevaría a cabo el desarrollo de composiciones como las sugeridas en el documento D1 a partir de los péptidos predichos con el documento D2, valiéndose de las habilidades rutinarias en el campo técnico. Por lo cual, la solución propuesta por la solicitante se considera obvia y sin altura inventiva.

9. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Stevanovic 2002	1 – 2	Nivel inventivo
D2	Parker et al. 1994	1 – 2	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2014-10-21

Elaboró: Edna Carolina Bonilla Leon

Revisó: Consuelo Leguizamon Leguizamon

IDENTIFICATION OF TUMOUR-ASSOCIATED T-CELL EPITOPES FOR VACCINE DEVELOPMENT

Stefan Stevanovic

Ten years ago, the first melanoma patient was successfully treated by vaccination with a short peptide, which was, in fact, the first tumour-specific T-cell epitope ever defined — MAGE. Since then, a number of clinical vaccination studies have underlined the potential of tumour-specific T-cell epitopes. But, how can we identify more epitopes to improve their efficacy as an anticancer treatment?

HLAs

Human leukocyte antigens, which are molecules of the human major histocompatibility complex.

CD8

A surface molecule that is expressed exclusively by cytotoxic T cells.

CD4

A surface molecule that is expressed exclusively by T-helper cells.

MHC CLASS II MOLECULES

Peptide receptors, similar to class I molecules in structure and function, but exclusively expressed by a small set of immune cells. They mainly present peptides from extracellular proteins to T-helper cells.

University of Tübingen,
Institute for Cell Biology,
Department of Immunology,
Auf der Morgenstelle 15,
D-72076 Tübingen,
Germany.
e-mail: stefan.stevanovic@
uni-tuebingen.de
doi:10.1038/nrc841

The first indication that tumours were immunogenic came from animal models, in which tumours were rejected. There are, however, marked differences in the degree of immunogenicity between different tumours. Some are easily rejected, whereas others escape destruction by the immune system. So, can we use this knowledge to increase tumour immunogenicity as a therapeutic strategy? A large number of pre-clinical and clinical trials in humans have demonstrated the feasibility of antitumour vaccinations. Vaccination is usually well tolerated, which highlights the safety of this approach.

Tumour vaccines make use of tumour antigens — which are, in general, derived from proteins that are produced by the tumour — and aim to trigger a cellular immune response that is executed by T cells. Tumour antigens can be either tumour specific, so are expressed exclusively in tumour tissue, or tumour associated, so are highly overexpressed in tumours but can also be found in normal tissue; differentiation antigens are overexpressed in tumours and are found only in certain types of normal tissue (see below, and TABLE 1). Peptides from tumour antigens, like those from every other cellular protein, are released from their source proteins during antigen processing, mainly by the proteasome, and are presented by human leukocyte antigen (HLA) class I molecules on the cell surface. Such HLA–peptide complexes are under the surveillance of the cellular immune system. The cellular immune response consists of a cytotoxic

effector function, which is carried out by cytotoxic T cells that are positive for the CD8 surface marker and recognize peptides that are presented by HLA class I molecules, and a helper function, which is executed by CD4-positive T cells that are restricted by major histocompatibility complex (MHC) CLASS II MOLECULES (see BOX 1). The type of immune reaction that is initiated therefore depends on which MHC molecule presents a peptide from the antigen. During the 1990s, when tumour antigens and their peptide fragments — epitopes, which are presented by MHC molecules — were extensively characterized, much of the work focused on CYTOTOXIC T LYMPHOCYTES (CTLs) and the MHC CLASS I antigen-processing system. In fact, owing to experimental limitations, our knowledge of MHC class-II-restricted T-helper epitopes is still rather limited.

Many epitopes have been reported, some of which are derived from the classical tumour antigens that were identified a long time ago — such as p53, RAS and carcinoembryonic antigen — but others are from recently discovered tumour antigens. More than 70 tumour antigens that are recognized by (mainly CD8⁺) T cells were listed in a recent review¹. In principle, a short peptide that is derived from the sequence of a tumour antigen and presented by an HLA class I molecule represents a potential tumour vaccine. We do not know exactly how many times an individual overcomes tumours during their lifetime. The risk of immunosuppressed individuals developing certain virus-associated tumours — such as non-Hodgkin's lymphoma and Kaposi's sarcoma —

Summary

- T-cell epitopes from tumour antigens have been included in many vaccination studies, and their potential to induce antitumour immune responses has become manifest. Nevertheless, the clinical outcome of such studies has to be improved.
- The number of known epitopes is still limited; therefore, some tumours cannot be treated by immunotherapeutic approaches. For others, the efficacy is not yet optimal.
- T-cell epitopes from tumour antigens can be defined by two principal strategies: one starts from an existing T-cell response and identifies the target of the response, whereas the other uses the sequence of a tumour antigen and employs epitope prediction to identify the relevant epitopes.
- New tumour antigens can be discovered by analysing the specificity of existing T-cell responses, or by screening strategies such as the SEREX programme, comparative proteome analysis or gene-expression profiling.
- To improve the clinical outcome of antitumour vaccinations, tumour-escape mechanisms have to be avoided by the use of efficient, multitarget vaccines.
- With the growing number of T-cell epitopes, it will become feasible to design patient-specific, individual vaccines that address several different antigens from one tumour and several HLA specificities, including class-II-restricted epitopes.

is rather high, but, for most kinds of tumour, impaired functions of the immune system do not seem to be important for suppressing tumour formation. Nevertheless, we have to realize that the immune system has failed when tumours develop. This might be because the tumour originates from ‘self’ and is therefore not immunogenic. It could also be that the very low number of antigens that are present in the early stages of tumour development does not induce an immune response, as is the case with low doses of pathogens.

The wide variety of antitumour vaccines that have already been applied show that the perfect vaccine — with respect to the selection and number of antigens, and the application strategy — has still to be designed. One of the most favoured applications uses the most efficient professional antigen-presenting cells, DENDRITIC CELLS (DCs), pulsed with peptides from tumour antigens. The

number of cells and the number of different — in part, ‘engineered’ — peptides administered, and the application of adjuvants or enhancers differs among all studies. Although a complete tumour regression has been reported several times^{2,3}, reports of a poor clinical outcome unfortunately also exist^{4,5}, and the first critical opinions have already been voiced; these speak of disappointing trials⁶. There are several possible reasons for the failure of antitumour vaccination, one of which is loss of the antigen from the tumour, which leads to tumour-escape variants⁷. But the loss or downregulation of specific molecules in the antigen-processing pathway has also been reported^{8,9}. In addition, immune responses occasionally even fail if antigen, MHC and specific T cells are present, which indicates that tumours can also use other mechanisms to exert a certain form of immunosuppression.

The use of T-cell epitopes in immunotherapy of tumours still presents us with the problem that individual differences between patients — both in the type of HLA molecules and in the epitopes that are presented and recognized — have not yet been finely ascertained. Patient-specific expression of tumour-associated antigens, quantitative differences in HLA-restricted presentation of epitopes and T-cell pools that vary among individuals might all have a significant role. For example, in the SYFPEITHI database¹⁰ (see online links box), which lists cancer-related T-cell epitopes, 11 different HLA-A*0201-restricted CTL epitopes from ERBB2 (HER2/neu) are listed, and we do not know which is most efficient in the struggle against an individual tumour (see BOX 2). A significant limitation of many clinical trials is their use of one or a few epitopes that is presented by only one HLA restriction element, as this facilitates tumour escape because loss of only one antigen suffices. To promote the exciting option of defeating tumours by ‘self’, the basic strategies must now be corrected. The future of a cancer immunotherapy that exploits T-cell epitopes from tumour antigens lies in

CYTOTOXIC T LYMPHOCYTES (CTLs; killer cells, cytotoxic T cells). These control all major histocompatibility complex class-I-expressing body cells for the presence of abnormal (viral, tumour-associated) peptides.

MHC CLASS I MOLECULES Highly polymorphic glycoproteins that are expressed by every nucleated body cell of vertebrates, and that are encoded by the gene cluster ‘major histocompatibility complex’ (MHC). The human MHC molecules are termed HLA (human leukocyte antigen) molecules. MHC class I molecules mainly present peptides from intracellular proteins to cytotoxic T cells.

DENDRITIC CELLS (DCs). These present T-cell epitopes very efficiently and are able to activate cytotoxic T lymphocytes.

Table 1 | A selection of tumour antigens and their T-cell epitopes

Antigen class	Tumour antigen	CTL epitope (sequence)	HLA	Helper epitope known	References
Cancer-testis antigen	MAGE1	EADPTGHSY	A*0101	+	17
	MAGE3	FLWGPRLV	A*0201	+	51
	NY-ESO-1	SLLMWITQC	A*0201	+	52
Mutated protein	CDK4	ACDPHSGHFV	A*0201	–	53
	β-Catenin	SYLDSGIHF	A*2402	–	54
	Caspase-8	FPSDSWCYF	B*3501	–	55
Tumour virus	HPV16 E7	TLGIVCPI	A*0201	–	56
Differentiation antigen	Tyrosinase	SEIWRDIDF	B*4402	+	57
	GP100	ALLAVGATK	A*0301	+	58
	PSMA	ALFDIESKV	A*0201	–	59
Overexpressed protein	ERBB2	KIFGSLAFL	A*0201	–	60
	MUC1	LLLLTVLTV	A*0201	–	30
	CEA	HLFGYSWYK	A*0301	–	61

Three classes of tumour antigens — cancer-testis antigens, mutated antigens and tumour-virus antigens — are termed tumour specific, as they occur exclusively in tumour tissue. All other antigens are referred to as tumour associated. The largest group so far is represented by overexpressed antigens. T-helper-cell epitopes have been characterized for a small number of tumour antigens only. A comprehensive listing of tumour antigens can be found in REF. 1. CDK4, cyclin-dependent kinase 4; CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocyte; HLA, human leukocyte antigen; HPV, human papillomavirus; MAGE, melanoma antigen; MUC1, mucin 1; PSMA, prostate-specific membrane antigen.

Box 1 | Tumours and the immune system

The crucial problem of tumour immunology is that tumours develop from 'self', but the immune system has been trained to exclusively recognize 'non-self' structures. Many of the immune system's components therefore remain idle when faced with tumour cells. For the fight against tumours, the immune system must learn to recognize 'self' structures that are usually invisible, but — following mutation, differentiation or overexpression — can become targets for recognition (TABLE 1). The immune system is armed with:

Cytotoxic T lymphocytes (CTLs)

CTLs record every change within the protein reservoir of body cells, as it is presented to them by means of peptide fragments and the human leukocyte antigen (HLA) class I receptors — a highly polymorphic system of cell-surface proteins. If HLA molecules present an abnormal peptide, CTLs are able to recognize this and destroy the target cell, by driving it into apoptosis. CTLs, like all T cells, tolerate all peptides from proteins that belong to the usually expressed self-protein pool of the organism.

Antibodies

Several tumour-reactive antibodies have been described and some of them have been developed into new cancer therapies — for example, **Herceptin**. However, it is often difficult for antibodies to reach a tumour in the periphery *in vivo*. The B cells, which produce antibodies, cannot act on their own, but require the help of T-helper cells.

T-helper cells

One subgroup — the T-helper 1 (T_H1) cells — is necessary to induce and propagate an inflammatory immune response in which CTLs are the main players. T-helper 2 (T_H2) cells favour the humoral immune response, which is supported by B cells and antibodies. T-helper cells recognize their antigen only in the form of an HLA class-II-presented peptide.

Natural killer (NK) cells

NK cells recognize and destroy body cells that do not express HLA molecules. Nevertheless, tumour cells often escape NK-cell recognition after HLA loss.

individual, patient-specific analysis and therapy, and in using several tumour antigens and epitopes that are presented by more than two HLA molecules. However, the number of tumour-specific T-cell epitopes that are known today is still too low, with the possible exception of those for malignant MELANOMA, which remains the model system for tumour vaccines. There is an urgent need for more epitopes so that several tumour antigens can be addressed with one vaccine, and to expand the applicability to a larger number of different tumours. So, which strategies will allow us to define new tumour antigens and T-cell epitopes?

Tumour antigens

Tumour immunologists have defined five different classes of antigens that are associated with tumours (TABLE 1) and that can be targets of antitumour T-cell responses if short peptide fragments that are derived from their sequences are presented by HLA class I molecules. However, we still do not know if most of them are also capable of delivering antigenic peptides to MHC class II molecules. So, what are the characteristics of tumour antigens that make them useful in antitumour vaccines? Most important are tumour-specific antigens: cancer-testis antigens, protein variants that are created by somatic mutations within tumour cells, and proteins from tumour viruses. These are found only in tumour tissue¹, and therefore do not encompass the risk of

autoimmune reactions during immunotherapy. Antigens that are highly overexpressed in tumours are referred to as tumour associated and might also serve as targets of a cellular immune response. The occurrence of the antigen within the population is also a measure of how successful a vaccine might be. Ubiquitous or shared tumour antigens are expressed by most tumour-bearing individuals and include **telomerase**¹¹ and **survivin**¹², but antigens that are expressed by only a small percentage of tumours — such as the MAGE gene family^{13–15} — might also be used. So, in present clinical studies, tumour specificity and widespread expression have to be considered to further the development of tumour vaccines. An ideal treatment would include only tumour antigens that are expressed by the respective tumour.

Identifying T-cell epitopes from tumour antigens Two experimental strategies are currently being used. One begins with a documented T-cell reaction against tumour cells, and characterizes T-cell specificity and the nature of the antigen that is recognized. The other uses known tumour antigens as its starting point, and uses the 'reverse immunology' approach to predict *in silico* T-cell epitopes from the sequence of such antigens, according to allele-specific peptide motifs of HLA molecules. T cells are subsequently induced by stimulation with synthetic peptides, and peptide-specific T cells are then tested for the recognition and killing of tumour cells (FIG. 1).

From T-cell recognition to T-cell epitopes. The classical approach is to investigate the specificity of T cells that recognize tumour cells in order to define the epitope (FIG. 1a). The first tumour-specific T-cell epitope that was identified was from **MAGE1** (REF. 16), and was presented by HLA-A*0101. It was defined by narrowing down the complexity of antigens that produced the immune response in the target **melanoma** cell. Thierry Boon and colleagues expressed human cDNA libraries that were generated from melanoma cells, in COS cells that were transfected with the HLA-A*0101 gene, until the T-cell reaction focused on a single gene. Using truncation experiments, a small region of DNA was identified that harboured the relevant T-cell recognition site¹⁷. Since then, the methods for expression cloning have been substantially improved; a very efficient expression library has recently been described, which enables identification of T-cell specificity after enrichment steps¹⁸.

An alternative approach, which also starts from T-cell function, uses biochemical methods to purify MHC molecules that present the relevant peptide epitope. After acid elution from the MHC molecule, the highly complex mixture of MHC ligands is fractionated by chromatography (capillary high-performance liquid chromatography) and the fraction that is recognized by T cells is then analysed by mass spectrometry¹⁹. The low number of tumour-specific peptides among thousands of inconspicuous peptides, the often low affinity of T cells for tumour epitopes, and the very low number of peptides, impede this molecular characterization. Less than 10 picograms of peptide have to be analysed, which requires highly sensitive analytical techniques.

MELANOMA
Skin cancer, originating from transformed melanocytes.

MAGE
Melanoma antigen; a family of proteins that are expressed only in testis or tumour cells.

PROTEOMICS

Analysis of the entirety of proteins from a tissue sample, separated by two-dimensional gel electrophoresis. Each individual protein spot can be identified after tryptic digestion and mass spectrometrical analysis of the resulting peptides.

MICROARRAY ANALYSIS

'DNA chips' contain many thousands of oligonucleotides that represent fragments of genes, immobilized on a solid surface. They are probed with cDNA that is prepared from mRNA of tissue samples and so give quantitative information about gene expression.

Box 2 | Individual differences in humans

Humans are largely heterogeneous organisms when compared with inbred laboratory mouse strains. Human leukocyte antigen (HLA) polymorphisms contribute to this: one individual HLA pattern — that consists of two alleles each of HLA-A, -B, -C, -DR, -DQ and -DP — occurs only once among tens of thousands of humans. The major histocompatibility complex class I molecule HLA-A*0201 is the most commonly expressed allele; one-third of Caucasians are positive for this allele. Nevertheless, the density of HLA molecules varies greatly on the surface of normal cells, and especially on tumour cells.

But it is not only in the HLA system — and in minor histocompatibility antigens — that tumours vary; tumours also vary greatly with respect to the expression of tumour-associated antigens. Although each tumour results from a monoclonal situation, the thousands of chromosomal abnormalities that are acquired during tumour development guarantee that each tumour is both unique and heterogeneous. Individual mutations provide us with the opportunity of using these as the target of immunotherapies. For optimal treatment, individual differences between patients must therefore be ascertained with extreme precision. This involves consideration of the following features:

- Expression of two HLA-A and two HLA-B alleles: type and density
- Expression of known tumour antigens
- Proteins that are specifically overexpressed in the individual tumour
- HLA-presented peptides from known tumour antigens or overexpressed proteins.

This strategy is very tedious and risky, for all these reasons, and such cases remain rare exceptions in tumour-epitope characterization.

From antigens to T-cell epitopes. Immediately after publication of the first HLA-allele-specific peptide motifs²⁰, scientists used the motifs to predict T-cell epitopes from antigen sequences^{21,22}. This approach has been termed 'reverse immunology'²³ and usually starts from a known tumour antigen, but without pre-existing T cells (FIG. 1b). Epitope prediction has now been carried out for more than ten years, and most tumour-specific T-cell epitopes known today have been defined with the help of peptide motifs. Several programmes offer free epitope predictions for a wide range of MHC class-I- and class-II-restricted

epitopes from different organisms — the most commonly used are BIMAS²⁴ and SYFPEITHI. In addition, programmes have evolved during the past two years that offer the prediction of proteasomal cleavages^{25,26}: an overview on prediction programmes is given in REF. 27. The experimental verification of proteasomal cleavage sites²⁸ or the selected potential epitopes starts with peptide synthesis. Synthetic peptides might be used to immunize transgenic mice to produce a T-cell response²⁹, or to restimulate precursor T cells *in vitro*, which are derived from either healthy blood donors or tumour patients³⁰. The final step to establishing tumour-specific T cells that have a distinct specificity is accomplished when the raised T cells recognize tumour cells that express the respective antigen. A direct demonstration of natural processing of the predicted epitopes can be achieved by the 'predict, calibrate and detect' method³¹, which uses synthetic peptide candidates as calibrants for a capillary-chromatography-mass-spectrometric system, and by comparing the results with those obtained after acid elution of naturally processed peptides from HLA molecules of tumour cells.

New antigens as a source of new epitopes

With the development of screening methods to identify tumour-associated antigens, huge data sets had to be evaluated. The first screening method, SEREX, uses antibodies from sera of tumour patients to determine potential tumour antigens³²: all proteins recognized by such antibodies (but not by antibodies from healthy persons) have been included in the **SEREX database** (see online links box), which now contains many hundreds of antigens. Two other screening methods — PROTEOMICS^{33–35} and MICROARRAY ANALYSIS^{36,37} — investigate the differences between healthy tissue and tumour tissue at the protein or mRNA level, respectively. With this type of differential analysis, several dozen to several hundred tumour-associated antigens can be defined within one assay, depending on the experimental thresholds. After screening a series of samples, scientists need to interpret large amounts of data in the light of published work and database contents. Results from complementary methods have to be

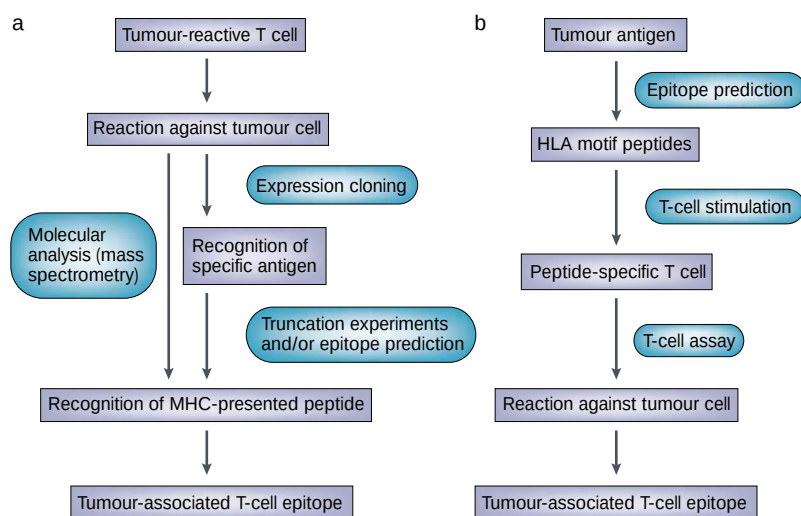


Figure 1 | Strategies for the characterization of T-cell epitopes. Depending on the starting point, which is either a T-cell reaction (a) or a known tumour antigen (b), two different strategies can be followed. **a** | The specificity of a T-cell receptor can be characterized using expression libraries, in order to identify the relevant antigen and its T-cell epitope. **b** | Potential T-cell epitopes can be predicted from the sequence of a tumour antigen. Their ability to induce peptide-specific T cells is assayed, and the tumour specificity of such T cells is verified by assaying tumour-cell recognition. HLA, human leukocyte antigen; MHC, major histocompatibility complex.

Table 2 | Outcome of vaccine trials performed with different modes of application of tumour-associated T-cell epitopes

Tumour	Source of epitope(s)	Carrier/adjuvant	Cytokine	Helper epitope	Admin.	Patients	PR/CR	References
Melanoma	Tyrosinase	Peptide alone	GM-CSF	–	6 x i.d.	18	0	62
Melanoma	RAS	Peptide alone	GM-CSF	–	6 x i.d.	10	0	63
Melanoma	GP100	Montanide ISA-51/QS-21	–	TT	2–7 x s.c.	22	n.a.	40
Melanoma	MART1 or GP100 mod.	Autologous DC	–	–	4 x i.v.	10	0	5
Melanoma	Several (pool)	Autologous DC	–	–	4–12 x i.v.	14	1	64
Glioma	Tumour extract	Autologous DC	–	–	3 x s.c.	9	0	65
Pancreatic adenocarcinoma	Mutated RAS	Peptide alone	GM-CSF	–	6 x i.d.	48	5	66
Melanoma	MAGE3 or MART1	Autologous PBMC	IL-12	–	s.c.	8	2	67
Medullary thyroid carcinoma	Calcitonin, CEA	Autologous DC	–	–	7–14 x i.d.	7	1	68
Metastatic-NY-ESO-1-expressing cancers	NY-ESO-1	Peptide alone + DMSO	–	–	4–12 x i.d.	12	0	69
Melanoma	GP100 mod.	Peptide alone	IL-2 or GM-CSF or IL-12	–	s.c.	43	6	70
Prostate cancer	PSMA	Autologous DC	–	–	6 x i.v.	37	11	71
Cervical carcinoma	HPV16 E7	Montanide ISA-51	–	+	s.c.	19	0	39
Gastrointestinal carcinoma	MAGE3	Autologous DC	–	–	4 x i.v.	12	0	72
Breast, ovarian cancer	ERBB2, MUC1	Autologous DC	–	PADRE	3–9 x s.c.	10	1	41

This survey lists a selection of clinical trials that are performed with synthetic peptides. Admin., number and route of applications (i.d., intradermal; i.v., intravenous; s.c., subcutaneous); CEA, carcinoembryonic antigen; DC, dendritic cell; DMSO, dimethyl sulphoxide; GM-CSF, granulocyte-macrophage colony-stimulating factor; HPV, human papillomavirus; IL, interleukin; MAGE, melanoma antigen; MART1, melanoma-associated antigen recognized by T-cells 1; mod., modified; MUC1, mucin 1; n. a., not applicable; PADRE, pan-DR epitope; PBMC, peripheral-blood mononuclear cell; PR/CR, partial response/complete response according to the World Health Organization definitions; PSMA, prostate-specific membrane antigen.

combined to obtain reliable information. For example, the combination of differential display at the protein level, and the recognition of antigens by patients' antibodies might reveal interesting candidate antigens³⁸, as well as the combination of expression profiling using DNA microarrays with the analysis of peptides presented by HLA molecules of the same tumour sample. After establishing a new tumour-associated antigen, the search for T-cell epitopes from this antigen might start anew by employing reverse immunology³⁶.

Help is required

Vaccinations that are aimed at recruiting cytotoxic T cells for tumour destruction or prevention have been successful but, unfortunately, the success has been limited to just a few cases. The response rates that have been documented in many clinical studies are encouraging, but must be increased. In addition to tumour-escape mechanisms, inefficient triggering of the T-cell system might be a reason for low response rates. In order to mimic the concerted action of the immune system against pathogens, epitopes that stimulate **CD4⁺** T-helper cells have recently been included in antitumour peptide vaccination studies^{39–41}. Ideally, the epitopes that are recognized by CTLs and T-helper cells should be derived from the same tumour antigen. However, although many tumour-specific CTL epitopes are known, only a very small number of HLA class-II-presented, tumour-specific

T-helper epitopes have been characterized. Increased efforts are therefore being made to identify tumour-associated epitopes that are presented by class II molecules^{42–45}, which would activate CD4⁺ helper cells to induce and maintain CD8⁺ killer cells⁴⁶. In principle, the strategies that are used for the characterization of CTL epitopes can also be applied to helper epitopes. This holds true for the classical approach in particular, which starts from T-cell recognition, although transfection of MHC class-II-expressing antigen-presenting cells with expression libraries represents a specific problem. An elegant strategy directs the antigen of interest to the lysosomal/endosomal compartment, where antigen processing leads to the generation of MHC class-II-presented epitopes^{47–50}. Performing reverse immunology for MHC class-II-restricted antigen presentation is far more difficult, for two reasons. First, the peptide motifs of MHC class II molecules are more degenerate compared with class I motifs and therefore do not allow for similarly precise predictions. Second, it is impossible, at present, to predict the proteolytic events that take place during antigen processing within the MHC class II pathway.

Diverse clinical applications: partial success

The first vaccination that was carried out with a tumour-specific T-cell epitope was in a melanoma patient. Since then, melanoma has represented the model system for cancer immunotherapy. So far, most T-cell epitopes from

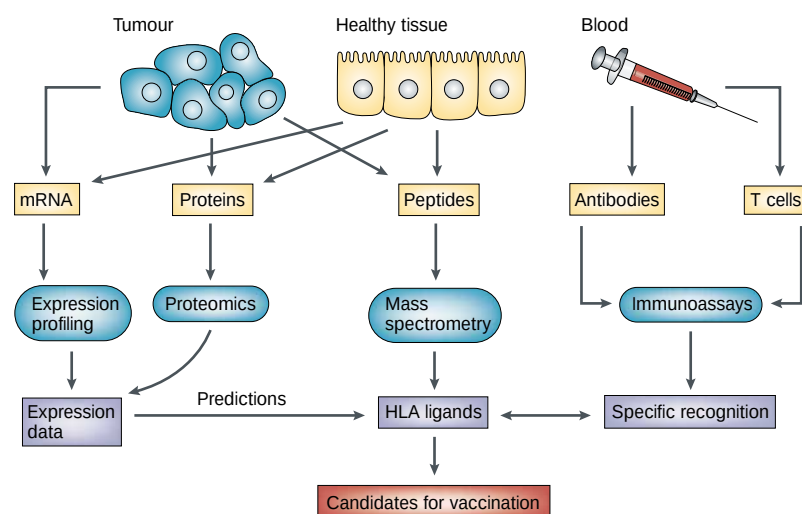


Figure 2 | Complementary information leads to the identification of useful tumour epitopes. As much information as possible has to be compiled about the presence of antigens or epitopes in a given tumour in order to identify new tumour antigens, and new tumour-associated T-cell epitopes that can be used in clinical studies. This strategy could lead to the characterization of an individual, patient-specific T-cell epitope pattern, which represents the prescription to treat this tumour. HLA, human leukocyte antigen.

MELANOSOMES
Subcellular organelles in melanocytes, which contain the skin's pigments.

PERIPHERAL-BLOOD MONONUCLEAR CELLS (PBMCs). Includes B cells, T cells and monocytes. These can be obtained from whole blood after ficoll density-gradient centrifugation.

tumour antigens have been derived from melanomal or MELANOSOMAL proteins. With the growing number of T-cell epitopes identified, the studies have now been expanded to other tumours. Clinical studies have been carried out in more than ten tumour entities, including cancers of the **breast, cervix, gastrointestinal duct, lung, ovary, pancreas, prostate, kidney and medullary thyroid**.

Although many cancer patients have been treated successfully, these represent just a small percentage of all individuals who underwent vaccine therapy (TABLE 2). Most patients that were included in clinical studies did not benefit from treatment with peptide epitopes that were identified from tumour antigens. We can speculate about the possible reasons that lead to such a disappointing outcome, but it is important to keep in mind that terminal patients — who have already undergone therapies in which their tumours proved to be resistant — are most frequently included in trials. It would be very interesting to compare these results with a first-line immunotherapeutic treatment of low-risk patients.

An additional factor is the individual differences between people (BOX 2), which have never been assessed in their entirety. We are also still unsure of the optimal application of tumour-associated T-cell epitopes: the many possible methods of vaccination have never been compared systematically in humans. Even if we leave out viral or other vectors, proteins and DNA, and only consider the

application of synthetic peptides, each protocol that has been applied — use of PERIPHERAL-BLOOD MONONUCLEAR CELLS (PBMCs) or DCs as carriers; peptide only, intradermal or subcutaneous administration; or addition of different adjuvants, cytokines or helper epitopes — has a limited, but comparable success (TABLE 2). It is therefore impossible to state that one thing works and another does not. Most application protocols have been shown to induce T-cell responses in patients, but tumour regression has been observed in only a few cases.

New approaches, future directions

There is much scope for the successful application of tumour-associated T-cell epitopes in cancer therapy, as the proof of principle was established long ago: immune responses are induced in most cases, and most tumour types are potential targets of immunotherapy. The problems, however, include the unsolved question of optimal clinical application, unknown escape mechanisms of tumours, and the low number of T-cell epitopes that have been identified for non-melanoma tumours. It has often been argued that HLA polymorphisms are a significant drawback in tumour immunology, but this is not as hopeless as it might seem at first glance: the most abundant HLA molecules have been investigated thoroughly, and their peptide motifs or even crystal structures have been determined. Our knowledge includes peptide motifs of HLA molecules that are expressed in more than 95% of Caucasians.

Activities must now focus on the identification of more tumour-associated antigens, and T-cell epitopes that are derived from their sequences. FIG. 2 shows how many different methods can be used to obtain complementary information about the characteristics of a given tumour. The combination of proteomics and antibody screening has been reported³⁸, and the combination of expression profiling, HLA-ligand characterization and T-cell assays from blood has been established in our own department (Weinschenk, T. *et al.*, manuscript in preparation). A modern anticancer vaccine should not target one or a few antigens only. It is also important to leave the model system of HLA-A*0201, and to characterize T-cell epitopes that can be presented by many different HLA class I or class II molecules. The most promising strategy seems to be an individual analysis of every tumour that is accessible, and individual treatment of every tumour patient (see also BOX 2 and FIG. 2). The modern peptide vaccine should consist of tumour-associated CTL epitopes and helper epitopes from various tumour antigens that are restricted to more than two HLA class I molecules and one HLA class II molecule. Hopefully, these developments will lead to real benefits for patients with cancer.

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Review

Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens

Markus Schirle, Toni Weinschenk, Stefan Stevanović *

Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany

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Abstract

The identification of T cell epitopes from immunologically relevant antigens remains a critical step in the development of vaccines and methods for monitoring of T cell responses. This review presents an overview of strategies that employ computer algorithms for the selection of candidate peptides from defined proteins and subsequent verification of their *in vivo* relevance by experimental approaches.

Several computer algorithms are currently being used for epitope prediction of various major histocompatibility complex (MHC) class I and II molecules, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides. Moreover, the analysis of proteasomal digests of peptides and whole proteins has led to the development of algorithms for the prediction of proteasomal cleavages. In order to verify the generation of the predicted peptides during antigen processing *in vivo* as well as their immunogenic potential, several experimental approaches have been pursued in the recent past. Mass spectrometry-based bioanalytical approaches have been used specifically to detect predicted peptides among isolated natural ligands. Other strategies employ various methods for the stimulation of primary T cell responses against the predicted peptides and subsequent testing of the recognition pattern towards target cells that express the antigen. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: T cell; Epitope prediction; HLA; Peptide; Computer algorithm

Abbreviations: ANN, artificial neural network; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HLA, human leukocyte antigen; MHC, major histocompatibility complex; MSMS, tandem mass spectrometry; RT-PCR, reverse transcriptase-polymerase chain reaction; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; TCR, T cell receptor; WWW, world wide web.

* Corresponding author. Tel.: +49-7071-2987645; fax: +49-7071-295653.

E-mail address: stefan.stevanovic@uni-tuebingen.de (S. Stevanović).

1. Introduction

Major histocompatibility complex (MHC) molecules are highly polymorphic cell surface molecules that present peptidic ligands to cells of the T cell compartment of the immune system. MHC class I ligands have a typical length of 8–12 amino acids and are derived from endogenously expressed proteins that are degraded by cytosolic proteases, most notably the proteasome. The proteolytic fragments are transported into the endoplasmic reticulum in an

ATP-dependent fashion by the transporter associated with antigen processing (TAP), where they bind to newly synthesized empty MHC class I molecules. The MHC-peptide complex is subsequently transported to the cell surface and can be recognized by the T cell receptor (TCR) of CD8⁺ cytotoxic T cells (CTL). MHC class II ligands have a more variable length of 9–25 amino acids and are derived mainly from exogenous or transmembrane proteins, but also from cytosolic proteins that are degraded by various proteases which originate from the lysosomal compartment. After binding to MHC class II molecules in the late endosomal/lysosomal compartment, the MHC-peptide complex is also transported to the cell surface, where it can be recognized by the TCR of CD4⁺ T cells.

Recognition of a peptide derived from a disease-associated protein, e.g., a viral or a tumor-specific protein, in the presence of a costimulatory signal leads to T cell activation and triggers a T cell-mediated immune response. Therefore, which peptide fragments of immunologically relevant antigens are available in the context of a certain MHC-molecule for recognition by T cells is crucial for the development of peptide-based or other defined antigen-based vaccines. Moreover, tools for monitoring specific T cell responses, such as MHC-peptide tetramers, intracellular cytokine staining or ELISPOT assays, depend on the identification of the relevant T cell epitope.

A major breakthrough in the identification of T cell epitopes was the discovery that ligands of a certain MHC-molecule carry chemically related amino acids in certain positions of their primary sequence, which permits the definition of a “peptide motif” for every MHC allele (Falk et al., 1991). This information was rapidly used to predict potential epitopes from protein sequences (Pamer et al., 1991; Rötzschke et al., 1991) and was the start signal for the so-called “reverse immunology” approach (Celis et al., 1994), which has since become the most successful strategy for the identification of T cell epitopes.

The first step of such studies is usually the computer-based prediction of potential MHC ligands from a protein of interest, which is followed by experiments to verify the natural processing of the predicted peptides and their recognition by T cells. The

classical and most straightforward approach relies on the stimulation of primary T cell responses against the predicted peptides *in vitro* or in (transgenic) animals and subsequent testing of the recognition pattern of the generated T cells towards target cells that endogenously express the antigen. Alternatively, patient-derived T cells, which might have been already primed *in vivo*, are used. This “pure” reverse immunology approach has been used extensively in the recent past for the identification of CTL and, to a lesser extent, helper T cell epitopes from a wide variety of immunologically relevant proteins. However, a major drawback of this labor- and resource-intensive approach is the high failure rate, even if the peptide in question binds well to the MHC molecule and the generated T cells recognize target cells loaded with the synthetic peptide. The reason for this is either that the peptide is not produced by the processing machinery of the cell, or the T cells raised by primary peptide stimulation *in vitro* are of low affinity, or a combination of both.

In consequence, various groups have made attempts to decrease the number of candidate peptides by applying additional criteria to epitope prediction. For example, the increasing knowledge about specificities of other cellular components of the antigen processing machinery, most notably the proteasome and TAP, has made it possible to apply these specificities as additional filters to the selection of candidate peptides. Moreover, experimental selection criteria, such as MHC-peptide binding assays and the bioanalytical verification of presentation of predicted MHC-ligands, have been applied successfully.

This review tries to give a comprehensive overview of available computer-based prediction algorithms and strategies that combine the predictive power of these theoretical approaches with experimental approaches for a reliable and rapid identification of T cell epitopes.

Since it has been written from an immunologist’s point of view, this review focuses mostly on prediction programs that have already been applied to the identification of novel epitopes. Furthermore, it should be noted that there are tremendous differences between different prediction softwares in terms of availability. Some programs are easily accessible for free on the world wide web (WWW); others are available from the authors of the corresponding pub-

lications; still others are available on a commercial basis only.

2. Computational predictions

2.1. Epitope prediction

The recognition of MHC-peptide complexes by the T cell receptor (TCR) is the first step in a series of cellular processes that lead to the initiation of T cell-mediated immunity. It is, therefore, not surprising that as soon as the peptidic nature of MHC ligands was discovered and before the rules for the binding of peptides to MHC molecules were elucidated, first attempts for the prediction of T cell antigenic structures from protein sequences were published. These predictions were based solely on comparisons of precursor peptide sequences known to contain T cell epitopes. The suggested characteristics of T cell epitopes were predicted secondary structures (amphipathic helices) (DeLisi and Berzofsky, 1985) or short primary sequence motifs (Rothbard and Taylor, 1988; Sette et al., 1989). It was the discovery of allele-specific motifs shared by eluted natural MHC ligands (Falk et al., 1991) that finally allowed the exact prediction of peptides from a given protein sequence presented on MHC class I molecules (Pamer et al., 1991; Rötzschke et al., 1991).

For MHC class II ligands, the elucidation of peptide motifs turned out to be more difficult due to the variable length of the ligands as well as the more degenerate anchor positions, which are sometimes not used at all. Several methods for the determination of MHC class II peptide motifs have been based on sequence information of natural ligands, including the alignment of natural ligands, pool sequence analysis, or substitution variants of known ligands (Rudensky et al., 1992; Chiczy et al., 1992; Hunt et al., 1992a,b; Falk et al., 1994; Leighton et al., 1991). In 1992, the first HLA-DR-motif based on binding assays using a phage peptide display library was published (Hammer et al., 1994a,b).

In the beginning of the 1990s, the selection of candidate peptides was done manually or using simple pattern search programs by scanning protein sequences for main anchor positions only. However,

the constant refinement of peptide motifs and knowledge about secondary anchor amino acids, disfavored amino acids and other chemical interactions important for binding to the MHC molecule quickly led to the development of more complex computational predictions.

Depending on the focus of research, the determination of peptide motifs has been approached from two sides.

On the one hand, the binding properties of particular MHC molecules have been analyzed by determining the binding of synthetic peptides from protein antigens using MHC-peptide binding assays (Ruppert et al., 1993; Kubo et al., 1994; Parker et al., 1994; Hammer et al., 1994a,b). The most refined descriptions of such “peptide-binding motifs” have been achieved by using positional scanning with random synthetic peptide libraries (Stryhn et al., 1996; Fleckenstein et al., 1996; Udaka et al., 2000).

On the other hand, the analysis of natural ligands by individual ligand analysis as characterized by Edman degradation (Falk et al., 1991; DiBrino et al., 1993; Corr et al., 1993; Falk et al., 1994) and tandem mass spectrometry (MSMS) (Hunt et al., 1992a,b) as well as Edman pool sequencing (Falk et al., 1991) leads to so-called “natural ligand motifs” that give information about the natural peptide repertoire presented by a certain MHC molecule. These motifs are, therefore, not only a result of the binding properties of this MHC molecule, but reflect also—to a certain extent—features of the antigen processing machinery, e.g., cleavage specificities of the proteasome or sequence requirements for transport into the ER by TAP. Indeed, MHC-binding motifs derived from random peptide libraries show significant differences, especially for the C-terminal amino acids, compared to motifs derived from natural ligands (Davenport et al., 1997). This raises the question of the *in vivo* relevance of motifs based on the binding properties of synthetic peptides.

2.1.1. MHC class I

Historically, MHC class I epitope prediction had a headstart over its MHC class II counterpart. The reasons for this include the defined length of MHC class I ligands of mostly nine amino acids and the more clearcut rules of MHC class I motifs that have been more clearly characterized. Currently, there are

two predictive algorithms available on the world wide web that allow unrestricted predictions free of charge: “BIMAS”, developed by Parker et al. (1994) (<http://www-bimas.dcrn.nih.gov/molbio/hla-bind/>) and “SYFPEITHI”, developed by our group (Rammensee et al., 1999) (<http://www.syfpeithi.de>). SYFPEITHI uses motif matrices deduced from refined motifs based on the pool sequence and single peptide analysis exclusively of natural ligands. Potential binders for various human, mouse and rat MHC class I molecules are ranked according to the presence of primary and secondary anchor amino acids as well as favored and disfavored amino acids.

BIMAS ranks potential human leukocyte antigen (HLA) binders according to predicted half-time dissociation of MHC-peptide complexes. While the motif matrix for HLA-A2 is based solely on binding studies, other matrices have been deduced from reported motifs, regardless of the method used for their identification. This might explain the similar results that were obtained with both algorithms in a recent study that identified HLA-B7-presented epitopes from the tumor-associated antigen (TAA) carcinoembryonic antigen (CEA) (Lu and Celis, 2000) since for HLA-B7 the reported motifs are based on natural ligands. Both algorithms have been used successfully during recent years for the identification of CTL epitopes from a wide range of antigens, e.g., from the human TAAs telomerase (BIMAS) (Vonderheide et al., 1999) and TRP2 (SYFPEITHI) (Sun et al., 2000).

Other prediction programs that have been used for the identification of MHC class I-presented epitopes are distributed on disk (e.g., “MOTIFS” (D’Amaro et al., 1995)), part of commercial websites [e.g., “EPIMATRIX” (De Groot et al., 1997) (http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html)] or are currently not publicly available (Sette et al., 1994).

The motif-based algorithms so far described are still relatively simple programs that consider every amino acid position in a peptide individually. For every position, a coefficient is assigned to each amino acid and the overall score or binding value of the peptide are determined by addition or multiplication of amino acid coefficients. Moreover, there are several reports employing artificial neural networks (ANN) (Brusic et al., 1994; Adams and Koziol, 1995; Gulukota et al., 1997; Milik et al., 1998) or the polynomial method (Gulukota et al., 1997). These approaches open up the possibility to consider amino acid preferences that depend on the properties of amino acids in other positions of the peptide.

Still another approach is based on structural information obtained by X-ray crystallography of peptide–MHC complexes (Altuvia et al., 1997; Schueler-Furman et al., 2000). While none of these latter, non motif-based approaches has actually been used to date for the identification of novel CTL epitopes, they could prove useful in the future.

Table 1 demonstrates the similarities and differences of some of the currently used MHC class I

Table 1

Comparison of MHC class I predictions

Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge on interactive websites: the epitope prediction programs BIMAS and SYFPEITHI and a program for prediction of proteasomal cleavage sites PAPROC.

Sequence	Source (position)	HLA molecule	Program used for identification	Reference	SYFPEITHI rank/score	BIMAS rank/value	PAPROC *
ILAKFLHWL	human TERT (540–548)	A* 0201	BIMAS	(Vonderheide et al., 1999)	1/30	1/1745.714	No
KVLEYVIKV	human MAGE-A1 (278–286)	A* 0201	SYFPEITHI	(Pascolo et al., 2001)	3/26	1/743.189	Yes
ILHNGAYSL	human Her-2/neu (435–443)	A* 0201	Sette et al.	(Rongcun et al., 1999)	3/27	16/36.316	Yes
HLSTAFARV	human G250 (254–262)	A* 0201	MOTIFS	(Vissers et al., 1999)	19/22	38/4.493	No

* Prediction of proteasomal generation of C-terminus by human proteasome wild-type III.

prediction programs. It shows selected CTL epitopes that were identified using four different computer programs and also lists the corresponding results from SYFPEITHI, BIMAS and a prediction algorithm for proteasomal cleavage, “PAPROC” (see below), i.e., programs that are available at interactive websites at no cost. However, it should be noted that some studies do not give the results of the original prediction procedure, such as ranks or values, but only lists of peptides that were selected after performing additional tests.

2.1.2. MHC class II

The main obstacle for the prediction of MHC class II ligands has been the differing degree of degeneration of motifs. While some alleles, e.g., HLA-DRB1*0405, show a strong preference for certain related amino acids in the anchor positions that is comparable to MHC class I motifs (in this case D, E in P9) (Rammensee et al., 1999), other alleles make the definition of primary anchor amino acids virtually impossible (e.g., for HLA-DRB1*0401 all amino acids except G, P, F, W have been assigned anchor amino acids in P9) (Rammensee et al., 1999). To date, the focus has been on predictions of HLA-DR ligands, with only one report on predictions of HLA-DQ-restricted epitopes (Godkin et al., 1998). The first matrix-based prediction algorithms for HLA-DRB1*0401 were based on the results of side-chain scanning experiments using simplified, polyalanine-based peptide libraries (Hammer et al., 1994a,b; Marshall et al., 1995). The work by Hammer and colleagues resulted in the commercial program “TEPITOPE”, which focuses on promiscuous HLA-DR binding peptides, but also allows allele-specific predictions. It has been used in several studies, e.g., in the identification of an HLA-DR11-presented epitope from MAGE-A3 (Manici et al., 1999).

Another matrix-based HLA-DRB1*0401-prediction algorithm, also based on binding studies using synthetic peptides (Southwood et al., 1998) has been used for the retrospective calculation of an epitope from human gp100 (Touloukian et al., 2000) that had been identified previously (Halder et al., 1997). This program is available free on request from the Surgery Branch of the NCI.

Still another customized computer algorithm also based on published HLA-DR binding motifs has been used for the identification of HLA-DR-presented promiscuous T cell epitopes from *Plasmodium falciparum* (Doolan et al., 2000). The first program for prediction of MHC class II based on natural ligands of two HLA-DR13 alleles was the PAP program (Davenport et al., 1995). This program, which can be obtained free from the author, uses corrected amino acid yields from Edman pool sequencing directly as coefficients in motif matrices. One of the following publications also reports for the first time predictions for HLA-DQ2 and -DQ8 and the identification of a novel HLA-DQ2-presented epitope from gliadin, the antigen of celiac disease (Godkin et al., 1998).

Currently, SYFPEITHI is the only interactive website offering unrestricted MHC class II predictions free of charge. As for MHC class I, the available predictions for HLA-DR and several mouse MHC class II alleles are based on motif matrices deduced from natural ligands by single ligand analysis and Edman pool sequencing.

For HLA-DRB1*0401, predictions by artificial neural networks have already been applied successfully to the identification of helper T cell epitopes. Zarour and colleagues have used an ANN, based on the work of Honeyman et al. (1998) and Brusic et al. (1998), for the identification of epitopes from the TAAs Melan-A/MART-1 (Zarour et al., 2000a,b) and NY-ESO-1 (Zarour et al., 2000a,b). These prediction programs are currently not publicly available.

Finally, several publications have explored the possibilities of other more complex computational predictions. For example, Mallios (1999) has described an iterative algorithm to align and optimize an MHC class II-binding matrix based on sequences of known class II ligands alone or in combination with suggested motifs. Up to now, none of these predictions have been applied to the identification of T cell epitopes.

Table 2 shows examples of helper T cell epitopes that were identified by four different computer programs and also lists the corresponding results from SYFPEITHI and the HLA-DRB1*0401 prediction program from the NCI. However, in contrast to MHC class I, comparisons of MHC class II predictions can be even more difficult for several reasons.

Table 2

Comparison of MHC class II predictions

Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge either on an interactive website (SYFPEITHI) or by email (NCI).

Epitope sequence	Source (position)	HLA molecule	Reference	Program used for identification	SYFPEITHI rank/source	NCI rank/value
WNRQLYPEWTEAQRLD	human gp 100 (44–59)	DRB1* 0401	(Touloukian et al., 2000)	NCI	2/26	1/0.0195
RNGYRALMDKSLHVGTCALTRR	human MART-1 (51–73)	DRB1* 0401	(Zarour et al., 2000)	ANN	1/22	– / –
TSYVVKVLHHMVKISG -----	human MAGE-A3 (281–295)	DRB1* 1101	(Manici et al., 1999)	TEPITOPE	8/22 * *	n.a.

Putative core sequences are underlined; * * best ranked peptides 284–298 (score 28) and 280–294 (score 24) also contain other putative core sequences (– – –); n.a., not available.

First, due to the variable length of MHC class II ligands, the identified epitopes may harbor more than one putative core sequence. Furthermore, some studies focus on promiscuous MHC class II-binding peptides, while others use allele-specific predictions.

2.2. Predictions of antigen processing

Much of the work combining computational predictions and applied immunology has focussed on MHC-peptide binding only. However, our increasing knowledge about the specificities of other cellular components involved in antigen processing, such as the proteasome and TAP, has been used to create comparable prediction algorithms with the future prospect of fusing them to epitope prediction programs.

The proteasome is a cytosolic multi-subunit protease that is, among other functions, involved in the generation of peptide ligands for MHC class I molecules and responsible for the generation of the correct C-terminus of CTL epitopes (Craiu et al., 1997; Stoltze et al., 1998). The proteolytically active core complex (20S proteasome) can associate with the 19S cap complex to form the 26S proteasome, which is responsible for the degradation of ubiquitin-marked proteins. The three different active sites show different proteolytic specificities, cleaving after large, hydrophobic amino acids, basic amino acids and acidic amino acids, respectively. Upon exposure of cells to interferon γ (IFN γ), the catalytically active subunits are replaced by three IFN γ -inducible subunits. This results in a shift in the overall cleavage specificity of this immunoproteasome towards hydrophobic amino acids, while cleavages after acidic amino acids are disfavored. This corresponds to the fact that almost all reported peptide motifs of vertebrate MHC molecules demand hydrophobic or basic C-terminal anchor amino acids.

An experimental approach was chosen by Kessler et al. (2001) for the identification of HLA-A*0201-presented CTL epitopes from the TAA PRAME by an extended reverse immunology strategy that incorporates proteasomal digest analysis. Predicted HLA-A*0201-binding peptides were tested in binding and stability assays, followed by an analysis of in vitro proteasome-mediated digestions of 27mer precursor

peptides encompassing candidate epitopes. For only 4 out of 19 high affinity binders, efficient generation of the correct C-terminus was observed and all four peptides were found to be efficient CTL epitopes.

The first published prediction algorithm for cleavages by 20S proteasomes, “FRAGPREDICT”, is based on published peptide cleavage data (Holzhütter et al., 1999; <http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html>). The PAPROC-algorithm (Nussbaum et al., 2001) <http://www.paproc.de>), developed by our group, is based on an evolutionary algorithm trained on cleavage data of 20S proteasomal digests of a whole protein substrate [enolase (Nussbaum et al., 1998)], and additional peptide digest data from the literature. While the currently available prediction programs are based only on semiquantitative data from human or yeast constitutive 20S proteasomal digests, more accurate predictions based on fully quantified protein digestion data as well as immunoproteasomal digestion data (Toes et al., 2001) are currently being developed. Another program, “NETCHOP” (Kesmir et al., manuscript submitted; <http://www.cbs.dtu.dk/services/NetChop/>) is an ANN that was trained on data from the analysis of residues at the termini and flanking regions of known natural MHC class I ligands. All these programs for the prediction of proteasomal cleavages are available free of charge on interactive websites.

The overall goal of combining proteasomal prediction algorithms with epitope prediction is already possible using combinations of FRAGPREDICT and PAPROC with SYFPEITHI and BIMAS at the website of the Max-Planck-Institute for Infection Biology (www.mpiib-berlin.mpg.de/MAPPP/). It should be noted that this program also predicts C-terminally extended precursor peptides.

An ANN-based device has also been reported for the prediction of human TAP peptide-binding affinities using a polyalanine-based peptide library (Daniel et al., 1998). The program predicted a higher affinity to TAP for eluted natural ligands compared to randomly selected peptides with similar binding affinities to the same HLA molecule, indicating that such predictions might be—at least for some HLA-alleles—a valuable addition for epitope prediction tools. However, this would probably require a more profound investigation of peptide translocation by TAP,

not only binding of peptides. The reported preferences of TAP for peptide translocations (Momburg et al., 1994) have not yet led to a prediction program.

3. Experimental verification of predicted epitopes

3.1. Analysis of binding properties

In most cases, epitope prediction is followed by binding studies with synthetic peptides in order to reduce the number of potential candidate peptides by discarding nonbinding peptides for the final analysis of recognition by peptide-specific T cells.

Binding of synthetic peptides to MHC molecules is mainly studied by assays based on competition (Sette et al., 1994; Olsen et al., 1994; Vogt et al., 1994), reconstitution of MHC-peptide complexes on the cell surface (Stuber et al., 1994; Zeh et al., 1994; Storkus et al., 1993), or stabilization of MHC-peptide complexes on TAP-deficient cells (Stuber et al., 1992).

In competition assays, the studied peptides compete with a labeled reference peptide of intermediate affinity for binding to MHC molecules. Such assays are performed either using whole cells or purified MHC molecules in solution. In both cases, the amount of labeled MHC-bound peptide is measured. For MHC class II-binding assays usually fluorescence dyes, like fluorescein or 7-amino-4-methylcoumarin-3-acetic acid (AMCA), are used for labeling the N-terminal amino acid of the MHC ligand. For MHC class I-binding studies, amino acid side chains have to be labeled by fluorescent dyes or by radioactive isotopes, such as ^{125}I without interfering with the more restricted binding properties of MHC class I molecules. Performing in-solution assays using purified MHC molecules, MHC-peptide complexes have to be separated from unbound free peptide by gel filtration (Sette et al., 1994), spun columns (Stryhn et al., 1997) or high-performance size exclusion chromatography (HPSEC) (Vogt et al., 1994). In addition, enzyme linked immunosorbent assay (ELISA)-based assays (Jensen, 1991; Hammer et al., 1995) or the more sensitive time-resolved fluorimetry in a europium fluoroimmunoassay (Tompkins et

al., 1993) have been used as the readout. Competition-based binding assays result in IC_{50} values, which do not represent an absolute dissociation constant since they are dependent on the reference peptide. However, they allow a better comparison of binding affinities of different peptides than the results obtained by reconstitution or stabilization assays. Competition assays using purified MHC molecules employ soluble MHC molecules, which might have a slightly different structure compared to native molecules embedded in the cell membrane. Whole cell assays overcome these problems by using MHC molecules on the surface of intact cells. As an additional feature, mild acid treatment of the cells can be used to remove peptides from the MHC-binding groove. Cells are then incubated with a fluorescence-labeled reference peptide together with different concentrations of the peptides of interest (van der Burg et al., 1995). The amount of bound reference peptide can be measured by FACS analysis. As peptide-depleted MHC molecules have a short half-life and β_2 -microglobulin dissociates rapidly, only membrane-anchored MHC- α -chains are left and, thus, this competition assay might also represent a reconstitution assay.

In classical reconstitution assays, cells expressing the appropriate HLA alleles are stripped by incubation at pH 3.2 for a short time, then the peptide of interest and a conformation-dependent monoclonal antibody are added. The difference in fluorescence intensity determined by FACS analysis between cells incubated with and without peptide after staining with fluorescence-labeled secondary antibody is used to determine peptide binding (Storkus et al., 1993).

The stabilization assay was first described by Stuber et al. (1992). Cell lines with defective peptide loading mechanisms, like the TAP-deficient murine cell line RMA-S (Ljunggren and Karre, 1985) or the human cell lines T2 (Cerundolo et al., 1990) or ST-EMO (de la Salle et al., 1994), display a low level of mostly empty MHC class I molecules on their surface due to the rapid turnover of empty MHC molecules. The addition of exogenous binding peptide to the cells, however, leads to stabilization and accumulation of MHC molecules, which can be determined using monoclonal antibodies and FACS analysis. Due to its simplicity, this assay can serve for screening large numbers of peptides. Moreover,

transfection of any human cell with cDNA of the immediate early protein ICP47 of the Herpes simplex virus generates a TAP-deficient phenotype and, thus, permits the extension of the stabilization assay to virtually any HLA allele (Gatfield et al., 1998).

3.2. Analysis of presentation

Since the first identification of a natural MHC class I ligand by tandem mass spectrometry (MSMS), this technique has become the bioanalytical method of choice for high sensitivity sequencing of natural MHC ligands. This has led to the development of approaches that combine epitope prediction and HPLC-MS and MSMS for specific detection and identification of disease-associated MHC class I ligands. Brockman et al. (1999) used a simple main anchor-based epitope prediction to identify masses of candidate H2-K^b-ligands from *Trypanosoma cruzi*. The corresponding mass chromatograms showed in two cases significant peaks and subsequent HPLC-MSMS analysis revealed indeed two naturally processed peptides. A variant of this approach is the “Predict, Calibrate, Detect” (PCD) method (Schirle et al., 2000), which permits a more rapid and sensitive screening of tumor samples by calibrating the HPLC-MS system with synthetic analogs of predicted potential HLA-A2 ligands from tumor-associated proteins. HLA-A2 ligands from TAAs p53, CEA and MAGE-A1 have been identified using PCD (Schirle et al., 2000; Pascolo et al., 2001); the MAGE-A1-derived peptide has been verified as a CTL epitope using an HLA-A2 transgenic mouse model (Pascolo et al., 2001).

These approaches are not a substitute for testing peptide-specific recognition of tumor cells by CTL; however, they allow further focussing of these efforts to those peptides that are not only binding to the MHC molecule, but are actually processed and presented.

3.3. Analysis of T cell recognition

The final experimental verification of a predicted epitope is achieved by the identification of T cells, which specifically recognize the naturally processed epitope in an HLA-restricted fashion. One major

source for T cells are peripheral blood mononuclear cells (PBMC) from patients or healthy donors, which are either already primed in vivo or which can be primed in vitro, respectively. In addition, immunization of various HLA class I and class II transgenic mice (reviewed in Faulkner et al., 1998 and Sonderstrup et al., 1999) can be used to generate specific T cells. T cell responses after stimulation can be measured by means of lytic activity, proliferation and cytokine production. Lytic activity of cytotoxic T lymphocytes has been analyzed since 1968 using the ⁵¹Cr-release assay (Brunner et al., 1968; Cerottini and Brunner, 1974); however, this assay requires the cumbersome in vitro expansion of specific T cells from PBMCs. Nevertheless, it is still widely used. As an alternative, T cell proliferation has been investigated by radioactive ³H-thymidine incorporation. To determine peptide-specific T cell responses, a sensitive proliferation assay was established in 1996. This assay avoids radioactivity and uses BrdU incorporation instead (Mayer et al., 1996). In our experience, this proliferation assay has a much higher sensitivity than the ⁵¹Cr-release assay. The first quantitative assay for IL-2 production by activated T cells described in 1978 is still used today (Gillis et al., 1978). This sensitive method uses an IL-2-dependent mouse T cell line, CTLL, as the target cell population, which proliferates and incorporates ³H-thymidine in response to IL-2 in culture medium. After IL-2- and IFN γ -specific antibodies became available, enzyme-linked immunosorbent assays (ELISA) were established to measure T cell responses (Gehman and Robb, 1984; Van der Meide et al., 1985; Troye-Blomberg et al., 1985). The advantages of ELISA procedures are their simple setup and the capacity to handle many samples in parallel.

None of the methods discussed so far is able to detect cytokine production by T cells at a single cell level. The first application to address this question was the ELISPOT assay reported in 1988 for enumerating IFN γ -secreting lymphocytes (Czerkinsky et al., 1988; Versteegen et al., 1988), originally developed for the detection of single specific antibody-secreting cells (Czerkinsky et al., 1983). In this assay, activated T cells are plated in anti-IFN γ antibody-coated wells; single IFN γ -secreting cells are detected as spots after staining trapped IFN γ with an insoluble dye generated by an enzyme coupled to the

second anti-IFN γ antibody. Peptide-specific T cells can be assessed in this way with higher sensitivity compared to the ^{51}Cr -release assay (Miyahira et al., 1995), even without the need for prior *in vitro* expansion, which may bias the result. ELISPOT has been used to measure TNF α secretion (Herr et al., 1996) or IFN γ secretion (Scheibenbogen et al., 1997; Schmittl et al., 1997) as markers for T cell activation. The tedious visual counting of spots was no longer necessary after the introduction of a computer-assisted image analysis system, which has made data analysis convenient, objective and suitable for handling large sample pools (Cui and Chang, 1997).

Another way to assess cytokine production at the single cell level is achieved by intracellular cytokine staining and flow cytometry analysis. After stimulation in the presence of monensin, T cells are fixed by paraformaldehyde and permeabilized by saponin to allow antibodies to penetrate through the cell membrane, the cytosol and the membranes of the endoplasmic reticulum and Golgi organelle. Monensin interrupts intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex and, thus, an increase in the signal-to-noise ratio leads to a higher sensitivity (Sander et al., 1989, 1991; Jung et al., 1993; Mascher et al., 1999; Pala et al., 2000). Intracellular cytokine staining allows high throughput of samples and multiparameter characterization of cytokine production as well as parallel detection of cell surface markers on a single cell basis without the need for prolonged *in vitro* culture and cloning. However, the sensitivity is limited by the fluorescence-activated cell-sorting analysis.

Similar to the intracellular cytokine staining approach is the cell surface affinity matrix technology (Manz et al., 1995). Instead of detecting cytokines in the cytoplasm, secreted cytokines are measured on live single cells. After antigenic restimulation, secreted IFN γ or other cytokines are captured by a cell surface affinity matrix (Brosterhus et al., 1999). This matrix comprises bispecific antibody–antibody conjugates directed against CD45 for cell membrane anchoring and against the cytokine of interest. Detection of secreted, matrix bound cytokine is achieved by fluorescence-labeled antibody and flow cytometry. In contrast to intracellular cytokine staining, this technology allows enrichment and isolation of living cells, which can be further cultured and analyzed.

Visualization of antigen-specific T cells is possible by staining T cell receptors with MHC/peptide tetrameric complexes (Altman et al., 1996; Galimore et al., 1998; Murali-Krishna et al., 1998; Letvin et al., 1999). These tetramers are made by *in vitro* folding MHC heavy chain in the presence of β_2 -microglobulin and a specific peptide ligand. The heavy chain is engineered in a way that the purified MHC-peptide complex can be biotinylated and then tetramerized via fluorochrome dye-coupled avidin. Tetramerization enhances the affinity of the $\alpha\beta$ TCRs to soluble peptide-loaded MHC molecules. By this method, a quantitation of T cells displaying TCRs specific for the analyzed peptide is possible; however, nothing can be said about the functionality of these T cells. The tetramer approach is well established for MHC class I molecules; however, detection of antigen-specific CD4 $^{+}$ T cells with soluble MHC class II tetramers is now in progress (Crawford et al., 1998; Novak et al., 1999; Kotzin et al., 2000; Kuroda et al., 2000; Reichstetter et al., 2000; Kwok et al., 2000; Meyer et al., 2000). Application of MHC class I tetramers in the field of virus immunology has been reviewed by Doherty and Christensen (2000).

Probably, the most sensitive method for detecting T cell responses is the quantitation of induced cytokine mRNA after activation by quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR). This quantitative method is based on the 5' nuclease assay in which the 5' $\rightarrow$ 3' endonuclease activity of the Taq DNA polymerase cleaves a hybridized sequence-specific fluorogenic internal oligonucleotide probe during PCR. Relative increases in fluorescence emission from the reporter dye are detected online in an analytical thermal cycler (Heid et al., 1996). This assay has been used to quantify cytokine mRNA expression (IL-2, IL-4, IL-10, TNF α , IFN γ) in previously frozen whole blood samples (Kruse et al., 1997). More recently, this very sensitive and highly reproducible method was adapted by normalizing copy numbers of cytokine mRNAs to CD8 mRNA copy numbers in order to analyze specific T cell responses in fresh PBMCs without any further stimulation (Kammula et al., 1999, 2000). This way, it was possible to monitor the specific T cell response after peptide vaccination in melanoma patients. Though this is no single cell assay, it is very

sensitive, highly reproducible and permits a high sample throughput.

4. Outlook

While computational predictions have already become a familiar tool for many immunologists searching for T cell epitopes from immunologically relevant proteins, there is the constant need to use new data to update and extend the available programs. Reliable epitope prediction is still only available for a limited number of organisms and alleles because little or no information is available about the corresponding peptide specificities. However, several studies have shown both for MHC class I and II that the knowledge of which amino acids occupy those positions of the MHC polypeptide chain responsible for the interaction with peptide ligands make it possible, in some cases, to predict the peptide specificities for MHC molecules with as yet unknown motifs (Seeger et al., 1999; Sturniolo et al., 1999).

Furthermore, predictions for other characteristics of antigen processing, such as proteasomal cleavage specificities, are still in their infancy, with programs that are based on quantified cleavage data from immuno- and constitutive proteasomes not yet being publicly available. On the other hand, the development of prediction programs for MHC class II that are able to take antigen processing into consideration are still in the distant future.

Since the increasing usage of screening techniques, such as DNA microarrays (Lockhart et al., 1996) and serological analysis of recombinant cDNA expression libraries (SEREX) (Türeci et al., 1996), provide a rapidly growing pool of candidate protein antigens, interactive combinations of computational epitope predictions with these approaches, such as the recently described combination of DNA microarray analysis and TEPITOPE (Sturniolo et al., 1999), are expected to provide a rapidly growing number of epitopes for use in immunotherapy of a wide range of diseases.

However, while the described strategies combining computational prediction and experimental methods are likely to provide a rapidly increasing number of T cell epitopes as potential tools for therapeutic and diagnostic purposes, the identified epitopes still

have to pass the ultimate test: they have to prove to be useful in the *in vivo* situation.

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