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International Immunology, Vol. 9, No. 8, 1997, pp 1085-1093 Int. J. Cancer, Vol. 68, No. 4, 1996, pp 471-478 Eur. J. Immunol., Vol. 26, 1996, pp 435-443
Eur. J. Immunol., Vol. 23, No. 10, 1993, pp 2687-2691
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Journal of Protein Chemistry, Vol. 8, No. 1, 1989, pp 79-86 Dev. Oncol. 1985, 28 (RNA tumor viruses, oncog. hum. cancer AIDS) pp 151-167 Proc. Natl. Acad. Sci., Vol. 81, No. 16, August 1984, pp 522 7-5231

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(54) Abstract Title
Peptides based on the p21 ras proto-oncogene protein for the treatment of cancer

(57) A peptide capable of inducing specific cytotoxic T cell responses (CD 8+) comprises 8 to 10 amino acids of the p21 ras proto-oncogene protein including position 12, 13 or 61 wherein an amino acid substitution has been made at the latter position. These peptides may be used as cancer vaccines and in compositions for anti-cancer treatment.

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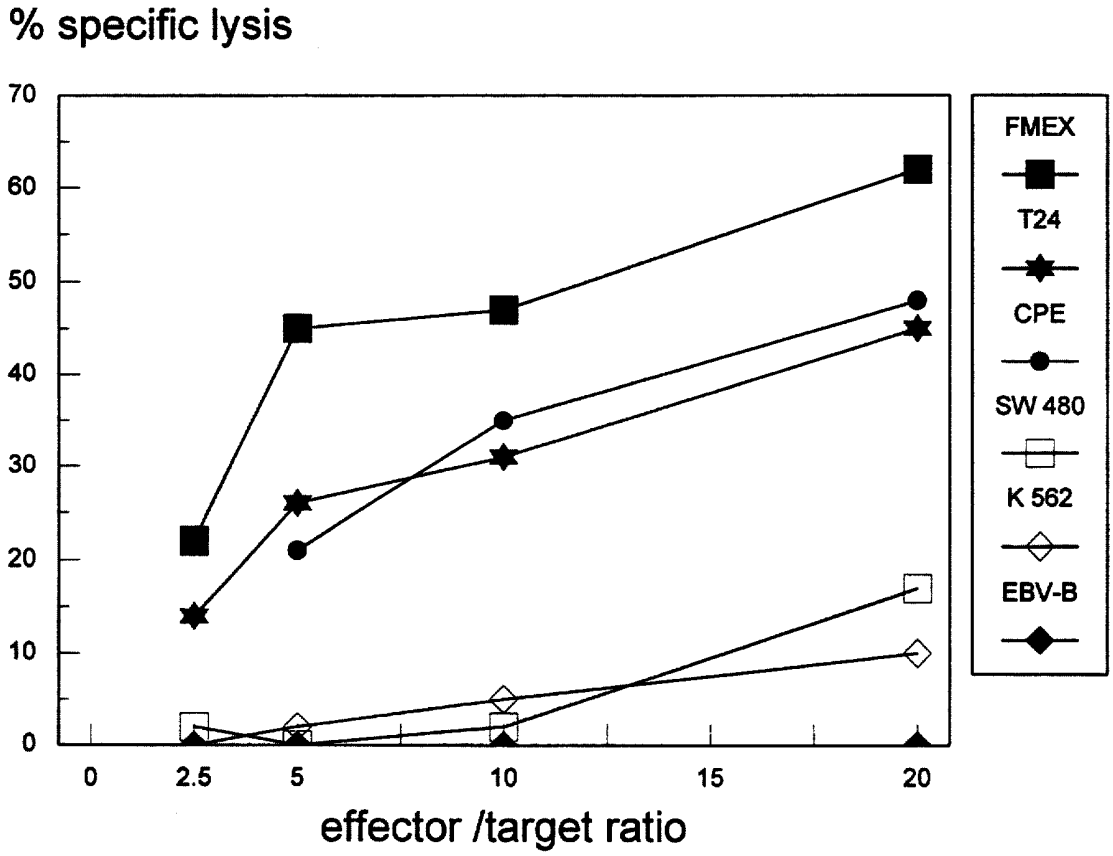
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60
50
40
30
20
10
0
0
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5
10
15
20
effector /target ratio
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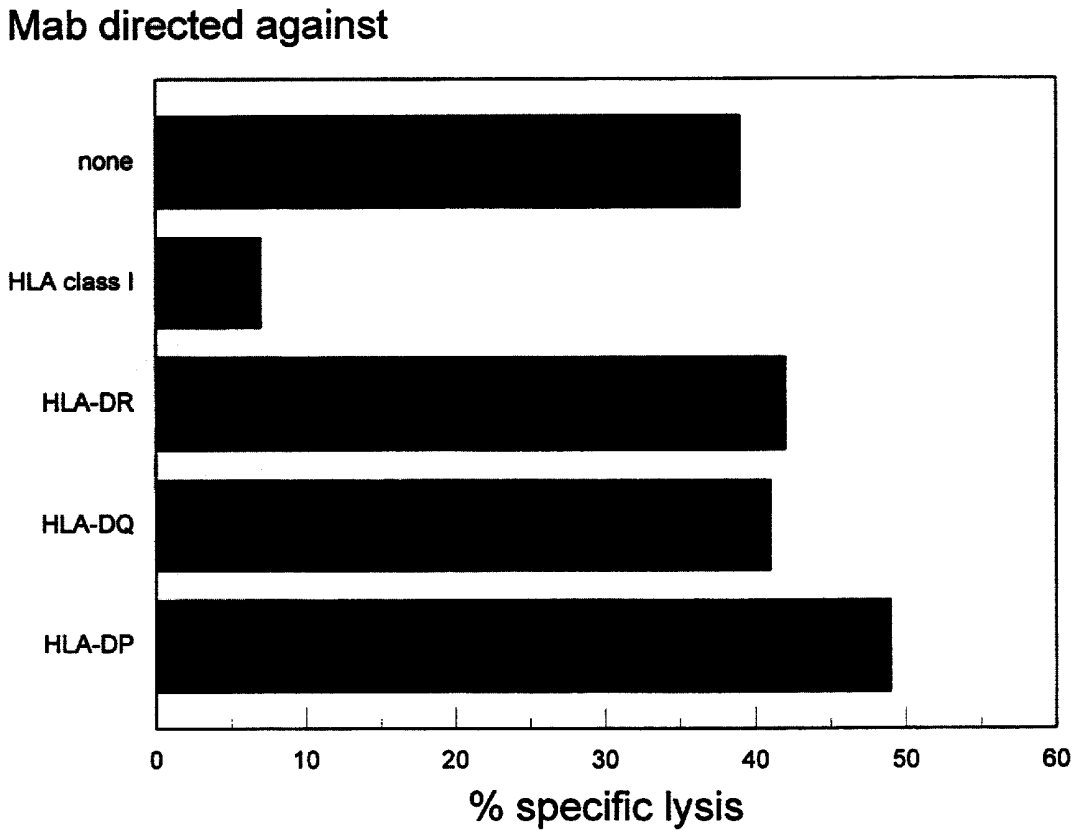
T24
CPE
SW 480
K 562
EBV-B

Fig. 1


Mab directed against
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HLA-DQ
HLA-DP
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20
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40
50
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% specific lysis

Fig. 2

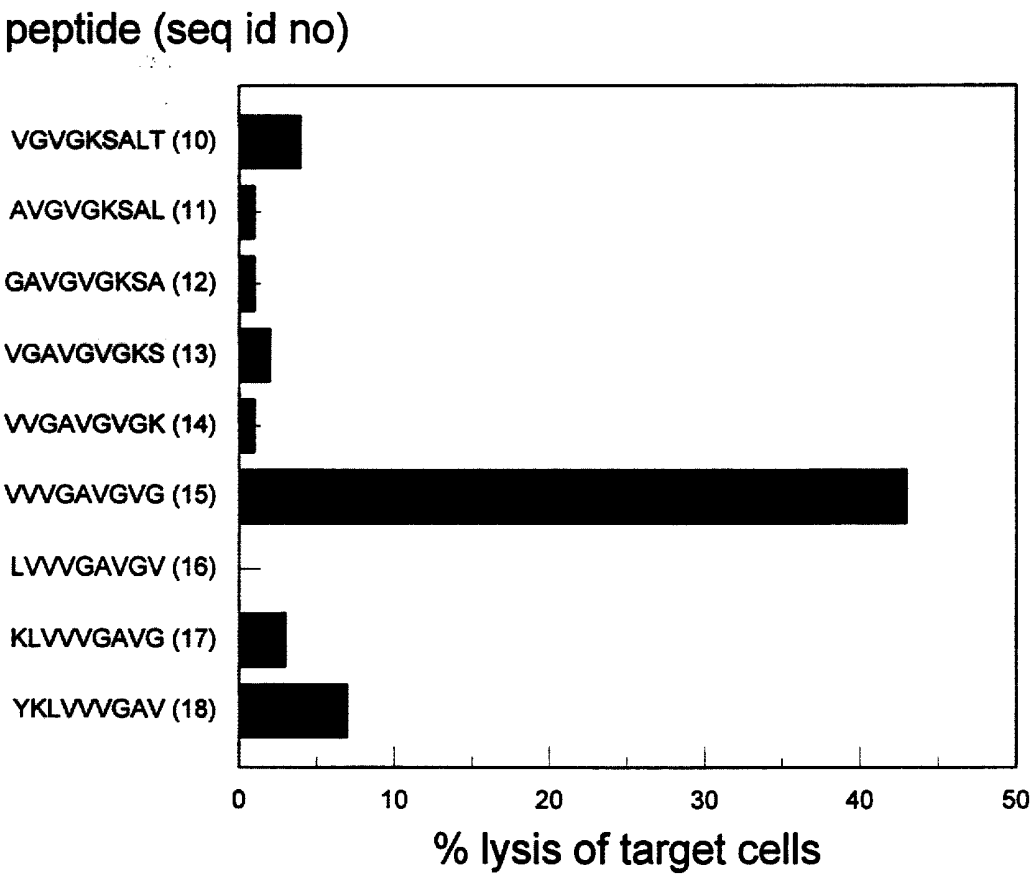
peptide (seq id no)
VGVGKSALT (10)
AVGVGKSAL (11)
GAVGVGKSA (12)
VGAVGVGKS (13)
VVGAVGVGK (14)
VVVGAVGVG (15)
LVVVGAVGV (16)
KLVVVGAVG (17)
YKLVVVGAV (18)
0
10
20
30
40
50
% lysis of target cells

Fig. 3

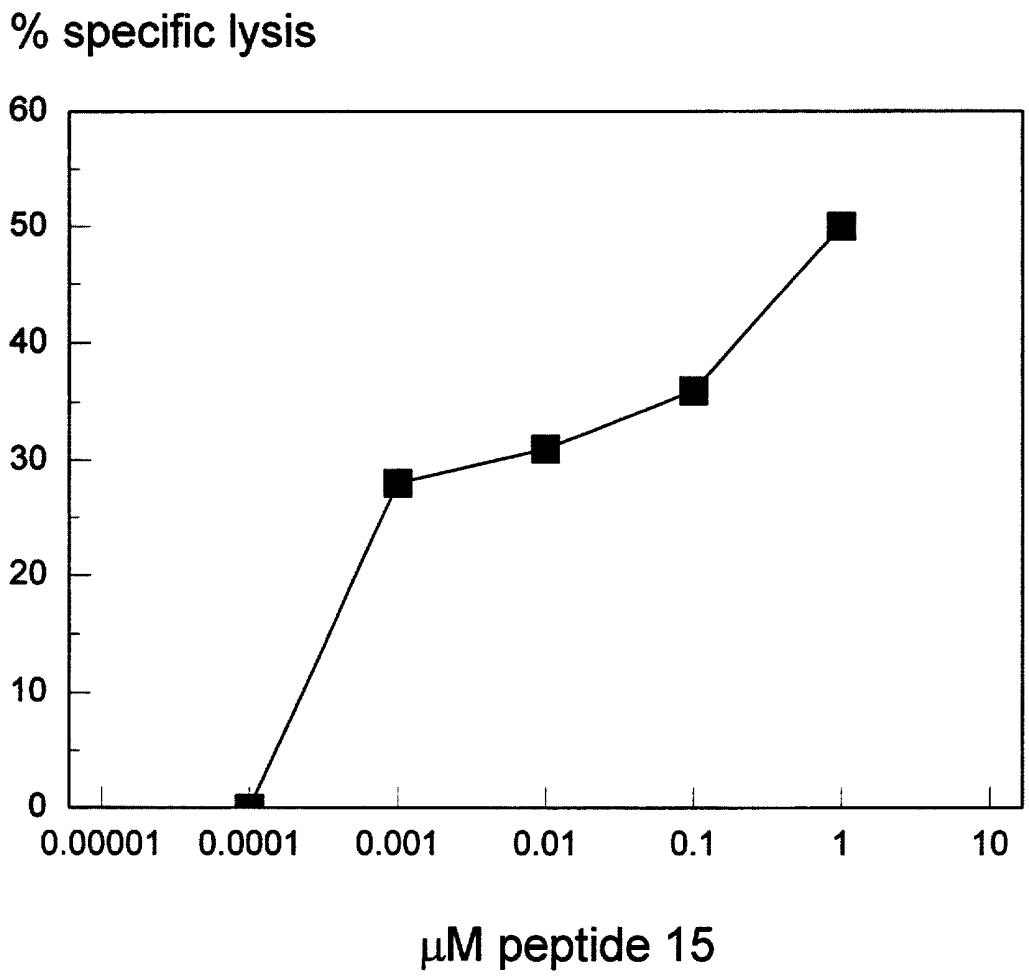
% specific lysis
60
50
40
30
20
10
0
0.00001
0.0001
0.001
0.01
0.1
1
10
µM peptide 15

Fig.4

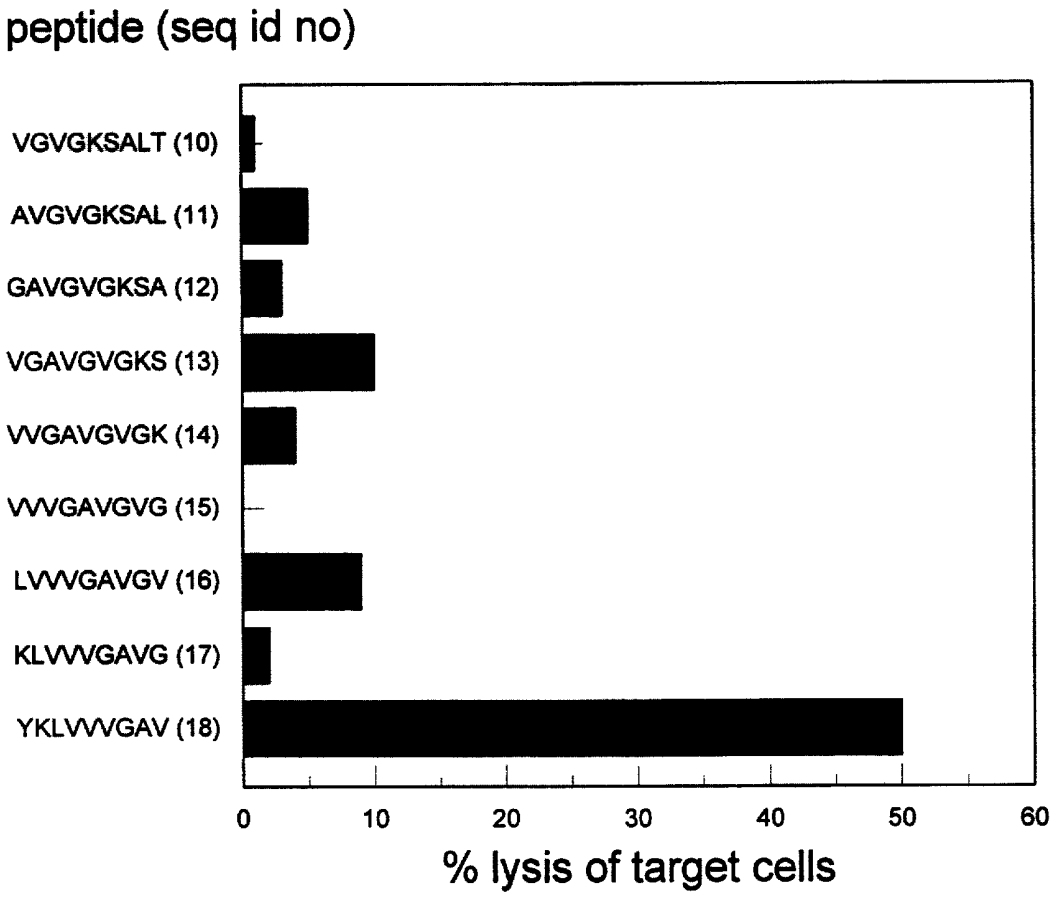
peptide (seq id no)
VGVGKSALT (10)
AVGVGKSAL (11)
GAVGVGKSA (12)
VGAVGVGKS (13)
VVGAVGVGK (14)
VVVGAVGVG (15)
LVVVGAVGV (16)
KLVVVGAVG (17)
YKLVVVGAV (18)
0
10
20
30
40
50
60
% lysis of target cells

Fig. 5

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P E P T I D E S

Summary of the invention

This invention relates to synthetic peptides corresponding to p21 ras oncogene protein products which elicit cytotoxic T cellular immunity, and to cancer vaccines and compositions for anti-cancer treatment comprising said peptides, as well as methods for the treatment or prophylaxis of cancers arising from activated ras oncogenes.

The present invention represents a further development of anti-cancer treatment or prophylaxis based on using the body's own immune system through an activation and strengthening of the immune response from specific cytotoxic T cells.

Technical Background

The genetic background for the onset of cancer are proto-oncogenes and oncogenes. Proto-oncogenes are normal genes of the cell which have the potential of becoming oncogenes. All oncogenes code for and function through a protein. Oncogenes arise in nature from proto-oncogenes through point mutations or translocations, thereby resulting in a transformed state of the cell harbouring the mutation. Cancer develops through a multistep process involving several mutational events and oncogenes.

In its simplest form a single base substitution in a proto-oncogene may cause the resulting gene product to differ in one amino acid only.

It has been shown that point mutations in intracellular "self"-proteins may give rise to tumour rejection antigens, consisting of peptides differing in a single amino acid from the normal peptide. The T cells which recognise these peptides in the context of the major histocompatibility (MHC) molecules on the surface of the tumour cells, are capable of killing the tumour cells and thus rejecting the tumour from the host.

In contrast to antibodies produced by the B cells, which typically recognise an antigen in its native conformation, T cells recognise an antigen only if the antigen is bound and presented by a MHC molecule. Usually this bonding will take place only after appropriate antigen processing, which comprises a proteolytic fragmentation of the protein, so that the peptide fits into the groove of the MHC molecule. Thereby T cells are enabled to also recognise peptides derived from intracellular proteins. T cells can thus theoretically recognise aberrant peptides derived from anywhere in the tumour cell, in the context of MHC molecules on the surface of the tumour cell, and can subsequently be activated to eliminate the tumour cell harbouring the aberrant oncogene.

M.Barinaga, Science, 257, 880-881, 1992 offers a short review of how MHC binds peptides. A more comprehensive explanation of the Technical Background for this Invention may be found in D. Male et al, Advanced Immunology, 1987, J.B.Lippincott Company, Philadelphia. Both references are hereby included in their entirety.

The MHC molecules in humans are normally referred to as HLA (human leukocyte antigen) molecules. They are encoded by the HLA region on the human chromosome No 6.

The HLA molecules appear as two distinct classes depending on which region of the chromosome codes for them and which T cell subpopulations they interact with and thereby activate primarily. The HLA class I molecules are encoded by the HLA A, B and C subloci and they primarily activate CD8+ cytotoxic T cells. The HLA class II molecules are encoded by the DR, DP and DQ subloci and primarily activate CD4+ T cells.

Normally every individual has six different HLA Class I molecules, usually two alleles from each of the three subgroups A,B and C. However in some cases the number of different HLA Class I molecules is reduced due to occurrence of the same HLA allele twice.

All the gene products are highly polymorphic. Different individuals thus express distinct HLA molecules that differ from those of other individuals. This is the basis for the difficulties in finding HLA matched organ donors in transplantations. The significance of the genetic variation of the HLA molecules in immunobiology is reflected by their role as immune-response genes. Through their peptide binding capacity, the presence or absence of certain HLA molecules governs the capacity of an individual to respond to specific peptide epitopes. As a consequence, HLA molecules determine resistance or susceptibility to disease.

T cells may inhibit the development and growth of cancer by a variety of mechanisms. Cytotoxic T cells, both HLA class I restricted CD8+ and HLA Class II restricted CD4+, may directly kill tumour cells presenting the appropriate tumour antigens. Normally, CD4+ helper T cells are needed for cytotoxic CD8+ T cell responses, but if the appropriate peptide antigen is presented, cytotoxic CD8+ T cells can be activated directly, which results in a quicker, stronger and more efficient response.

While the peptides that are presented by HLA class II molecules are of varying length (12-25 amino acids), the peptides presented by HLA class I molecules must normally be exactly nine amino acid residues long in order to fit into the HLA binding groove. A longer peptide will result in non-binding if it cannot be processed internally by an APC or target cell, such as a cancer cell, before presenting in the HLA groove. Only a very limited number of deviations from this requisition of nine amino acids have been reported, and in those cases the length of the presented peptide has been either eight or ten amino acid residues long.

The explanation for this difference in necessary peptide length for binding, is found in the nature of the peptide binding grooves of HLA class I and II molecules. The peptide binding groove of HLA class I is closed at both ends, whereas the peptide binding groove of HLA class II is open at both ends and therefore allows the binding of longer peptides that protrude from the binding groove.

A requirement for both HLA class I and II binding is that the peptides must contain a binding motif, which normally is different for different HLA groups and subgroups (alleles). A binding motif is characterised by the requirement for specific amino acids in some positions of the peptide so that a narrow fit with the pockets of the HLA binding groove is achieved. Further, it is necessary to avoid some specific amino acids at other positions of the peptide since they cause steric hindrance for binding. The result of this, taken together with the peptide length restriction, is that it is quite unlikely that a peptide binding to one type of HLA class I molecules will also bind to another type. Thus, for example, it may very well be that the peptide binding motif for HLA-A1 and HLA-A2 molecules, which both belong to the class I gender, is as different as the motifs for the HLA-A1 and HLA-B1 molecules.

In order to use oncogene derived peptides as vaccines or anti-cancer agents to generate anti tumour CD8+ cytotoxic T cells, it is therefore necessary to investigate the oncogenic antigene in question and identify individual peptides that can bind to the various types of HLA class I molecules. Effective vaccination of an individual can only be achieved if at least one HLA class I allele on an APC can bind a vaccine peptide.

Thus this clearly differs from the situation with HLA class II molecules where it is possible to extend the peptides at both terminals, which makes it possible to

design longer peptides that contain epitopes for different types of HLA class II molecules.

Transforming ras genes are the oncogenes most frequently identified in human cancer. It has been established that many of the common cancers such as pancreatic, ovarial, colon rectal, lung and biliary tract carcinomas, result from mutations in ras genes in a high percentage of the patients having such cancers. The protein encoded for by such oncogenes will carry mutations almost exclusively in the positions 12 or 13 or 61 whereas the remaining amino acids in the sequence correspond to the ones found in the p21 ras proto-oncogene protein.

As a consequence synthetic *ras* peptides can be used as anticancer therapeutical agents or vaccines with the function to trigger the cellular branch of the immune system (T-cells) against cancer cells in patients afflicted with cancers that arise from activated *ras* oncogenes.

In the present description and claims, the amino acids are represented by their three or one letter abbreviation as known in the art.

Prior art

Scott I. Abrams et al, Eur. J. Immunol. 1996, *26:* 435-443 have published results of immunisation of mice with a 4-12 fragment of p21 ras protein having a substitution of Val for Gly at position 12 which resulted in cytotoxic T cell

responses (CD8+). These data demonstrate that mutant p21 ras having a Val substitution at position 12 contains a peptide sequence which exhibits specific binding to a murine MHC class I molecule.

The finding that a mouse strain can be immunised is not relevant for the present invention for the following reasons:

It is a general observation in mice that strains with different H-2 MHC types recognise different sets of peptides from the same protein, [S.S.Zamvil et al, J.Exp.Med, Vol. 168, (1988), 1181-1186], thus a peptide which elicits an immune response in a mouse of one strain, may not stimulate T cells from another, closely related mouse strain. Also in experimental models, T cells from mice, rats and human beings are known to recognise different, non overlapping epitopes of the same protein. The explanation for this is thought to reside in differences between the species in their antigen processing machinery and peptide binding capabilities of their MHC molecules.

From PCT/NO92/00032 it is known that synthetic peptides spanning the positions 1-25 of p21 ras proteins and fragments having a mutation in positions 12, 13 or 61 can be used to elicit CD4+ T cell immunity against cancer cells harbouring said mutated p21 ras oncogene protein through the administration of such peptides in vaccination or cancer therapy schemes.

Although the prior art has identified p21 ras protein fragments that give rise to CD4+ T cell immunity, no previous studies have defined the correct antigens or antigenic sites giving rise to tumour specific cytotoxic CD8+ T cell immunity in humans.

Definition of Problem solved by the Invention.

Thus, although a CD4+ T cell immunity has been achieved and cancer treatment of patients suffering from tumours deriving from p21 ras oncogenes is at present investigated, the activation of the cells capable of killing the tumour cells, namely the cytotoxic T cells, has been difficult to achieve in a sufficient strength. Further, the cytotoxic T cell activation, as achieved indirectly through a first CD4+ T cell activation, is rather slow. This is a serious problem especially for inoperable patients with a short life time expectation.

Therefore there is a need for an anticancer treatment or vaccinating agent, which will establish a strong cytotoxic T cell response against tumours harbouring mutated ras oncogenes in a quick and reliable manner in order to improve the activity of anti-cancer treatment or prophylaxis based on peptides derived from mutated p21 ras proteins.

Definition of the Invention

It has now according to the present invention been found a group of synthetic peptides which solve the above mentioned problems through the direct activation of cytotoxic CD8+ T cells against tumours harbouring an activated ras oncogene. These peptides are from 8-10 amino acids long and have been shown to be identical to naturally processed epitopes as presented by HLA class I molecules in a human patient suffering from such a tumour.

Thus, the peptides according to this invention are characterised in that they

a) contains 8-10 amino acids, and encompasses the position 12 and/or 13, or 61 of a p21 ras proto-oncogene protein, and has an amino acid substitution in position 12 or 13 or 61, while the remaining amino acids correspond to the ones found in the same positions of said protein;

and

b) if the peptide encompasses the positions 12 and 13, they are not both Gly;
and
if the amino acid in position 13 is Gly, the amino acid in position 12 can be any amino acid except Gly;
or

if the amino acid in position 12 is Gly, the amino acid in position 13 can be any amino acid except Gly

or

if the peptide encompasses the position 61, the amino acid in this position can be any amino acid except Gln;

and

c) induces specific cytotoxic T cell (CD8+) responses.

The most preferred peptides according to this invention are the peptides consisting of nine amino acids.

Through the peptides of the invention the following advantages are achieved:

- it is possible to design a stronger anticancer therapy and vaccination;
- the direct activation of the cytotoxic CD8+ T cells results in a quicker establishment of the killer cells necessary to kill the tumour cells;
- a more direct therapy and prophylaxis directed against the specific genetic alterations presented by neoplastic cells is possible.

According to one aspect of the present invention a pharmaceutical composition is prepared which comprises a peptide of the present invention. The pharmaceutical composition can be used to treat a human patient afflicted

with a cancer harbouring a ras oncogene with a mutation in position 12, 13 or 61.

As used in this specification and in the claims the term pharmaceutical composition should not only encompass a composition usable in treatment of cancer patients, but also compositions useful in connection with prophylaxis, i.e. vaccine compositions.

Thus, in another aspect of the present invention, the pharmaceutical composition can be used to vaccinate a human being in order to obtain resistance against cancers arising from ras oncogenes with a mutation in position 12, 13 or 61.

A third aspect of the present invention is the use of the peptides defined above to prepare a pharmaceutical composition for eliciting cytotoxic T cell responses in the treatment or prophylaxis of cancers arising from activated ras oncogenes.

A further aspect of the present invention is a method for the treatment of a human patient afflicted with cancer which comprises administering at least one peptide of the invention in an amount effective to elicit a cytotoxic (CD8+) T cell response.

Yet another aspect of the invention is a method for the vaccination of a human being in order to obtain resistance against cancers arising from activated ras oncogenes, which comprises administering at least one peptide of the

invention, in an amount effective to elicit a cytotoxic T cell response.

In another aspect of the present invention the peptides of the invention are administered in a pharmaceutical composition or in the methods for the treatment or prophylaxis described above as a mixture of peptides. The mixture may either be:

(a) a mixture of peptides having different mutations in one position, i.e. position 12 or position 13 or position 61.

or

(b) a mixture of peptides having the same mutation, but suitable to fit different HLA alleles

or

(c) a mixture of both mixtures (a) and (b)

or

(d) a mixture of several mixtures (a)

or

(e) a mixture of several mixtures (b)

or

(f) a mixture of several mixtures (a) and several mixtures (b).

Alternatively the peptides in the mixtures may be covalently linked with each other to form larger polypeptides or even cyclic polypeptides.

The amino acids chosen in position 12, 13 or 61 in the above mentioned mixtures would be the most commonly found mutations in a specific cancer. Such mixture or mixtures

would then be suitable for the treatment of a patient afflicted with said cancer or for the prophylaxis of a person belonging to a risk group for said cancer.

In the prophylaxtic treatment of persons not belonging to any specific risk group, but which may still be in the danger of becoming ill from a cancer harbouring mutated ras oncogene, the administration of a mixture as defined in abstract (f) is considered useful.

In this manner it is possible to adopt the present invention to the different aspects mention above.

It is a purpose of the present invention to produce a cancer therapy or vaccine for cancers harbouring mutated ras oncogenes, by inducing T cell immunity either in vitro, ex vivo or in vivo with the peptides according to the present invention.

Another purpose of the present invention is to design an anti-cancer treatment or prophylaxis specifically adapted to a human individual in need of such treatment or prophylaxis, which comprises administering at least one peptide according to this invention. The administration may take place one or several times as suitable to establish and/or maintain the wanted cytotoxic T cell immunity.

It is further anticipated that the power of an anticancer vaccine or peptide drug as disclosed in the above mentioned PCT/NO92/00032 application, can be enhanced if

the peptides of this invention were included. This is based on the assumption that if both specific CD8+ T cells (cytotoxic T cells) and specific CD4+ T cell responses may be induced at the same time, it will lead to an even stronger T cell immunity. Thus in another embodiment of the present invention peptides of the present invention are administered together with, either simultaneously or in optional sequence, the peptides disclosed in PCT/NO92/00032.

Embodiments

The most preferred peptides according to the invention are those which carry the amino acids substitutions most commonly found in human cancers arising from mutated ras oncogenes. In position 12 of p21 ras proteins the most commonly found mutations are Asp, Val, Arg, Cys, Ala and Ser. In position 13 the most commonly found mutations are Asp and Val. In position 61 the most commonly found mutations are Arg, His, Lys and Leu.

One group of preferred peptides according to this invention are the following peptides, wherein X_1 represents position 12 in the p21 ras protein and can be any amino acid except Gly:

X_1GVGKSALT,

AX_1GVGKSAL,

GAX_1GVGKSA,

VGAX_1GVGKS,

VVGAX_1GVGK,

VVVGAX_1GVG,

LVVVGAX_1GV,

KLVVVGAX_1G,

YKLVVVGAX_1

The most preferred peptides of the above group are those wherein X_1 is Asp, Val, Arg, Ala, Cys or Ser.

A further group of peptides of this invention are the following, wherein X_1 represents position 12 in a p21 ras protein and can be any amino acid except Gly:

X_1GVGKSAL

AX$_1$GVGKSA,

GAX$_1$GVGKS,

VGAX$_1$GVGK,

VVGAX$_1$GVG,

VVVGAX$_1$GV,

LVVVGAX$_1$G,

KLVVVGAX$_1$

The most preferred peptides of the above group are those wherein X_1 is Asp, Val, Arg, Ala, Cys or Ser.

A further group of peptides of this invention are the following, wherein X_1 represents position 12 of a p21 ras protein and can be any amino acid except Gly:

X_1GVGKSALTI,

AX$_1$GVGKSALT,

GAX$_1$GVGKSAL,

VGAX$_1$GVGKSA,

VVGAX$_1$GVGKS,

VVVGAX$_1$GVGK,

LVVVGAX_1GVG,

KLVVVGAX_1GV,

YKLVVVGAX_1G,

EYKLVVVGAX_1

The most preferred peptides of the above group are those wherein X_1 is Asp, Val, Arg, Ala, Cys or Ser.

A second group of especially preferred peptides according to this invention are the following wherein X_2 represents position 13 of the p21 ras protein and can be any amino acid except Gly:

X_2VGKSALTI,

GX_2VGKSALT,

AGX_2VGKSAL,

GAGX_2VGKSA,

VGAGX_2VGKS,

VVGAGX_2VGK,

VVVGAGX_2VG,

LVVVGAGX_2V,

KLVVVGAGX_2

The most preferred peptides of the above group are those wherein X_2 is Asp or Val.

A further group of peptides of the invention are the following wherein X_2 represents position 13 of the p21 ras protein and can be any amino acid except Gly:

X_2VGKSALT,

GX_2VGKSAL,

AGX_2VGKSA,

GAGX_2VGKS,

VGAGX_2VGK,

VVGAGX_2VG,

VVVGAGX_2V,

LVVVGAGX_2

The most preferred peptides of the above group are those wherein X_2 is Asp or Val.

A further group of peptides of the invention are the following wherein X_2 represents position 13 of the p21 ras protein and can be any amino acid except Gly:

X_2VGKSALTIQ

GX_2VGKSALTI

AGX_2VGKSALT

GAGX_2VGKSAL

VGAGX_2VGKSA

VVGAGX_2VGKS

VVVGAGX_2VGK

LVVVGAGX_2VG

KLVVVGAGX_2V

YKLVVVGAGX_2

The most preferred peptides of the above group are those wherein X_2 is Asp or Val.

A third group of preferred peptides according to this invention are the following wherein X_3 represents position 61 of the p21 ras protein and can be any amino acid except Gln:

X_3EEYSAMRD

GX_3EEYSAMR

AGX_3EEYSAM

TAGX_3EEYSA

DTAGX_3EEYS

LDTAGX_3EEY

ILDTAGX_3EE

DILDTAGX_3E

LDILDTAGX_3

The most preferred peptides of the above group are those wherein X_3 is Arg, Lys, His or Leu.

A further group of peptides of the invention are the following wherein X_3 represents position 61 of the p21 ras protein and can be any amino acid except Gln:

X_3EEYSAMR,

GX_3EEYSAM,

AGX_3EEYSA,

TAGX_3EEYS,

DTAGX_3EEY,

LDTAGX_3EE,

ILDTAGX_3E,

DILDTAGX_3

The most preferred peptides of the above group are those wherein X_3 is Arg, Lys, His or Leu.

A further group of peptides of the invention are the following wherein X_3 represents position 61 of the p21 ras protein and can be any amino acid except Gln:

X_3EEYSAMRDQ,

GX_3EEYSAMRD,

AGX_3EEYSAMR,

TAGX_3EEYSAM,

DTAGX_3EEYSA,

LDTAGX_3EEYS,

ILDTAGX_3EEY,

DILDTAGX_3EE,

LDILDTAGX_3E,

LLDILDTAGX_3

The most preferred peptides of the above group are those wherein X_3 is Arg, Lys, His or Leu.

As appears from the listing of peptides above, the peptides according to the present invention may be symmetrical or unsymmetrical around each of the positions where the mutations are found in the oncogene proteins.

It is considered that the peptides may be administered together, either simultaneously or separately, with compounds such as cytokines and/or growth factors, i.e. interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte macrophage colony stimulating factor (GM-CSF) or the like in order to strengthen the immune response as known in the art.

The peptides according to the present invention can be used in a vaccine or a therapeutical composition either alone or in combination with other materials, such as for instance in the form of a lipopeptide conjugate which as known in the art can induce high-affinity cytotoxic T cells (K. Deres, Nature, Vol.342, (nov.1989)).

The peptides according to the present invention may be useful to include in either a synthetic peptide or recombinant fragment based vaccine.

The peptides of the present invention are particularly suited for use in a vaccine capable of safely eliciting cytotoxic CD8+ T cell immunity:

(1) the peptides are synthetically produced and therefore do not include transforming cancer genes or other sites or materials which might produce deleterious effects,

(2) the peptides may be used alone to induce cytotoxic T cellular immunity,

(3) the peptides may be targeted for cytotoxic T cell responses without the side effects of other unwanted responses.

The peptides according to the present invention can be included in pharmaceutical compositions alone or together with usual pharmaceutically acceptable additives, adjuvants, diluents, stabilisers, carriers or the like as known in the art.

The peptides of the invention can be administered in an amount in the range of 1μg - 1g to an average human patient or individual to be vaccinated. It is more preferred to use a smaller dose in the range of 1μg - 10mg for each administration.

A person skilled in the art will find other possible modes of using the peptides of this invention, and these are meant to be encompassed by the present claim.

A cancer therapy according to the present invention may be administered both in vivo, ex vivo or in vitro having as the main goal the raising of specific cytotoxic T cell lines or clones against the gene product of the oncogene responsible for the cancer type with which the patient is afflicted.

The peptides according to this invention may be produced by conventional processes as known in the art, and this is elucidated in the description of the synthesis below.

The invention is further described in the claims.

BIOLOGICAL EXPERIMENTS

In order for a cancer vaccine and methods for specific cancer therapy based on specific T cell immunity to be effective, three conditions must be met:

1. The peptide used must correspond to the processed p21 ras oncogene protein fragment as presented by a HLA Class I molecule on the cancer cell or on professional antigen presenting cells,
2. The peptides used must be bound to a HLA Class I molecule in an immunogenic form, and
3. Cytotoxic T-cells (CD8+) capable of recognising and responding to the HLA Class I/peptide complex must be present in the circulation of the human being.

It has been established that all these conditions are met for the peptides according to the present invention. The peptides according to the present invention give rise to

specific cytotoxic T cell immune responses in vitro. HLA Class I molecules capable of binding the peptides were determined. It has been established that the synthetic peptides according to this invention correspond to the processed oncogene protein fragments. This is exemplified with synthetic p21 ras peptide fragments having a mutation in position 12. The specificity of cytotoxic T cells induced in vivo by ras peptide vaccination was determined with the peptides of the invention. This is a clear indication that the cancer patient's T cells had been activated by the identical peptide fragments in vivo.

Description of the Figures

Figure 1 shows that a $CD8^+$ cytotoxic T cell clone (CTL 69-30) which was obtained from peripheral blood from a pancreatic carcinoma patient after 12Val mutant ras peptide vaccination, can recognize and kill different tumor cell lines expressing 12Val mutated p21 ras. The cytotoxic T cell clone was obtained after cloning of T-cell blasts present in peripheral blood mononuclear cells (PBMC) from a pancreatic carcinoma patient after position 12 Val mutant ras peptide vaccination. The peptide vaccination protocol included several infusions of large amounts of peptide- loaded autologous professional antigen-presenting cells (APC). Cloning of T cells was performed by plating responding T cell blasts at 5 blasts per well onto Terasaki plates. Each well contained 2×10^4 autologous, irradiated (30 Gy) PBMC as feeder cells, and the

cells were propagated with the 12Val peptide at 25 mM and 5 U/ml recombinant interleukin-2 (rIL-2) (Amersham, Aylesbury, UK) in a total volume of 20 mL. After 9 days T cell clones were transferred onto flat-bottomed 96-well plates (Costar, Cambridge, MA) with 1 mg/ml phytohemagglutinin (PHA, Wellcome, Dartford, UK), 5 U/ml rIL-2 and allogeneic irradiated (30 Gy) PBMC (2 x 10^5) per well as feeder cells. Growing clones were further expanded in 24-well plates with PHA / rIL-2 and 1 x 10^6 allogeneic, irradiated PBMC as feeder cells and screened for peptide specificity after 4 to 7 days. T cell clone 69-30 was selected for further characterisation. It was found that it expresses the cell-surface phenotype CD3, CD8 and TcR ab. When tested at different effector to target ratios, it was found that CTL 69-30 exhibits lysis of autologous tumour cell targets, which indicates that it is directed against a tumour derived antigen, such as mutant ras.

In order to verify that the antigen recognised is associated with mutant ras, and to identify the HLA class I molecule presenting the putative mutant ras peptide to the cytotoxic T cell clone, different 12Val p21 *ras* expressing tumour cell lines carrying one or more HLA class I molecules in common with those of the patient, were used as target cells in cytotoxicity assays. Target cells were labelled with

3H-thymidine (9.25×10^4 Bq/mL) over night, washed once and plated 5000 cells per well in 96 well plates. T cells were added at different effector to target ratios and the plates were incubated for 4 hours at 37°C and then harvested before counting in a liquid scintillation counter (Packard Topcount). Data represent percent specific lysis of ^{3}H-thymidine labelled target cells in a 4h assay at different effector/target ratios. Values are expressed as the mean of triplicate cultures ± SD. T cell clone 69-30 demonstrated lysis of the bladder carcinoma cell line T24 (12Val$^+$, HLA-A1$^+$, B35$^+$) and the melanoma cell line FMEX (12Val$^+$, HLA-A2$^+$, B35$^+$), but not of the colon carcinoma cell line SW 480 (12Val$^+$, HLA-A2$^+$, B8$^+$). The autologous EBV-B cells (12Val$^-$, HLA-A1$^+$, A2$^+$, B8$^+$, B35$^+$) and the natural killer target K562 used as controls, were not lysed. These results suggest that T cell clone 69-30 recognises an endogenously-processed 12Val epitope in the context of HLA-B35.

Figure 2 further demonstrates the HLA class I restriction of T cell clone 69-30 by blocking experiments. The results show that the cytolytic effect of T cell clone 69-30 on autologous pancreatic carcinoma cells (CPE) could be blocked by a panreactive HLA class I mAb (W6/32), but remained unaltered in the presence of monoclonal antibodies directed against HLA class II DR, DQ and DP antigens. Taken together with the

results obtained with the different 12Val expressing tumour cell lines, these data demonstrate HLA class I restriction and indicate that HLA-B35 is the restricting molecule of T cell clone 69-30. Specific lysis of CPE-targets was HLA class I restricted as demonstrated by experiments involving monoclonal antibodies directed against HLA class I (W6/32) and class II (B8/11, SPV-L3 and B7/21) antigens. The cytotoxic T cell clone activity against the autologous tumour cell line was evaluated using monoclonal antibodies directed against HLA class I and class II molecules at a final concentration of 10 mg/ml. Assays were set up in triplicate in 96 well plates and the target cells were preincubated for 30 minutes at 37°C before addition of T cells. Results obtained with an effector/target ratio of 10/1 are shown. Data represent percent specific lysis against ^{3}H-thymidine labelled CPE targets and the various mAbs in a 4h assay, with activity expressed as the mean ± SD of triplicate cultures.

Figure 3 shows the fine specificity of T cell clone 69-30 in peptide pulsing experiments. To identify the mutant ras peptide actually beeing recognised by T cell clone 69-30, the panel of nonamer peptides; peptide 10-18, spanning positions 4 to 20 of p21 ras containing the Val substitution at position 12, was tested. Only peptide 15 was capable of stimulating T cell clone 69-30 activity in these experiments.

^{3}H-thymidine labelled, mild acid eluted autologous EBV-B cells were plated 2500 cells per well in 96 well plates and pulsed with the peptides at a concentration of 1 mM together with b2-microglobulin (2.5 mg/mL) in a 5% CO_2 incubator at 37°C before addition of the T cells. Assays were set up in triplicate in 96 well plates and incubated for 4 hours with an effector to target ratio of 5 to 1. The specificity of cytotoxic T cell clone recognition for the appropriate mutant peptide was illustrated by the absence of lysis observed with the peptide expressing normal ras sequence. Controls included T cell clone cultured alone, with APC in the absence of peptides or with an irrelevant melanoma associated peptide MART-1/Melan-A peptide. Data are given as mean of triplicate cultures.

Figure 4 shows the sensitivity of the T cell clone 69-30 to peptide 15. The data show that an anti-ras cytotoxic T cell activity was detectable over a range of several log units, with maximal lysis at 1×10^{-6} M and half maximal response at 1×10^{-9} M peptide concentration. This was examined in a dose-response experiment using peptide sensitised EBV-B cells as target cells. The target cells were pulsed with peptide 15 as described in Figure 3, with the exception that the peptides were added at different concentrations before the addition of T cells. Controls included target cells alone

and target cells pulsed with the irrelevant melanoma associated peptide Melan-A/Mart-1. Data are expressed as the mean of triplicate cultures ± SD.

Figure 5 shows the fine specificity of T cell clone 42-33 in peptide pulsing experiments. T cell clone 42-33 was also obtained from a vaccinated patient. Of the panel of nonamer peptides; peptide 10-18, only peptide 18 was capable of stimulating T cell clone 42-33. In the experiments the TAP deficient T2 cell line was used as antigen presenting cells. This cell line expresses only small amounts of HLA-A2 antigen, but increased levels of HLA class I antigens at the cell surface can be induced by addition of b2-microglobulin. ^{3}H-labelled target cells were incubated with the different test peptides and control peptides at a concentration of 1 mM together with b2-microglobulin (2.5 mg/mL) for one hour at 37°C. After peptide pulsing, the target cells were washed extensively, counted and plated 2500 cells per well in 96 well plates before addition of the T cells. The plates were incubated for 4 hours at 37°C in 5% CO_2 before harvesting. Controls included T cell clone cultured alone or with target cells in the absence of peptides. Assays were set up in triplicate in 96 well plates with an effector to target ratio of 20 to 1.

Synthesis

The peptides were synthesised by using continuous flow solid phase peptide synthesis (9050 PepSynthesizer, MilliGen or Novasyn Crystal peptide synthesiser, Novabiochem). N-α-Fmoc-amino acids with appropriate side chain protection (Ser(tBu), Thr(tBu), Tyr(tBu), Lys(Boc), His(Trt), Arg(Pmc), Cys(Trt), Asp(O-tBu), Glu(O-tBu)) were used. The Fmoc-amino acids were activated by TBTU prior to coupling. 20% piperidine in DMF was used for selective removal of Fmoc after each coupling. Detachment from the resin and final removal of side chain protection was performed by 95% TFA (aq.). The peptides were purified and analysed by reversed phase (C18) HPLC (Shimadzu LC8A). The identity of the peptides was confirmed by using electro-spray mass spectroscopy (Finnigan mat SSQ710).

The peptides which were synthesised by this method are listed in the Sequence ID listing.

Claims

1. A peptide characterised in that it

a) contains 8-10 amino acids, and encompasses the position 12 and/or 13, or 61 of a p21 ras proto-oncogene protein, and has an amino acid substitution in position 12 or 13 or 61, while the remaining amino acids correspond to the ones found in the same positions of said protein;

and

b) if the peptide encompasses the positions 12 and 13, they are not both Gly;
and
if the amino acid in position 13 is Gly, the amino acid in position 12 can be any amino acid except Gly;
or
if the amino acid in position 12 is Gly, the amino acid in position 13 can be any amino acid except Gly
or
if the peptide encompasses the position 61, the amino acid in this position can be any amino acid except Gln;

and

c) induces specific cytotoxic T cell (CD8+) responses.

2. A peptide according to claim 1 characterised in that it consists of 9 amino acids.

3. A Peptide according to claim 2 characterised in that it is selected from the following group:

$\mathbf{X_1}$GVGKSALT

A$\mathbf{X_1}$GVGKSAL

GA$\mathbf{X_1}$GVGKSA

VGA$\mathbf{X_1}$GVGKS

VVGA$\mathbf{X_1}$GVGK

VVVGA$\mathbf{X_1}$GVG

LVVVGA$\mathbf{X_1}$GV

KLVVVGA$\mathbf{X_1}$G

YKLVVVGA$\mathbf{X_1}$

wherein $\mathbf{X_1}$ can be any amino acid except Gly, but $\mathbf{X_1}$ is most preferred Asp, Val, Arg, Ala, Cys or Ser.

4. A Peptide according to claim 2 characterised in that it is selected from the group consisting of:

$\mathbf{X_2}$VGKSALTI

G$\mathbf{X_2}$VGKSALT

AG$\mathbf{X_2}$VGKSAL

GAG$\mathbf{X_2}$VGKSA

VGAG$\mathbf{X_2}$VGKS

VVGAG$\mathbf{X_2}$VGK

VVVGAG$\mathbf{X_2}$VG

LVVVGAG$\mathbf{X_2}$V

KLVVVGAG$\mathbf{X_2}$

X_2 can be any amino acid except Gly, but X_2 is most preferred Asp or Val.

5. A Peptide according to claim 2 characterised in that it is selected from the group consisting of:

X_3EEYSAMRD

GX_3EEYSAMR

AGX_3EEYSAM

TAGX_3EEYSA

DTAGX_3EEYS

LDTAGX_3EEY

ILDTAGX_3EE

DILDTAGX_3E

LDILDTAGX_3

X_3 can be any amino acid except Gln, but X_3 is most preferred Arg, Lys, His or Leu.

6. A pharmaceutical composition comprising at least one peptide according to any of the claims 1-5 and a pharmaceutically acceptable carrier or diluent.

7. A pharmaceutical composition according to claim 6 for the treatment of a human patient afflicted with a cancer associated with activated ras oncogenes.

8. A pharmaceutical composition according to claim 7 for the treatment of a patient afflicted with any of the following: pancreatic cancer, colo-rectal cancer, lung cancer, malignant melanoma, ovarial cancer, and biliary tract carcinomas.

9. A pharmaceutical composition according to claim 6 for the prophylactic treatment of a human being, to obtain resistance against a cancer associated with activated ras oncogenes.

10. A pharmaceutical composition according to claim 9 for the prophylactic treatment of a human being to obtain resistance against pancreatic cancer, colo-rectal cancer, lung cancer, malignant melanoma, ovarial cancer, and biliary tract carcinomas.

11. A pharmaceutical composition comprising a combination of at least one peptide according to claims 1-5 and at least one peptide according to PCT/NO92/00032.

12. Pharmaceutical composition comprising a mixture of peptides according to the claims 1-5.

13.Use of a peptide according to any of the claims 1-5 for the preparation of a pharmaceutical composition for eliciting specific cytotoxic (CD8+) T-cell responses in the treatment or prophylaxis of cancers associated with activated ras oncogenes.

14. Method for the treatment of a patient afflicted with cancer associated with p21 ras oncogenes, by eliciting specific cytotoxic (CD8+) T-cell responses through stimulating in vivo, ex vivo or in vitro with a peptide according to the claims 1-5.

15. Method for the vaccination of a human being in order to obtain resistance against cancers associated with activated ras oncogenes, by eliciting specific cytotoxic (CD8+) T-cell responses through stimulating in vivo, ex vivo or in vitro with a peptide according to the claims 1-5.

The Patent Office

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Application No: GB 9718110.1 **Examiner:** S J Pilling
Claims searched: 1 to 15 **Date of search:** 19 January 1998

Patents Act 1977
Search Report under Section 17

Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.P): C3H (HA3, HA5)

Int Cl (Ed.6): C07K 7/08, A61K 38/08, 39/00

Other: ONLINE: CAS ONLINE

Documents considered to be relevant:

Category	Identity of document and relevant passage	Relevant to claims
E,X	WO 97/40156 A1 (GOVERNMENT OF THE USA) see page 10 line 16 to page 11 line 29 and the examples.	1-3
X	WO 92/14756 A1 (NORSK HYDRO A.S.) see page 23 lines 18 to 19 and the claims.	1, 6-15
X	Int. J. Cancer, Vol. 72, No. 5, 1997, M K Gjertsen *et al*, "*Cytotoxic CD4+ and CD8+ T lymphocytes, generated by mutant p21-ras (12 Val) peptide vaccination of a patient, recognize 12 Val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying the mutation*", pages 784 to 790, see particularly Table 1, the Materials and Methods and Discussion.	1-3,6-15
X	International Immunology, Vol. 9, No. 8, 1997, M Charles Smith *et al*, "*Oncogenic mutations in ras create HLA-A2.1 binding peptides but affect their extracellular processing*", pages 1085 to 1093 see particularly Table 1.	1,6-15
X	Int. J. Cancer, Vol. 68, No. 4, 1996, A Juretic *et al*, "*Cytotoxic T-lymphocyte responses against mutated p21 ras peptides: an analysis of specific T-cell-receptor gene usage*", pages 471 to 478, see particularly Table 1.	1-3,6-15

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.

Application No: GB 9718110.1 **Examiner:** S J Pilling
Claims searched: 1 to 15 **Date of search:** 19 January 1998

Category	Identity of document and relevant passage	Relevant to claims
X	Eur. J. Immunol., Vol. 26, 1996, S I Abrams *et al*, "*Identification of overlapping epitopes in mutant ras oncogene peptides that activate CD4+ and CD8+ T cell responses*", pages 435 to 443, see particularly Table 1.	1-3,6-15
X	Eur, J. Immunol., Vol. 23, No. 10, 1993, B Fossum *et al*, "*Overlapping epitopes encompassing a point mutation (12 Gly-Arg) in p21 ras can be recognized by HLA-DR, -DP and -DQ restricted T cells*", pages 2687 to 2691 see particularly Figure 2.	1,6-15
X	Eur. J. Immunol. Vol. 23, No. 3, 1993, T Gedde-Dahl III *et al*, "*T cell clones specific for p21 ras-derived peptides: characterization of their fine specificity and HLA restriction*", pages 754 to 760 see particularly Figure 1.	1-3
X	Journal of Protein Chemistry, Vol. 8, No. 1, 1989, P W Brandt-Rauf *et al*, "*Conformational effects of amino acid substitutions at positions 10, 12 and 13 in the P21 protein*", pages 79 to 86, see the Methods.	1
X	Dev. Oncol. 1985, 28 (RNA tumor viruses, oncog. hum. cancer AIDS) A Thor *et al*, "*Monoclonal antibodies generated to a synthetic peptide define ras gene expression at the single gene level in human colon and mammary carcinomas*", pages 151 to 167, see particularly the Procedure.	1
X	Proc. Natl. Acad. Sci. USA, Vol. 81, No. 16, August 1984, P Horan Hand *et al*, "*Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas*", pages 5227 to 5231, see particularly the Materials and Methods.	1

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Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
&	Member of the same patent family	E	Patent document published on or after, but with priority date earlier than, the filing date of this application.