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(54) Title: IMMUNOTHERAPEUTIC METHODS USING TUMOR ANTIGENIC PEPTIDES COATED ON CELLS AND VACCINES MADE THEREOF

(57) Abstract

A novel method of immunization, which can be used either prophylactically or therapeutically, is described. The method comprises coating of antigen presenting cells with a peptide and administering the peptide-coated cells to a mammalian subject to provoke an immune response. Useful peptides are those derived from fusion proteins resulting from chromosomal translocations and spanning the translocation breakpoint. Immunogens, constituted by the peptide-coated cells, are also described.
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IMMUNOTHERAPEUTIC METHODS USING TUMOR ANTIGENIC PEPTIDES COATED ON CELLS AND VACCINES MADE THEREOF

RELATED APPLICATIONS

The present application extends the general subject matter disclosed in international application PCT/US94/02551, published September 29, 1994 as WO 94/21287. This prior application is relied upon for general teachings related to the subject matter of the present invention and is hereby incorporated, in its entirety, by reference.

TECHNICAL FIELD

The present invention pertains to novel immunotherapeutic methods and immunogens that utilize irradiated, peptide-pulsed antigen presenting cells (APCs) to elicit an immune response in a patient.

BACKGROUND ART

Cellular immunity is a key component of the mechanism of tumor rejection. No previous cancer vaccine has shown much success in treating cancer. Most previous cancer vaccines that have been tried have involved whole cancer cells or cell extracts, which are poorly defined mixtures of many proteins. The present invention provides a cancer vaccine or immunotherapy that stimulates CD8⁺ cytotoxic T
lymphocytes (CTL) that are specific for cells of the tumor target. The general considerations of the present invention are described in WO 94/21287. Prior methods to induce CD8+ CTL with synthetic peptides have been limited to antigens from foreign microbial pathogens, such as viruses and bacteria. Present theories of tumor initiation and progression hold that tumor cells arise from mutational events, either inherited or somatic, that occur in a normal cell. These events lead to escape from normal control of proliferation in the cell population which contains the tumorigenic mutation(s). In many instances, mutations resulting in substitution of a single amino acid are sufficient to convert a normal cellular protein into an oncogenic gene product. The target antigens of the present invention are fusion proteins that are expressed from oncogenes created by chromosomal translocations.

Ewing’s sarcoma and related subtypes of neuroectodermal tumors share a specific translocation t(11;22)(q24;q12) (Delattre et al., Nature 359:162 (1992). Alveolar rhabdomyosarcoma tumors often exhibit a specific translocation t(2;3)(q35;q14) (D.N. Shapiro et al., Cancer Res. 53:5108-5112 (1993); N. Galili et al., Nature Genetics 5:230-235 (1993)).

SUMMARY OF THE INVENTION

The present invention is concerned with providing novel immunophylactic or immunotherapeutic methods for use in mammals, preferably humans, which methods are based solely or partially on immunizing said mammal with synthetic or recombinant peptides to induce cytotoxic T lymphocytes. The methods are advantageously applicable to the prevention or treatment of cancer(s) in said mammals, since cytotoxic T lymphocytes may be the primary means of host defense against cancer cells.

Our present results show that peptides having an amino acid sequence that encompass the fusion joint of a
tumor-specific fusion protein encoded by a human chromosomal translocation are useful in the immunization method of the invention. Two preferred fusion protein targets for the immunization are created by the human chromosomal translocations t(11;22)(q24;q12) and t(2;3)(q35;q14).

Thus, the present invention provides a method of immunizing a subject with a safe, non-toxic synthetic peptide, in the absence of harmful adjuvants or live viral vectors, to induce CTL that can specifically lyse tumor cells expressing a gene product of a chromosomal translocation, preferably a gene product encoded by the human chromosome translocations t(11;22)(q24;q12) and t(2;3)(q35;q14).

Exemplary of the immunoprophylactic and immunotherapeutic methods encompassed by the present invention are those which comprise a method for eliciting tumor-specific CD8+ cytotoxic T lymphocytes in a human or other mammal, comprising the steps of (1) determining the nucleotide sequence of a chromosomal translocation breakpoint encoding a fusion protein that is expressed by a cell harboring the translocation to identify the breakpoint, (2) selecting a synthetic peptide spanning the site of the protein fusion and representing the amino acid sequence of the breakpoint region of the fusion protein, (3) coating an autologous or syngeneic lymphoid or myeloid cell population containing antigen presenting cells, preferably containing dendritic cells, with the synthetic peptide by incubation with the peptide in vitro, (4) irradiating the cells with between 1,000 and 3,500 rad gamma irradiation, and (5) injecting said peptide-coated cells intravenously into the recipient person or other mammal. A preferred irradiation dose is 1500-3500 rad.

Immunogens encompassed by the present invention are those containing an autologous or syngeneic myeloid or lymphoid cell population coated with a synthetic peptide, in combination with a pharmaceutically acceptable carrier.
Preferable immunogens encompassed by the present invention are those prepared as follows:

1. Sequencing nucleic acid from a tumor sample to identify chromosomal translocation mutations,

2. Selecting a synthetic peptide corresponding to the site of a "breakpoint" fusion joint of a fusion protein product encoded by the chromosomal translocation;

3. Coating an autologous or syngeneic myeloid or lymphoid cell population which contains antigen presenting cells, preferably containing dendritic cells, with the synthetic peptide by incubation with the peptide in vitro for several hours,

4. Irradiating the cells with between 1,000 and 3,500 rad gamma irradiation, and

5. Combining with a pharmaceutically acceptable carrier.

A preferred composition according to the present invention is thus one comprising an irradiated autologous or syngeneic myeloid or lymphoid cell population, preferably containing dendritic cells, wherein the cells are coated with a peptide corresponding to the site of a "breakpoint" fusion joint of a fusion protein product encoded by a chromosomal translocation, wherein the cells have been irradiated with between 1,000 and 3,500 rad gamma irradiation. Preferably the peptide coating the cells is one spanning the breakpoint of a fusion protein created by the human chromosomal translocation t(11;22)(q24;q12) or t(2;3)(q35;q14). The composition can further comprise a pharmaceutically acceptable diluent or carrier, preferably one suitable for intravenous injection.

In embodiments of the invention related to or using peptide coated cells, the peptide can also be displayed upon the surface of the cells as a result of intracellular processing of an antigen expressed from DNA resident in the cell. For example, peripheral or splenic mononuclear
cells that have been transfected with a recombinant DNA that encodes a target fusion protein can be cultured, then irradiated, without prior coating with a solution of the peptide.

The peptide presented by the mononuclear cells is bound by the MHC class I and/or by the MHC class II molecules on the surface of the mononuclear cells.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**Figure 1.** Recognition of EWS/FLI 1 Type 8 peptide-pulsed EL4 target cells by αEWS/FLI type 8 CTL. At day 5; ○, α EWS/FLI 1 (type 8) CTL stimulated with 20 μM peptide against targets pulsed with 10 μM peptide; ●, α EWS/FLI 1 (type 8) CTL stimulated with 20 μM peptide against targets not exposed to peptide.

**Figure 2.** In vitro recognition and lysis of peptide-pulsed tumor targets by αPAX-3/FKHR cytotoxic T cells generated by peptide-pulsed spleen cell immunization. Specific in vitro recognition and lysis of tumor cell targets was measured using a 6-hr $^{51}$Cr release assay.

**Figure 3.** In vitro recognition and lysis of peptide-pulsed tumor targets by αPAX-3/FKHR cytotoxic T cells generated by peptide-pulsed spleen cell immunization. Specific in vitro recognition and lysis of L cell tumor cell targets was measured using a 6-hr $^{51}$Cr release assay.

**Figure 4.** In vitro recognition and lysis of peptide-pulsed tumor targets by αEWS/FLI 1 type 1 cytotoxic T cells generated by peptide-pulsed dendritic cell immunization. Specific in vitro recognition and lysis of tumor cell targets was measured using a 6-hr $^{51}$Cr release assay.
DETAILED DESCRIPTION OF THE INVENTION

The invention comprises a method of immunization for therapeutic or prophylactic purposes and also vaccines to be employed in the immunization method. In particular, the immunogen is made up of antigen-presenting cells which have been coated with peptides that bind to class I MHC molecules on the surface of the antigen-presenting cells. The peptides are derived from fusion proteins encoded by chromosomal translocations that specifically occur in tumor cells and span the breakpoint of the translocation. The peptides thus present an epitope present in a mutated protein expressed by tumor cells growing within a host.

The peptides to be employed may be obtained by any of the commonly known methods in the art; for example, but not limited to, total organic synthesis. In selecting the peptide(s) to be employed, the practitioner would seek to provide an epitope which is not normally present in the recipient of the peptide-coated cells. Such a peptide is provided by one spanning the breakpoint of a chromosomal translocation that forms a nucleotide sequence encoding a fusion protein and occurs in a tumor cell. Another consideration is that the peptides should preferably have an amino acid sequence that includes a motif recognized by CD8+ CTL and more preferably also includes a motif recognized by CD4+ helper T lymphocytes.

Identification of proteins which are produced in a tumor cell that are not normally present in the host can be accomplished by several methods, including a comparison by electrophoresis of the total protein profile of the tumor cells and comparing that profile to that of a normal cell of the same tissue. However, it is more convenient to identify mutations in normal cellular proteins that have led to the tumor phenotype. This is accomplished by sequencing of a nucleic acid obtained from a sample of the tumor tissue. The tumor tissue is preferably obtained by cone biopsy, open biopsy or fine needle aspiration. Also, frozen tissue samples can be used.
The nucleic acid obtained from a tumor sample is preferably DNA, but RNA can also be used. The nucleic acid can be sequenced by any of the methods well-known in the art. For rapid sequencing of DNA from a known gene region, the polymerase chain reaction (PCR) is commonly used. Rapid RT-PCR sequencing of chromosomal translocation breakpoints is described, for example, by Downing et al. (D.R. Downing et al., Am. J. Pathol. 143:1294-1300 (1993)). For designing primers for use in the PCR, the practitioner would preferably choose sequences expected to be 100-300 bases apart in the nucleic acid to be amplified. The separation can be varied considerably, however. Primers are typically about 20 residues in length, but this length can be modified as well-known in the art, in view of the particular sequence to be amplified. Also, the primers should not contain repetitive or self-complementary sequences and should have a G+C content of approximately 50%. A computer program for designing PCR primers is available (OLIGO 4.0 by National Biosciences, Inc., 3650 Annapolis Lane, Plymouth, MI).

In the embodiments of the invention in the present application, mutations which are useful to identify are those which result in the fusion of two proteins which are separated in a normal cell. The immunizing peptide is then made to represent a portion of the mutant protein which includes the "breakpoint" region.

Rapid methods for sequencing p53 mutations from tumors have been developed (D. D’Amico et al., Oncogene 7, 339-346 (1992)). It is expected that these methods can easily be used to identify the sequences of other known genes. Thus, it is entirely feasible to sequence the protein coding region of a number of probable genes to search for mutations which are present in the genome of cells from a tumor biopsy sample. In particular, the availability of PCR primers which saturate the protein coding regions of known protooncogenes and tumor
suppressor genes, since the DNA sequence of many of these
genes are known, allows the rapid determination of the
sequence of their gene products from DNA isolated from a
biopsy specimen. The nucleotide sequence of many fusion
proteins resulting from chromosomal translocations are
also known. Exemplary are the bcr-abl fusion and the
fusions shown in Table 1. Rapid DNA sequencing technology
is well-known in the art.

Nucleotide sequences determined on biopsy specimens
or tumors resected at surgery could be used to design
synthetic peptides for immunization for immunotherapy, or
after surgery as "adjuvant" immunotherapy. Although
immunization with autologous peripheral blood cells
incubated briefly in peptide and reinfused may be more
cumbersome than immunization with an "off-the-shelf"
vaccine, as a form of immunotherapy, it certainly requires
less effort and expense than in vitro expansion of tumor
infiltrating lymphocytes (TIL) for reinfusion, or other
similar forms of adoptive cellular immunotherapy. As a
preliminary step, one could also determine whether CTL
specific for the mutant oncogene peptide already existed
in a patient's peripheral blood or tumor-infiltrating
lymphocytes. If so, peptide immunization might boost an
inadequate response to levels capable of rejecting the
tumor, or to a level sufficient for clearing
micrometastases after resection of the primary tumor. If
not, peptide immunization might still be efficacious,
because cells pulsed with high concentrations of the
peptide may be more immunogenic than the tumor cell.

Methods for assaying CTL function in blood samples are
well-known in the art (see, for example, M.A. Alexander-
Miller et al., Internat. Immunol. 8:641-649 (1996)).

When choosing the peptide to synthesize, the
practitioner should design the sequence so that it is
soluble. Also it is desirable that the peptide sequence
be one that is easily synthesized, that is, lacks highly
reactive side groups. Furthermore, the peptide need not
be the minimal peptide that will bind to the MHC protein. That is, the peptide need not be the shortest sequence that is bound by the MHC protein. The radiation dose that is used in the irradiation step is one which is sufficient to inactivate the genomic DNA, preventing proliferation of the coated cells. However, the metabolism of the irradiated cells remains intact and so longer peptide antigens expressed in the cells, for example after transfection with a recombinant DNA encoding a fusion protein target, can be expressed in the cells and they will properly process them for presentation by the surface MHC molecules. Such a mode of presentation is an alternate to coating of the cells with added peptides. Transfection of mononuclear cells with recombinant DNA constructs is considered known in the art.

The general aspects of eliciting CTL by immunization with peptide-coated antigen presenting cells, including peptide synthesis, preferred cell populations, irradiation doses, conditions for coating the cells with peptide, routes of administration of the coated cells, and the like, are all described in WO 94/21287.

**MODES FOR CARRYING OUT THE INVENTION**

**EXAMPLE 1**

*In vitro Recognition and Lysis of Peptide-pulsed Tumor Targets by Cytotoxic T Cells Generated by Peptide-pulsed Spleen Cell Immunization*

An approach to cancer immunotherapy is to identify a gene product that is mutated in the cancer cell that might serve as a specific antigenic marker for malignant cells. Promising candidates for this purpose are the products of dominant and recessive oncogenes ("tumor suppressor genes"). Some oncogene products are formed by fusion of two proteins which are normally separate entities as a result of chromosomal rearrangements. An example of such a fusion oncogene is the bcr-abl oncogene.
Hence, an element that makes malignant cells different from the normal cells is the presence of a mutated cellular gene product. We propose, therefore, that eliciting a cytotoxic T-lymphocyte (CTL) immune response to mutated cellular gene products, particularly gene products that are proteins that represent a fusion of portions of one or more proteins that are separated in normal cells, can give rise to effective tumor therapy.

Because CTL recognize fragments of endogenously synthesized cell proteins brought to the cell surface by class I MHC molecules (A. Townsend & H. Bodmer, Annu. Rev. Immunol. 7, 601-624 (1989); O. Rötzschke & K. Falk Immunol. Today 12, 447-455 (1991); J.J. Monaco, Immunol. Today 13, 173-179 (1992)), the mutated gene product does not have to be expressed intact on the cell surface to be a target for CTL. A crucial requirement for such an approach is that an intracellular protein be broken down, processed, and presented by class I MHC molecules.

Ewing's sarcoma (ES) and alveolar rhabdomyosarcoma (ARMS) are both small, blue, round cell tumors of childhood that have recently been found to harbor tumor-specific translocations thought to play a role in the pathogenesis of these tumors. To develop new therapeutic approaches to the treatment of these tumors, we have determined that cytotoxic T lymphocytes (CTL) can be generated against peptides derived from the tumor-specific fusion proteins generated by the t(1;12)(q24;q12) giving rise to a EWS/FLI-1 chimeric protein in the case of ES or the t(2;13)(q35;q14) giving rise to the PAX-3/FKHR chimeric protein in the case of ARMS (see, Table 1).
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<td>EWS/FLI 1 (type 1)*</td>
<td>SSSYGQQN/PSYDSVRRGA</td>
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<td>-DR3,-DR(2,5,7),-DR7</td>
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<td>EWS/FLI 1 (type 8)</td>
<td>SSSYGQPN/PYQILGPTSS</td>
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<td>-DR1,-DR3/DRW52,-DPW4,-DR(2,5,7),-DR7</td>
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<td>PAX-3/FKHRb</td>
<td>TIGNGLSPQ/NSIRHNLSL</td>
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<td>H-2Dd,Ld,c I-EK</td>
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* - t(11;22)(q24;q12) Ewing's Sarcoma (ES)

b - t(2;13)(q35;q14) Alveolar Rhabdomyosarcoma (ARMS)

c - not predicted; determined by restriction
Since CTL recognize fragments of peptides brought to the cell surface by class I MHC molecules during normal degradation processes, a requirement for the success of this approach is that the peptides bind class I MHC molecules. We noted that these fusion proteins peptides with potential binding motifs for MHC class I and class II molecules. To test this, mice were immunized with synthetic peptides spanning the translocation breakpoints. CTL were generated which were capable of specifically recognizing and lysing peptide-pulsed targets in vitro. We have subsequently shown that a mouse colon adenocarcinoma cell line (CT26.W, which does not express either of the EWS/FLI-1 or the PAX-3/FKHR fusion proteins) that has been transfected with a PAX-3/FKHR expression vector can also be recognized and lysed by CTL derived from mice immunized with a synthetic peptide derived from the PAX-3/FKHR fusion protein.

**In vitro recognition and lysis of peptide-pulsed tumor targets by αEWS/FLI I type 8 cytotoxic T cells generated by peptide-pulsed spleen cell immunization**

Specific in vitro recognition and lysis of tumor cell targets was measured using a 6-hr \(^{51}\)Cr release assay. Briefly, for target cells, EL4 (H-2\(^b\)) tumor cells were labeled with 300 \(\mu\)Ci sodium chromate \((^{51}\)Cr\) and pulsed with 10 \(\mu\)M EWS/FLI 1 type 8 peptide for 2 hr at 37°C. The cells were washed to remove unincorporated \(^{51}\)Cr and unbound peptide and incubated in the presence of cytotoxic T cells (CTL) generated by peptide-pulsed spleen cell immunization of BALB.B mice at the effector to target cell ratios (E:T) indicated in Figure 1.

To obtain CTL, mice were immunized by intravenous administration of syngeneic spleen cells pulsed for 2 hours with 20 \(\mu\)M peptide and then irradiated with 2000 rad gamma radiation, essentially as described in WO 94/21287 (for instance, in Example 1 therein). Spleen cells were obtained 4 weeks later and restimulated in vitro with
irradiated, syngeneic spleen cells pulsed for 2 hours with 20 μM peptide and then irradiated with 2000 rad gamma radiation. After restimulation, the spleen cells were cultured for 5 days and then assayed for CTL activity. The results are shown in Figure 1.

**EXAMPLE II**

**Adoptive Transfer Studies**

Two studies have been conducted to ascertain the efficacy of tumor suppression by peptide-pulsed spleen cells. In the first study, BALB/c mice were immunized with PAX-3/FKHR peptide-pulsed spleen cells, prepared as in Example I, or mock immunized with HBSS. Four weeks later, these animals were challenged by intravenous administration of 5 x 10⁵ of either CT26.W or CT26P/F8 tumor cells. Fifteen days after tumor challenge, the animals were euthanized, the lungs were stained and metastatic tumor nodules were counted. Results of this study are presented in Table 2.

In the second study, the adoptive transfer was accomplished after transplantation of the tumor cells. BALB/c mice were challenged with either CT26.W or CT26P/F8 (Day 0). Three days later (D3), mice received 2 x 10⁷ spleen cells (i.v.) obtained from animals immunized with PAX-3/FKHR peptide-pulsed spleen cells or mock immunized with HBSS (4 wk post-immunization). Nine days later (D12), the animals were euthanized, the lungs were stained and metastatic tumor nodules were counted. Results of this study are presented in Table 3.
Table 2: Summary of PAX-3/FKHR in vivo tumor challenge study

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunization</td>
<td>Mock</td>
<td>Mock</td>
<td>PAX-3/FKHR</td>
<td>PAX-3/FKHR</td>
</tr>
<tr>
<td>Tumor Challenge</td>
<td>CT26.W</td>
<td>CT26P/F8</td>
<td>CT26.W</td>
<td>CT26P/F8</td>
</tr>
<tr>
<td>5</td>
<td>300(^a)</td>
<td>100 + (\mu)(^b)</td>
<td>300</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>213</td>
<td>250</td>
<td>300</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>111 + (\mu)</td>
<td>181</td>
<td>70</td>
</tr>
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<td>134</td>
<td>104 + (\mu)</td>
<td>300</td>
<td>17</td>
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<td></td>
<td>239</td>
<td>132 + (\mu)</td>
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<td>17</td>
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<tr>
<td>10</td>
<td></td>
<td></td>
<td>276.2</td>
<td>43.2(^c)</td>
</tr>
<tr>
<td>15 mean</td>
<td>237.2</td>
<td>141.4</td>
<td>276.2</td>
<td>43.2(^c)</td>
</tr>
</tbody>
</table>

\(^a\) The maximum number of metastatic lung nodules able to be discerned was 300.

\(^b\) The presence of micrometastatic disease was obvious, but only semiquantitative.

\(^c\) The mean of treatment group D differs from the means of groups A, B, and C at the \(P = 0.05\) level. The means of groups A, B, and C do not differ significantly from each other.
Table 3: Summary of adoptive transfer study involving PAX-3/FKHR

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor Challenge</td>
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<td>CT26P/F8</td>
<td>CT26.W</td>
<td>CT26P/F8</td>
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<tr>
<td>Adoptive Transfer</td>
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<td>naive</td>
<td>immunized</td>
<td>immunized</td>
</tr>
<tr>
<td></td>
<td>100 IC(^b)</td>
<td>16 + μ(^c)</td>
<td>300(^d)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>300(^c)</td>
<td>36</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>200 + μ</td>
<td>200 IC</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>200 + μ</td>
<td>300</td>
<td>142</td>
</tr>
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<td></td>
<td>300</td>
<td>87</td>
</tr>
<tr>
<td>mean</td>
<td>250</td>
<td>113</td>
<td>277.4</td>
<td>62.4(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Mice received 2 \times 10^7 spleen cells i.v. from either unimmunized animals or animals that had been immunized with PAX-3/FKHR peptide-pulsed spleen cells (10mM pulse; 1 \times 10^7 spleen cells i.v.) 4 weeks prior to transfer.

\(^b\) Incomplete staining of lung, only stained sections were quantitated.

\(^c\) The presence of micrometastatic disease was obvious, but only semiquantitative.

\(^d\) The maximum number of metastatic lung nodules able to be discerned was 300.

\(^e\) The mean of treatment group D differs from the means of groups A and C at the P = 0.05 level.
These in vivo studies demonstrate that mice immunized with PAX-3/FKHR peptide-pulsed spleen cells were partially protected when challenged with CT26P/F8 but were not protected if challenged with CT26.W cells. Furthermore, unimmunized animals were not protected when challenged with CT26.W or CT26P/F8. These results show that immunization offers tumor-specific therapeutic benefit.

In addition, animals bearing established disease, CT26.W or CT26P/F, were treated with adoptively transferred cells obtained from either unimmunized mice or from mice immunized with PAX-3/FKHR peptide-pulsed spleen cells. Those animals which had established disease from CT26P/F8 and received cells adoptively transferred from immunized animals had no disease or greatly reduced tumor burden. These data demonstrate the potential for the treatment of established disease and for recurrent disease, which is a particular problem with these tumors. This approach also provides a specific treatment for these devastating childhood tumors with much less toxicity than chemotherapy.

**EXAMPLE III**

*In Vitro Recognition and Lysis of Peptide-pulsed and of Transfected Cells by αPAX-3/FKHR CTL*

CTL were generated by preparing spleen cell suspensions from BALB/c mice immunized 4 weeks prior to splenectomy with syngeneic spleen cells pulsed 2 hrs. with 10 μM PAX-3/FKHR peptide (Table 1). The suspended cells were then restimulated in vitro with irradiated syngeneic spleen cells pulsed 2 hr with 10 μM peptide and cultured for five days.

Specific in vitro recognition and lysis of tumor cell targets was measured using a 6-hr $^{51}$Cr release assay. Briefly, CT26 (H-2$d$) tumor cells were labeled with 300 μCi sodium chromate ($^{51}$Cr ) and pulsed with 1.0 μM PAX-3/FKHR peptide for 2 hr at 37°C. In addition, CT26P/F8, a cloned transfectant of CT26 which expresses the entire PAX-3/FKHR
gene, were also used as targets. Target cells were washed to remove unincorporated \(^{51}\)Cr and unbound peptide and incubated at the effector to target cell ratios (E:T) indicated in Figure 2.

**EXAMPLE IV**

**Specificity of the MHC Molecule Presenting PAX-3/FKHR Peptide**

Specificity of the MHC molecule presenting the PAX-3/FKHR peptide was tested by assaying the PAX-3/FKHR-specific lysis by CTL generated as in Example III against peptide-pulsed cell lines expressing a single type of MHC molecule. L cell transfectants which express a single H-2\(^d\) molecule (H-D\(^d\), H-L\(^d\), or H-K\(^d\)) were labeled with 300 \(\mu\)Ci sodium chromate (\(^{51}\)Cr) and pulsed with 10 \(\mu\)M PAX-3/FKHR peptide for 2 hr at 37\(^\circ\)C. The cells were washed to remove unincorporated \(^{51}\)Cr and unbound peptide and incubated at the effector to target cell ratios (E:T) indicated in the presence of cytotoxic T cells (CTL) generated as described in Example III. The results are shown in Figure 3 and demonstrate that the response is restricted to H-2L\(^d\).

**EXAMPLE V**

**In Vitro Recognition and Lysis of Peptide-pulsed Tumor Cells by \(\alpha\)EWS/FLI 1 type 1-specific CTL Generated by Peptide-pulsed Dendritic Cell Immunization**

Specific in vitro recognition and lysis of tumor cell targets was measured using a 6-hr \(^{51}\)Cr release assay. CTL were obtained by immunizing BALB/c mice with irradiated syngeneic spleen cells pulsed 2 hr with 1.0 or 0.1 mM peptide. Spleen cell suspensions were prepared 4 weeks following immunization and were stimulated in vitro for 2 hrs. with EWS/FLI1 type 1 peptide (see Table 1) at 37 \(^\circ\)C. Cells were then cultured for 6 days.

P815 (H-2\(^d\)) tumor cells were labeled with 300 \(\mu\)Ci sodium chromate (\(^{51}\)Cr) and pulsed with 10 \(\mu\)M EWS/FLI1 type 1 peptide for 2 hr at 37\(^\circ\)C. The cells were washed to
remove unincorporated $^{51}$Cr and unbound peptide and incubated in the presence of cytotoxic T cells (CTL) generated by peptide-pulsed dendritic cell immunization of BALB/c mice at the effector to target cell ratios (E:T) indicated in Figure 4.

EXAMPLE VI

Recognition of a Translocation Breakpoint Peptide by Human Class I-MHC Restricted Helper T Lymphocytes

To assess the ability of human MHC molecules to recognize the cancer vaccine peptides according to the present invention, the proliferative response of human HLA-DR1 $^{+}$ CD4 $^{+}$ T cells was measured. HLA-DR1 $^{+}$ CD4 $^{+}$ dendritic cells from human peripheral blood were cultured for 7 days, then stimulated one time in vitro with the EWS/FLI 1 type 8 peptide (Table 1) added to the culture medium as free peptide to 10 $\mu$M. As a positive control experiment, a second sample of the cells was stimulated with peptide HP-56, derived from a hepatitis C virus protein and known to bind to HLA-DR1. Proliferation of the cells was measured by assaying incorporation of $[^{3}H]$-thymidine into DNA by standard methods. Results are shown in Table 4.

The results show a difference in the mean of the proliferative response between the EWS/FLI 1 type 8 peptide-stimulated cells and the unstimulated cells that is similar to that observed for the positive control. However, the result is not statistically significant, suggesting that a second in vitro stimulation is necessary to obtain a clear signal of binding. It is of interest that Gambacorti-Passerini et al. have shown that the EWS/FLI 1 type 2 peptide shown in Table 1 binds to the HLA-Cw4 molecule (C. Gambacorti-Passerini et al., J. Am. Assoc. Cancer Res. 36:231, abstr. 1379 (1995)).
Table 4: Proliferative Response of HLA-DR1+ Human CD4+ T Cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Proliferative Response ('H incorporation)</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 EWS/FLI 1 V8</td>
<td>823 802 5762 1408</td>
<td>1521</td>
</tr>
<tr>
<td>(10.0 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Peptide</td>
<td>713 908 870 773</td>
<td>812</td>
</tr>
<tr>
<td>Positive Control</td>
<td>1376 632 1494 1646</td>
<td>1209</td>
</tr>
<tr>
<td>(10.0 μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the claims below.
CLAIMS

What is claimed is:

1. A method for immunization, which comprises:
   (i) exposing splenic or peripheral blood mononuclear cells to a peptide, either by contact with a solution of the peptide or by intracellular processing of an antigen expressed from within the cell, whereby said peptide binds to at least one of MHC class I molecules and MHC class II molecules on the surface of said mononuclear cells, and wherein said peptide has an amino acid sequence that encompasses the fusion joint of a tumor-specific fusion protein encoded by a human chromosomal translocation.

2. The method of claim 1, where in said human chromosomal translocation is the human chromosomal translocation t(11;22)(q24;q12) or t(2;3)(q35;q14).

3. The method of claim 1 or claim 2, which further comprises:
   (ii) irradiating said mononuclear cells having said peptide bound to said at least one of MHC class I molecules and MHC class II molecules on their surface; and
   (iii) administering to a mammal the irradiated mononuclear cells having said peptide bound to said at least one of MHC class I molecules and class II MHC molecules on the their surface.

4. The method of claim 1 or claim 3, wherein said human chromosomal translocation is the t(11;22)(q24;q12) translocation.

5. The method of claim 1 or claim 3, wherein said human chromosomal translocation is the t(2;13)(q35;q14) translocation.
6. The method of claim 1 or claim 3, wherein said peptide has an amino acid sequence comprising a sequence selected from the group consisting of SSSYGQQNPSYDSVRGA, SSSYGQQSSLLAYNT, SSSYGQQSPPLGGAQTI, SSSYGQQNPYQILGPTSS and TIGNGLSPQNSIRHNLSL.

7. The method of claim 5, wherein said peptide has an amino acid sequence comprising the sequence TIGNGLSPQNSIRHNLSL.

8. The method of claim 5, wherein said peptide has an amino acid sequence comprising the sequence SSSYGQQNPYQILGPTSS.

9. The method of any one of claims 1 to 8, wherein said mononuclear cells are dendritic cells.

10. The method of any one of claims 1 to 5, or 9 wherein said peptide is a minimal peptide which can bind to said MHC class I molecule.

11. The method of any one of claims 1 to 5, 9 or 10, wherein said peptide is a peptide which adopts an amphipathic helical conformation in solution.

12. The method of any one of claims 1 to 11, wherein said peptide is bound to MHC class I molecules on the surface of said mononuclear cells.

13. The method of any one of claims 1 to 12, wherein said mononuclear cells are contacted with a solution of said peptide.

14. The method of any one of claims 1 to 13, wherein said mononuclear cells are irradiated with gamma radiation at a dose of 1500-3500 rad.
15. The method of any one of claims 1 to 14, wherein said cells are administered intravenously.

16. An immunogen which comprises a population of peripheral blood mononuclear cells coated with a peptide which is bound to at least one of MHC class I molecules and MHC class II molecules on the surface of said mononuclear cells, wherein said peptide has an amino acid sequence that encompasses the fusion joint of a tumor-specific fusion protein encoded by a human chromosomal translocation, and a pharmaceutically acceptable carrier.

17. An immunogen according to claim 16, wherein said human chromosomal translocation is \( t(11;22)(q24;q12) \) or \( t(2;3)(q35;q14) \).

18. An immunogen according to claim 16 or claim 17, wherein said peptide has an amino acid sequence comprising a sequence selected from the group consisting of \( \text{SSYGQQNPSYDSVRRGA, SSSYGQQSSLLAYNT, SSSYGQQSPPLGGAQTI, SSSYGQQNPYQILGPTSS and TIGNGLSPQNSIRHNLSL.} \)

19. An immunogen according to any one of claims 16 to 18, wherein said peptide is bound to MHC class I molecules on the surface of said mononuclear cells.

20. A peptide having an amino acid sequence selected from the group consisting of \( \text{SSYGQQNPSYDSVRRGA, SSSYGQQSSLLAYNT, SSSYGQQSPPLGGAQTI, SSSYGQQNPYQILGPTSS and TIGNGLSPQNSIRHNLSL.} \)

21. A pharmaceutical composition comprising a peptide having an amino acid sequence selected from the group consisting of \( \text{SSYGQQNPSYDSVRRGA, SSSYGQQSSLLAYNT, SSSYGQQSPPLGGAQTI, SSSYGQQNPYQILGPTSS and} \)
TIGNGLSPQNSIRHNLSL, and a pharmaceutically acceptable carrier.

22. A pharmaceutical composition according to claim 21, wherein said pharmaceutically acceptable carrier comprises an adjuvant or dendritic cells obtained from peripheral blood of a subject.

23. Use of a peptide having an amino acid sequence selected from the group consisting of SSSYGQQNPYQILGPTSS and TIGNGLSPQNSIRHNLSL, to make a vaccine against Ewing's sarcoma or alveolar rhabdomyosarcoma.
Recognition of EWS/FLI 1 V1 Pulsed P815 Tumor Targets by anti-EWS/FLI 1 V1 Specific CTL Generated by Peptide-Pulsed Dendritic Cell Immunization of BALB/C Mice

FIG. 4
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/82 A61K39/00 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>WO 94 21287 A (US HEALTH) 29 September 1994 cited in the application see page 13, line 27 - line 36; claims; examples ---</td>
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<td>X</td>
<td>WO 93 23549 A (CENTRE NAT RECH SCIENT; AURIAS ALAIN (FR); DELATTRE OLIVIER (FR);) 25 November 1993 see claims 16-18, 36, 37; figures 13, 14, 16, 18; examples ---</td>
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<td>A</td>
<td>WO 91 17187 A (UNIV CALIFORNIA) 14 November 1991 see claims; examples ---</td>
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
  *E* earlier document but published on or after the international filing date
  *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  *O* document referring to an oral disclosure, use, exhibition or other means
  *P* document published prior to the international filing date but later than the priority date claimed

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<td>6 February 1997</td>
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Name and mailing address of the ISA
European Patent Office, P.B. 5118 Patentlaan 2
NL - 2280 HV Rijswijk
T.l. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer
Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)
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<td>A</td>
<td>US 5 208 022 A (EGGERS ARNOLD E) 4 May 1993 see claims; examples</td>
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INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-15
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although these claims are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.: 

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 

Remark on Protest

☐ The additional search fees were accompanied by the applicant’s protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)
<table>
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