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(54) Title: METHOD FOR DIAGNOSING A DISORDER BY DETERMINING EXPRESSION OF GAGE TUMOR REJECTION ANTIGEN PRECURSORS

(57) Abstract

A new family of tumor rejection antigen precursors, and the nucleic acid molecules which code for them, are disclosed. These tumor rejection antigen precursors are referred to as GAGE tumor rejection antigen precursors, and the nucleic acid molecules which code for them are referred to as GAGE coding molecules. Various diagnostic and therapeutic uses of the coding sequences and the tumor rejection antigens, and their precursor molecules are described.

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METHOD FOR DIAGNOSING A DISORDER BY DETERMINING EXPRESSION OF GAGE TUMOR REJECTION ANTIGEN PRECURSORS

RELATED APPLICATION

This application is a continuation-in-part of copending patent application Serial No. 08/096,039 filed July 22, 1993.

FIELD OF THE INVENTION

This invention relates to a nucleic acid molecule which codes for a tumor rejection antigen precursor. More particularly, the invention concerns genes, whose tumor rejection antigen precursor is processed, inter alia, into at least one tumor rejection antigen that is presented by HLA-Cw6 molecules. The genes in question do not appear to be related to other known tumor rejection antigen precursor coding sequences.

BACKGROUND AND PRIOR ART

The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T lymphocyte, or "T cell" response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cells and HLA/peptide complexes is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses

to cellular abnormalities. Much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, Science 257: 880 (1992); Fremont et al., Science 257: 919 (1992); Matsumura et al., Science 257: 927 (1992); Latron et al., Science 257: 964 (1992).

The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs cytolytic T lymphocytes, or "CTLs" hereafter. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., Immunogenetics 35: 145 (1992); van der Bruggen et al., Science 254: 1643 (1991), for further information on this family of genes. Also, see U.S. patent application Serial Number 807,043, filed December 12, 1991, now U.S. Patent No. _____.

In U.S. patent application Serial Number 938,334, the disclosure of which is incorporated by reference, it is explained that the MAGE-1 gene codes for a tumor rejection antigen precursor which is processed to nonapeptides which are presented by the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind to one HLA molecule, but not to others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one

particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent Application Serial Number 008,446, filed January 22, 1993 and incorporated by reference, the fact that the MAGE-1 expression product is processed to a second TRA is disclosed. This second TRA is presented by HLA-C clone 10 molecules. The disclosure shows that a given TRAP can yield a plurality of TRAs.

In U.S. Patent Application Serial Number 994,928, filed December 22, 1992, and incorporated by reference herein teaches that tyrosinase, a molecule which is produced by some normal cells (e.g., melanocytes), is processed in tumor cells to yield peptides presented by HLA-A2 molecules.

In U.S. patent application Serial No. 08/032,978, filed March 18, 1993, and incorporated by reference in its entirety, a second TRA, not derived from tyrosinase is taught to be presented by HLA-A2 molecules. The TRA is derived from a TRAP, but is coded for by a non-MAGE gene. This disclosure shows that a particular HLA molecule may present TRAs derived from different sources.

In U.S. patent application Serial No. _____, filed June 17, 1993 and incorporated by reference herein, an unrelated tumor rejection antigen precursor, the so-called "BAGE" precursor is described. The BAGE precursor is not related to the MAGE family.

The work which is presented by the papers, patent, and patent applications cited supra deals, in large part, with the MAGE family of genes, and the unrelated BAGE gene. It has now been found, however, that additional tumor rejection antigen precursors are expressed by cells. These tumor rejection antigen precursors are referred to as "GAGE" tumor rejection antigen precursors. They do not show homology to either the MAGE family of genes or the BAGE gene. Thus the present invention relates to genes encoding such TRAPs, the tumor rejection antigen precursors themselves as well as applications of both.

The invention is elaborated upon further in the

disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 sets forth lysis studies using CTL clone 76/6.

Figure 2 shows tumor necrosis factor ("TNF") release assays obtained with various transfectants and controls.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

A melanoma cell line, MZ2-MEL was established from melanoma cells taken from patient MZ2, using standard methodologies. This cell line is described, e.g., in PCT Application PCT/US92/04354, filed May 22, 1992, published November 26, 1992, and incorporated by reference in its entirety. Once the cell line was established, a sample thereof was irradiated, so as to render it non-proliferative. These irradiated cells were then used to isolate cytolytic T cell clones ("CTLs") specific thereto.

A sample of peripheral blood mononuclear cells ("PBMCs") was taken from patient MZ2, and contacted to the irradiated melanoma cells. The mixture was observed for lysis of the melanoma cells, which indicated that CTLs specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown in vitro, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μCi/ml of $Na(^{51}Cr)O_4$. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 ul aliquots containing 10^3 cells, were distributed into 96 well

microplates. Samples of PBLs were added in 100 ul of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in a 8% CO_2 atmosphere.

Plates were centrifuged again, and 100 ul aliquots of supernatant were collected and counted. Percentage of ^{51}Cr release was calculated as follows:

$$\% \ ^{51}Cr \text{ release} = \frac{(ER-SR)}{(MR-SR)} \times 100$$

where ER is observed, experimental ^{51}Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells in 200 ul of medium alone, and MR is maximum release, obtained by adding 100 ul 0.3% Triton X-100 to target cells.

Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology. The CTL clone MZ2-CTL 76/6 was thus isolated. The clone is referred to as "76/6" hereafter.

The same method was used to test target K562 cells, as well as the melanoma cell line. Figure 1 shows that this CTL clone recognizes and lyses the melanoma cell line, i.e. MZ2-MEL but not K562. The clone was then tested against other melanoma cell lines and autologous EBV-transformed B cells in the same manner described supra. Figure 1 shows that autologous B cells, transformed by Epstein Barr Virus ("EBV") were not lysed, and that while MZ2-MEL 3.0 was lysed by CTL clone 76/6, the cell line MZ2-MEL.4F$^-$ a variant which does not express antigen F was not. Hence, the clone appears to be specific for this antigen.

The results presented supra are inconclusive as to which HLA molecule presents the TRA. The lysed cell line, i.e., MZ2-MEL, is known to express HLA-A1, HLA-A29, HLA-B37, HLA-B44, HLA-Cw6, and HLA-C clone 10. In experiments not reported here but which follow the protocol of this example, a subline of MZ2-MEL was tested, which had lost expression of HLA molecules A29, B44, and C clone 10. The subline was lysed, thus indicating that the presenting molecule should be one of

A1, B37, or Cw6.

Example 2

Further studies were carried out to determine if 76/6 also produced tumor necrosis factor ("TNF") when contacted with target cells. The method used was that described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference. Briefly, samples of the CTL line were combined with samples of a target cell of interest in culture medium. After 24 hours, supernatant from the cultures was removed, and then tested on TNF-sensitive WEHI cells. Cell line MZ2-MEL 43, a subclone of the MZ2-MEL cell line discussed supra as well as in the cited references, gave an extremely strong response, and was used in the following experiments.

Example 3

The results from Example 2 indicated that MZ2.MEL.43 presented the target antigen of interest. As such, it was used as a source of total mRNA to prepare a cDNA library.

Total RNA was isolated from the cell line. The mRNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the mRNA was secured, it was transcribed into cDNA, via reverse transcription, using an oligo dT primer containing a NotI site, followed by second strand synthesis. The cDNA was then ligated to a BstXI adaptor, digested with NotI, size fractionated on a Sephacryl S-500 HR column, and then cloned, undirectionally into the BstXI and Not I sites of pcDNA-I-Amp. The recombinant plasmid was then electroporated into DH5α E. coli bacteria. A total of 1500 pools of 100 recombinant bacteria were seeded in microwells. Each contained about 100 cDNAs, because nearly all bacteria contained an insert.

Each pool was amplified to saturation and plasmid DNA was extracted by alkaline lysis and potassium acetate precipitation, without phenol extraction.

Example 4

Following preparation of the library described in Example 3, the cDNA was transfected into eukaryotic cells. The transfections, described herein, were carried out in duplicate. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 50 µl/well of DMEM medium containing 10% Nu serum, 400 µg/ml DEAE-dextran, and 100 µM chloroquine, plus 100 ng of the plasmids. As was indicated supra, the lysis studies did not establish which HLA molecule presented the antigen. As a result, cDNA for each of the HLA molecules which could present the antigen (A1, B37, Cw6) was used, separately, to cotransfect the cells. Specifically, one of 28 ng of cDNA for HLA-A1, cloned into pCD-SRα was used, as were 50 ng of cDNA for HLA-B37 in pcDNA-I-Amp, or 75 ng of cDNA for HLA-Cw6 in pcDNA-I-Amp, using the same protocol as was used for transfection with the library.

Transfection was made in duplicate wells, but only 500 pools of the HLA-Cw6 transfectants could be tested in single wells. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 µl of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 µl of DMEM supplemented with 10% FCS.

Following this change in medium, COS cells were incubated for 24-48 hours at 37°C. Medium was then discarded, and 1000-3000 cells of CTL clone 76/6 were added, in 100 µl of Iscove medium containing 10% pooled human serum supplemented with 20-30 U/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference.

The 1500 pools transfected with HLA-A1, and the 1500 pools transfected with HLA-B37 stimulated TNF release to a concentration of 15-20 pg/ml, or 2-6 pg/ml, respectively. Most of the HLA-Cw6 transfectants yielded 3-20 pg/ml, except

for one pool, which yielded more than 60 pg/ml. This pool was selected for further work.

Example 5

The bacteria of the selected pool were cloned, and 600 clones were tested. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL clone 76/6. Ninety-four positive clones were found. One of these, referred to as cDNA clone 2D6 was tested further. In a comparative test COS cells were transfected with cDNA clone 2D6 and the HLA-Cw6, HLA-Cw6 alone, or 2D6 alone. Control cell lines MZ2-MEL F^- and MZ2-MEL F^+ were also used. TNF release into CTL supernatant was measured by testing it on WEHI cells, as referred to supra. The optical density of the surviving WEHI cells was measured using MTT. Figure 2 shows that the COS cells transfected with HLA-Cw6 and cDNA-2D6, and the cell line MZ2-MEL F^+ stimulated TNF release from CTL clone 76/6, indicating that HLA-Cw6 presented the subject TRA.

Example 6

The cDNA 2D6 was sequenced following art known techniques. A sequence search revealed that the plasmid insert showed no homology to known genes or proteins. SEQUENCE ID NO: 1 presents cDNA nucleotide information for the identified gene, referred to hereafter as "GAGE". A putative open reading frame is located at bases 51-467 of the molecule.

Example 7

Following sequencing of the cDNA, as per Example 6, experiments were carried out to determine if cells of normal tissues expressed the gene. To determine this, Northern blotting was carried out on tissues and tumor cell lines, as indicated below. The blotting experiments used cDNA for the complete sequence of SEQ ID NO: 1. PCR was then used to confirm the results.

Table 1. Expression of gene GAGE

Normal tissues	
PHA-activated T cells	-
CTL clone 82/30	-
Liver	-
Muscle	-
Lung	-
Brain	-
Kidney	-
Placenta	-
Heart	-
Skin	-
Testis	+
Tumor cell lines	
Melanoma	7/16
Lung Carcinoma	1/6
Sarcoma	0/1
Thyroid medullary carcinoma	0/1
Tumor samples	
Melanoma	1/1

Example 8

Detailed analysis of normal tissues and tumors was carried out by applying polymerase chain reaction ("PCR") and the GAGE gene information described supra.

First, total RNA was taken from the particular sample, using art recognized techniques. This was used to prepare cDNA. The protocol used to make the cDNA involved combining 4 ul of reverse transcriptase buffer 5x, 1 ul of each dNTP, (10 mM), 2 ul of dithiothreitol (100 mM), 2 ul of dT-15 primer (20 um), 0.5 ul of RNasin (40 units/ul), and 1 ul of M-MLV reverse transcriptase (200 units/ul). Next, 6.5 ul of template RNA (1 ug/3.25 ul water, or 2 ug total template RNA) was added. The total volume of the mixture was 20 ul. This was mixed and incubated at 42°C for 60 minutes, after which it was chilled on ice. A total of 80 ul of water was then added, to 100 ul total. This mixture was stored at -20°C until used in PCR.

To carry out PCR, the primers

5′-AGA CGC TAC GTA GAG CCT-3′

(sense)

and

5′-CCA TCA GGA CCA TCT TCA-3"

(antisense)

SEQ ID NOS: 2 and 3, respectively, were used. The reagents included 30.5 ul water, 5 ul of PCR buffer 10x, 1 ul of each dNTP (10 uM), 2.5 ul of each primer (20 uM), and 0.5 ul of polymerizing enzyme "Dynazyme (2 units/ul). The total volume as 45 ul. A total of 5 ul of cDNA was added (this corresponded to 100 ng total RNA). The mixture was combined, and layered with one drop of mineral oil. The mixture was transferred to a thermocycler block, preheated to 94°C, and amplification was carried out for 30 cycles, each cycle consisting of the following:

first denaturation:	94°C, 4 min.
denaturation:	94°C, 1 min.
annealing:	55°C, 2 min.
extension:	72°C, 3 min.
final extension:	72°C, 15 min.

Following the cycling, 10 ul aliquots were run on a 1.5% agarose gel, stained with ethidium bromide.

cDNA amplified using the primers set forth supra yields a 238 base pair fragment. There is no amplification of contaminating genomic DNA, if present.

The results are presented in Table 2, which follows. They confirm that the only normal tissue which expresses GAGE is testis, whereas a number of tumors, including melanoma, lung, breast, larynx, pharynx, sarcoma, testicular seminoma, bladder and colon express the gene. Thus, any one of these tumors can be assayed for by assaying for expression of the GAGE gene.

Table 2

RT-PCR analysis of the expression of gene GAGE

NORMAL TISSUES	
Heart	-
Brain	-
Liver	-
Lung	-
Kidney	-
Spleen	-
Lymphocytes	-
Bone marrow	-
Skin	-
Naevus	-
Melanocytes	-
Fibroblasts	-
Prostate	-
Testis	+
Ovary	-
Breast	-
Adrenals	-
Muscle	-
Placenta	-
Umbilical Cord	-

TUMORS	Cell lines	Tumor samples	
Melanoma	40/63	46/146	(32%)
Lung cancer			
Epidermoid carcinoma		10/41	(24%)
Adenocarcinoma		4/18	
Small Cell Lung Cancer	6/23	0/2	
Breast cancer		15/146	(10%)
Head and Neck tumor			
Larynx		6/15	(40%)
Pharynx		3/13	
Sarcoma	1/4	6/18	(33%)
Testicular seminoma		6/6	(100%)
Bladder cancer		5/37	(14%)
Prostate cancer		2/20	
Colon carcinoma	5/13	0/38	
Renal cancer	0/6	0/45	
Leukemia	3/6	0/19	

The foregoing examples show the isolation of a nucleic acid molecule which codes for a tumor rejection antigen precursor. This "TRAP" coding molecule, however, is not homologous with any of the previously disclosed MAGE and BAGE coding sequences described in the references set forth supra. Hence, one aspect of the invention is an isolated nucleic acid molecule which comprises the nucleotide sequence set forth in SEQ ID NO: 1. This sequence is neither a MAGE nor a BAGE coding sequence, as will be seen by comparing it to the sequence of any of these genes as described in the cited references. Also a part of the invention are those nucleic acid sequences which also code for a non-MAGE and non-BAGE tumor rejection antigen precursor but which hybridize to a nucleic acid molecule containing the described nucleotide sequence, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. More specifically, stringent conditions, as used herein, refers to hybridization in 1M NaCl, 1% SDS, and 10% dextran sulfate. This is followed by two washes of the filter at room temperature for 5 minutes, in 2xSSC, and one wash for 30 minutes in 2xSSC, 0.1% SDS. There are other conditions, reagents, and so forth which can be used, which result in the same or higher degree of stringency. The skilled artisan will be familiar with such conditions, and, thus, they are not given here.

It will also be seen from the examples that the invention embraces the use of the sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., E. coli), or eukaryotic (e.g., CHO or COS cells). The expression vectors require that the pertinent sequence, i.e., those described supra, be operably linked to a promoter. As it has been found that human leukocyte antigen HLA-Cw6 presents a tumor rejection antigen derived from these genes, the expression vector may also include a nucleic acid sequence coding for HLA-Cw6. In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The tumor

rejection antigen precursor coding sequence may be used alone, when, e.g., the host cell already expresses HLA-Cw6. Of course, there is no limit on the particular host cell which can be used. As the vectors which contain the two coding sequences may be used in HLA-Cw6 presenting cells if desired, and the gene for tumor rejection antigen precursor can be used in host cells which do not express HLA-Cw6.

The invention also embraces so called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of each of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

To distinguish the nucleic acid molecules and the TRAPs of the invention from the previously described MAGE and BAGE materials, the invention shall be referred to as the GAGE family of genes and TRAPs. Hence, whenever "GAGE" is used herein, it refers to the tumor rejection antigen precursors coded for by the previously described sequences. "GAGE coding molecule" and similar terms, are used to describe the nucleic acid molecules themselves.

The invention as described herein has a number of uses, some of which are described herein. First, the invention permits the artisan to diagnose a disorder characterized by expression of the TRAP. These methods involve determining expression of the TRAP gene, and/or TRAs derived therefrom, such as a TRA presented by HLA-Cw6. In the former situation, such determinations can be carried out via any standard nucleic acid determination assay, including the polymerase chain reaction, or assaying with labelled hybridization probes. In the latter situation, assaying with binding partners for complexes of TRA and HLA, such as antibodies, is especially preferred. An alternate method for determination is a TNF release assay, of the type described supra. To carry out the assay, it is preferred to make sure that testis cells are not present, as these normally express GAGE. This is not

essential, however, as one can routinely differentiate between testis and other cell types. Also, it is practically impossible to have testis cells present in non-testicular sample.

The isolation of the TRAP gene also makes it possible to isolate the TRAP molecule itself, especially TRAP molecules containing the amino acid sequence coded for by SEQ ID NO: 1. These isolated molecules when presented as the TRA, or as complexes of TRA and HLA, such as HLA-Cw6, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the TRAP molecule. In addition, vaccines can be prepared from cells which present the TRA/HLA complexes on their surface, such as non-proliferative cancer cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to provide a CTL response, or be cells which express both molecules without transfection. Further, the TRAP molecule, its associated TRAs, as well as complexes of TRA and HLA, may be used to produce antibodies, using standard techniques well known to the art.

When "disorder" is used herein, it refers to any pathological condition where the tumor rejection antigen precursor is expressed. An example of such a disorder is cancer, melanoma in particular. Melanoma is well known as a cancer of pigment producing cells.

Therapeutic approaches based upon the disclosure are premised on a response by a subject's immune system, leading to lysis of TRA presenting cells, such as HLA-Cw6 cells. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs in vitro. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the

type described supra. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. COS cells, such as those used herein are widely available, as are other suitable host cells.

To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Riddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex, where the complex contains the pertinent HLA molecule. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

The foregoing therapy assumes that at least some of the subject's abnormal cells present the relevant HLA/TRA complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA of the pertinent sequences, in this case a GAGE sequence. Once cells presenting the relevant complex are identified via the foregoing screening methodology, they can be combined with a sample from a patient, where the sample contains CTLs. If the complex presenting cells are lysed by the mixed CTL sample, then it can be assumed that a GAGE derived, tumor rejection antigen is being presented, and the subject is an appropriate candidate for the therapeutic approaches set forth supra.

Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked in vivo, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon supra. The cells used in this approach may be those that normally express the complex,

such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a Vaccinia virus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining the tumor rejection antigen or the precursor itself with an adjuvant to facilitate incorporation into HLA-Cw6 presenting cells which then present the HLA/peptide complex of interest. The TRAP is processed to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing.

Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

(1) GENERAL INFORMATION:

(i) APPLICANTS: Boon-Falleur, Thierry; Van den Eynde, Benoit

(ii) TITLE OF INVENTION: ISOLATED NUCLEIC ACID MOLECULES CODING FOR GAGE TUMOR REJECTION ANTIGEN PRECURSORS

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(B) COMPUTER: IBM PS/2
(C) OPERATING SYSTEM: PC-DOS
(D) SOFTWARE: Wordperfect

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: 08/250,162
(B) FILING DATE: 27-MAY-1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA;
(A) APPLICATION NUMBER; 08/096,039
(B) FILING DATE; 22-JULY-1993
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Hanson, Norman D.
(B) REGISTRATION NUMBER: 30,946
(C) REFERENCE/DOCKET NUMBER: LUD 323.1

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INFORMATION FOR SEQUENCE ID NO: 1:
SEQUENCE CHARACTERISTICS:
(A) LENGTH: 648 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY linear
SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AGCTGCCGTC CGGACTCTTT TTCCTCTACT GAGATTCATC TGTGTGAAAT ATGAGTTGGC   60
GAGGAAGATC GACCTATCGG CCTAGACCAA GACGCTACGT AGAGCCTCCT GAAATGATTG  120
GGCCTATGCG GCCCGAGCAG TTCAGTGATG AAGTGGAACC AGCAACACCT GAAGAAGGGG  180
AACCAGCAAC TCAACGTCAG GATCCTGCAG CTGCTCAGGA GGGAGAGGAT GAGGGAGCAT  240
CTGCAGGTCA AGGGCCGAAG CCTGAAGCTG ATAGCCAGGA ACAGGGTCAC CCACAGACTG  300
GGTGTGAGTG TGAAGATGGT CCTGATGGGC AGGAGATGGA CCCGCCAAAT CCAGAGGAGG  360
TGAAAACGCC TGAAGAAGAG ATGAGGTCTC ACTATGTTGC CCAGACTGGG ATTCTCTGGC  420
TTTTAATGAA CAATTGCTTC TTAAATCTTT CCCCACGGAA ACCTTGAGTG ACTGAAATAT  480
CAAATGGCGA GAGACCGTTT AGTTCCTATC ATCTGTGGCA TGTGAAGGGC AATCACAGTG  540
TTAAAAGAAG ACATGCTGAA ATGTTGCAGG CTGCTCCTAT GTTGGAAAAT TCTTCATTGA  600
AGTTCTCCCA ATAAAGCTTT ACAGCCTTCT GCAAAGAAAA AAAAAAAA                648
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INFORMATION FOR SEQUENCE ID NO: 2:
SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGA CGC TAC GTA GAG CCT 18

INFORMATION FOR SEQUENCE ID NO: 3:
SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCA TCA GGA CCA TCT TCA 18

We claim:

1. An isolated nucleic acid molecule consisting of the nucleotide sequence set forth in SEQ ID NO: 1.

2. An isolated nucleic acid molecule which hybridizes, under stringent conditions, to the nucleic acid sequence set forth in SEQ ID NO: 1, and codes for a tumor rejection antigen precursor, with the proviso that said isolated nucleic acid molecule does not code for a MAGE tumor rejection antigen precursor or a BAGE tumor rejection antigen precursor.

3. An isolated nucleic acid molecule consisting of nucleotides 51-467 of SEQ ID NO: 1.

4. An isolated mRNA molecule which is complementary to the nucleic acid molecule of claim 1.

5. A host cell transfected with the nucleic acid molecule of claim 1.

6. A host cell transfected with the nucleic acid molecule of claim 2.

7. A host cell transfected with the nucleic acid molecule of claim 3.

8. An expression vector comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter.

9. An expression vector comprising the isolated nucleic acid molecule of claim 2 operably linked to a promoter.

10. An expression vector comprising the isolated nucleic acid molecule of claim 3 operably linked to a promoter.

11. The host cell of claim 5, wherein said host cell is a mammalian cell which expresses HLA-Cw6.

12. The host cell of claim 6, wherein said host cell is a mammalian cell which expresses HLA-Cw6.

13. The host cell of claim 7, wherein said host cell is a mammalian cell which expresses HLA-Cw6.

14. The expression vector of claim 8, further comprising a nucleic acid molecule which codes for HLA-Cw6.

15. The expression vector of claim 9, further comprising a nucleic acid molecule which codes for HLA-Cw6.

16. The expression vector of claim 10, further comprising a nucleic acid molecule which codes for HLA-Cw6.

17. Expression kit comprising a separate portion of each of:

(i) the isolated nucleic acid molecule of claim 1, and

(ii) a nucleic acid molecule which codes for HLA-Cw6.

18. Expression kit comprising a separate portion of each of:

(i) the isolated nucleic acid molecule of claim 2, and;

(ii) a nucleic acid molecule which codes for HLA-Cw6.

19. Expression kit comprising a separate portion of each of:

(i) the isolated nucleic acid molecule of claim 3, and;

(ii) a nucleic acid molecule which codes for HLA-Cw6.

20. An isolated tumor rejection antigen precursor coded for by the nucleic acid molecule of claim 1, 2 or 3.

21. Method for treating a subject with a disorder characterized by expression of a GAGE tumor rejection antigen precursor which is processed to a tumor rejection antigen presented by HLA-Cw6 molecules comprising: administering to said subject an amount of cytolytic T cells which are specific for complexes of said GAGE-derived tumor rejection antigen and HLA-Cw6 molecules and which lyse cells presenting said complexes, sufficient to ameliorate said disorder.

22. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule and comprising nucleotides sequence 51-476 of SEQ ID NO: 1, comprising administering to said subject an amount of cytolytic T cells specific to complexes of an HLA molecule and a tumor rejection antigen derived from said tumor rejection antigen precursor, sufficient to ameliorate said disorder.

23. Method for treating a subject with a disorder characterized by expression of a GAGE tumor rejection antigen precursor which is processed to a tumor rejection antigen presented by HLA-Cw6 molecules, comprising administering to said subject an amount of an agent which provokes an immune response to complexes of said GAGE-derived tumor rejection antigen and HLA-Cw6 molecules sufficient to provoke said response against cells presenting said complex.

24. Method for treating a subject with a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule comprising nucleotides 51-476 of SEQ ID NO: 1, comprising administering to said subject an amount of an agent which provokes an immune response to complexes of an HLA molecule and a tumor rejection antigen precursor,

sufficient to provoke said immune response against cells presenting said complexes.

25. Method for determining a disorder, comprising contacting a sample which does not contain normal testis cells with a probe nucleic acid molecule which hybridizes to a nucleic acid molecule which codes for GAGE, and determining hybridization of said probe to said nucleic acid molecule as determining said disorder.

26. Method for diagnosing a disorder characterized by expression of a tumor rejection antigen precursor coded for by a nucleic acid molecule comprising nucleotides 51-476 of SEQ ID NO: 1, comprising contacting a sample from a subject with an agent specific for said sequence or an expression product thereof, and determining interaction between said agent and said sequence or said expression product as a determination of said disorder.

27. The method of claim 25, wherein said disorder is melanoma.

28. The method of claim 26, wherein said disorder is melanoma.

29. The method of claim 25, wherein said disorder is breast cancer.

30. The method of claim 26, wherein said disorder is breast cancer.

31. The method of claim 25, wherein said disorder is a larynx or pharynx tumor.

32. The method of claim 26, wherein said disorder is a larynx or pharynx tumor.

33. The method of claim 25, wherein said disorder is sarcoma.

34. The method of claim 26, wherein said disorder is sarcoma.

35. The method of claim 25, wherein said disorder is testicular seminoma.

36. The method of claim 25, wherein said disorder is testicular seminoma.

37. The method of claim 25, wherein said disorder is bladder cancer.

38. The method of claim 26, wherein said disorder is bladder cancer.

39. The method of claim 25, wherein said disorder is colon carcinoma.

40. The method of claim 26, wherein said disorder is colon carcinoma.

41. Isolated nucleic acid molecule selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 3.

FIG. 1A

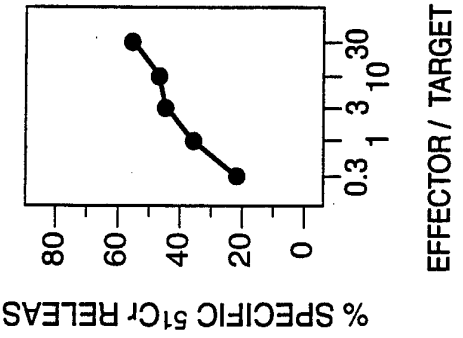


FIG. 1B

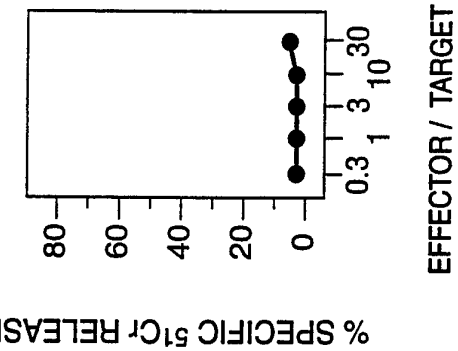


FIG. 1C

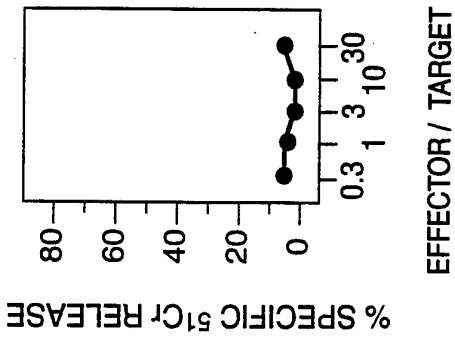


FIG. 1D

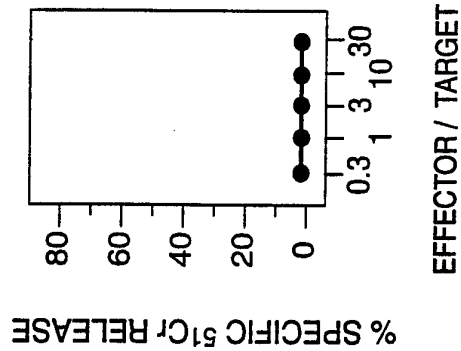
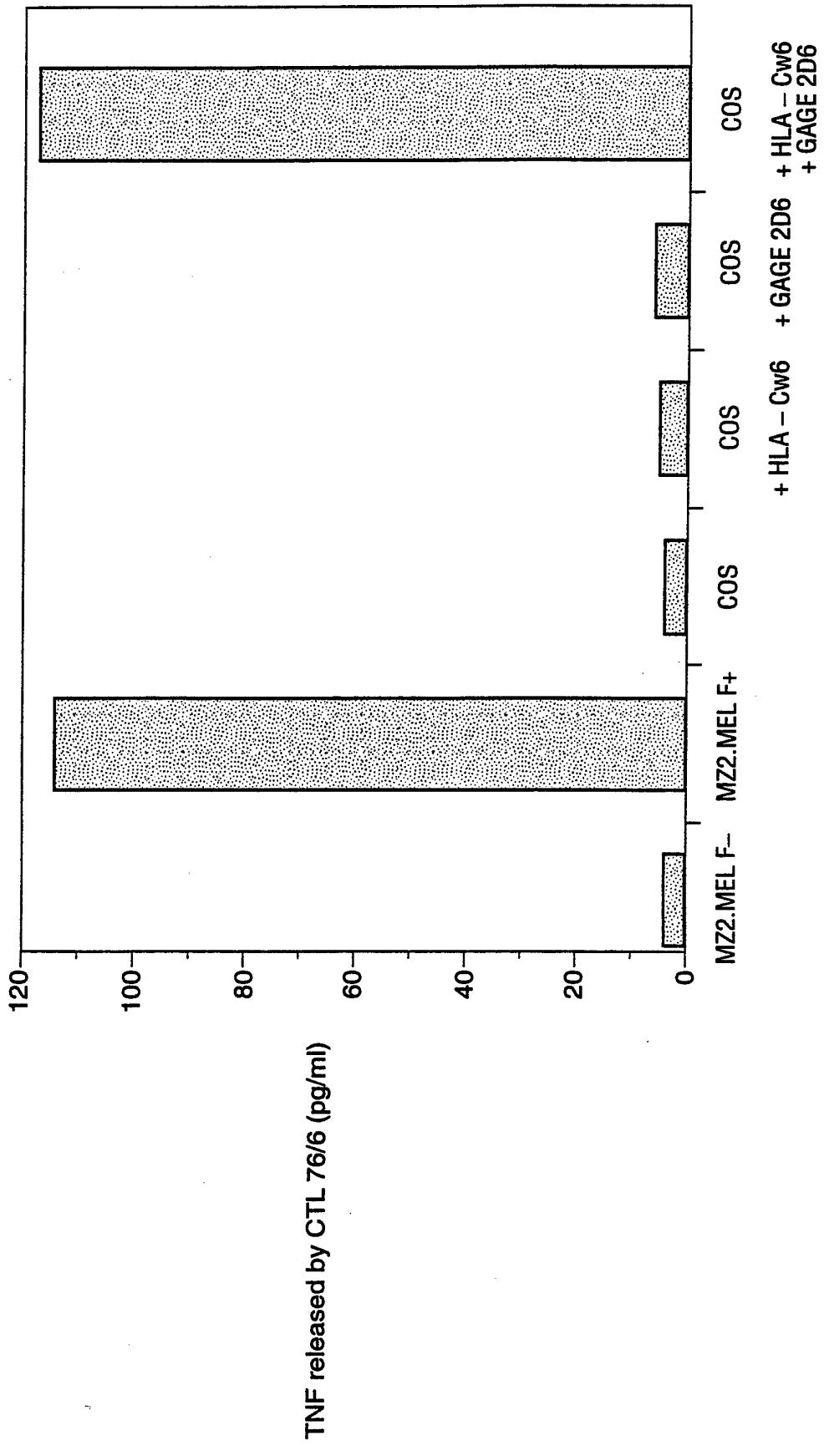


FIG. 2


TNF released by CTL 76/6 (pg/ml)
120
100
80
60
40
20
0
MZ2.MEL F–
MZ2.MEL F+
COS
COS
COS
COS
+ HLA – Cw6
+ GAGE 2D6
+ HLA – Cw6
+ GAGE 2D6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07878

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/6, 7, 69.1, 69.3, 320.1; 514/2; 530/350; 536/23.5
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)

U.S. : 435/6, 7, 69.1, 69.3, 320.1, 514/2; 530/350, 536/ 23.5

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 practicable, search terms used)

APS, Medline, Biosis, WPI
Search terms: GAGE, tumor, rejection, antigen, cancer, carcinoma, seminoma, trap

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 342, issued 23 November 1989, H. BODMER ET AL., "Anti-HLA-A2 antibody enhancement of peptide association with HLA-A2 as detected by cytotoxic T lymphocytes", pages 443-446, see entire document.	1-20, 41
Y	Immunogenetics, Volume 35, issued 1992, C. TRAVERSARI ET AL., "Transformation and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes", pages 145-152, see especially page 151.	1-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family 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 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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
01 SEPTEMBER 1994

Date of mailing of the international search report
16 NOV 1994

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07878

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Exp. Med., Volume 176, issued November 1992, C. TRAVERSARI ET AL., "A Nonapeptide Encoded by Human Gene Mage-1 Is Recognized on HLA-A1 by Cytolytic T lymphocytes Directed Against Tumor Antigen MZ2-E", pages 1453-1457, see especially 1456.	1-41
Y	Science, Volume 254, issued 13 December 1991, P. van der BRUGGEN ET AL., "A Gene Encoding an Antigen Recognized by Cytolytic T lymphocytes on a Human Melanoma", pages 1643-1647, see the entire document.	1-41
Y	Proc. Natl. Acad. Sci., Volume 84, issued November 1987, B. S. KWON ET AL., "Isolation and Sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus", pages 7473-7477, see entire document.	1-20, 41
A	J. Exp. Med., Volume 170, issued September 1989, T. WÖLFEL ET AL., "Lysis of human melanoma cells by autologous cytolytic cell clones" pages 797-810, see the entire document.	1-41
A	Int. J. Cancer, Volume 39, issued 1987, M. HERIN ET AL., "Production of Stable Cytolytic T-Cell Clones Directed Against Autologous Human Melanoma", pages 390-396, see the entire document.	1-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07878

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

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:

Please See Extra Sheet.

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07878

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/00; C12P 21/00; C12N 15/00; A61K 37/00; C07K 13/00; C07H 15/00

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-19 and 41, drawn to DNA molecules and expression kits.

Group II, claim 20, drawn to an antigen.

Group III, claims 21-24, drawn to methods of treatment.

Group IV, claims 25-40, drawn to methods of diagnosis.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of groups I and II are distinct as group I is drawn to DNA which has properties and uses that are distinct from the antigen of group II. The methods of groups III and IV are distinct as the method of group III and IV each have a different purpose and goal. The inventions of groups I and II and are distinct from the methods of group III and IV as the methods require special technical features that are not included in the products.