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(54) **Generation of immune responses to prostate-specific antigen (SPA)**

Herstellung von einer Immunantwort gegen Prostat-spezifisches Antigen (SPA)

Génération de réponses immunitaires à l'antigène spécifique de la prostate (SPA)

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Description

FIELD OF THE INVENTION

[0001] The present invention relates generally to generation of cellular and humoral immune responses to a mammalian prostate-specific antigen (PSA).

BACKGROUND OF THE INVENTION

[0002] Cancer of the prostate is the most commonly diagnosed cancer in men and is the second most common cause of cancer death (Carter, et al., 1990; Armbruster, et al., 1993). If detected at an early stage, prostate cancer is potentially curable. However, a majority of cases are diagnosed at later stages when metastasis of the primary tumor has already occurred (Wang, et al., 1982). Even early diagnosis is problematic because not all individuals who test positive in these screens develop cancer. Present treatment for prostate cancer includes radical prostatectomy, radiation therapy, or hormonal therapy. No systemic therapy has clearly improved survival in cases of hormone refractory disease. With surgical intervention, complete eradication of the tumor is not always achieved and the observed re-occurrence of the cancer (12-68%) is dependent upon the initial clinical tumor stage (Zietman, et al., 1993). Thus, alternative methods of treatment including prophylaxis or prevention are desirable.

[0003] Prostate specific antigen (PSA) is a 240 amino acid member of the glandular kallikrein gene family. (Wang, et al., 1982; Wang, et al., 1979; Bilhartz, et al., 1991). PSA is a serine protease, produced by normal prostatic tissue, and secreted exclusively by the epithelial cells lining prostatic acini and ducts (Wang, et al., 1982; Wang, et al., 1979; Lilja, et al., 1993). Prostate specific antigen can be detected at low levels in the sera of healthy males without clinical evidence of prostate cancer. However, during neoplastic states, circulating levels of this antigen increase dramatically, correlating with the clinical stage of the disease (Schellhammer, et al., 1993; Huang, et al., 1993; Kleer, et al., 1993; Oesterling, et al., 1991). Prostate specific antigen is now the most widely used marker for prostate cancer. The tissue specificity of this antigen makes PSA a potential target antigen for active specific immunotherapy (Armbruster, et al., 1993; Brawer, et al., 1989), especially in patients who have undergone a radical prostatectomy in which the only PSA expressing tissue in the body should be in metastatic deposits. Recent studies using in-vitro immunization have shown the generation of CD4 and CD8 cells specific for PSA (Peace et al., 1994; Correale et al., 1995). However, although weak natural killer cell responses have been occasionally documented in prostate cancer patients (Choe, et al., 1987), attempts to generate an *in vivo* immune response have met with limited success. For example, several attempts to actively immunize patients with prostate adenocarcinoma cells admixed with Bacillus Calmette-Guerin (BCG) have shown little or no therapeutic benefit (Donovan, et al., 1990). The ability to elicit an immune response as a result of exposure to PSA in vivo would be extremely useful. WO-A-95/04548 refers to the use of PSA in producing an immune response.

[0004] Vaccinia virus has been used in the world-wide eradication of smallpox. This virus has been shown to express a wide range of inserted genes, including several tumor associated genes such as p97, HER-2/neu, p53 and ETA (Paoletti, et al., 1993). Other pox viruses that have been suggested as useful for expression of multiple genes include avipox such as fowl pox. Cytokines expressed by recombinant vaccinia virus include IL-1, IL-2, IL-5, IL-6, TNF-α and IFN-γ (Paoletti, et al., 1993). Recombinant pox viruses, for example vaccinia viruses, are being considered for use in therapy of cancer because it has been shown in animal models that the co-presentation of a weak immunogen with the highly immunogenic poxvirus proteins can elicit a strong immune response against the inserted gene product (Kaufman, et al., 1991; Paoletti, et al., 1993; Kantor, et al., 1992a; Kantor, et al., 1992b; Irvine, et al., 1993; Moss, et al., 1993). A recombinant vaccinia virus containing the human carcinoembryonic antigen gene has just completed phase 1 clinical trials in carcinoma patients with no evidence of toxicity other than that observed with the wild type smallpox vaccine (Kantor, et al., 1992b).

[0005] Currently, models for the evaluation of prostate therapeutics include the canine (McEntee, et al., 1987), and the Dunning rat (Isaacs, et al., 1986); neither of these models, however, are practical for the study of PSA-recombinant vaccines due to the very low homology of rat and canine PSA to human PSA (Karr, et al., 1995; Schroder, et al., 1982). In contrast, the prostate gland of the rhesus monkey is structurally and functionally similar to the human prostate (Wakui, et al., 1992). At the molecular level, there is 94% homology between either the amino acid or nucleic acid sequences of rhesus PSA (Gauthier, et al., 1993) and those sequences of human prostate specific antigen (Karr, et al., 1995; Lundwall, et al., 1987). Thus, human PSA is essentially an autoantigen in the rhesus monkey. Accordingly, the rhesus monkey can serve as a model for autologous anti-PSA immune reactions.

SUMMARY OF THE INVENTION

[0006] The present invention is defined in and by the appended claims.

[0007] We have discovered that by using a recombinant viral vector, preferably a pox virus vector having at least one

insertion site containing a DNA segment encoding prostate-specific antigen (PSA), or a cytotoxic T-cell eliciting epitope thereof, operably linked to a promoter capable of expression in the host, a specific humoral and cellular immune response to PSA can be generated. The method preferably comprises introducing a sufficient amount of the recombinant pox virus vector into a host to stimulate the immune response, and contacting the host with additional PSA at periodic intervals thereafter. The additional PSA, or a cytotoxic T-cell eliciting epitope thereof, may be added by using a second pox virus vector from a different pox genus. In another embodiment, additional PSA can be added by contacting the host with PSA by a variety of other methods, including in one preferred embodiment adding PSA. The PSA may be formulated with an adjuvant or in a liposomal formulation.

[0008] In a further embodiment, an immune response to PSA can be generated by contacting the host initially with a sufficient amount of PSA, or a cytotoxic T-cell eliciting epitope thereof, to stimulate an immune response and at periodic intervals thereafter contacting the host with additional PSA. The additional PSA, or a cytotoxic T-cell generating fragment thereof, may be added using a pox virus vector as discussed above.

[0009] We have also discovered that human cytotoxic T-cells specific for PSA can be produced using a cytotoxic T-cell eliciting epitope of the PSA and that these cells have the ability to lyse PSA-expressing human prostate carcinoma cells.

[0010] As used herein the term "prostate specific antigen" includes the native protein whether purified from a native source or made by recombinant technology, as well as any polypeptide, mutein or portion derived therefrom that is capable of generating an immune response to a native conformationally correct PSA. For example, one can make conservative amino acid substitutions in the molecule without adversely affecting the ability to use the recombinant to generate an antibody that will also recognize native PSA.

[0011] The pox virus is selected from the group of pox viruses consisting of fowlpox, canary pox and pigeon pox. A more preferred avipox is fowlpox.

[0012] Vaccinia viral vectors may elicit a strong antibody response. Thus while numerous boosts with vaccinia vectors are possible, its repeated use may not be preferred in certain instances. We have discovered that by using pox from different genera to boost, this sensitivity problem can be minimized.

[0013] Adjuvants include, for example, RIBI Detox, QS21, and incomplete Freund's adjuvant. Liposomal formulations can also be used.

[0014] Human cytotoxic T-cells specific for PSA produced in accordance with the present invention can be isolated from a human host. These cells can be used in drug assays, used to map cytotoxic T-cells eliciting antigen epitopes or in adoptive cell therapy.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Figure 1 shows a Western blot of PSA from rV-PSA infected BSC-40 cells. Lanes 2-4 are extracts from supernatant fluid from cells infected overnight with rV-PSA at an MOI of 1, while Lanes 7-9 are extracts from the corresponding infected cells. Lanes 1 and 7 are supernatant extracts and cell extracts from V-Wyeth infected cells. Blot was developed using a specific MAb for human PSA. This blot illustrates that cells infected with rV-PSA authentically express and secrete the 33 kD PSA protein.

Figures 2A, 2B and 2C show the manifestation of rV-PSA immunization. In Figure 2A, the area of lesions was measured 7 days following each inoculation of rhesus monkeys with either V-Wyeth (open circles) or rV-PSA (closed circles). In Figure 2B, the duration of the lesion was monitored as time of scab disappearance. In Figure 2C, the extent of lymph node swelling was recorded and characterized as very swollen (3+), i.e., more than two axillary nodes swollen; swollen (2+), i.e., one or two nodes easily palpable; marginally swollen (1+), i.e., one node was barely palpable; or not swollen (O), 7 days following inoculation with vaccinia virus. Each symbol represents one monkey.

DETAILED DESCRIPTION OF THE INVENTION

[0016] We have induced an immune response specific to PSA in the rhesus monkey model by placing the PSA gene into a recombinant viral vector, i.e, a pox vector.

[0017] Additionally, an immune response to PSA can be generated by contacting the host initially with a sufficient amount of PSA, or a cytotoxic T-cell eliciting epitope thereof, to stimulate an immune response and at periodic intervals thereafter contacting the host with additional PSA. The additional PSA, or a cytotoxic T-cell generating fragment thereof, may be added using a pox virus vector.

[0018] A DNA fragment encoding the open reading frame of human PSA can be obtained, for example, from total

RNA extracted from the human metastatic prostate adenocarcinoma cell line, LNCaP.FGC (CRL 1740, American Type Cell Culture (ATCC), Rockville, MD) by reverse transcriptase PCR using PSA specific oligonucleotide primers 5' TCTA-GAAGCCCCAAGCTTACCACCTGCA 3' (SEQ. ID. NO.:1), 5' TCTAGATCAGGGGTTGGCCACGATGGTGTCCTT-GATCCACT 3' (SEQ. ID. NO.:2). The nucleotide sequence of the PSA cDNA has been published (Lundwall, et al., 1987).

[0019] Recombinant human PSA can be obtaining using a baculovirus expression system in accordance with the method of Bei et al., J. Clin. Lab. Anal., 9:261-268 (1995), the disclosure of which is herein incorporated by reference.

Viral vector

[0020] Basic techniques for preparing recombinant DNA viruses containing a heterologous DNA sequence encoding the carcinoma self-associated antigen or cytotoxic T-cell eliciting epitope are known to the skilled artisan and involve, for example, homologous recombination between the viral DNA sequences flanking the DNA sequence in a donor plasmid and homologous sequences present in the parental virus (Mackett, et al., Proc. Natl. Acad. Sci. USA 79: 7415-7419 (1982)). For example, recombinant viral vectors such as a pox viral vector can be used in delivering the gene. The vector can be constructed for example by steps known in the art, e.g. analogous to the methods for creating synthetic recombinants of the fowlpox virus described in U.S. Patent No. 5,093,258, the disclosure of which is incorporated herein by reference. Other techniques include using a unique restriction endonuclease site that is naturally present or artificially inserted in the parental viral vector to insert the heterologous DNA.

[0021] Avipox vectors used in the present invention are fowlpox, canary pox and pigeon pox. The preferred avipox is fowlpox.

[0022] For example, the DNA gene sequence to be inserted into the virus can be placed into a donor plasmid, e.g., an *E. coli* plasmid construct, into which DNA homologous to a section of DNA such as that of the insertion site of the poxvirus where the DNA is to be inserted has been inserted. Separately the DNA gene sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by DNA homologous to a DNA sequence flanking a region of pox DNA which is the desired insertion region. With a parental pox viral vector, a pox promoter is used. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria and isolated. Preferably, the plasmid also contains an origin of replication such as the *E. coli* origin of replication, and a marker such as an antibiotic resistance gene for selection and propagation in *E. coli.*

[0023] Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the parental virus, e.g., poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome, at a site which does not affect virus viability.

[0024] As noted above, the gene is inserted into a region (insertion region), in the virus which does not affect virus viability of the resultant recombinant virus. The skilled artisan can readily identify such regions in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, the TK gene has been found in all pox virus genomes examined [leporipoxvirus: Upton, et al., J. Virology, 60:920 (1986) (shope fibroma virus); capripoxvirus: Gershon, et al., J. Gen. Virol., 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, et al., J. Virol., 46:530 (1983) (vaccinia); Esposito, et al., Virology, 135:561 (1984) (monkeypox and variola virus); Hruby, et al., PNAS, 80:3411 (1983) (vaccinia); Kilpatrick, et al., Virology, 143:399 (1985) (Yaba monkey tumor virus); avipoxvirus: Binns, et al., J. Gen. Virol. 69:1275 (1988) (fowlpox); Boyle, et al., Virology, 156:355 (1987) (fowlpox); Schnitzlein, et al., J. Virological Methods, 20:341 (1988) (fowlpox, quailpox); entomopox (Lytvyn, et al., J. Gen. Virol. 73:3235-3240 (1992)].

[0025] In fowlpox, in addition to the TK region, other insertion regions include, for example, the BamHI J fragment [Jenkins, et al., AIDS Research and Human Retroviruses 7:991-998 (1991)] the *Eco*RI-*Hind*III fragment, *Eco*RV-*Hind*III fragment, *Bam*HI fragment and the *Hind*III fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, et al., J. of Virol. 67:3069-3076 (1993); Taylor, et al., Vaccine 6:497-503 (1988); Spehner, et al., (1990) and Boursnell, et al., J. of Gen. Virol. 71:621-628 (1990)].

[0026] In addition to the requirement that the gene be inserted into an insertion region, successful expression of the inserted gene by the modified poxvirus requires the presence of a promoter operably linked to the desired gene, i.e., in the proper relationship to the inserted gene. The promoter must be placed so that it is located upstream from the gene to be expressed. Promoters are well known in the art and can readily be selected depending on the host and the cell type you wish to target. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, 40K or fowlpox promoters such as FPV C1A. Enhancer elements can also be used in combination to increase the level of expression. Furthermore, the use of inducible promoters, which are also well known in the art, in some embodiments are preferred.

[0027] A specific immune response for PSA can be generated by administering between about 10^5-10^9 pfu of the recombinant pox virus, constructed as discussed above to a host, more preferably one uses 10^7 pfu. The preferred host

is a human. At least one interval thereafter, which is preferably one to three months later, the immune response is boosted by administering additional antigen to the host. More preferably there is at least a second "boost" preferably one to three months after the first boost. The antigen may be administered using the same pox virus vector. The antigen may preferably be administered using a second pox virus vector from a different pox genera, or may be administered directly using, for example, an adjuvant or liposome. Cytokines, e.g., IL-2, IL-6, IL-12 or costimulatory molecules, e.g., B7.1, B7.2, may be used as biologic adjuvants and can be administered systemically to the host or co-administered via insertion of the genes encoding the molecules into the recombinant pox vector.

[0028] Adjuvants include, for example, RIBI Detox (Ribi Immunochemical), QS21 and incomplete Freund's adjuvant.

Generation of Cytotoxic T-Cells

[0029] Cytotoxic T-cells specific for PSA can be established from peripheral blood mononuclear cells (PBMC) obtained from a host immunized as discussed above. For example, PBMC can be separated by using Lymphocyte Separation Medium gradient (Organon Teknika, Durham, NC, USA) as previously described [Boyum, et al., Scand J. Clin Lab Invest 21: 77-80 (1968)]. Washed PBMC are resuspended in a complete medium, for example, RPMI 1640 (GIBCO) supplemented with 10% pool human AB serum (Pel-Freeze Clinical System, Brown Dear, WI, USA), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin (GIBCO). PBMC at a concentration of about 2 x 10^5 cells in complete medium in a volume of, for example, 100 μl are added into each well of a 96-well flat-bottom assay plate (Costar, Cambridge, MA, USA). The antigen or peptides are added into the cultures in a final concentration of about 50 μg/ml and incubated at 37°c in a humidified atmosphere containing 5% CO_2 for 5 days. After removal of peptide containing media, the cultures are provided with fresh human IL-2 (10U/ml) after 5 days and replenished with IL-2 containing medium every 3 days. Primary cultures are restimulated with the same peptide (50 μg/ml) on day 16. 5 x 10^5 irradiated (4,000 rad) autologous PBMC are added in a volume of about 50 μl complete medium as antigen-presenting cells (APC). About five days later, the cultures are provided with human IL-2 containing medium as described previously. Cells are restimulated for 5 days at intervals of 16 days.

Epitope mapping

[0030] The cytotoxic T-cells can be used to determine the epitope of the PSA that elicits a cytoxic T-cell. For example, one can cut the PSA into numerous peptide fragments. Alternatively, the fragments can be chemically synthesized. Cytotoxic T-cells can then be plated and different fragments added to different wells. Only T-cells which recognize one of the pre-selected peptide fragments as an epitope will continue to expand, thereby permitting ready identification.

[0031] These fragments can then be used to elicit cytotoxic T-cell instead of using the whole protein. Additionally, one can prepare other fragments containing the epitope to enhance its ability to elicit a cytoxic T-cell response. Modifications to these fragments are well known in the art and include the use of conjugates, specific amino acid residues such as cystines, etc.

Drug Assay

[0032] The cytotoxic T-cell can also be used to screen for compounds which enhance the ability of the antigen to create a cytotoxic T-cell response. For example, cytotoxic T-cells can be incubated with a selected epitope, for example, in a microtiter plate. The compound to be tested, e.g. a drug, is then added to the well and the growth of the T-cells is measured. T-cell expansion indicates that the test compound enhances the T-cell response. Such compounds can be further evaluated.

Therapy

[0033] The cytotoxic T-cell can be cultured to amplify its number and then injected back into the host by a variety of means. Generally, between 1 x 10^5 and 2 x 10^{11} cytotoxic T-cells per infusion are administered in, for example, one to three infusions of 200 to 250 ml each over a period of 30 to 60 minutes. After the completion of the infusions, the patient may be treated with recombinant interleukin-2 with a dose of 720,000 IU per kilogram of body weight intravenously every eight hours; some doses can be omitted depending on the patient's tolerance for the drug. In addition, after infusion, additional antigen or fragments containing T-cell eliciting epitope(s) may be administered to the patent to further expand the T-cell number. The antigen or epitope may be formulated with an adjuvant and/or may be in a liposomal formulation.

[0034] The cytotoxic T-cells can also be modified by introduction of a viral vector containing a DNA encoding TNF and reintroduced into a host in an effort to enhance the anti-tumor activity of the cells. Other cytokines can also be used.

[0035] The recombinant vector can be administered using any acceptable route, including, for example, scarification and injection, e.g., intradermal, subcutaneous, intramuscular, intravenous or intraperitoneal.

[0036] For parenteral administration, the recombinant vectors will typically be injected in a sterile aqueous or non-aqueous solution, suspension or emulsion in association with a pharmaceutically-acceptable carrier such as physiological saline.

[0037] In the following Reference Example and Examples, discussion of vectors other than fowlpox, canary pox and pigeon pox is for comparison and illustration only.

REFERENCE EXAMPLE 1

CONSTRUCTION OF VECTORS

Pox Viruses

[0038] A number of pox viruses have been developed as live viral vectors for the expression of heterologous proteins (Cepko et al., Cell 37:1053-1062 (1984); Morin et al., Proc. Natl. Acad. Sci. USA 84:4626-4630 (1987); Lowe et al., Proc. Natl. Acad. Sci. USA, 84:3896-3900 (1987); Panicali & Paoletti, Proc. Natl. Acad. Sci. USA, 79:4927-4931 (1982); Mackett et al., Proc. Natl. Acad. Sci. USA, 79:7415-7419 (1982)). Representative fowlpox and swinepox virus are available through the ATCC under accession numbers VR-229 and VR-363, respectively.

DNA Vectors For *In Vivo* Recombination With A Parent Virus

[0039] Genes that code for desired carcinoma associated antigens are inserted into the genome of a pox virus in such a manner as to allow them to be expressed by that virus along with the expression of the normal complement of parent virus proteins. This can be accomplished by first constructing a DNA donor vector for *in vivo* recombination with a pox virus.

[0040] In general, the DNA donor vector contains the following elements:

(i) a prokaryotic origin of replication, so that the vector may be amplified in a prokaryotic host;
(ii) a gene encoding a marker which allows selection of prokaryotic host cells that contain the vector (e.g., a gene encoding antibiotic resistance);
(iii) at least one gene encoding a desired protein located adjacent to a transcriptional promoter capable of directing the expression of the gene; and
(iv) DNA sequences homologous to the region of the parent virus genome where the foreign gene(s) will be inserted, flanking the construct of element (iii).

[0041] Methods for constructing donor plasmids for the introduction of multiple foreign genes into pox virus are described in WO91/19803, the techniques of which are incorporated herein by reference. In general, all DNA fragments for construction of the donor vector, including fragments containing transcriptional promoters and fragments containing sequences homologous to the region of the parent virus genome into which foreign genes are to be inserted, can be obtained from genomic DNA or cloned DNA fragments. The donor plasmids can be mono-, di-, or multivalent (i.e., can contain one or more inserted foreign gene sequences).

[0042] The donor vector preferably contains an additional gene which encodes a marker which will allow identification of recombinant viruses containing inserted foreign DNA. Several types of marker genes can be used to permit the identification and isolation of recombinant viruses. These include genes that encode antibiotic or chemical resistance (e.g., see Spyropoulos et al., J. Virol., 62:1046 (1988); Falkner and Moss., J. Virol., 62:1849 (1988); Franke et al., Mol. Cell. Biol., 5:1918 (1985), as well as genes such as the *E. coli lacZ* gene, that permit identification of recombinant viral plaques by colorimetric assay (Panicali et al., Gene, 47:193-199 (1986)).

Integration Of Foreign DNA Sequences Into The Viral Genome And Isolation Of Recombinants

[0043] Homologous recombination between donor plasmid DNA and viral DNA in an infected cell results in the formation of recombinant viruses that incorporate the desired elements. Appropriate host cells for *in vivo* recombination are generally eukaryotic cells that can be infected by the virus and transfected by the plasmid vector. Examples of such cells suitable for use with a pox virus are chick embryo fibroblasts, HuTK143 (human) cells, and CV-1 and BSC-40 (both monkey kidney) cells. Infection of cells with pox virus and transfection of these cells with plasmid vectors is accomplished by techniques standard in the art (Panicali and Paoletti, U.S. Patent No. 4,603,112, WO89/03429).

[0044] Following *in vivo* recombination, recombinant viral progeny can be identified by one of several techniques. For example, if the DNA donor vector is designed to insert foreign genes into the parent virus thymidine kinase (TK) gene, viruses containing integrated DNA will be TK and can be selected on this basis (Mackett et al., Proc. Natl. Acad. Sci. USA, 79:7415 (1982)). Alternatively, co-integration of a gene encoding a marker or indicator gene with the foreign gene

(s) of interest, as described above, can be used to identify recombinant progeny. One preferred indicator gene is the *E. coli lacZ* gene: recombinant viruses expressing β-galactosidase can be selected using a chromogenic substrate for the enzyme (Panicali et al., Gene, 47:193 (1986)).

Characterizing The Viral Antigens Expressed By Recombinant Viruses

[0045] Once a recombinant virus has been identified, a variety of methods can be used to assay the expression of the polypeptide encoded by the inserted gene. These methods include black plaque assay (an *in situ* enzyme immunoassay performed on viral plaques), Western blot analysis, radioimmunoprecipitation (RIPA), and enzyme immunoassay (EIA).

EXAMPLE I

Generation of PSA Specific Immune Response

Materials and Methods

Recombinant Vaccinia Virus

[0046] A 786 bp DNA fragment encoding the entire open reading frame of human prostate specific antigen was amplified by reverse transcriptase PCR (GeneAmp RNA PCR Kit, Perkin Elmer, Norwalk, CT) from total RNA extracted from the human metastatic prostate adenocarcinoma cell line, LNCaP.FGC (CRL 1740, American Type Culture Collection (ATCC), Rockville, MD). The predicted amino acid sequence derived from the PSA coding sequence was shown to be nearly identical to the published sequence (Lundwall, et al., 1987), differing only in a change from asparagine to tyrosine at position 220. The PSA DNA fragment, containing the entire coding sequence for PSA, 41 nucleotides of the 5' untranslated region, and 520 nucleotides of the 3' untranslated region, was inserted into the Xba I restriction endonuclease cleavage site of the vaccinia virus transfer vector pT116. The resulting plasmid, designated pT1001, contains the PSA gene under the control of the vaccinia virus 40K promoter (Gritz, et al. 1990) and the *E. coli lacZ* gene under the control of the fowlpox virus C1 promoter (Jenkins, et al., 1991). The foreign genes are flanked by DNA sequences from the Hind III M region of the vaccinia genome. A plaque-purified isolate from the Wyeth (New York City Board of Health) strain of vaccinia was used as the parental virus in the construction of the recombinant vaccinia virus. The generation of recombinant vaccinia virus was accomplished via homologous recombination between vaccinia sequences in the Wyeth vaccinia genome and the corresponding sequences in pT1001 in vaccinia-infected RK_{13} cells (CCL 37, ATCC) transfected with pT1001. Recombinant virus was identified using a chromogenic assay, performed on viral plaques *in situ,* that detects expression of the *lacZ* gene product in the presence of halogenated indolyl-beta-D-galactoside (Bluo-gal), as described previously (Panacali, et al., 1986). Appropriate blue recombinant viruses were purified by four rounds of plaque-purification. Virus stocks were prepared by clarifying infected RK_{13} cell lysates followed by centrifugation through a 36% sucrose cushion.

Characterization of Recombinant Virus

Southern Analysis of DNA recombination

[0047] The recombinant vaccinia genome was analyzed by viral DNA extraction, restriction endonuclease digestion with Hind III, and Southern blotting as previously described (Kaufman *et al.,* 1991).

Western Analysis of protein expression

[0048] Confluent BSC-40 cells were infected with either parental wild type vaccinia virus (designated V-Wyeth) or recombinant vaccinia-PSA (designated rV-PSA) at an MOI of 1 in Dulbecco's Modified Eagle's Medium containing 2% fetal bovine serum. After an overnight infection, the medium was removed from the cells, and an aliquot was methanol precipitated to assay for the presence of secreted PSA. The infected cells were lysed in hypotonic lysis buffer (150 mM NaCI, 0.05% EDTA, 10 mM KCI, 1 mM PMSF) and then sonicated. Cell lysates and culture media were electrophoresed on an SDS-10% acrylamide gel. The proteins were transblotted to nitrocellulose, and the blot was incubated with a rabbit antibody specific for PSA (P0798, Sigma Chemical Co., St. Louis, MO) for 4 hours at ambient temperature, washed, and then incubated with goat anti-rabbit phosphatase-labeled secondary antibody (AP, Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed according to the manufacture's instructions.

Generation of B-cell lines

[0049] Monkey autologous B lymphoblastoid cell lines (BLCL) were established by infecting $1x10^5$ freshly isolated PBMCs in 100 ml of RPMI 1640 supplemented with L-glutamine, gentamicin, and 10% FCS (Biofluids, Rockville, MD) with 100 ml supernatant from S594 cells (kindly provided by Dr. M. D. Miller, Harvard Medical School, New England Regional Primate Research Center, Southborough, MA), which contains the baboon herpesvirus *Herpes papio,* in a 96 well, flat-bottomed plate (Costar, Cambridge, MA). Following transformation, cells were expanded, and media changed once weekly.

Immunization of Monkeys

[0050] Twelve juvenile male rhesus monkeys (*Macaca mulatta*), ages 1 to 2 years, were assigned to three vaccination groups of four animals each. One animal from each group was prostatectomized. Animals were immunized 3 times on days 1, 29, and 57. Doses of either 1×10^7 or 1×10^8 PFU of rV-PSA were administered to 4 animals by skin scarification. V-Wyeth (1×10^8 PFU) was administered to 4 animals as controls. The animals were housed and maintained at the Toxicology Research Laboratory, University of Illinois at Chicago (TRL/UIC) in accordance with the guidelines of the National Cancer Institute Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services Publication NIH 85-23, revised 1985 by the FDA Center for Biologics Evaluation and Research Office of Biological Product Review, Division of Product Quality Control, Pathology and Primatology Laboratory, Bethesda, MD).

Toxicology

[0051] Physical examinations were performed on ketamine (Ketamine® HCl, 10 mg/kg I.M.) sedated animals. Rectal temperatures and weights were recorded for each monkey on a weekly basis. The vaccination site was observed and erythema and swelling were measured by caliper. Each animal was examined for regional lymphadenopathy, hepatomegaly, and splenomegaly. Any other gross abnormalities were also recorded.

[0052] Blood was obtained by venipuncture from the femoral vein of ketamine sedated animals before and after each immunization. A complete blood count, differential, hepatic and renal chemistry evaluation was performed on each monkey by TRL/UIC. Results were compared to normal primate values (Kantor *et al.,* 1992b). Circulating levels of PSA before and after immunization were analyzed by radioimmunoassay (Tandem™, Hybritech, San Diego, CA).

Measurement of Antibody Titers

[0053] Prior to each immunization and 2 weeks following each immunization, anti-PSA antibody was quantified by ELISA. Microtiter plates were coated with purified PSA (100 ng/well, Calbiochem, La Jolla, CA), ovalbumin (100 ng/well, Sigma), or 1×10^7 PFU/well UV-inactivated V-Wyeth in PBS. The plates were blocked with 2% BSA in PBS, dried, and stored at -20° C until used. The plates were incubated with serum diluted 1:5, as well as a monoclonal antibody for PSA (DAKO M750, Denmark) as a standard control, for 24 hours at 4° C. Plates were washed several times with PBS containing 1 % BSA, and incubated at 37° C for 45 min with horseradish peroxidase-conjugated goat anti-human IgG or IgM heavy chain specific antiserum (1:8000) (Southern Biotechnology Associates, Birmingham, AL) and antibody detected by HRP substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacture's instructions. The absorbance of each well was read at 405 nm using a Bio-Tek EL310 microplate ELISA reader (Winooski, VT).

Lymphoproliferative Assay

[0054] Autologous monkey BLCL were plated at a density of 3×10^6 cells/well in 24 well plates with 160 mg/well purified PSA (Fitzgerald, Concord, MA) or 160 mg/well ovalbumin (Sigma) at 37° C for 24 hours. Cells were then γ-irradiated (14000 rad), harvested, washed and suspended at a final concentration of 1×10^7/ml. Fresh monkey PBMCs from heparinized blood, 6 weeks to 7 months after the last immunization, were isolated on lymphocyte separation medium (Organon Teknika, West Chester, PA). Lymphoproliferative responses were evaluated by co-culturing 1.5×10^5 cells with 5×10^5 cells/well of autologous BLCL in 0.2 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum in flat-bottomed 96 well plates (Costar) for 5 days. PBMCs were cultured with 2×10^7 PFU/ml UV-inactivated V-Wyeth as a recall antigen or 2 mg/ml Con-A as positive controls. Cells were labeled for the final 12-18 h of the incubation with 1 mCi/well [^{3}H]thymidine (New England Nuclear, Wilmington, DE) and harvested with a PHD cell harvester (Cambridge Technology, Cambridge, MA). The incorporated radioactivity was measured by liquid scintillation counting (LS 6000IC; Beckman, Duarte, CA). The results from triplicate wells were averaged and are reported as mean $\pm$ SEM.

Results

Generation and Characterization of Recombinant Virus

[0055] The cDNA fragment encoding the open reading frame of human PSA was obtained by reverse transcriptase PCR using PSA specific oligonucleotide primers 5' TCTAGAAGCCCCAAGCTTACCACCTGCA 3' (SEQ. ID. NO.:1), 5' TCTAGATCAGGGGTTGGCCACGATGGTGTCCTTGATCCACT 3' (SEQ. ID. NO.:2), and ligated into the vaccinia virus transfer vector pT106. This vector contains a strong vaccinia virus early/late promoter (designated P40) upstream of the multiple cloning site to drive the synthesis of the inserted gene product. The ligation and orientation of the PSA DNA fragment, as well as promoter position were verified by PCR and sequencing. The chimeric vector construct was inserted into the vaccinia virus genome Hind III M site by homologous recombination as previously reported (Kaufman, et al., (1991)), and confirmed by Southern analysis probing with ^{32}P radiolabeled DNA corresponding to PSA sequences and vaccinia sequences in the Hind III M region (data not shown). The entire cDNA sequence of PSA in the vaccinia virus clone was shown to be nearly identical to the published sequences (Lundwall, et al., 1987).

[0056] Expression of recombinant protein was confirmed by western blot analysis of supernatant fluids and protein extracts from rV-PSA infected BSC-40 cells. These cells are routinely used for the evaluation of recombinant vaccinia products (Moss, et al., 1993). Incubation of cell supernatant blots from rV-PSA infected cells with rabbit anti-PSA antibody revealed a single immunoreactive polypeptide of approximately 33,000 daltons (Figure 1, lanes 2-4). Similarly, incubation of protein extract blots from rV-PSA infected cells revealed a single band of the same molecular weight (Figure 1, lanes 7-9). This is consistent with the predicted size of the PSA molecule (Armbruster, et al., 1993; Wang, et al., 1982). Cell supernatant blots (lane 1) or protein extract blots (lane 6) from cells infected with parental strain V-Wyeth remained negative for expression of PSA. These results thus demonstrate that a recombinant vaccinia virus can faithfully express the human PSA gene product.

Rhesus Monkey Model

[0057] The prostate gland of the rhesus monkey is structurally and functionally similar to the human prostate (Wakui, et al., 1992). At the molecular level, there is 94% homology between both the amino acid and nucleic acid sequences of rhesus PSA (Gauthier, et al., 1993) and human prostate specific antigen (Karr, et al., 1995; Lundwall, et al., 1987). Human PSA is essentially an autoantigen in the rhesus monkey.

Experimental Design

[0058] Table 1 delineates the protocol used in the immunization of 12 rhesus monkeys with either rV-PSA or the control V-Wyeth by skin scarification. Three groups of 4 animals were immunized with either rV-PSA at $1x10^7$ PFU/dose, rV-PSA at $1x10^8$PFU/dose, or V-Wyeth at 10^8PFU/dose 3 times at 4 week intervals. These doses were chosen to ascertain the maximum tolerated dose for safety as well as to obtain maximum humoral and cell-mediated responses to PSA.

[0059] The rhesus monkeys were divided into 3 groups: high dose V-Wyeth, low dose rV-PSA, and high dose rV-PSA. One animal in each group was surgically prostatectomized to parallel two situations with regard to potential therapy in humans: (a) prostate intact, with primary and/or metastatic disease; or (b) patients prostatectomized with prostate cancer metastatic deposits. The presence of an intact prostate gland could conceivably serve as an antigen 'sink', either inducing anergy through persistence of antigen, or masking immunological effects by sequestering reactive cells or antibodies.

Physical Consequence of Immunization

[0060] The area of the lesions induced by rV-PSA or V-Wyeth was analyzed 7 days following each inoculation. In general, more induration was seen after the first inoculation, compared to the second inoculation (Figure 2A). After the third inoculation, there was no swelling of the vaccination site. The duration of the lesion following each immunization was shorter after each inoculation (Figure 2B). Regional lymph node swelling following vaccination was greater in most monkeys following the first immunization, compared to the second, or third immunization (Figure 2C). In general, no differences were seen in these parameters with the use of rV-PSA or V-Wyeth. Monkeys receiving V-Wyeth were compared with those receiving rV-PSA with respect to constitutional symptoms. Mild temperature elevations were seen in all animals following vaccination. There was no evidence of weight loss, hepatomegaly or splenomegaly in any of the animals, and there was no differences between V-Wyeth or rV-PSA treated animals (data not shown). Animals were tested for complete blood count, differential, and hepatic and renal chemistries. Complete blood counts remained within normal limits throughout the study in both V-Wyeth and rV-PSA immunized animals (Table 2). Hepatic and renal functions were assessed prior to immunization and 12 weeks following primary immunization (Table 3). Parameters analyzed

included alkaline phosphatase, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and creatine and creatine kinase levels. There was no significant difference between animals receiving V-Wyeth or rV-PSA. There was no detectable PSA in the circulation of any of these monkeys after any immunization (detection limit was 0.1 ng/ml). At this time, which is 54 weeks post all immunizations, no toxicities were observed in monkeys of any of the groups, including those which were prostatectomized.

PSA Specific Humoral Responses

[0061] As indicated in Table 1, monkeys 1-4 were administered V-Wyeth while monkeys 5-12 were administered rV-PSA. Sera from each of these monkeys were analyzed by ELISA for immunoreactivity to PSA or UV-inactivated V-Wyeth, and ovalbumin as control antigen. Sera obtained from monkeys prior to vaccination were negative for reactivity to PSA (Table 4, PI). Fifteen days following primary immunization, monkeys in both the $1x10^8$ and $1x10^7$ dose rV-PSA groups developed low titer IgM antibodies specific for PSA (titers were determined at a 1:5 serum dilution). Although other isotopes of antibody were analyzed (IgG, IgA, IgM), only IgM was induced by rV-PSA throughout the observation period of 270 days. The antibody titers decreased over the 4 weeks prior to the next inoculation. Prior to the second vaccination on day 29, 3 of 4 animals in the $1x10^7$rV-PSA group remained positive for PSA antibody, while 4 of 4 animals remained positive in the $1x10^8$rV-PSA group. Anti-PSA antibody titers increased after the second vaccination on day 29, but remained static after the third vaccination on day 57. By 270 days after the primary immunization, all animals were negative for PSA IgM antibody. Monkeys remained negative for IgG specific for PSA throughout the observation period (data nor shown). There was no correlation between rV-PSA dose and anti-PSA IgM titer, nor was there any apparent effect of prostatectomy. All monkey sera were negative for IgG or IgM to ovalbumin at all time points; as a positive control, however, the IgG titer in all three treatment groups to vaccinia virus was greater than 1:2000 as early as 29 days after the primary immunization (data not shown).

[0062] In general, vaccinia virus is a weak human pathogen (Paoletti *et al.,* 1993). Following vaccination, local erythema, induration, low-grade fever, and regional lymphadenopathy are common. The virus replicates in the epidermal cells of the skin and the virus is usually cleared within 14 days. All monkeys, whether given V-Wyeth or rV-PSA, exhibited the usual low grade constitutional symptoms of a vaccinia virus infection (Figure 2). There was no evidence of any adverse effects as indicated by changes in blood counts, differentials, hepatic and renal chemistries (Tables 2-3). The monkeys appeared healthy, without any physical signs of toxicity, throughout the 54 weeks of observation.

[0063] Although the rV-PSA construct was unable to elicit an anti-PSA IgG response, PSA specific IgM responses were noted in all rV-PSA immunized monkeys regardless of dose level (Table 4). These antibody responses were of low titer, short lived and could not be boosted, indicating induction of a primary response but not memory B-cells or affinity maturation.

PSA Specific Lymphoproliferative Assay

[0064] PSA specific T-cell responses in monkeys immunized with rV-PSA or V-Wyeth were analyzed using a lymphoproliferative assay. As seen in Table 5, the PBMCs from all monkeys analyzed responded, regardless of whether they received rV-PSA or V-Wyeth, to the lymphocyte mitogen concanavalin-A, as well as with the recall antigen UV-inactivated V-Wyeth. Differential responses to PSA *versus* medium alone or ovalbumin were seen in 1 animal (number 6) in the 1×10^7 PFU rV-PSA group. All PBMCs from animals in the 1×10^8 PFU rV-PSA group, however, responded to PSA in this assay. This experiment was repeated 5 times with similar results and data shown in Table 5 is from PBMCs isolated from monkeys 270 days after the primary immunization. No differences in PSA specific T-cell responses were seen in the prostatectomized monkeys.

[0065] To investigate cell mediated responses to the administration of rV-PSA, lymphoproliferative assays were performed using PBMCs from animals receiving the recombinant vaccine. One of four monkeys receiving the lower dose of rV-PSA (1×10^7 PFU) and four of four receiving the higher dose (1×10^8 PFU) maintained specific T-cell responses to PSA protein up to 270 days following primary immunization as indicated by the lymphoproliferative assay (Table 5). Prostatectomy appeared to have no effect on either the humoral or cellular responses of monkeys receiving rV-PSA. Evidence of PSA specific T-cell responses in monkeys lacking mature antibody isotopes could be due to two distinct events following vaccination with rV-PSA: a T-cell independent event, leading to IgM production, and a T-cell dependent event, leading to specific lymphoproliferative responses.

Table 1 Inoculation protocol of rhesus monkeys with the PSA recombinant and wild-type vaccinia virus

Monkey	Prostate	Immunogen	Dose* (PFU)
1	Yes	V-Wyeth	$1x10^8$

(continued)

Monkey	Prostate	Immunogen	Dose* (PFU)
2	Yes	V-Wyeth	$1x10^8$
3	Yes	V-Wyeth	$1x10^8$
4	No	V-Wyeth	$1x10^8$
5	Yes	rV-PSA	$1x10^7$
6	Yes	rV-PSA	$1x10^7$
7	Yes	rV-PSA	$1x10^7$
8	No	rV-PSA	$1x10^7$
9	Yes	rV-PSA	$1x10^8$
10	Yes	rV-PSA	$1x10^8$
11	Yes	rV-PSA	$1x10^8$
12	No	rV-PSA	$1x10^8$

* All animals received 3 immunizations at 4 week intervals.

Table 2

Mean WBC count, hematocrit, and differential count in rhesus monkeys receiving recombinant or wild-type vaccin

		V-Wyeth (*n*=4)		rV-PSA (*n*=8)	
Test	Normal ranges	Before immunization[a]	After immunization[b]	Before immunization	After immunization
WBC	7-15 x 10^3	5.0 ± 0.8	5.1 ± 0.5	5.2 ± 0.7	5.8 ± 0.9
Hematocrit (vol. %)	33-43	37.4 ± 0.2	37.0 ± 0.1	37.8 ± 0.4	37.0 ± 0.5
Lymphocytes	1-7 x 10^3	2.8 ± 0.7	3.9 ± 0.5	2.2 ± 0.4	3.5 ± 0.8
SEGS[c] (%)	3-69	2.0 ± 0.2	0.78 ± 0.2	2.9 ± 0.6	1.9 ± 0.3
Monocyles (%)	0-8	0.1 ± 0.05	0.2 ± 0.04	0.1 ± 0.04	0.2 ± 0.50
Eosinophils (%)	0-8	0.1 ± 0.02	0.2 ± 0.10	0.1 ± 0.03	0.1 ± 0.02

[a] 1 week prior to primary immunization
[b] 12 weeks following primary immunization
[c] Segmented lymphocytes

Table 3 Mean serum chemistry values in rhesus monkeys receiving recombinant or wild-type vaccine

		V-Wyeth (*n*=4)		rV-PSA (*n*=8)	
Test	Normal ranges	Before immunization[a]	After immunicaion[b]	Before immunization	After immunization
ALKP[c] (u/l)	200-800	451 ± 48	610 ± 33	339 ±74	454 ± 47
BUN[d] (mg/dl)	12-30	19.0 ± 3.0	17.8 ± 0.9	17.1 ± 0.6	20.5 ± 1.0
ALT[e] (u/l)	20-60	25.2 ± 1.9	22.8 ± 1.0	28.9± 5.3	25.8 ± 1.6
AST[f] (u/l)	40-80	37.8 ± 2.3	31.8 ± 4.4	37.9 ± 3.6	31.9 ± 2.4
LDH[g] (u/l)	200-500	194 ± 20	212 ± 21	236 ± 41	194 ± 13
Creatine (mg/dl)	0.5-1.0	0.9 ± 0.10	0.8 ± 0.03	0.8 ± 0.05	0.8 ± 0.02

(continued)

Test	Normal ranges	V-Wyeth (*n*=4) Before immunization[a]	V-Wyeth (*n*=4) After immunicaion[b]	rV-PSA (*n*=8) Before immunization	rV-PSA (*n*=8) After immunization
Creatine Kinase (u/l)	500-2000	662 ± 112	466± 119	498 ± 120	563 ± 81

[a] 1 week prior to primary immunization
[b] 12 weeks following primary immunization
[c] Alkaline phosphatase
[d] Blood urea nitrogen
[e] Alanine aminotransferase
[f] Aspartate aminotransferase
[g] Lactate dehydrogenase

Table 4 Primate IgM[a] Response to Inoculation with rV-PSA

Monkey	Immunogen	Dose (PFU)	Days Post Immunication[b] PI[d]	15	29[e]	43	57[e]	71	270
1	V-Wyeth	1 x 10^8	ND[f]	ND	ND	ND	ND	ND	ND
2	V-Wyeth	1 x 10^8	ND	ND	ND	ND	ND	ND	ND
3	V-Wyeth	1 x 10^8	ND	ND	ND	ND	ND	ND	ND
4[c]	V-Wyeth	1 x 10^8	ND	ND	ND	ND	ND	ND	ND
5	rV-PSA	1 x 10^7	ND	>40	5	20	>40	>40	ND
6	rV-PSA	1 x 10^7	ND	>40	ND	ND	20	20	ND
7	rV-PSA	1 x 10^7	ND	>40	5	20	20	20	ND
8[c]	rV-PSA	1 x 10^7	ND	>40	5	10	>40	>40	ND
9	rV-PSA	1 x 10^8	ND	20	5	20	10	10	ND
10	rV-PSA	1 x 10^8	ND	20	5	40	>40	NT[g]	ND
11	rV-PSA	1 x 10^8	ND	>40	>40	>40	>40	>40	ND
12[c]	rV-PSA	1 x 10^8	ND	>40	20	40	20	20	ND

[a] All monkey seras were negative for IgG to PSA at all time points; All seras were positive for IgG to vaccinia virus (> 1:2000) at day 71.
[b] Monkeys received vaccinations on days 1, 29, and 57. Sera (1:5) was tested by ELISA. Titers were calculated using an O.D. of 0.4.
[c] Animal was prostatectomized.
[d] PI, Pre-immune.
[e] Animals bled before boosting.
[f] ND, not detectable; limit of detection wns <1:5 dilution.
[g] NT, not tested.

Table 5 PSA Specific Lymphoproliferative T-cell Responses of Rhesus PBMCs 270 Days Following Inoculation with rV-PSA

Monkey	Immunogen	Dose (PFU)	Antigen[a] Medium	Con A	Oval	UV-Wyeth	PSA[d]
1	V-Wyeth	1 x 10^8	397	65701	376	24785	414
2[b]	V-Wyeth	1 x 10^8	NT	NT	NT	NT	NT
3	V-Wyeth	1 x 10^8	450	84860	522	18859	413
4[c]	V-Wyeth	1 x 10^8	532	107840	553	16571	387
5	rV-PSA	1 x 10^7	412	85276	408	6040	539
6	rV-PSA	1 x 10^7	401	96398	404	7776	3,134

(continued)

Monkey	Immunogen	Dose (PFU)	Antigen[a] Medium	Con A	Oval	UV-Wyeth	PSA[d]
7	rV-PSA	1 x 10^7	417	90801	522	10908	434
8[c]	rV-PSA	1 x 10^7	1069	99216	744	15346	484
9	rV-PSA	1 x 10^8	384	106248	386	14499	10,635
10	rV-PSA	1 x 10^8	432	92263	404	19872	18,561
11	rV-PSA	1 x 10^8	411	94055	1063	5124	16,245
12[c]	rV-PSA	1 x 10^8	420	124896	392	11944	12,945

[a] Antigen concentrations were: Con a (2 μg/ml); Ovalbamin (100 μg/ml); UV-Wyeth (2x 19^7 pfu/ml); and PSA (100 μg/ml). Each value represents a mean CPM of triplicate samples. Standard deviation never exceeded 10%.
[b] NT, Not Tested. B-cells were not transformed for this animal.
[c] Animal was prostatectomized.
[d] Values in bold are significant when compared to their respective medium control values ($p < 0.001$).

EXAMPLE II

Identification Of Potential Prostate Specific Antigen (PSA) Specific T Cell Epitopes

[0066] Since the entire amino acid sequence of human PSA is known and human class 1 HLA A2 consensus motifs have been described, studies were undertaken to identify a series of peptides that would potentially bind class 1 A2 molecules. A2 was chosen since it is the most common HLA class 1 molecule being represented in approximately 50% of North American Caucasians and 34% of African Americans. The peptide sequence of PSA was thus examined for matches to the consensus motifs for HLA A2 binding peptides. Peptides were only selected if their sequence diverged sufficiently from the PSA-related human glandular kallikrein (HGK) gene and pancreatic kallikrein antigen (PKA) sequences.

[0067] The amino acid sequence of human PSA was scanned using a predictive algorithm that combines a search for anchor residues with numerical assignments to all residues at all positions. The T2 cell binding assay was then used to determine which peptides bound human HLA A2 molecules. As can be seen in Table 6, PSA peptides 141-150, 154-163 and 146-154 scored positive in this assay (Nijman, H. W., et al., Eur. J. Immunol. 23:1215-1219, 1993). Table 7 gives the amino acid sequence of these peptides and compares them to corresponding sequences of HGK and PKA.

Table 6

PSA peptide binding assay		
Antigen		MAb A2, 69
None		127.25[a]
PSA 141-150	230.34	
PSA 146-154	223.97	
PSA 154-163	182.30	

Peptides were used at a concentration of 50μg/ml [a]Mean channel fluorescent intensity.
CIRA2 cell line was used as positive control for anti-A2 staining [99.4 (241.15)].

Table 7

PSA peptide amino acid sequence

PSA	141-150	F	L	T	P	K	K	L	Q	C	V
HGK		-	-	R	-	R	S	-	-	-	-
PKA		-	S	F	-	D	D	-	-	-	-

(continued)

PSA peptide amino acid sequence											
PSA	146-154	K	L	Q	C	V	D	L	H	V	
HGK		S	-	-	-	-	S	-	-	L	
PKA		D	-	-	-	-	-	-	K	I	
PSA	154-163	V	I	S	N	D	V	C	A	Q	V
HGK		L	L	-	-	-	M	-	-	R	A
PKA		I	L	P	-	-	E	-	E	K	A

EXAMPLE III

Establishment of Human T Cell Lines Cytolytic For Human Tumor Cells Expressing PSA

[0068] PBMC from normal healthy donors expressing the HLA A2 class 1 allele were used in an attempt to determine if PSA specific peptides are immunogenic for humans. Peptides 141-150 and 154-163 were used in this study. The methodology used for the establishment of these cell lines involves pulsing of PBMC with peptide and IL-2 as previously described (*Tsang, K. Y.*, *et al. JNCI, in press* and in U.S. Application Serial No. 08/396,385, the disclosure of which is herein incorporated by reference). T cell lines were able to be established from 5/6 normal donors using PSA peptide 141-150 and from 6/6 normal donors using PSA peptide 154-163. Moreover, PBMC were obtained from two prostate cancer patients. T cell lines were established from these PBMC cultures using peptide 154-163.

[0069] Some of these T cell lines have been phenotyped. As seen in Table 8, one cell line designated T-866, which was derived from pulsing with peptide 141-150, contains appreciable amounts of CD4+/CD8+ double positive cells and another cell line, designated T-1538, derived from pulsing with peptide 154-163, shows a similar phenotype.

[0070] Four of the T cell lines derived from three different individuals were then assayed for their ability to lyse human cells (Table 9). As seen in Table 9, the T cell line designated T-866, derived from peptide 141-150, was able to lyse T2 cells when pulsed with the appropriate peptide (141-150). No lysis was seen using the PSA negative human colon cancer cell line COLO-205. While 80% lysis was seen using the LNCAP PSA containing human prostate cancer cell line. When employing the NK target K562, which measures non-specific lysis due to NK cell activity, only 23% lysis was obtained. Similar results were seen employing a different T cell line obtained from the same patient which was derived from pulsing with PSA peptide 154-163. Two additional T cell lines which were derived from peptide 154-163 were also analyzed. One was from a normal donor (T-1538) and one was from a prostate cancer patient (T-PC2). As can be seen in Table 9, employing both of these T cell lines, enhanced lysis was seen when the T2 cell line was pulsed with the 154-163 peptide and enhanced lysis was seen when employing the PSA expressing prostate specific cell line LNCAP, as compared to COLO-205 or K562. These studies demonstrate that T cell lines can be established using the peptides and protocols generated here which have the ability to lyse PSA expressing human prostate carcinoma cells.

Table 8

Flow cytometry analysis of PSA peptide specific T cells						
T-cell Line	PSA Peptide	CD3	CD4	CD8	CD4/CD8	CD56
T-866	141-150	96	35	6.5	59	0
T-1538	154-163	94	5.2	32	62	0

Table 9

Cytotoxic effects of PSA peptide specific T cells % specific release (lysis)						
T-cell Line	PSA Peptide	T2	T2+ peptide	LNCAP	K562	COLO-205
T-866	141-150	10[a]	40	80	23	7
T-866	154-160	16	35	60	22	10
T-1538	154-160	10	40	29	3	10

(continued)

Cytotoxic effects of PSA peptide specific T cells % specific release (lysis)						
T-cell Line	PSA Peptide	T2	T2+ peptide	LNCAP	K562	COLO-205
T-PC2	154-160	15	35	35	2	8
[a]Percent of ^{111} In specific release 24 hour cytotoxic assay (E:T ratio, 25:1) (SD < 2.5%)						

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SEQUENCE LISTING

[0072]

(1) GENERAL INFORMATION:

(i) APPLICANT: THERION BIOLOGICS CORPORATION, ET AL.

(ii) TITLE OF INVENTION: GENERATION OF IMMUNE RESPONSES TO PROSTATE-SPECIFIC ANTIGEN (PSA)

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: SEWALL P. BRONSTEIN; DIKE, BRONSTEIN, ROBERTS & CUSHMAN
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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 26 JUNE 1996
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/500,306
(B) FILING DATE: 10 JULY 1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: RESNICK, DAVID S.
(B) REGISTRATION NUMBER: 34,235
(C) REFERENCE/DOCKET NUMBER: 45394-PCT

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(A) TELEPHONE: (617) 523-3400
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
TCTAGAAGCC CCAAGCTTAC CACCTGCA 28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
TCTAGATCAG GGGTTGGCCA CGATGGTGTC CTTGATCCAC T 41

[0073] This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the scope of the invention as set forth in the claims.

Claims

1. Pharmaceutical compositions for use In stimulating an antl-PSA immune response in a man, comprising:

a first composition comprising a pharmaceutical carrier and a recombinant first pox virus vector having at least one insertion site containing a DNA segment encoding essentially autoantigenic prostate specific antigen (PSA) or a cytotoxic T-cell eliciting epitope thereof operably linked to a promoter and capable of expression in the man to stimulate an immune response to the PSA or epitope on introduction of the first pox virus vector into the man, wherein the first pox virus is fowlpox, canary pox or pigeon pox; and
a second composition, to be contacted with the man at at least one periodic time interval after the first composition, comprising a pharmaceutical carrier and a recombinant second pox virus vector having at least one insertion site containing a DNA segment encoding essentially autoantigenic PSA or a cytotoxic T-cell eliciting epitope thereof operably linked to a promoter and capable of expression in the man to stimulate an immune response to the PSA or epitope on introduction of the second pox virus vector into the man, wherein the second pox virus is fowlpox, canary pox or pigeon pox and is different from the first pox virus.

2. Pharmaceutical compositions for use in stimulating an anti-PSA immune response in a man, comprising:

a first composition comprising a pharmaceutical carrier and a recombinant pox virus vector having at least one Insertion site containing a DNA segment encoding essentially autoantigenic PSA or a cytotoxic T-cell eliciting epitope thereof operably linked to a promoter and capable of expression in the man to stimulate an immune response to the PSA or epitope on introduction of the pox virus vector into the man, wherein the pox virus is fowlpox, canary pox or pigeon pox; and
a second composition, to be contacted with the man at at least one periodic time interval after the first composition, comprising a pharmaceutical carrier and essentially autoantigenic PSA or a cytotoxic T-cell eliciting epitope thereof.

3. Pharmaceutical compositions for use in stimulating an anti-PSA immune response in a man, comprising:

a first composition comprising a pharmaceutical carrier and essentially autoantigenic PSA or a cytotoxic T-cell eliciting epitope thereof; and
a second composition, to be contacted with the man at at least one periodic time interval after the first composition, comprising a pharmaceutical carrier and a recombinant pox virus vector having at least one insertion site containing a DNA segment encoding essentially autoantigenic PSA or a cytotoxic T-cell eliciting epitope thereof operably linked to a promoter and capable of expression in the man to stimulate an immune response to the PSA or epitope on introduction of the pox virus vector into the man, wherein the pox virus is fowlpox, canary pox or pigeon pox.

4. Pharmaceutical compositions as claimed in claim 2, wherein the PSA or T-cell eliciting epitope thereof is formulated with an adjuvant or is in a liposomal formulation.

5. Pharmaceutical compositions as claimed in claim 4, wherein the adjuvant is selected from the group consisting of RIBI Detox, QS21 and incomplete Freund's adjuvant.

6. Pharmaceutical compositions as claimed in claim 4, wherein the adjuvant is a biologic adjuvant.

7. Pharmaceutical compositions as claimed in claim 6, wherein the biologic adjuvant is selected from the group consisting of a cytokine, e.g. interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-12) and costimulatory molecules, e.g. B7.1, B7.2.

8. Pharmaceutical compositions as claimed in claim 1, wherein the recombinant second pox virus vector has an inserted gene encoding a biologic adjuvant selected from the group consisting of a cytokine, e.g. interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 (IL-12) and costimulatory molecules, e.g. B7.1, B7.2, whereby the biologic adjuvant is co-expressed on administration to the man.

Patentansprüche

1. Pharmazeutische Zusammensetzungen zur Verwendung zur Stimulierung einer Anti-PSA-Immunantwort beim Menschen umfassend:

   eine erste Zusammensetzung enthaltend einen pharmazeutischen Träger und einen rekombinanten ersten Pockenvirusvektor mit mindestens einer Insertionsstelle, die ein DNA-Segment enthält, das im Wesentlichen autoantigenes prostataspezifisches Antigen (PSA) oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon enthält operativ verbunden mit einem Promotor und fähig zur Expression im Menschen, um eine Immunantwort gegen das PSA oder das Epitop bei Einführung des ersten Pockenvirusvektors in den Menschen zu stimulieren, wobei das erste Pokkenvirus Geflügelpest-, Kanarienpocken- oder Taubenpockenvirus ist und eine zweite Zusammensetzung, die während mindestens eines periodischen Zeitintervalls nach der ersten Zusammensetzung mit dem Menschen in Kontakt gebracht wird, die einen pharmazeutischen Träger und einen rekombinanten zweiten Pockenvirusvektor enthält mit mindestens einer Insertionsstelle, die ein DNA-Segment enthält, das im Wesentlichen autoantigenes PSA oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon codiert, operativ verbunden mit einem Promotor und fähig zur Expression im Menschen, um eine Immunantwort gegen PSA oder ein Epitop davon zu stimulieren bei Einführung des zweiten Pockenvirusvektors in den Menschen, wobei das zweite Pokkenvirus Geflügelpest-, Kanarienvogelpocken- oder Taubenpockenvirus ist und sich von dem ersten Pockenvirus unterscheidet.

2. Pharmazeutische Zusammensetzungen zur Verwendung zur Stimulierung einer Anti-PSA-Immunantwort bei einem Menschen umfassend:

   eine erste Zusammensetzung enthaltend einen pharmazeutischen Träger und einen rekombinanten Pockenvirusvektor mit mindestens einer Insertionsstelle, die ein DNA-Segment enthält, das im Wesentlichen autoantigenes PSA oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon codiert, operativ verbunden mit einem Promotor und fähig zur Expression im Menschen, um eine Immunantwort gegen PSA oder ein Epitop zu stimulieren bei Einführung des Pockenvirusvektors in den Menschen, wobei das Pockenvirus Geflügelpest-, Kanarienpocken- oder Taubenpokkenvirus ist und
   eine zweite Zusammensetzung, die mit dem Menschen in mindestens einem periodischen Zeitintervall nach der ersten Zusammensetzung in Kontakt gebracht wird, die einen pharmazeutischen Träger und im Wesentlichen autoantigenes PSA oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon enthält.

3. Pharmazeutische Zusammensetzungen zur Verwendung zur Stimulierung einer Anti-PSA-Immunantwort beim Menschen umfassend:

   eine erste Zusammensetzung enthaltend einen pharmazeutischen Träger und im Wesentlichen autoantigenes PSA oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon und
   eine zweite Zusammensetzung, die mit dem Menschen in mindestens einem periodischen Zeitintervall nach der ersten Zusammensetzung in Kontakt gebracht wird, enthaltend einen pharmazeutischen Träger und einen rekombinanten Pockenvirusvektor mit mindestens einer Insertionsstelle, die ein DNA-Segment enthält, das im Wesentlichen autoantigenes PSA oder ein cytotoxisches eine T-Zellantwort hervorrufendes Epitop davon codiert, operativ verbunden mit einem Promotor und fähig zur Expression im Menschen, um eine Immunantwort gegen PSA oder ein Epitop zu stimulieren bei Einführung des Pockenvirusvektors in den Menschen, wobei das Pockenvirus Geflügelpest-, Kanarienvogelpocken- oder Taubenpockenvirus ist.

4. Pharmazeutische Zusammensetzungen nach Anspruch 2, wobei das PSA oder das eine T-Zellantwort hervorrufende Epitop davon mit einem Adjuvans formuliert wird oder sich in einer liposomalen Formulierung befindet.

5. Pharmazeutische Zusammensetzungen nach Anspruch 4, wobei das Adjuvans ausgewählt ist aus der Gruppe bestehend aus RIBI Detox, QS21 und inkomplettem Freund's Adjuvans.

6. Pharmazeutische Zusammensetzungen nach Anspruch 4, wobei das Adjuvans ein biologisches Adjuvans ist.

7. Pharmazeutische Zusammensetzungen nach Anspruch 6, wobei das biologische Adjuvans ausgewählt ist aus der Gruppe bestehend aus einem Cytokin, z.B. Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-12 (IL-12) und co-stimulatorischen Molekülen, z.B. B7.1, B7.2.

8. Pharmazeutische Zusammensetzungen nach Anspruch 1, wobei der rekombinante zweite Pockenvirusvektor ein Gen insertiert hat, das ein biologisches Adjuvans codiert ausgewählt aus der Gruppe bestehend aus einem Cytokin, z.B. Interleukin-2 (IL-2), Interleukin-6 (IL-6), Interleukin-12 (IL-12) und costimulatorischen Molekülen (z.B. B7.1, B7.2), wobei das biologische Adjuvans bei Verabreichung im Menschen coexprimiert wird.

Revendications

1. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme, comprenant :

   une première composition comprenant un véhicule pharmaceutique et un vecteur recombinant d'un premier virus de variole ayant au moins un site d'insertion comprenant un segment d'ADN codant essentiellement l'antigène autoantigénique spécifique de la prostate (PSA) ou son épitope cytotoxique déclenchant une cellule T, relié fonctionnellement à un promoteur et capable d'expression chez l'homme pour stimuler une réponse immunitaire au PSA ou à l'épitope par l'introduction du vecteur du premier virus de variole chez l'homme, le premier virus de variole étant la variole de la poule, la variole du canari ou la variole du pigeon ;
   une seconde composition à mettre en contact avec l'homme à au moins un certain intervalle de temps après la première composition, comprenant un véhicule pharmaceutique et un vecteur recombinant d'un deuxième virus de variole ayant au moins un site d'insertion contenant un segment d'ADN codant essentiellement du PSA autoantigénique ou son épitope cytotoxique déclenchant une cellule T, relié fonctionnellement à un promoteur et capable d'expression chez l'homme, pour stimuler une réponse immunitaire au PSA ou à l'épitope par introduction du vecteur du deuxième virus de variole chez l'homme, le deuxième virus de variole étant la variole de la poule, la variole du canari ou la variole du pigeon et étant différent du premier virus de variole.

2. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme comprenant :

   une première composition comprenant un véhicule pharmaceutique et un vecteur recombinant d'un premier virus de variole ayant au moins un site d'insertion comprenant un segment d'ADN codant essentiellement le PSA autoantigénique ou son épitope cytotoxique déclenchant une cellule T, relié fonctionnellement à un promoteur et capable d'expression chez l'homme, pour stimuler une réponse immunitaire au PSA ou à l'épitope par l'introduction du vecteur du premier virus de variole chez l'homme, le premier virus de variole étant la variole de la poule, la variole du canari ou la variole du pigeon ;
   une deuxième composition à mettre en contact avec l'homme au moins à un intervalle de temps après la première composition, comprenant un véhicule pharmaceutique et du PSA essentiellement autoantigénique ou son épitope cytotoxique déclenchant une cellule T.

3. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme, comprenant :

   une première composition comprenant un véhicule pharmaceutique et du PSA essentiellement autoantigénique ou son épitope cyclotoxique déclenchant une cellule T ; et
   une seconde composition à mettre en contact avec l'homme à au moins un certain intervalle de temps après la première composition, comprenant un véhicule pharmaceutique et un vecteur recombinant d'un deuxième virus de variole ayant au moins un site d'insertion contenant un segment d'ADN codant essentiellement du PSA autoantigénique ou son épitope cytotoxique déclenchant une cellule T, relié fonctionnellement à un promoteur et capable d'expression chez l'homme pour stimuler une réponse immunitaire au PSA ou à l'épitope par introduction du vecteur du deuxième virus de variole chez l'homme, le deuxième virus de variole étant la variole de

la poule, la variole du canari ou la variole du pigeon.

4. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme suivant la revendication 2, dans lesquelles le PSA ou son épitope déclenchant une cellule T est formulé avec un adjuvant ou dans une formulation liposomique.

5. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme suivant la revendication 4, dans lesquelles l'adjuvant est choisi dans le groupe consistant en RIBI Detox, QS21 et l'adjuvant de Freund incomplet.

6. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme suivant la revendication 4, dans lesquelles l'adjuvant est un adjuvant biologique.

7. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme suivant la revendication 6, dans lesquelles l'adjuvant biologique est choisi dans le groupe consistant en une cytokine par exemple l'interleukine-2 (IL-2), l'interleukine-6 (IL-6), l'interleukine-12 (IL-12) et des molécules costimulantes, par exemple B7.1, B7.2.

8. Compositions pharmaceutiques à utiliser dans la stimulation d'une réponse immunitaire anti-PSA chez l'homme suivant la revendication 1, dans lesquelles le vecteur recombinant du deuxième virus de la variole a un gène inséré codant un adjuvant biologique choisi dans le groupe consistant en une cytokine, par exemple l'interleukine-2 (IL-2), l'interleukine-6 (IL-6), l'interleukine-12 (IL-12) et des molécules costimulantes, par exemple B7.1, B7.2, l'adjuvant biologique étant coexprimé par administration à l'homme.

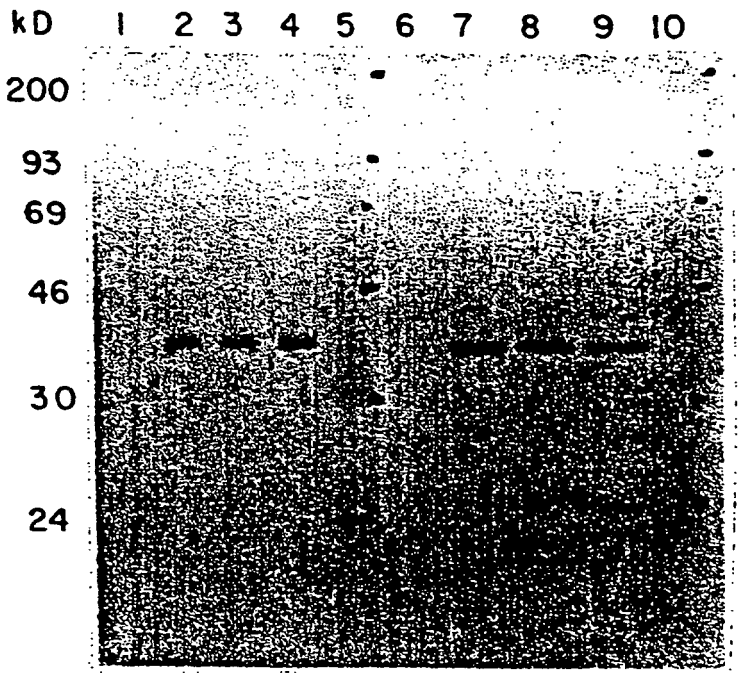


FIG. 1

FIG.2A

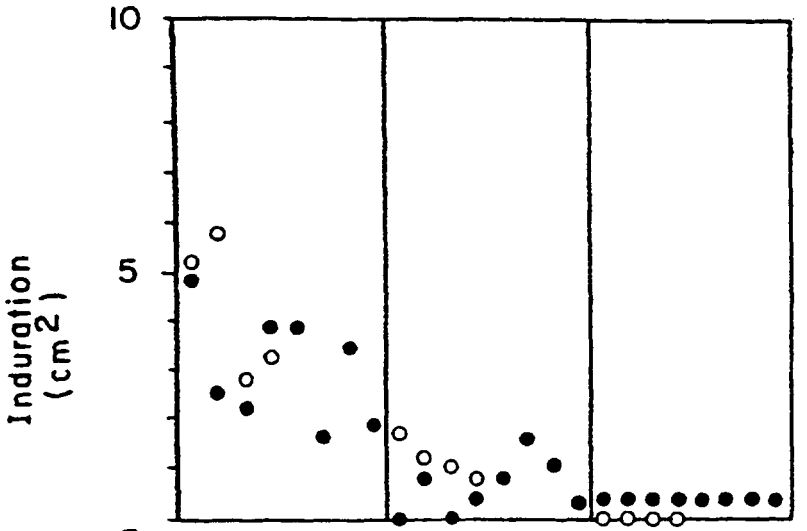


FIG.2B

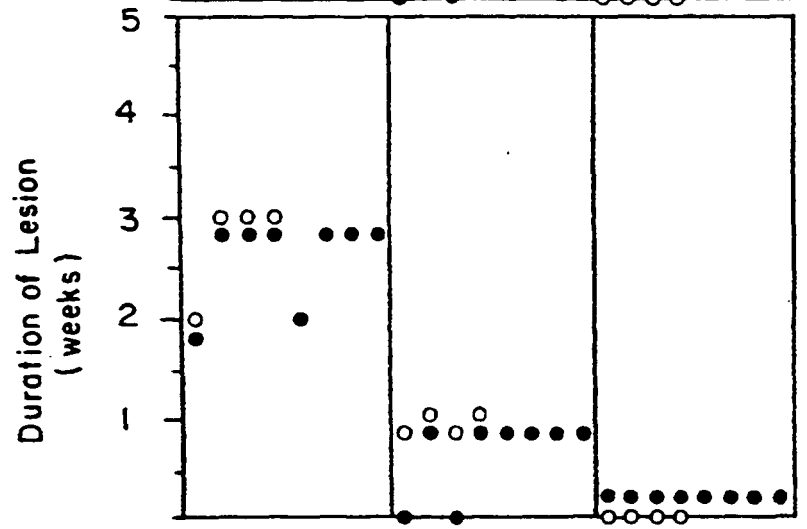
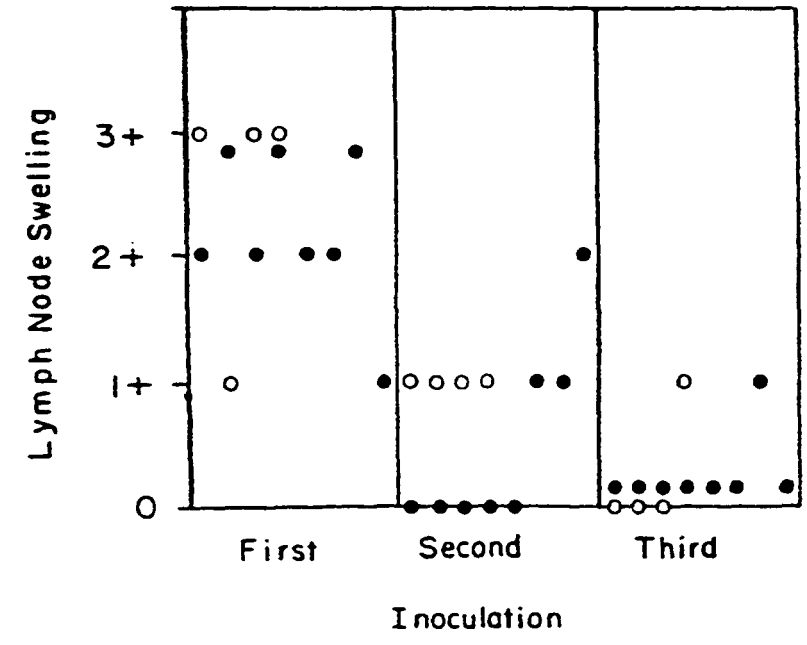


FIG.2C



REFERENCES CITED IN THE DESCRIPTION

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