A method of enhancing an immune response comprising the step of exposing an immune cell to an antigen presenting cell having cytokine adsorbed.
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DEVELOPMENT

Methods and Products for Enhancing Immune Responses

Field of the Invention

The present invention relates to cytokine related products and methods useful for enhancing immune responses.

Background of the Invention

Recovery from infection and specific protection against reinfection depend upon an organism's immune system and in particular the activity of lymphocytes and cytokines. Inactive B and T lymphocytes are specifically activated when their clonally distributed receptors are exposed and bound to a corresponding specific antigen. Cytokines are produced in abundance by a subset of T lymphocytes after the lymphocytes are activated and facilitate the two events that comprise activation of lymphocytes: (1) clonal expansion so that specific lymphocytes increase in frequency; and (2) differentiation so that mechanisms to effect immunity are manufactured by the cell.

Interleukin 2 (IL-2) is a cytokine that greatly enhances immune responses and that markedly stimulates cell proliferation and differentiation of a variety of different immune cells including T cells, B cells, macrophages, monocytes, natural killer cells, and granulocytes (K. Smith, Science, 240:11691 (1988)). The capacity for IL-2 to induce proliferative signals in T lymphocytes is the property that accounts for the cytokine's discovery (S. Gillis and K. Smith, Nature, 268:154 (1977); and D. Morgan et al., Science, 193:1007 (1976)). Conditioned supernatants from lectin-stimulated peripheral blood mononuclear cells were shown to enhance the proliferation of T cells in culture. Later the substance most responsible for this activity was defined as IL-2. The identity

A number of different expression systems for IL-2 have been developed (S. Rosenberg et al., Science, 223:1412 (1984); G. Smith et al., Proc Natl. Acad. Sci. USA, 82:8404 (1985); D. Williams et al., Nucl. Acids Res. 16:10453 (1988); E. Bender et al., Gene, 86:227 (1990); T. Williams et al., Anal. Biochem., 176:28 (1989); B. Cullen, DNA, 7:645 (1988); and A. Gulino et al., Biochimica et Biophysica Acta, 1087:7 (1990)). IL-2 has been produced in large quantities in bacteria, yeast, insect cells, and eukaryotic cells. Because IL-2 was produced as a purified homogeneous product, the activities seen could be clearly assigned to the molecule and the potent activity of IL-2 in inducing T cell proliferation was confirmed. It was also observed that the cytokine strongly promoted the growth of natural killer cells and markedly enhanced the level of cytotoxicity that both natural killer cells and T lymphocytes exhibited (G. Trinchieri et al., J. Exp. Med., 160:1147 (1984); E. Grimm et al., J. Exp. Med., 155:1823 (1982); B. Vose and G. Bonnard, J. Immunol., 130:687 (1983); R. Dempsey et al., J. Immunol., 129:2504 (1982); F. Erard et al., J. Exp. Med., 160:584 (1984); and S. Wee et al., J. Immunol., 134:310 (1985)).

Vectors directing the expression of IL-2 have been utilized in a murine model for solid tumors. Cultured cell lines that produce tumors upon inoculation into naive mice have been transfected with vectors that encode for the expression of IL-2 and given to mice subcutaneously. The mice injected with unmodified cells produced tumors whereas the IL-2 producing tumor cells did not result in any solid tumors. Animals that received the IL-2 producing tumor cells were able to reject unaltered tumor cells that were subsequently injected and no IL-2
dependent toxicity was observed (B. Gansbacher et al., J. Exp. Med., 172:1217 (1990)).

The capacity of IL-2 to enhance cytotoxicity has been used to justify the intravenous inoculation of tumor patients. The injected cytokine has enhanced anti-tumor immunity and locally injected IL-2 has been shown to be effective in stimulating anti-tumor effects. The direct injection of IL-2 at the site of tumors provides a local concentration of the cytokine, and several investigators have demonstrated tumor regression after injection of IL-2 at the site of tumor growth (G. Cortesina et al., Cancer, 62:2482 (1988); G. Forni et al., J. Immunol., 138:4033 (1987); R. Steis et al., Proc. Am. Soc. Clin. Oncol., 6:250 (1987); and K. Yasumoto et al., Cancer Res., 47:2184 (1987)). Similarly, intradermal injection of recombinant IL-2 in patients with lepromatous leprosy has produced a significant systemic effect in decreasing the total body burden of Mycobacterium laprae and no drug toxicity was observed (G. Kaplan et al., J. Exp. Med., 173:993 (1991)).

Other potential therapies for immune disorders include a variety of techniques for providing IL-2 to a patient. Some of these techniques involve the transfer of cells for the purpose of inducing immunity and include therapies involving the inoculation of a patient with autologous tumor cells that have been manipulated to enhance their immunogenicity. Tumors have been surgically removed and genetically altered with gene vectors that encode for IL-1, IL-2, IL-4, IL-7, GM-CSF, allogeneic HLA antigens, B7, or IGF-1 antisense. In all of these instances, genetic alteration of tumor cells has made the cells immunogenic and therapies based on these findings are currently in clinical trials.

The use of IL-2 in liposomes has also been used to deliver the cytokine to a specific site. The local delivery of liposome formulations of IL-2 gave significantly greater anti-tumor effects compared to empty liposomes or to soluble IL-2 and was seen to be superior
to systemic inoculation of the cytokine (P. Anderson et al., Cancer Res., 50:1853 (1990)). The inclusion of IL-2 in liposomes along with a variety of different immunogens such as the polysaccharide from Pseudomonas aeruginosa (E. Abraham and S. Shah, J. Immunol., 149:3719 (1992)), recombinant herpes simplex virus glycoprotein D (R. Ho et al., Vaccine, 10:209 (1992)), and the lipoprotein of Gram-negative bacterial cell wall (T. Utsugi et al., Lympho. Cyto. Res., 10:487 (1991)) resulted in enhancement of the specific immune responses. In these cases the liposomes were given locally, in the peritoneal cavity, intranasal, or subcutaneously and the delivery of the cytokine was co-localized with the antigen.

Encapsulation of the cytokine produced a marked depot effect when the cytokine was given intrathoracically, subcutaneously, or intraperitoneally. The biodistribution and pharmacokinetics of the drug were greatly influenced by both encapsulation and by route of administration (P. Anderson et al., J. Immunother., 12:19 (1992)). Liposome encapsulated IL-2 maintained higher serum levels than free IL-2 when injected intravenously (M. Joffret et al., Vaccine, 8:385 (1990)).

In an effort to produce vaccines that would better elicit protective immunity, investigators have engineered live viruses so that infection would result in the local production of IL-2. Recombinant vaccinia viruses have been produced that include IL-2 in an expression cassette so that cells infected with the virus produced IL-2 locally (C. Flexner et al., Nature, 330:259 (1987); and I. Ramshaw et al., Nature, 329:545 (1987)). The recombinant vaccinia virus was more effective than the parental virus in acting as a vaccine in immunodeficient mice and IL-2 dependent toxicity was not observed.

Another method that co-localizes the cytokine with antigen for vaccination involves the production of a chimeric protein which includes both an IL-2 domain and an antigen domain. A fusion protein consisting of a fully
functional IL-2 domain and an epitopically intact herpes simplex virus type 1 glycoprotein D domain has been produced (S. Hinuma et al., FEBS, 288:138 (1991)). This fusion protein elicited high titers of specific antibodies and strong cell-mediated immunity without any adjuvants. A fusion protein consisting of both IL-2 and an immunoglobulin chain specific for carcinoma cells has also been produced (H. Fell et al., J. Immunol., 146:2446 (1991)). The activity of the IL-2 and the immunoglobulin remained intact in the chimeric protein and consequently it enhanced the in vitro cytotoxic destruction of tumor cells expressing the relevant antigen.

Since recombinant IL-2 is poorly soluble at neutral pH and has a short half-life in serum, investigators have chemically modified IL-2 with an active ester of polyethylene glycol (M. Knauf et al., J. Biol. Chem.; 263:15064 (1988)). This modification did not alter the molecule's capacity to induce T cell proliferation or to enhance cytotoxicity. However, its serum half-life was greatly increased and its capacity to inhibit the growth of a sarcoma cell line in mice was likewise increased. Polyethylene glycol modified IL-2 also reduced the immunogenicity of the cytokine (N. Katre, J. Immunol., 144:209 (1990)). Characterization of the modified molecule was performed with the recognition of the multi-dimensional nature of pharmacological parameters and it has been shown to be superior to unmodified cytokine in mediating inhibition of tumor growth (M. Knauf et al., J. Biol. Chem.; 263:15064 (1988); and N. Katre et al., Biochem., 84:1487 (1987)). However, IL-2 chemically modified with polyethylene glycol possessed toxicity similar to the parent compound (H. Moon et al., Biotech. Ther., 1:203 (1989)). Polyethylene glycol modified IL-2 has been injected directly into tumors instead of using an intravenous route of delivery and the conjugated IL-2 both caused tumor regression and induced the development of specific immunity against the tumor that was transferred
to naive animals by lymphocytes (L. Balemans et al., Canc. Immunol. Immunother., 37:7 (1993)).

IL-2 has been bound to solid phase Sepharose beads via a standard chemical reaction for covalent coupling in order to assess the potential bioactivity of IL-2 in vitro. (David R. Kaplan, Molecular Immunology, 28(11):1255-1261 (1991)). The IL-2 was spontaneously released into culture medium in active form during short-term incubation and the release was dependent upon the availability of soluble protein in the incubation medium as well as the incubation temperature. Id. IL-2 coupled to polystyrene beads to form a solid phase of the cytokine was able to increase the cytotoxic activity of rat spleen cells in vitro and a single injection of IL-2 coupled beads into the peritoneal cavities of normal rats resulted in the in vivo activation of cytotoxicity of peritoneal exudate cells. (Edward D. Crum et al., Cancer Research, 51:875-879 (1991)). When IL-2 coupled beads were mixed with methylcholanthrene-induced Mc7 or Mc107 sarcoma cells and injected into normal syngeneic Wistar rats, the growth of the tumor was suppressed. Id.

The art described in this background is not admitted to be prior art to the invention.

Summary of the Invention

The present invention concerns products and methods for enhancing immune responses and is based on the discovery that immune responses may be enhanced by adsorbing a cytokine to an antigen presenting cell. The adsorption procedure involves incubating cytokine with cell surfaces either in the presence or absence of proteins. Enhancement of immune responses may be achieved through the delivery of a powerful immunostimulatory molecule, such as IL-2, to the site of activation of the immune response by adsorption of the cytokine to the cell surface of the appropriate antigen presenting cell. For tumor immunotherapy that cell would be the tumor cell or
a tumor cell fused with an antigen presenting cell and for vaccines that cell would be an antigen presenting cell exposed to the relevant antigen such as influenza or tetanus toxoid. Cytokine can also be adsorbed to a T cell that functions not as an antigen presenting cell but is used for adoptive immunotherapy.

Thus, in a first aspect the present invention features a method for enhancing an immune response by exposing an immune cell to an antigen presenting cell having adsorbed cytokine.

By "enhancing" is meant increasing in any way and specifically includes both promoting a pre-existing immune response and generating a new immune response where none existed before. Specific examples of how enhancing an immune response could be measured would vary depending upon the particular immune response, but may include detecting the number of T-cells, B-cells, natural killer cells, monocytes, or granulocytes, the concentration of circulating antibodies or observing certain clinical conditions associated with an immune response.

Immune response is the response of the whole or part of an immune system of an organism, i.e., cellular and humoral systems in vivo or in vitro. In preferred embodiments the immune response is either the proliferation or differentiation of a cell selected from the group that consists of T-lymphocytes, B-lymphocytes, natural killer cells, monocytes and granulocytes. Proliferation is clonal expansion of a cell line such that there is an increase in the frequency or number of cells by cell division, or growth such that there is an increase in cell size. The rate of cell proliferation may be measured by counting the number of cells produced in a given unit of time. Differentiation encompasses changes by which cells manufacture mechanisms to effect immunity through a humoral or cellular response. Examples of such changes include, but are not limited to production of antibody molecules, production of cytokines, production of
receptors for cytokines, production of proteins that are responsible for cytotoxicity, various alterations in cell morphology, and change in cell type. T-lymphocytes are thymus-derived lymphocytes that are capable of inducing a cell-mediated immune response. Examples of T-lymphocytes include helper T-cells, suppressor T-cells and cytotoxic T-cells. B-lymphocytes are Ig⁺, Thy⁻ lymphocytes that are capable of inducing a humoral antibody response. Natural killer cells are lymphocyte-like cell that kills antibody-coated eukaryotic cells and can also kill a variety of tumor and virus-infected cells in culture in the absence of an antibody. Monocytes are nonlymphoid cells, in particular a type of white blood cell that become tissue macrophages, which phagocytose and digest invading microorganisms, foreign bodies, and senescent cells. Granulocytes are nonlymphoid cells, in particular a type of white blood cell that is filled with granules containing degradative enzymes that are secreted as part of an immune response.

By "exposing" is meant creating conditions either in vitro or in vivo in which an immune cell is brought into close proximity to a cytokine adsorbed antigen presenting cell such that cytokine is released from the antigen presenting cell and can interact with receptors on the immune cell.

An immune cell is a cell that is part of an organism's immune system and includes B lymphocytes, T lymphocytes, natural killer cells, monocytes and granulocytes. In preferred embodiments the immune cell is a peripheral blood mononuclear cell.

By "cytokine" is meant a molecule which facilitates both proliferation and differentiation of immune cells. The cytokine may be IL-2 or self-associated IL-2. Other cytokines include but are not limited to: IL-1, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 and gamma interferon.

By "adsorbed" is meant linked to the surface of the antigen presenting cell, for example either by association
with a portion of a cytokine receptor complex, via another cytokine binding molecule, via intercalation into the lipid bilayer of the cell or via self-association with bound cytokine. The cytokine may be adsorbed by incubating cells, preferably at concentrations ranging from $10^6$/ml to $50 \times 10^6$/ml, most preferably $10 \times 10^6$/ml, in media containing cytokine, preferably between 100-10,000 nM of recombinant cytokine (e.g., IL-2), most preferably 2,000 nM of recombinant cytokine for approximately at least 1/2-4 hours at 0-10 degrees Celsius. Adsorption is a process where the cytokine contacts the cell surface of the antigen presenting cell on the extracellular side of the cell membrane, in contrast to intracellular delivery of the cytokine to the cell surface. Adsorption is preferably carried out in vitro.

For IL-2 some means of adsorption are via an alpha chain of an IL-2 receptor complex or via other IL-2 binding molecules. Self-association of the IL-2 molecule may occur upon binding or adsorption. Adsorption via the alpha chain can be distinguished from an alpha chain independent mechanism (i.e., by other IL-2 binding molecules) by sensitivity to treatment with acid, which strips IL-2 from the cells expressing the alpha chain but not the cells which do not express the alpha chain. These mechanisms may also be distinguished by the binding of biotinylated or iodinated IL-2 which binds to the cells expressing the alpha chain but not to cells that do not express the alpha chain. Binding of IL-2 to cells that do not express the alpha chain is sensitive to treatment with trypsin or β-galactosidase indicating that binding is dependent on glycoproteins on the cell surface.

An antigen presenting cell is any cell capable of presenting antigen to an immune cell so as to initiate or modulate (enhance or diminish) an immune response. Examples of conventional antigen presenting cells include B-cells, dendritic cells, macrophages, and endothelial cells.
In a preferred embodiment the immune response may be enhanced by the localized slow release of the cytokine. Localized slow release is a mechanism for delivery to an antigen presenting cell that is positioned in close proximity to the immune cell and occurs over an extended period of time. For example, over a period of days at 37°C in culture. *In vivo*, release would be at least over a period of several days.

By "antigen" is meant a molecule which participates in the induction or modulation of an immune response through determinant groups that can interact with specific receptors for the antigen. Examples of antigens include: fungal antigens, bacterial antigens, parasitic antigens, viral antigens, allogeneic antigens, a xenogeneic antigens and an autologous tumor antigens, including antigens from human immunodeficiency virus, hepatitis B virus, and antigens from allogeneic or xenogeneic tissue.

By "allogeneic" is meant from a genetically different member of the same species.

By "xenogeneic" is meant from a member of a different species.

By "autologous tumor antigen " is meant a tumor antigen derived from the same tumor that is in a patient.

In yet other preferred embodiments the method involves exposure of the immune cell to the antigen presenting cell *in vivo*. The immune cell may be a peripheral blood mononuclear cell, the antigen presenting cell may be a B cell, tumor cell (e.g., an autologous tumor cell), dendritic cell, monocytic cell, Epstein-Barr virus transformed B cell, or endothelial cell. The cytokine may be IL-2, a self-associated cytokine, self-associated IL-2, or a mixture of IL-2 and non-IL-2 cytokines.

Peripheral blood mononuclear cells include lymphocytes and monocytes.

By "B cell" is meant a cell that has the potential to produce immunoglobulin.
By "tumor cell" is meant a cell the exhibits unregulated growth.

By "autologous tumor cell" is meant a tumor cell derived from the same tumor that is in a patient.

By "dendritic cell" is meant an antigen presenting cell with multiple cytoplasmic extensions circulating in the peripheral blood and expressing Class II MHC molecules.

By "monocytic cell" is meant a mononuclear phagocytic cell circulating in the blood and expressing Class II MHC molecules and CD14.

By "Epstein-Barr virus transformed B cell" is meant a B cell immortalized by infection with the Epstein-Barr virus.

By "endothelial cell" is meant a cell lining a vascular space.

In vivo exposure of an immune cell to the antigen presenting cell occurs in the body of the organism.

IL-2 is interleukin 2 and encompasses all or any portion of the molecule encoded by the IL-2 gene, and any variation capable of stimulating immune cells. The IL-2 molecule has an approximate molecular weight of 15,000, and is produced by T-cells. The cDNA for IL-2 has been cloned (Taniguchi et al., Nature, 302:305 (1983); Devos et al., Nucl. Acids Res. 11:4307 (1983); and Clark et al., Proc. Natl. Acad. Sci. USA 81:2543 (1984)). The sequence of IL-2 is hereby incorporated by reference. The local production of interleukin 2 (IL-2) by T lymphocytes acts to enhance the immune response by inducing growth, differentiation and/or cytotoxicity of a variety of immune cells.

Self-association occurs when two or more molecules of the same type interact with each other so as to noncovalently bind together. By the same type is meant that the molecules have the same function. Thus, self-associated IL-2 refers to multiple molecules of IL-2 interacting with each other, and non-IL-2 self-associated
cytokines refers to other cytokines that interact with each other.

More than one adsorbed cytokine can be present on an antigen presenting cell. By utilizing a mixture of more than one cytokine (e.g., IL-2 and non-IL-2 cytokines) for adsorption, such an antigen presenting cell can be produced.

In preferred embodiments adsorption of IL-2 to the surface of the antigen presenting cell is via an alpha chain of an IL-2 receptor complex, via any other IL-2 binding molecule, via intercalation of into the lipid bilayer of the cell, or via self-association.

An IL-2 receptor complex consists of an α chain and a β chain. The α chain of an IL-2 receptor complex is a 55 kd protein which binds IL-2 with a Kd of approximately 10^{-8} M. The β chain is involved in signal transduction and is not involved with loading IL-2 onto cells. Binding to other IL-2 binding molecules refers to molecules that bind IL-2 other than the α chain. Applicant has found that binding of IL-2 to cells can occur independent of the α chain.

Intercalation into the lipid bilayer is anchoring in the lipid bilayer of the plasma membrane that is based on non-specific hydrophobic interacts and does not involve specific binding to proteins.

Binding by self-association involves binding of additional cytokine molecules to cytokine molecules already bound to an antigen presenting cell. The molecules already bound may have become bound by any of the discussed methods, including self-association.

In further preferred embodiment the cytokine adsorbed antigen presenting cell may be also provided to immunocompromised patients.

By "immunocompromised" is meant a patient with an abnormality in the immune system, such that the ability of the immune system to generate a response is diminished. Immunocompromised patients may be identified, for example,
by reduced white blood counts and other techniques well-known in the art.

In other aspects the invention features an artificial antigen presenting cell having adsorbed cytokine.

By "artificial antigen presenting cell" is meant an antigen presenting cell that does not have cytokine naturally bound to its surface or the cytokine is bound in different manner than occurs in nature or the cytokine is bound at greater amounts than naturally occurs on that cell. Preferably the cell has been manipulated in vitro to load the cytokine onto the cell surface.

The present invention also features methods for producing an antigen presenting cell having cytokine adsorbed by utilizing the process of adsorption.

Adsorption includes binding of cytokine to a cytokine receptor on the cell surface, binding of cytokine to any cytokine binding molecule on the cell surface, intercalation of the cytokine into the lipid bilayer of the cell, and self-association of the cytokine.

In another aspect the invention features a method for enhancing immune response by promoting the self-association of IL-2.

By "promoting self-association of IL-2" is meant incubating the cells with IL-2 under conditions that would favor self-association such as concentrations of IL-2 in the self-associating range of from 100 nM to 10,000 nM. Additionally, it may be possible to further promote self-association by changing the primary sequence of IL-2.

In a further aspect the invention features a method for adoptive immunotherapy comprising adsorbing IL-2 onto an activated T cell and introducing the T cell into a patient.

An activated T-cell is a T-cell specific for an antigen, such as a tumor or a virus. Adsorption of IL-2 onto the T cell is carried out by using any of the described adsorption procedures. Introduction into a
patient of the cytokine adsorbed activated T cell is by standard methods known by those who practice the art.

Adoptive immunotherapy involves the introduction of activated T-cells into a patient so as to enhance the immune response of the patient. Since T cells are dependent on IL-2 for their survival, pre-loading of these cells with IL-2 prior to inoculation into a patient should increase their survival in vivo and thus increase their capacity to mediate immunity in vivo.

There are several advantages to the present invention. One advantage of the present invention is that it enhances the capacity of cells to vaccinate individuals to selected antigens such as influenza or tetanus toxoid. Adsorption of cytokine to cells that have been pulsed with antigen or antigenic peptides will permit greater immunogenic potential.

Other advantages of the present invention are based on the local delivery of cytokine and can be more easily understood in light of the fact that cytokines, such as IL-2, do not naturally circulate systemically, but are produced physiologically for local consumption. Thus, the therapeutic potential of intravenous IL-2 therapy has been considerably restricted by the extraordinary toxicity that is associated with its systemic inoculation (S. Rosenberg et al., N. Engl. J. Med., 316:889 (1987); W. Urba et al., Cancer Res., 50:185 (1990); and K. Margolin et al., J. Clin. Oncol., 7:484 (1989)). Intravenous injections of IL-2 for treatment of patients with solid tumors is limited by the dose of cytokine that can be safely given.

IL-2 causes a vascular leak syndrome that can lead to severe hypotension and consequently systemic delivery of the cytokine can be life-threatening. Moreover, there are multiple other serious side effects of systemic levels of IL-2 that have hampered the development of clinical cytokine treatments.

By recognizing the regional bias of IL-2, alternative strategies for therapeutic purposes have been developed
and are described herein. Thus, one advantage is that the present invention overcomes the problem of systemic toxicity by local delivery of the molecule. Moreover, since delivery is specifically localized to the surface of the cell that bears the antigenic peptides, it is expected that there is greater specificity in the immune response that is elicited. In addition, since release is from a cell localized at the site at which the immune response is generated, levels of IL-2 and the modes of deliver of IL-2 may more closely mimic physiological conditions.

Another method that provides for the local delivery of IL-2 involves the use of genetically altered cells. The difficulty with the use of genetically altered cells to treat solid tumors in this way involves the requirement for the in vitro isolation and growth of appropriate tumor cells, the procedure of transferring genes and selecting for transfectants, and the reinfusion of these genetically altered cells in a safe way. Since naturally occurring tumors often comprise cells of different levels of differentiation, the outgrowth of cells in culture may not fully represent the tumor cells that exist in the patient. Moreover, not all tumors give growing cells in culture and for tumors that do grow, the in vitro procedure will take considerable time. In addition, therapies based on the transfer of genes require the time consuming and labor intensive procedures of transfer of genes and selection for transfectants. In patients with tumors it is optimal to begin therapy as soon as possible, thus these procedures are disadvantageous because of the length of time they require. Additionally, the reinfusion of cells that have been genetically altered entails safety considerations since the introduced genes will not be totally controlled in their production of protein or in their potential to recombine.

The present invention is advantageous as cell growth is not essential for adsorption of cytokine to the surface
and adsorption does not entail the introduction of exogenous genes into the patient.

Other objectives, features, and advantages of the invention will be found throughout the following description and claims.

**Brief Description of The Drawings**

Figures 1A and 1B present histograms showing IL-2 adsorption to K562 cells immediately after adsorption and after 2 days at 37°C. The histograms with the area under the curves filled in represent control K562 cells without any IL-2 in the medium and the histograms with the area under the curves open represent K562 cells incubated with IL-2. The x axis represents increasing fluorescence intensity on a log scale indicating more IL-2 adsorption the further to the right. The y axis represents the number of cells.

Figure 2 is a graph comparing the amount of thymidine incorporation over time for peripheral blood mononuclear cells exposed to JY stimulators not pre-adsorbed with IL-2 (solid circles) and cultured JY stimulators that were pre-adsorbed with IL-2 (solid squares). The y axis represents tritiated thymidine incorporation (dpm x 1000). The x axis represents days of incubation.

Figure 3 is a graph comparing the amount of thymidine incorporation over time for peripheral blood mononuclear cells exposed to K562 stimulators not pre-adsorbed with IL-2 (solid circles) and K562 stimulators that were pre-adsorbed with IL-2 (solid squares). The y axis represents tritiated thymidine incorporation (dpm x 1000). The x axis represents days of incubation.

Figure 4 is a graph comparing the amount of thymidine incorporation for IL-2 receptor positive T cells exposed to K562 cells pre-incubated with various concentrations of IL-2 or soluble IL-2. Solid hexagons represent T cells incubated with soluble IL-2, 20 pM. Solid diamonds represent K562 cells not incubated with IL-2. Solid
triangles (down) represent K562 cells pre-incubated with 250 nM IL-2. Solid triangles (up) represent K562 cells pre-incubated with 500 nM IL-2. Solid squares represent K562 cells pre-incubated with 1000 nM IL-2. Solid circles represent K562 cells pre-incubated with 2000 nM IL-2. The y axis represents tritiated thymidine incorporation (dpm x 1000). The x axis represents days of incubation.

Figure 5A and 5B present histograms showing IL-2 adsorption to dendritic cells. In Figure 5A the cells were freshly isolated. In Figure 5B the cells were cultured for 2 days at 37°C. The histograms with the area under the curves filled in represent dendritic cells not incubated with IL-2 and the histograms with the area under the open curve represent dendritic cells incubated with IL-2 prior to staining with anti-IL-2 antibodies. The x axis represents increasing fluorescence intensity on a log scale indicating more IL-2 adsorption the further to the right. The y axis represents the number of cells.

Description of the Preferred Embodiments

The present invention is directed to methods for cellular modulation with a focus on cells used for inducing or enhancing an immune response and involves cytokine adsorbed antigen presenting cells, their methods of production and use. The method is applicable to, but not restricted to, clinical situations that require immunostimulation, such as tumor immunotherapy and vaccination. The present invention is predicated on the discovery of the utility of adsorbing cytokine to the cell surface of an antigen bearing cell. This technology utilizes the self-associative properties of the molecule which greatly enhance the capacity of cytokine to adsorb to cell surfaces. The presence of protein surrounding the cells after injection favor the slow dissolution and release of the IL-2.

Various cytokines, various antigen presenting cells, and various combinations of the two are encompassed by the
present invention, although one preferred cytokine is IL-2, a molecule that powerfully enhances immune responses. IL-2 adsorbed to an antigen presenting cell induces a more powerful proliferative response than the same antigen presenting cell without adsorbed IL-2. The delivery of IL-2 to a cell surface is predicated upon the discovery of the capability of cell surfaces to load IL-2, taking into account the propensity of the IL-2 molecule to self-associate upon binding to the surface. Self-association allows for large amounts of IL-2 to be localized to the cell surface where the antigen is also present.

The present invention may involve adsorbing cytokine to the surface of cells in vitro. A pharmacologically active cytokine composition comprises an cytokine peptide that acts to enhance the immune response of cells by binding to specific surface receptors on the immune cell. A broad array of different IL-2 muteins can be used to mediate these effects. Adsorption of cytokine to the cell surface may be accomplished by incubating high concentrations of cytokine with the desired cell, either in the presence or absence of protein. To adhere to the cell the IL-2 should be a form of the molecule that self-associates such as the native form. Modifications that enhance self-association can be used for adsorption. Upon inoculating an individual with the cell possessing surface adsorbed cytokine, the cytokine is slowly released from the cell so that local concentrations can be maintained for a long period at a high level.

IL-2 can be readily adsorbed to the surface of cells, especially peripheral blood antigen presenting cells (dendritic cells, monocytes, and B lymphocytes) and tumor cells. The IL-2 in a biologically active form is gradually released over days in culture. These cells do not express the α chain of the IL-2 receptor and thus adsorption occurs by an α chain independent mechanism. All cells do not have the capacity to adsorb IL-2 via this α chain independent mechanism. Red blood cells, several
different tumor lines, and freshly isolated peripheral blood T lymphocytes and natural killer cells are unable to adsorb the cytokine.

The amount of IL-2 adsorbed is estimated to be approximately 50-100,000 molecules per cell. The amount can be varied by incubating the cell with various concentrations of IL-2. As few as 100 cells with adsorbed IL-2 can initiate significant proliferation in IL-2 receptor positive cells when co-cultured. IL-2 adsorbed to cells that do not possess the α chain of the receptor complex cannot be removed by acid treatment with pH 2.5 medium. IL-2 adsorbed to cells through the α chain can be removed by treatment with acid, pH 4.0.

Without being limited to any particular mode of operation, the incubation procedure is believed to work because IL-2 self-associates upon adsorption to cells and thus, antigen presenting cells are preloaded with increased amounts of the cytokine (IL-2 or any other cytokine). Self-association is a naturally occurring event for IL-2 and thus there is no need to do anything to make the self-association occur.

Noncovalent self-association of IL-2 has been demonstrated by a number of different methods (T. Arakawa et al., Biochem., 25:8274 (1982); J. Fleischmann et al., Biochem. Biophys. Res. Commun., 152:879 (1986); and D. Kaplan, Mol. Immunol., 28:1255 (1991)). Self-association has been observed in gel filtration chromatography and in fluorescence quenching which has shown that the K₄ of self-association of 600 nM (J. Fleischmann et al., Biochem. Biophys. Res. Commun., 152:879 (1986)).

The classical IL-2 bioassay does not distinguish among self-associated and non-self-associated cytokine. In the prolonged proliferation after pulse assay, IL-2 receptor positive cells are pulsed with a source of IL-2 in serum-containing medium for 1 hour at 4°C and then they are plated in microtiter wells and thymidine incorporation is assessed every day for 1 week. Results indicate that
concentrations of IL-2 in the self-associating range, greater than 400 nM, give more proliferation in this assay than concentrations that give negligible self-association, for instance 100 nM, in the prolonged proliferation after pulse assay. In the classical IL-2 bioassay these two concentrations are indistinguishable (C. Bergmann et al., Cytokine, 4:192 (1992)).

Furthermore, prior uses of IL-2 have been predicated on the conception of the cytokine as an absolute monomer. Nevertheless, IL-2 spontaneously forms noncovalent self-associations. This characteristic has not been considered in designing the therapeutic protocols. The present invention uses this property for modifying and regulating the therapeutic responses.

Pharmaceutical Formulations and Modes of Administration

This invention encompasses the adsorption of cytokine to an antigen presenting cell and administration of the antigen presenting cell with cytokine adsorbed to a patient. Selection of the antigen presenting cell and the particular cytokine to be adsorbed, is based on the disease to be treated. For example treatment of a particular type of cancer entails isolation of tumor cells of the cancer, irradiation of these cells to prevent growth, and adsorption with IL-2. Inoculation of these IL-2 adsorbed tumor cells back into the patient acts to induce or enhance an immune response to the tumor and results in tumor regression. Immunodeficient patients, such as those infected with Human Immunodeficiency Virus (HIV), do not respond well to vaccines. In order to enhance their immune responsiveness, HLA-matched irradiated Epstein-Barr virus transformed B cells, non-transformed activated or nonactivated B cells, activated or nonactivated monocytes or dendritic cells pulsed with appropriate antigens such as influenza or tetanus toxoid peptides are used for IL-2 adsorption. Inoculation of these cells into immunodeficient patients will enhance
their immune responsiveness to the antigens presented. The invention can be utilized to enhance the immune response to any particular antigen by pulsing an antigen presenting cell with a particular antigen and then loading onto that cell the appropriate cytokine. Isolation of antigen presenting cells, culturing of cells, irradiation and pulsing with antigen are practices well known and routine in the art.

It is possible to adsorb onto antigen presenting cells other cytokines besides IL-2, independent of or in conjunction with IL-2.

After adsorption of cells with cytokine, the antigen presenting cells are washed in sterile medium and adjusted to an appropriate cell concentration. The cell suspension is injected into an appropriate site via standard procedures. Administration of antigen presenting cells with adsorbed cytokine will be intradermal, subcutaneous, or intramuscular and is conducted in a manner familiar to those who practice the art.

For use in adoptive immunotherapy, T cells are isolated and cultured in the presence of a particular antigen (e.g., virus or tumor) so as to induce proliferation. Such techniques are known and routine in the art. These T cells can be loaded with cytokine (e.g., IL-2) as disclosed in Bergmann et al. 4 Cytokine 192 (1992) and re-infused into patients for therapeutic purposes.

Derivatives of Cytokines

Also provided herein are functional derivatives of a cytokine. By "functional derivative" is meant a "chemical derivative," "fragment," "variant," "chimera," or "hybrid" of the cytokine, which terms are defined below. A functional derivative retains at least a portion of the function of the cytokine, for example binding activity, or the ability to enhance an immune response which permits its utility in accordance with the present invention.
A "chemical derivative" of the cytokine contains additional chemical moieties not normally a part of the protein. Covalent modifications of the cytokine are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the cytokine with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

Such derivatized moieties may improve the stability, solubility, adsorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the cytokine and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, PA (1990).

The term "fragment" is used to indicate a polypeptide derived from the amino acid sequence of the cytokine having a length less than the full-length cytokine from which it has been derived. Such a fragment may, for example, be produced by proteolytic cleavage of the full-length cytokine. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the proteins to delete one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such fragments may retain one or more characterizing portions of the native cytokine. Examples of such retained characteristics include: substrate specificity; interaction with other molecules in the intact cell; regulatory functions; or binding with an agent specific for the native cytokine, or an epitope thereof.

Another functional derivative intended to be within the scope of the present invention is a cytokine comprising at least one "variant" polypeptide which either lack one or more amino acids or contain additional or substituted amino acids relative to the native cytokine. The
variant may be derived from a naturally occurring cytokine component by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify codons for one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native cytokine, as described above.

A functional derivative of cytokines comprising proteins with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art. For example, the modified components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman et al., DNA 2:183 (1983)) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is produced, and thereafter expressing this recombinant DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above. Alternatively, components of functional derivatives of cytokines with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The functional derivatives of the cytokines typically exhibit the same qualitative biological activity as the native cytokines.

**Examples**

The following examples illustrate various aspects and embodiments of the present invention and are in no way intended to be limiting of the scope.

**Example 1. Adsorption of IL-2 to K562 cells**

K562 cells, a cultured erythroleukemia tumor line, do not express the α chain of the IL-2 receptor. Cells were incubated with 2000 nM IL-2 for 1 hour at 4°C. After
incubation, cells were washed and analyzed for IL-2 binding by utilizing specific anti-IL-2 antibodies and a fluoresceinated second antibody. The histogram in Figure 1A presents an analysis of the cells immediately after adsorption and the histogram in Figure 1B presents an analysis of the cells after 2 days of incubation at 37°C. This experiment indicates that IL-2 can be loaded onto cells via an alpha chain independent mechanism and that IL-2 is released from the loaded cells over time.

Example 2: Adsorption of Cells With IL-2 Enhances Proliferation of Peripheral Blood Mononuclear Cells In Vitro

Preadsorption of stimulator cells with IL-2 makes them significantly better stimulators of peripheral blood mononuclear cells in vitro. Either JY, a cultured Epstein-Bar virus transformed B cell line, or K562 irradiated stimulators were incubated with 2000 nM recombinant IL-2 for 1 hour at 4°C in buffered saline without the presence of protein. After this incubation the cells were extensively washed in protein-containing medium. Small numbers of these cells (250 cells for JY and 500 cells for K562) were used as stimulators for the proliferation of peripheral blood mononuclear cells (PBMC) isolated from a healthy donor. Proliferation was measured by incorporation of tritiated thymidine.

Mock-preadsorbed cells that did not possess IL-2 on their cell surface did not stimulate any significant proliferation; however, the IL-2 preadsorbed cells induced a marked proliferative response. JY cells, which are positive for both MHC class I and II, are usually excellent stimulators when 10,000 cells are used for stimulation. The addition of such a small number of untreated JY cells (250 cells) is insufficient to stimulate a proliferative response, but IL-2 adsorption converts these small numbers of cells into powerful stimulators (see Figure 2).
K562 cells, which are MHC class II negative, are poor stimulators at any concentration, but IL-2 adsorption converts these cells into powerful stimulators (see Figure 3). Therefore, the presence of IL-2 enhanced responsiveness to an antigen presenting cell that otherwise is nonstimulatory.

Example 3: Adsorption of Cells With IL-2 Enhances Proliferation of IL-2 Receptor Positive T Cells In Vitro

K562 cells were incubated with 2000 nM, 1000 nM, 500 nM, 250 nM of IL-2 or no IL-2 for 1 hour at 4°C. After washing the cells were irradiated and 10⁴ of these cells were added to cultures of IL-2 receptor positive T cells. Cultures of IL-2 receptor positive T cells were also incubated with 2 pM of soluble IL-2 in the place of K562 cells. Approximately 20 pM of soluble IL-2 is equivalent to 10⁴ K562 cells pre-incubated with 500 nM IL-2. IL-2 driven proliferation of the T cells was determined for 4 days by incorporation of tritiated thymidine. The results (see Figure 4) demonstrate that cell adsorbed IL-2 can mediate IL-2 bioactivity in culture.

Example 4: Absorption of IL-2 to Dendritic Cells

Dendritic cells were isolated from human peripheral blood mononuclear cells (PBMC). PBMC, obtained by discontinuous gradient centrifugation of blood over ficoll/hypaque, were cultured at 10⁶ cells/ml in 10% fetal bovine serum in RPMI 1640 overnight at 37°C in 7% CO₂. The next day nonadherent cells were collected and centrifuged over 13.7% metrizamide. The cells at the interface were collected and washed twice in culture medium (J. Virol. 66:2102 (1992)). Dendritic cells were identified by morphology to be greater than 80% pure. Dendritic cells are known to be the most potent antigen presenting cells.

Binding of IL-2 was accomplished using 2000 nM in 10% fetal bovine serum in phosphate buffered saline as
previously described in Example 1. Cells were assayed for IL-2 binding as in Example 1. This experiment indicates that IL-2 is bound to both freshly isolated dendritic cells (Fig. 5A) and cultured dendritic cells (Fig. 5B). Other embodiments are within the following claims.
Claims

1. A method of enhancing an immune response comprising the step of exposing an immune cell to an antigen presenting cell having adsorbed cytokine.

2. The method of claim 1 wherein said immune response is proliferation or differentiation of a cell selected from the group consisting of T-lymphocytes, B-lymphocytes, natural killer cells, monocytes, and granulocytes.

3. The method of claim 1 wherein said immune response is enhanced by a localized slow release of said cytokine.

4. The method of claim 1 wherein said antigen presenting cell presents an antigen selected from the group consisting of a viral antigen, a bacterial antigen, a fungal antigen, a parasitic antigen, an allogeneic antigen, a xenogeneic antigen and an autologous tumor antigen.

5. The method of claim 4 wherein said viral antigen is from human immunodeficiency virus.

6. The method of claim 4 wherein said viral antigen is from hepatitis B virus.

7. The method of claim 1 wherein said antigen presenting cell is exposed to said immune cell in vivo.

8. The method of claim 1 wherein said immune cell is a peripheral blood mononuclear cell.

9. The method of claim 1 wherein said antigen presenting cell is selected from the group consisting of a B cell, a tumor cell, a dendritic cell, a monocytic
cell, an Epstein-Barr virus transformed B cell, and an endothelial cell.

10. The method of claim 1 wherein said cytokine is IL-2.

11. The method of claim 1 wherein said cytokine is self-associated.

12. The method of claim 1 wherein said cytokine is self-associated IL-2.

13. The method of claim 1 wherein said cytokine comprises a mixture of IL-2 and non-IL-2 cytokines.

14. The method of claim 10 wherein adsorption of said IL-2 to said surface of said antigen presenting cell is via an α chain of an IL-2 receptor complex.

15. The method of claim 10 wherein adsorption of said IL-2 to said surface of said antigen presenting cell is via any IL-2 binding molecule.

16. The method of claim 10 wherein adsorption of said IL-2 to said surface of said antigen presenting cell is via intercalation of the cytokine into the lipid bilayer of the cell.

17. The method of claim 10 wherein adsorption of said IL-2 to said cell surface of said antigen presenting cell is by self-association.

18. The method of claim 1 wherein said cytokine adsorbed antigen presenting cell is provided to immunocompromised patients.
19. An artificial antigen presenting cell useful for enhancing an immune response having adsorbed cytokine.

20. A method for producing a cytokine adsorbed antigen presenting cell comprising the step of adsorbing cytokine to the surface of an antigen presenting cell.

21. The method of claim 20, wherein adsorbing is by binding to a cytokine receptor.

22. The method of claim 20, wherein adsorbing is by binding to any cytokine binding molecule.

23. The method of claim 20, wherein adsorbing is by intercalation of said cytokine into the lipid bilayer of the cell.

24. The method of claim 20, wherein adsorbing is by self-association of said cytokine.


26. A method for adoptive immunotherapy comprising the steps of:

   a) adsorbing IL-2 onto an activated T cell; and
   b) introducing said T cell into a patient.
FIGURE 4

Thymidine Incorporation x 1000

Incubation Time (days)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER


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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO,A,93 07906 (SAN DIEGO REGIONAL CANCER CENTER) 29 April 1993 see page 3 - page 5 see example 1 ---</td>
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<td>WO,A,92 05262 (THE JOHN HOPKINS UNIVERSITY) 2 April 1992 see example 1 ---</td>
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<td>X</td>
<td>EP,A,0 611 263 (CELLIFE BIOTECHNOLOGIE PER LA VITA S.R.L.) 17 August 1994 see the whole document ---</td>
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<td>X</td>
<td>US,A,5 270 038 (GARY R. KLIMPEL) 14 December 1993 see column 3, line 15 - line 35 see example 4 see column 6, line 62 - column 7, line 13 ---</td>
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Further documents are listed in the continuation of box C.

Patient family members are listed in annex.

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

Date of the actual completion of the international search: 15 March 1996

Date of mailing of the international search report: 26. 03. 96

Name and mailing address of the ISA:
European Patent Office, P.B. 5818 Patentlaan 2 NL.-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer:
Fernandez y Branas, F

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

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<td>MOLECULAR IMMUNOLOGY, vol. 28, no. 11, 1991 OXFORD, pages 1255-1261, KAPLAN, D.R. 'Solid phase interleukin 2' see the whole document</td>
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<td>JOURNAL OF IMMUNOTHERAPY, vol. 11, 1992 NEW YORK, pages 209-217, LOPEZ M. ET AL 'Adoptive immunotherapy with activated macrophages grown in vitro from blood monocytes in cancer patients: a pilot study' see page 211</td>
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<td>CANCER RESEARCH, vol. 51, 1991 PHILADELPHIA, pages 875-879, CRUM, E.D. ET AL 'In vivo activity of solid phase interleukin 2' see the whole document</td>
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<td>IVAN M. ROITT ET AL 'ENCYCLOPEDIA OF IMMUNOLOGY' 1992, ACADEMIC PRESS, LONDON see page 114 - page 115</td>
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INTERNATIONAL SEARCH REPORT

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

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

1. X Claims Nos.: 1-18, 25-26
   because they relate to subject matter not required to be searched by this Authority, namely:
   Remark: Although claims 1-18, 25-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. □ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

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

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

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

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

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

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

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

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA.210 (continuation of first sheet (1)) (July 1992)
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