(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/30300 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US00/41688

(22) International Filing Date: 27 October 2000 (27.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/429,511 28 October 1999 (28.10.1999) US

(71) Applicant *(for all designated States except US)*: **OPHIDIAN PHARMACEUTICALS, INC.** [US/US]; 5445 East Cheryl Parkway, Madison, WI 53711 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **STAFFORD, Douglas** [US/US]; 5743 Timber View Court, Madison, WI 53711 (US). **KINK, John, A.** [US/US]; 110 Wolf Street, Madison, WI 53717 (US).

(74) Agents: **CARROLL, Peter, G.** et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

(81) Designated States *(national)*: AU, CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/30300 A2

(54) Title: PREVENTION AND TREATMENT OF AUTOIMMUNE DISEASE WITH LUMINALLY ADMINISTERED POLYCLONAL ANTIBODIES

(57) Abstract: The prevention and treatment of autoimmune disease in humans (as well as other animals) is described through the use of ligands directed to cytokines. Antibodies and receptors to the proinflammatory cytokines IL-2, TNF, IL-12 and IFN-gamma are employed (along with other ligands to such cytokines). Such ligands administered luminally are effective (as demonstrated in two experimental models of autoimmune disease) at delaying the onset of autoimmune disease.

PREVENTION AND TREATMENT OF AUTOIMMUNE DISEASE WITH LUMINALLY ADMINISTERED POLYCLONAL ANTIBODIES

FIELD OF THE INVENTION

The present invention relates to therapeutics for the prevention and treatment of autoimmune disease, and in particular the prevention and treatment of autoimmune disease in humans through the use of luminally administered polyclonal antibody.

BACKGROUND OF THE INVENTION

A progressive and maintained response by the immune system against self-components is termed autoimmunity. Normally self-tolerance mechanisms prevent the immune response from acting on self-components. However, all mechanisms have a risk of breakdown and occasionally the immune system turns on its host environment in an aggressive manner as to cause disease. This breakdown leads to the copious production of autoreactive B cells producing autoantibodies and/or autoreactive T cells leading to destructive autoimmune disease. The cellular mechanisms of autoimunity are the same as those involved in beneficial immune responses to foreign components which include antibody-dependent cell cytotoxicity, delayed-type hypersensitivity (DTH), and T-cell lympholysis.

Human autoimmune diseases can be divided into two categories: organ-specific and systemic. In organ-specific autoimmune disease, autoreactivity is directed to antigens unique to a single organ. In systemic autoimmune disease, autoreactivity is largely directed toward a broad range of antigens and involves a number of tissues. Disease in either type results from the generation of one or both autoreactive cell types (B or T cells). Autoreactive B cells leads to the generation of autoantibodies or immune complexes. Autoreactive T cells leads to the cellular DTH responses from T_{DTH} cells or cytotoxic responses from T_C cells. Some autoimmune diseases in humans and the immune response and antigen(s) involved are shown in Table 1.

Table 1

Human Autoimmune Diseases

Disease	Self-Antigen	Immune Response
Organ-Specific Autoimmune Disease		
Addison's disease	Adrenal cells	Autoantibodies
Autoimmune hemolytic anemia	Red blood cells	Autoantibodies
Goodpasture's disease	Renal and lung membranes	Autoantibodies
Graves' disease	Thyroid-stimulating hormone receptor	Autoantibodies
Hashimoto's thyroiditis	Thyroid proteins	T_{DTH} cells, autoantibodies
Idiopathic thrombocytopenia	Platelet membranes	Autoantibodies
Insulin-dependent diabetes mellitus (IDDM)	Pancreatic beta cells	T_{DTH} cells, autoantibodies
Myasthenia gravis	Acetylcholine receptors	Autoantibodies
Myocardial infarction	Heart muscle	Autoantibodies
Pernicious anemia	Gastric intrinsic factor	Autoantibodies
Poststreptococcal glomerulonephritis	Kidney	Immune complexes
Spontaneous infertility	Sperm	Autoantibodies
Systemic Autoimmune Disease		
Ankylosing spondylitis	Vertebrae	Immune complexes
Multiple sclerosis	Brain or white matter	T_{DTH} and T_c cells, autoantibodies
Rheumatoid arthritis	Connective tissue	Autoantibodies, immune complexes
Scleroderma	Nuclei, heart, lungs, GI tract, kidney	Autoantibodies
Sjogren's syndrome	Salivary gland, liver, kidney, thyroid	Autoantibodies
Systemic lupus erythematosus (SLE)	DNA, nuclear protein, RBC and platelet membranes	Autoantibodies, immune complexes

The current view of the etiology of autoimmune diseases posulates that both autoreactive T and B cells exist normally in the body. Control of these cells involves immune surveillance mechanisms which can induce tolerance to these cells and/or the selective elimination of these cells. Factors which overcome immune surveillance are thought to be responsible for the proliferation of these autoreactive cells leading to autoimmune disease.

Immune surveillance can be circumvented in several proposed ways: (1) Autoreactive cells can be stimulated through molecular mimicry by cross-reactive microbial antigens. A number of viruses and bacteria have been shown to possess antigenic determinants that are identical to normal host-cell components. Thus, antibodies generated against these microbial antigens can also recognize and damage host cells. Cross-reacting antibodies to heart muscle developed after a *Streptococcus* infection, for example, is thought of be the cause of an rheumatic fever. (2) In some cases, foreign antigen can directly stimulate autoreactive cells. Lipopolysaccharides or viral antigens from Epstein-Barr virus (EBV) or cytomegalovirus causes the direct stimulation of certain B cells. During mononucleosis, a disease caused by EBV, a variety of autoantibodies reactive to self-components are generated. Specifically, EBV can activate B cells to produce autoantibodies to nuclear DNA and immune cells. (3) Release of antigens normally sequestered from the immune system is another example of the breakdown of immune surveillance leading to autoimmune disease. Experimentally, animals injected parenterally with basic myelin protein, an antigen primarily found in the brain, develop experimental autoimmune encephalomyelitis. (4) Expression of specific HLA alleles has been associated with autoimmune individuals. It is thought that cells expressing these HLA's may act as a prime target for autoreactive cells. Individuals with the B27 HLA allele has a 90% increased relative risk of developing ankyosing spondylitis.

Current therapies for autoimmune diseases are not cures, but are aimed at reducing symptoms to provide the patient with a acceptable quality of life. In organ-specific autoimmune disorders, the symptoms can be corrected by the removal of the organ if possible. In some autoimmune diseases such as myasthenia gravis some

success have been achieved by removing the thymus. In addition, in organ-specific autoimmune disorders, symptoms can be corrected by metabolic control with biologically active compounds. For example, hypothroidism can be controlled by the administration of thryroxine or perrrnicious anemia can be treated with injections of vitamin B_{12}. Drugs used in most cases of autoimmune disease, especially systemic autoimmune disease, provide general nonspecific suppression of the immune system. For the most part these drugs do not distinguish between the pathological immune response and the protective immune response. Immunosuppressive drugs (*e.g.*, corticosteroids, azathioprine, cyclophoshamide and cyclosporin) are often given to suppress the proliferation of autoreactive lymphocytes. Anti-inflammatory drugs also are prescribed to patients with rheumatoid arthritis. Unfortunately these drugs, besides not working in many patients, have very serious side-effects. The general suppression of the immune response puts the patient at greater risk to infection and cancer.

Clearly there is a significant need for agents capable of preventing and treating autoimmune diseases. It would be desirable if such therapy could be administered in a cost-effective and timely fashion, with a minimum of adverse side effects.

SUMMARY OF THE INVENTION

The present invention relates to therapeutics for the prevention and treatment of autoimmune disease (including but not limited to those diseases listed in Table 1). Specifically, the present invention contemplates the prevention and treatment of autoimmune disease in humans as well as other animals through the use of ligands directed to cytokines. The examples of the present invention demonstrate the production of antibodies to the proinflammatory cytokines IL-1, -2, -6, -8, -12, and -15, as well as TNF and IFN-gamma (although other ligands to such cytokines are also contemplated). The examples of the present invention demonstrate a novel finding that ligands (such as antibodies) against pro-inflammatory cytokines such IL-2 or IL-12 administered luminally are effective (as demonstrated in experimental models of both T cell-mediated and B cell-mediated autoimmune disease) at delaying the onset and reducing the severity of autoimune disease.

In one embodiment, the present invention contemplates a method of treatment, comprising: a) providing: i) a human patient who is either at risk for autoimmune disease or who has symptoms of autoimmune disease, ii) a therapeutic formulation comprising one or more ligands directed to a proinflammatory cytokine, and; b) administering said formulation to said patient. It is not intended that the present invention be limited to the type of patient. In one embodiment, the patient is a child.

The present invention is also not limited by the degree of benefit achieved by the administration of the anti-cytokine ligands. For example, the present invention is not limited to circumstances where all symptoms are eliminated. In one embodiment, said administering reduces said symptoms (*e.g.*, the amount of autoantibody is reduced and/or the amount of pain is reduced). In another embodiment, said administering delays the onset of symptoms.

It is not intended that the present invention be limited by the route of administration. In one embodiment, the formulation is administered to the lumen of the intestine. In a preferred embodiment, said administering is performed orally. However, said administering can also be performed parenterally.

Where antibodies are the ligand employed, the present invention is not limited to the source of the anti-cytokine antibodies. In one embodiment, said antibodies are avian polyclonal antibodies (including but not limited to chicken antibodies). In one embodiment, said antibodies are purified antibodies. In the case of chicken antibodies, it is convenient that said chicken antibodies are purified from chicken eggs.

In accordance with the present invention, one or more members from the class of IL receptors or IL receptor analogues are selectively employed in soluble form to treat autoimmune patients. The present invention also contemplates using soluble tumor necrosis factor (TNF) receptors, or TNF receptor analogues, to treat autoimmune patients. In another embodiment, the method comprises treating with a therapeutic preparation comprising, in combination, both soluble TNF and soluble IL receptors.

The existence of membrane receptors to cytokines is now well-established. Many of these receptors have now been cloned and expressed in high yield. See U.S.

Patents Nos. 4,968,607, 5,925,351, 5,919,903, 5,919,456, 5,965,704, 5,945,511, 5,945,397, 5, 925,735, all of which are hereby incorporated by reference. Such receptors are contemplated as ligands for use in the methods of the present invention.

DESCRIPTION OF THE INVENTION

The present invention contemplates the use of ligands (*e.g.*, receptors, receptor fragments, antibodies and antibody fragments) directed to proinflammatory cytokines and inflammatory mediators administered to or at the lumen to treat autoimmune diseases. In one embodiment antibodies (raised in birds or mammals) against the offending inflammatory mediators are contemplated and these antibodies can be administered systemically, orally or mucosally either prophylatically or therapeutically to the patient. A variety of classes of inflammatory mediators are contemplated to be important to generate antibodies useful in the prevention and treatment of inflammatory diseases. Illustrative cytokines and inflammatory mediators are shown in Table 2. It is envisioned that ligands against these mediators would be used either singly or in combination to treat a specific disease. Combination therapies would consist of ligands to several mediators within a given pathway.

It is not intended that the present invention be limited to a particular mechanism. Indeed, and understanding of the precise mechanism by which the administration of ligands to cytokines achieves a therapeutic benefit is not necessary in order to successfully practice the present invention. While not limited to any particular mechanism, the inventors believe that cytokines play a major role in the initiation and regulation in immune responses and that the dysregulation of the cytokine network may also lead to the activation of autoreactive T cells leading to autoimmune. Preferential activation of a specific set of T cells, T_H1, is thought to play a central role in the pathogenesis of a number of autoimmune diseases. T cells with the CD4 phenotype are divided into subsets referred as T_H1 and T_H2 based on the nature of their immune reactivity and their cytokine secretion profile. T_H1 cells are associated cell-mediated inflammatory reactions and act as effector cells in infectious disease. Secreted cytokines that set the T_H1 subset apart are interferon gamma, tumor

necrosis factor (TNF) interleukin-2 (IL-2) and upon activation interleukin-12 (IL-12). T_H1 cytokines are referred to as proinflammatory cytokines because they activate cytotoxic, inflammatory and delayed hypersensitiviy reactions. In contrast, T_H2 cells are associated with helper cell function and antibody production. T_H2 cells upon activation secrete interleukins 4 (IL-4), 5 (IL-5), and 10 (IL-10). Cytokines from a T_H1 cells tend to inhibit the actions of the T_H2 cell and vice versa. Whether the characterization of such cells is correct or not, the data shows that the therapeutic methods (described in more detail below) result in a dramatic delay and/or reduction in autoimmune symptoms and disease.

Table 2

Examples Of Inflammatory Mediators

Class of Mediator	Specific Name
Colony Stimulating Factors (CSF)	Granulocyte-Macrophage CSF, Macrophage Growth Factor (Mp-CSF), Granulocyte CSF, Erythropoietin
Transforming Growth Factor (TGF)	TGF beta 1, 2, and 3
Interferons (IFN)	IFN alpha, beta, gamma
Interleukins (IL)	IL-1, IL-2, IL-3, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15
Tumor Necrosis Factor (TNF)	TNF- alpha, beta
Adherence proteins	Intercellular Adhesion Molecule (ICAM), selections L, E, and P, Vascular Cell Adhesion Molecule (VCAM)
Growth Factors	Leukemia Inhibitory Factor (LIF), Macrophage Migration-Inhibiting Factor (MIF), Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (ILGF), Nerve Growth Factor (NGF), B-cell growth Factor (BCGF)
Chemokines	Monocyte chemoattractant proteins (MCP) -1, 2, and 3, Rantes, Macrophage Inflammatory Protein (MIP), IL-8, Growth-Related Oncogene (GRO-alpha), Gamma interferon-inducible protein (IP 10)
Leukotrienes (LTB)	Leukotriene B_4, Leukotriene D_4
Vasoactive Factors	histamine, bradykinin, platelet activating factor (PAF)
Prostaglandins (PG)	PGE_2

DEFINITIONS

The phrase "ligand directed to a cytokine" is herein defined as meaning any molecule having affinity for a cytokine. Ligands can be chemically synthesized or designed by molecular evolution. Alternatively, such ligands can be known biomolecules or portions thereof (*e.g.*, receptors, antibodies). It is not intended that the present invention be limited to the mechanism by which ligands achieve a therapeutic benefit. Ligands may be "antagonists" in that they neutralize the biological impact of a secreted cytokine. On the other hand, ligands may simply block recognition of cytokines or interfere with cell function. For example, ligands may interact with a cell-surface cytokine so as to result in immune cells not participating in autoimmune phenomenon (*e.g.*, the cells are caused to enter apoptosis, etc.).

The phrase "administered to or at the lumen" is herein defined as administration that preferentially delivers compound(s) to the space in the interior of the intestines at a concentration in excess of what is found in circulation. Such delivery can be achieved by a variety of routes (*e.g.*, oral, rectal, etc.) in a variety of vehicles (*e.g.*, tablet, suppository, etc.). By contrast, a parenteral administeration is not designed to preferentially deliver compounds to the lumen (although some incidental delivery can take place through normal biodistribution).

The phrase "symptoms of autoimmune disease" is herein defined as any abnormal symptoms than can be attributed to the generation of autoreactive B and/or T cells. For example, autoantibodies are a common symptom associated with autoimmune disease. Symptoms are "reduced" when the severity [as measured by frequency (*e.g.* the number of episodes over time), amount (*e.g.* the amount of autoantibody or other indicator) or other parameter] of one or more symptoms are reduced. All symptoms need not be reduced for symptoms to be "reduced." Moreover, symptoms need not be eliminated for symptoms to be "reduced."

The phrase "at risk for autoimmune disease" is herein defined as individuals with familial incidence of autoimmunity. For example, many autoimmune diseases are associated with genetic factors such as certain HLA specificities.

A "proinflammatory cytokine" is any cytokine that can activate cytotoxic, inflammatory or delayed hypersensitivity reactions. Examples of such cytokines are IL-2, TNF and INF-gamma. Examples of inflammatory mediators are found in Table 2.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Production of antibodies proinflammatory mediators to IL-1, IL-2, IL-6, IL-8, IL-12, IL-15, TNF, and IFN in the Hen

This example involved (a) preparation of the immunogen and immunization, (b) purification of anti- IL-1, IL-2, IL-6, IL-8, IL-12, IL-15, TNF, and IFN chicken antibodies from egg yolk (IgY), and (c) detection of specific antibodies in the purified IgY preparations.

(a) *Preparation of the immunogen and immunization.*

Recombinant human (rh)tumor necrosis factor alpha, (TNF), recombinant mouse interleukin 1 beta (IL-1), recombinant mouse Interleukin 2, (IL-2), recombinant human interleukin 6, (IL-6), recombinant human interleukin 8 (IL-8), recombinant mouse interferon-gamma, (IFN) and recombinant mouse interleukin 12 (heterodimer) were purchased (lyophilized without bovine serum albumin (BSA) and designated carrier-free) from R&D Systems Inc., Minneapolis, MN and produced in *E. coli*. Recombinant human IL-15 expressed in *E.coli* was purchased from PeproTech, Inc., Rocky Hill, NJ. The lyophilized cytokines were reconstituted in phosphate-buffered saline pH 7.2-7.5 (PBS) at least 50 ug/ml. From approximately 2 to 50 ug of each cytokine was used to immunize groups of hens. Each hen received one 0.5 ml sub-cutaneous injection containing the individual cytokine with 75 ug Quil A adjuvant

(Superfos Biosector, Denmark, distributed by Accurate Chem., Westbury, N.Y.) in PBS. The hens were immunized every 2 weeks for at least 3 times then placed on a maintenance immunization schedule where the hens were immunized every 4-6 weeks.

(b) *Purification of anti-cytokine chicken antibodies from egg yolk (IgY).*

Groups of eggs were collected per immunization group at least 3-5 days after the last booster immunization. The chicken yolk immunoglobulin (IgY) was extracted by a two-step polyethylene glycol (PEG) 8000 method performed according to a modification of the procedure of Polson *et al., Immunol. Comm.*, 9:495 (1980). The yolks were separated from the whites and the yolks were placed in a graduated cylinder. The pooled yolks were blended with 4 volumes of PBS and PEG was added to a concentration of 3.5%. When the PEG was dissolved, the protein and lipid precipitates that formed were pelleted by centrifugation at 9,000 x g for 15 minutes. The supernatants were decanted and filtered through 4 layers of gauze to remove the floating particulates and a second PEG step was performed by adding PEG to a final concentration of 12% (the supernatants were assumed to contain 3.5% PEG). After a second centrifugation, the supernatants were discarded and the IgY pellets were typically resuspended in PBS or 0.1 M carbonate buffer pH 9.5 at approximately 1/6 -1/8 the original yolk volume. The concentration of the fractionated IgY's were estimated by measuring the absorbance at 280nm (an optical density at 280 nm of 1.1 to1.3 equals 1 mg of IgY/ml. The antibody concentrations were about 20-40 mg/ml. The IgY's used for oral administration in the animal models were resuspended in high pH-carbonate buffer. This was done to to help increase the pH in the stomach and minimize the acid hydrolysis of the IgY. IgYs extracted from the eggs of immunized hens are designated as "immune IgY," while IgYs extracted from the eggs of unimmunized hens is designated "preimmune IgY."

(c) Detection of anti-cytokine antibodies in the purified IgY preparations.

In order to determine if an anti-cytokine response was generated and to determine relative levels of the response, enzyme-linked immunosorbent assays (EIA)

were performed. Briefly, ninety-six well Falcon Pro-bind micro-titer plates were coated overnight at 40°C with 100 ul/well with different cell mediators (IL-1, IL-2, IL-6, IL-8, IL-12, IL-15, TNF, and IFN) at 0.1-1.0 ug/ml PBS. The wells are then blocked with PBS containing 3% BSA and 0.05% Tween 20 and incubated for about 1 hour at 37°C. The blocking solution was removed and the immune or preimmune IgY was diluted antibody diluent (1% BSA with 0.05% tween 20 in PBS). The samples at a beginning concentration of 20-40 mg/ml were diluted 1:30 to 1:100 in antibody diluent then added in duplicate wells of the microtiter plate. Typically the samples were then serially diluted 5-fold within the plate and incubated for about 1 hour at 37°C. The plates were then washed 3 times with PBS containing 0.05% Tween 20 then three times with PBS alone. Alkaline phosphatase-conjugated anti-chicken IgG (Kirkegaard and Perry Labs (KPL), Gaithersburg, MD), generally diluted 1:1000 in antibody diluent, was added to the plates and incubated about 1 hour at 37°C. The plates were washed again as above and substrate was added. Substrate was prepared either using p-nitrophenyl phosphate (104 phosphatase, Sigma Chemicals, St. Louis, MO) at 1 mg/ml in 0.05 M Na_2CO_3, pH 9.5, 10 mM $MgCl_2$ or using the Phosphatase Substrate System from KPL. The plates were read in a Dynatech plate reader at 410 nm from 10-30 minutes after substrate addition.

Very good antibody titers was detected by EIA against all of the mediators except IL-1. Titers were defined as the reciprocal of the highest immune IgY generating a EIA signal about 3-fold higher than that of preimmune IgY. Titers values of the cellular mediators TNF, IL-2, IL-6, IL-8, IL-12, IL-15 and IFN were approximately 10,000 or more. Repeated immunizations of hens using up to 10 ug of IL-1 per hen failed to generate a detectable IgY response in the hens. Overall, though the rest of the inflammatory mediators with adjuvant proved to be immunogenic in the hens. Good antibody titers were generated using small amounts of antigen. Thus the avian system appears to be a well-suited approach to produce high titer antibodies against mammalian inflammatory mediators.

EXAMPLE 2

Determination Of Anti-TNF IgY Neutralizing
Ability In A Cell-Based Neutralization Assay

This example involved the testing of the anti-TNF IgY neutralizing ability in a cell-based neutralization assay. Bioactivity of the anti-TNF IgY antibody was evaluated in the murine L929 cell based neutralization assay as previously described (N. Mathews *et al.*, 1987, Lymphokines and Interferons). Briefly, murine L929 cells(ATCC, Rockville, Maryland), sensitive to the cytotoxic effects of recombinant human TNF (rhTNF), were grown in sterile conditions with Ham's F12 and Dulbecco's Modified Eagles media (1:1 vol:vol ratio), containing 1.2g/L sodium bicarbonate and 15mM Hepes (Life Technologies,Gaithersburg, Maryland) and supplemented with 10% fetal bovine serum (Life Technologies). Cells were harvested using trypsin:EDTA (Life Technologies), and $2X10^4$ cells were dispensed into each well of a 96-well flat-bottomed plate (Costar) and incubated for 20 hours in a humidified chamber at 37°C and 5% CO_2. A commercial anti-TNF antibody, Remicade (a humanized mouse monoclonal antibody to human TNF, Centocor, Malvern, PA) served as a positive control, and a preimmune antibody, served as a negative control.

The IgY's were serially diluted in PBS (Life Technologies) supplemented with 1% BSA (wt:vol)(Life Technologies) and 10 ug/ml actinomycin D (ICN, Costa Mesa,CA). To each well containing antibody, an equal volume of 1ng/ml rhTNF (R&D Systems, Minneapolis, MN) was added, including controls which received only rhTNF or only PBS diluent. The plate was then incubated for 1 hour at 37°C. Finally, the antigen-antibody mixture was added to the cells and incubated for 20 hours at 37°C, 5% CO_2 in a humidified chamber. Cell viability was measured using the chromogenic Cell Titre 96 Proliferation Assay (Promega Corporation, Madison, WI) recording the optical density at 490 nm. The amount of anti-TNF that resulted in the prevention of cell death in 50% and 90% of the cells, termed neutralization dose

50 and 90 (ND50 and ND90) was calculated for each antibody. The results demonstrated that the anti-TNF IgY was effective at neutralizing TNF compared to preimmune IgY in the L929 cell-based assay. The ND50 and ND90 was determined to be approximately 70 ng/ml and 100 ng/ml, respectively.

EXAMPLE 3

Determination Of Anti-IL-2 IgY Neutralizing Ability In A Cell-Based Neutralization Assay

This example involved the testing of the anti-IL-2 IgY neutralizing ability in a cell-based neutralization assay. The ability of anti-IL-2 IgY to neutralize the bioactivity of IL-2 was determined in a cell-based assay based on a protocol described in *Current Protocols in Immunology*, Vol. 1, Section 6.3.4, 1994. CTLL-2 cell line (ATCC) requiring rhIL-2 (R&D systems) as a growth factor at 4 ng/ml was grown in RPMI 1640 with 10 % fetal calf serum at 37°C at 5% CO_2. Avian anti-IL-2 IgY was diluted serially two-fold with culture media in a 96 well microtiter plate. Goat anti-rh IL-2 IgG (R&D Systems) and preimmune IgY at the same concentrations were also serial diluted and served as positive and negative controls, respectively. Recombinant human IL-2 at 0.2 ng/well was added and pre-incubated with antibody for 1 hour at 37°C. CTLL-2 cells at 10^4/well was then added and incubated for 20 hours at 37°C, 5% CO_2 in a humidified chamber. Cell viability was measured using the chromogenic Cell Titre 96 Proliferation Assay (Promega Corporation, Madison, WI) recording the optical density at 490 nm. The results demonstrated that the anti-IL-2 IgY was effective at neutralizing rhTNF compared to preimmune IgY in the CTLL-2 cell-based assay. The ND 90 of anti-IL-2 IgY was approximately 500 ug/ml.

EXAMPLE 4

Determination Of Anti-IL-12 Neutralizing
Ability In A Cell-based Neutralization Assay

This example involved the testing of the anti-IL-12 IgY neutralizing ability in a cell-based neutralization assay. To measure the ability of anti-IL-12 IgY (generated to the heterodimer isoform) to neutralize the bioactivity of rhIL-12 heterodimer a cell-based assay was performed using peripheral blood mononuclear cells, (PBMC's). PBMC's were purified and activated according to the procedure in *Current Protocols in Immunology*, Vol. 1, Section 6.16, 1994. Various concentrations of the anti-IL-12 IgY (2,500 ug/ml-0.032 ug/ml) were incubated with rhIL-12 (R&D Systems) at 1ng/ml for 1 hour at 37°C in a 96 well plate. All dilutions were performed in the assay medium which consisted of a 1:1 dilution of RPMI (Life Technologies) and Dulbecco's Modified Eagles Medium (Life Technologies) with L-arginine (Life Technologies)at 2.5 mg/ml, 10% D-glucose (Sigma), and 10% human serum (Irvine Scientific). As a positive control, a commercially available anti-IL-12 (R&D Systems) was used, and as a negative control, preimmune IgY antibodies were used. Following the preincubation, the PBMC's were added to the antigen-antibody mixture at a final concentration of $2x10^5$ cells/ml and incubated for 48 hours at 37°C with 5% CO_2. During the final two hours of the incubation, Cell Titre 96 Proliferation Assay (Promega Corporation) was added, and the optical density was read at 490 nm. The assay indicated that anti-IL-12 IgY could neutralize rh IL-12 compared to preimmune IgY with an ND_{50} estimated at 0.47 mg/ml. (Data not shown).

EXAMPLE 5

Determination Of Anti-IFN-Gamma Neutralizing Ability *In Vivo* With A Mouse Endotoxin Model

This example involved the testing of the anti-IFN-gamma IgY neutralizing ability in a mouse endotoxin shock model. A bacterial lipopolysaccaride (LPS) model of endotixin shock described by J. Roth *et al.*, Nature, 364:798-802, 1993, was used to determine if anti-IFN-gamma IgY possessed neutralizing antibodies to IFN-gamma. Antibodies neutralizing to proinflammatory cytokines such as TNF and IFN-gamma have been reported to be effective in this model (Roth *et al.*). Equal concentrations of either preimmune IgY or anti-IFN-gamma IgY were pretreated with 1.2 mg of LPS from Salmonella enteritidis (Sigma) and incubated for 1 hour at 37°C. This pretreatment mixture (pretreatment)was then administered intraperitoneally into 18-20 G C57BL/6 mice (Charles River). Survival after challenge with LPS indicated that the antibodies were neutralizing against the cytokine. Similar experiments were preformed by directly administering the LPS /antibody mixture into mice without preincubation (premix). Results are shown in Table 3. The results indicated that anti-IFN-gamma IgY could effectively neutralize endogenous IFN-gamma produced by the mouse compared to preimmune control IgY in the LPS -endotoxin shock model.

Table 3

Treatment	# of Survivors / # Tested	% Survival
Preimmune IgY (pretreatment)	1/11	9
Anti-IFN-gamma IgY(pretreatment)	3/3	100
Preimmune IgY (premix)	0/6	0
Anti-IFN-gamma IgY (premix)	8/9	88

EXAMPLE 6

Treatment With Oral Anti-Cytokine Therapy In An
Animal Model Of Insulin-Dependant Diabetes Mellitus

An autoimmune animal model of insulin-dependant diabetes mellitis (IDDM) was used to determine if luminally-administered anti-cytokine therapy may be effective at preventing or delaying the onset of disease. IDDM is an autoimmune disease that effects about 0.2 % of the population causes the destruction of the insulin-producing (beta) islet cells in the pancreas. This destruction involves the presence of pancreatic autoimmune antibodies and leukocytic infiltration (insulitis) of the pancreatic islet cells. These events effectively lower the amount of normal insulin needed to maintain normal glucose metabolism resulting in diabetes. Nonobese diabetic (NOD) mice spontaneously develop autoimmune T-cell mediated IDDM. Diabetes in the NOD mouse is similar to human IDDM in both genetics and autoimmune pathogenesis (C.J. Boitard *et al.*, Autoimmunity 15 (Suppl):12-13, 1993 and A.A. Rossini *et al.*, Annu. Rev. Immunol., 3:289-320, 1995). This mouse strain has provided an important model for dissecting the pathogenesis of autoimmune diabetes in humans. NOD mice spontaneously develop insulitis between 2-4 weeks of age and progresses to diabetes 10-20 weeks later in about 80% of the female mice and 20% of the male mice. Severe IDDM ensues that results in excessive urine production containing high levels of glucose. With time, severe IDDM eventually results in a death in a most of the NOD mice.

This example involves: a) description of establishment of the NOD model and treatment, b) Methods and results to determine oral anti-cytokine efficacy on the delay of onset of glycosuria (high levels of glucose in the urine and c) prevention of mortality from disease.

a) *The NOD Model And Treatment.* Six week old (about 20 gram) female NOD mice were purchased (The Jackson Laboratory, Bar Harbor ME) and maintained under specific pathogen free (SPF) conditions. The maintenance of the mice in SPF conditions was performed according to procedures described in *Current Protocols in Immunology*, (1997) 15.9.1-15.9.23 by Edward H. Leiter (John Wiley & Sons, Inc.). SPF conditions required the use of autoclaved cages and barrier protected isolator lids with autoclaved food and bedding. The drinking water was filtered and acidified to pH 2-3 with HCl to prevent the growth of Pseudomonas. Groups of NOD mice (7/group) were aseptically treated orally with antibodies to two proinflammatory mediators, IL-2 and IL-12. Treatment controls consisted of treating mice orally with either vehicle (0.1 M carbonate buffer pH 9.2-9.5) or preimmune IgY. The IgY's were diluted in 0.1 M carbonate buffer to minimize IgY hydrolysis in the stomach. Treatments were sterile filtered (0.4 () before use. Approximately 0.2 mls of a 20-40 mg/ml (200-400 mg/kg/day) of IgY solution was orally administered using a 20 G, 3.5 cm long feeding needle (Popper & Sons, New Hyde, NY). Treatments were administered orally once per day, five days (weekdays) a week. Mid-way through the study, one mouse in the vehicle-treated group and one from the anti-IL-12 treated group died from dosing unrelated to IDDM disease.

b) *Efficacy: Delay Of Onset Of Glycosuria.* The monitoring of the glycemic status began on shortly after arrival using reagent strips to semi-quantitatively measure urine glucose. Either Diastix (glucose) or Uristix (glucose and protein) reagent strips for urinalysis were used (Bayer Diagnostics, Elkhart, IN). The level of glycemia was determined colorimetrically from turquoise to dark brown with the strips representing glucose levels of 100, 250, 500, 1000, and 2000 mg of glucose/dl. Individual mice were placed in clean cages without bedding and a drop of urine was collected on the test area and read after 30 seconds. Mice are considered diabetic with glycosuria when urine glucose levels were > 250 mg/dl. Treatment results are shown in Table 4. The results indicate that oral anti-cytokine therapy using both anti-IL-2 and anti-IL-12 IgY could delay the onset of diabetes in the NOD mouse model. The

onset of glycosuria first appeared when the mice were approximately 14-15 weeks old after about 8 weeks of treatment. Glycosuria first appeared at the same time in some mice treated with vehicle, preimmune and anti-Il-12 IgY. Initial onset of glycosuria in the anti-IL-2 treated mice was delayed. During treatments from approximately week 11 to week 16, 67 % and 43 % of vehicle treated and preimmune treated mice were glycosuric. In contrast, only 14 % and 17 % of the anti-Il-2 and anti-Il-12 treated mice, respectively were glycosuric. After 16 weeks of treatment nearly all the mice in the vehicle treated (100 %) and preimmune treated mice (83 %) had diabetes. In contrast, roughly half of the anti-cytokine treated groups (42 % for anti-IL-2 and 67 % for anti-IL-12) were diabetic. Preimmune IgY treatment compared to vehicle treatment was found to have no therapeutic effect in terms of delay of diabetes. Results, however indicated that the onset of diabetes in NOD mice with was delayed approximately 6 weeks after treatment with oral anti-cytokine therapy.

c) *Efficacy: Prevention Of Mortality From Disease.* Results indicated that oral anti-cytokine therapy using anti-IL-2 and anti-IL-12 IgY prevented death due to the complications of diabetes in the NOD mice model (Table 5). The time of death in the mice during treatment was monitored and closely coincided with several weeks of high glucose levels in the blood (2000 mg/dl). Death from diabetes started in both in the vehicle and preimmune treated mice at 17 weeks of age after about 11 weeks of treatment. Sixty-seven % of vehicle treated and 57 % of preimmune treated NOD mice were dead after 22 weeks of age. Significantly, none of the anti-IL-2 treated and only 17 % anti-IL-12 treated were dead after 22 weeks (Table 5). The results showed that oral anti-IL-2 and anti-IL-12 therapy in NOD mice could prevent mortality from disease compared to the control treated mice.

EXAMPLE 7

Treatment With Oral Anti-Cytokine Therapy In
An Animal Model Of Systemic Lupus Erythematosus

An autoimmune animal model of Systemic Lupus Erythematosus (SLE) was used to determine if oral anti-cytokine therapy may be effective at preventing or delaying the onset of disease. SLE is a systemic autoimmune disease that usually appears in women from 20 and 40 years of age and is characterized by fever, weakness, joint pain, erythematous lesions, pleurisy and kidney disfunction. Affected individuals may produce autoantibodies to a vast array of self-components such as DNA, red blood cells, and platelets. Immune complexes of autoantibodies are formed which are deposited on blood vessels resulting in vasulitis and glomerulonephritis in the kidney. The New Zealand black x New Zealand white F_1 hybrid mouse (NZB/W^1) spontaneously develop severe autoimmune disease that closely resembles SLE in humans (A.N. Theofilopoulos and F.J. Dixon, Adv. Immunol., 37:269, 1985). This model in particular has been very useful in understanding the immunological defects involved in the development of SLE autoimmunity. NZB/W^1 mice develop immune complex-mediated glomerulonephritis, resulting in the excretion of high levels of protein in the urine and an anti-DNA IgG serum response. As true with SLE in humans, the incidence of autoimmunity in NZB/W^1 female mice is much higher than males. Mice eventually develop a fatal disease around 6-9 months in female animals.

This example involves a description of: a) the NZB model and treatment, b) methods and results to determine oral anti-cytokine efficacy on the delay of onset of proteinuria (high levels of protein in the urine, c) Reduction of an anti-double stranded (ds) DNA serum response and d) Prevention of mortality from disease.

a) *The NZB/W^1 Model And Treatment.* Six week old (about 20 gram) female NZB/W^1 mice were purchased (The Jackson Laboratory, Bar Harbor ME) and maintained under normal non-SPF conditions according to the breeder. Groups of NZB/W^1 mice (7/group) were treated orally with anti-IL-2 IgY or anti-IL-12 IgY

diluted in 0.1 M carbonate buffer pH 9.2-9.5. Treatment control mice were treated orally with either vehicle (0.1 M carbonate buffer pH 9.2-9.5) or preimmune IgY. Approximately 0.2 mls of a 20-40 mg/ml (200-400 mg/kg/day) of IgY solution was orally administered using a 20 G, 3.5 cm long feeding needle (Popper & Sons, New Hyde, NY). Treatments were administered orally once per day, five days (week days) a week.

b) *Efficacy: The Delay Of Onset Of Proteinuria.* The presence of proteinuria in the NZB/W[1] mice was detected using reagent strips to semi-quantitatively measure urine protein. Uristix (glucose and protein) reagent strips for urinalysis were used (Bayer Diagnostics, Elkhart, IN). The presence of measurable amounts of protein in the urine by the reagent strips is considered abnormal and indicative of disease. The level of proteinuria was determined colorimetrically from light green (negative-trace) to dark green with the strips measuring protein levels of 30, 100, 300, and >2000 mg of protein/dl of urine. Individual mice were placed in clean cages without bedding and a drop was collected on the test area and read after 30 seconds. Mice are considered to have proteinuria when urine protein levels were > 30 mg/dl. Treatment results are shown in Table 6. The results indicate that oral anti-cytokine therapy with either anti-IL-2 and anti-IL-12 could delay the onset of diabetes in the NZB/W[1] mouse model. The onset of proteinuria first appeared in the preimmune and vehicle-treated mice approximately when the mice were 14 weeks old after about 7 weeks of treatment. During treatments from week 7 to week 28 the majority (71%-100%) of the vehicle-treated or Preimmune-treated mice had proteinuria (>30 mg/dl). Significantly, proteinuria in the NZB/W[1] mice treated orally with either anti-IL-2 and anti-IL-12 IgY was not detected until week 21 of treatment. After 28 weeks of treatment most of the vehicle-treated and preimmune treated mice were proteinuric (86 % (6/7)). In contrast, at week 28 of treatment, only 29 % (2/7) of the anti-IL-2 treated mice and 43 % (3/7) of the anti-IL-12 IgY treated mice were proteinuric. These results indicate that oral anti-cytokine therapy to IL-2 and IL-12

could effectively delay the onset of proteinuria in NZB/W[1] mice compared to the control treated mice.

c) *Reduction Of An Anti-Double Stranded (ds) DNA Serum Response.* The presence of an anti-ds DNA response which is pathognomonic for SLE was determined using a commercial enzyme immunoasssay (EIA) kit designed to measure specific autoantibodies against ds DNA in human serum. Blood samples were taken at various times during treatment from each NZB/W[1] mouse from the tail vein. Serum was then collected after the blood was allowed to clot. The microtiter EIA assay (Bindazyme Anti-ds DNA EIA kit, The Binding Site, Birmingham, England) was performed on the serum samples according to manufacturers instructions except instead of using the anti-human peroxidase conjugate, a sheep anti-mouse IgG peroxidase (The Binding Site) was used. The sheep anti-mouse IgG peroxidase conjugate was diluted 1/10,000 in the assay as recommended by the manufacturer. Internal assay controls were run and serum samples were tested in duplicate. Tetramethylbenzidine substrate was used and after 10 minutes the plates were read at 450 nm. The higher the absorbance value the test serum at 450 nm, the higher the anti-ds DNA response. The average absorbance with the standard error of the mean of each treatment group at various times is shown in Table 7. The results shown a similar increase in an anti-ds DNA serum response in both the vehicle and preimmune treated mice. This response is roughly reduced by half in the anti-IL-2 and anti-IL-12 treated NZB/W[1] mice throughout the treatment regimen. The anti-ds DNA serum response in the anti-cytokine treated mice is delayed approximately 11 weeks comparing the results from week 17 to week 28. Results indicated that oral anti-cytokine therapy using anti-IL-2 and anti-IL-12 IgY could effectively reduce the anti-ds DNA response in NZB/W[1] mice compared to the control treated mice.

d) *Prevention Of Mortality From Disease.* Results indicated that oral anti-cytokine therapy using anti-IL-2 and anti-IL-12 could delay mortality due to the complications of SLE in the NZB/W[1] mice model (Table 8). The time of death in the

mice during treatment was monitored and closely coincided with high anti-ds DNA levels in the serum. Death began to occur in the vehicle and preimmune-treated NZB/W[1] mice at 26 weeks of age (20 weeks of treatment) matching previous reports on this model. At a week 36, 57 % (4/7) of vehicle treated and 71% (5/7) of preimmune-treated mice died from disease. In contrast, none of the mice in the anti-IL-2 have died and all still appeared healthy. While 28% of the mice treated with anti-IL-12 died, onset was delayed (the first animal in this group died at week 32 and the second at week 36). The results showed that oral anti-IL-2 and anti-IL-12 therapy in NZB/W[1] mice could prevent mortality from disease compared to the control treated mice.

EXAMPLE 8

Determination of anti-IL-6 IgY Neutralizing Ability in a Cell-based Neutralization Assay

This example involved the testing of the anti-IL-6 IgY neutralizing ability in a cell-based neutralization assay.

To measure the ability of the anti-IL-6 IgY to neutralize the bioactivity of rhIL-6, a standard IL-6 cell assay was performed. B9 cells (The Central Laboratory, Amsterdam, The Netherlands) and rhIL-6 a growth factor for these ceslls were incubated with various concentrations of the antibody (100 ug/ml - 0.2 ug/ml) for 1 hour at 37 C and 5% CO2, in a 96 well plate. As a positive control, a commercially available anti-IL-6 antibody (R&D Systems) was used, and as a negative control, preimmune IgY antibodies were used. All antibodies and antigens were diluted in RPMI 1640 (Gibco BRL, Life Technologies, Rockville, MD) with 5% fetal bovine serum (Life Technologies), and 50 mM 2-mercaptoethanol. The assay mixture, containing antibodies at the concentrations listed, rhIL-6 (R&D Systems) at 2.5 ng/ml, and cells at 1x105 cells/ml, was incubated at 37 C and 5% CO2 for 48 hours. For the last 2 hours of the incubation, Cell Titre 96 Proliferation Assay (Promega Corp. Madison WI) was added, and the optical density of the plate was read at 490 nm. The

neutralizing dose of anti-IL-6 IgYwhich neutralized approximately 50% of the IL-6 designated as the ND50 . The ND50 of anti-IL-6 IgY in the B9 cell assay was determined to be 10 ug/ml (data not shown). Preimmune IgY was unable to neutralize the effects of IL-6. These results indicated that the anti-IL-6 IgY generated was capable of neutralizing IL-6.

EXAMPLE 9

Determination of anti- IL-8 Neutralizing Ability
in a Cell-based Neutralization Assay

This example involved the testing of the anti-IL-8 IgY neutralizing ability in a cell-based neutralization assay.

To assess the neutralization capacity of the anti-IL-8 IgY antibodies a standard assay to measure IL-8 activity was performed. IL-8 can induce myeloperoxidase activity measured from human neutrophils, as previously described (Schroder, J., et al, J. Immunol., 139:3474-3483,1987). Anti-IL-8 IgY activity was determined indirectly by measuring the inhibition of myeloperoxidase activity. Briefly, human neutrophils were isolated from whole blood and treated with 5 ug/ml of cytochalasin B (Sigma)as described by Schroder et al. Recombinant human IL-8 (R&D Systems) at 1 ug/ml was added to various concentrations (from 7.6 mg/ml-0.95 mg/ml) of anti-IL-8 IgY antibody in Gey's buffer (Sigma) containing 1 mg/ml BSA (Sigma). This mixture was incubated for 30 minutes at room temperature in a 96 well plate. As a positive control and negative control in this assay a commercially available anti-IL-8 antibody (R&D Systems) and preimmune IgY antibodies were also tested. To each microtiter plate well, $7x10^6$ cells/ml containing the IL-8/antibody mixture was added at a final volume of 100 ul and incubated for one hour at room temperature. Supernatants were harvested, and myeloperoxidase activity was measured using 0.167 mg/ml o-dianisidine (Sigma), with 0.0005% hydrogen peroxide in a 50mM phosphate buffer, pH 6.0. The change in myeloperoxidase activity at 460 nm was measured over 1 minute. At the highest dose tested (7.6 mg/ml), anti-IL-8 IgY inhibited approximately 30% of the

bioactivity of the IL-8 (data not shown). Preimmune IgY at the same concentration was unable to inhibit myeloperoxidase activity. Thus, these results indicated that the anti-IL-8 IgY generated here was capable of neutralizing IL-8.

EXAMPLE 10

Treatment with oral anti-cytokine therapy in murine experimental allergic encephalomyelitis (EAE).

An autoimmune animal model of EAE was used to determine if luminally-adminstered anti-cytokine therapy may be effective at preventing the onset or reducing the severity of disease. EAE is a T cell-mediated autoimmune disease of the central nervous system (CNS), which has a number of characteristics to the human neurological disorder multiple sclerosis. The disease can be initiated in rodents by immunization with CNS tissue or neuroantigens, such as myelin basic protein or proteolipid protein (PLP). During the induction of EAE, sensitized T-cells to the neuroantigen migrate into the CNS and react with the neuroantigen found in the myelin sheath of nerve cells. The activated T-cells release potent pro-inflammatory mediators which result in the recruitment of additional inflammatory cells to the CNS. Consequently, an increase in vascular permeability occurs with fibrin depostion leading to damage of normal nerve conduction and results in the clinical signs of EAE. This animal model is also characterized by the multiple episodes of reoccurring disease (relapse). This chronic relapses-remitting course of disease is typically found in many MS patients.

This example involves: a) description of the murine EAE model and treatment, b) treatment results using oral anti-TNF, anti-IL-2 or anti- IL-6 IgY, c) treatment results using higher dosages of oral anti-TNF or anti-IL-12 IgY.

a) *The EAE model and treatment.*

Active EAE was induced in SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) essentially as described by Whitham RH et al. 1991. Eight to ten week-old female mice were each immunized with 150 ug of PLP peptide (residues 139-151) emulsified in approximately an equal volume of complete Freunds adjuvant (Gibco, BRL) supplemented with Mycobacterium tuberculosis H37 RA (400 ug final). Each mouse was inoculated subcutaneously in the back with 200 ul of the PLP immunogen. On day 0 and 2 each mouse also received and intraperitoneal injection of 200 ng of pertussis toxin (List Biologics, Campbell, CA) in 500 ul of sterile PBS.

Mice were evaulated daily and scored for the severity of clinical disease on a scale from 0-5 as previously reported by Godiska R. *et al., J. Neuroimmunology* 58:167-176 (1995). A clinical score for each examination was assigned as follows: 0, normal; 1, loss of motor control in the tail; 2, hindquarter weakness or the inability to turn over when placed on back; 3, total hindquarter paralysis; 4, total hindquarter paralysis with incontinence and/or forearm involvement; 5, death due to EAE. A spontaneous clinical relapse was defined as an increase in clinical score of 1 or more, developing after a period of stabilization or improvement lasting for at least 24 hours. The average clinical score with the standard error of the mean (SEM) was determined at different time points throughout the course of disease. The scores of mice that died from disease (score of 5) were throughout to determine the average clinical score. Data analysis was performed using the Mann Whitney test for statistical significance. A p value of 0.1 or less was considered significant.

SJL/J mice (7/group) were treated orally with either anti-TNF IgY, anti-IL-2 IgY, anti-IL-6 IgY or anti-IL-12 IgY diluted in 0.1 M carbonate buffer. Treatment control mice were treated either orally with carbonate buffer (untreated) or preimmune IgY. Approximately 0.2 mls of a 40-50 mg/ml IgY solution was administered using a 20 G., 3.5 cm feeding needle (Popper & Sons, New Hyde, NY). Treatments were administered once or twice per day, five days (week days) a week.

b) *treatment results using oral anti-TNF, anti-IL-2 or anti- IL-6 IgY.*

In the first experiment, mice were pretreated with IgY for 1 week, once per day prior to the induction of disease. Mice were treated with approximately 500 mg/kg/day. Test mice were pretreated with either anti-TNF, anti-IL-2 or anti-IL-6 IgY. After induction the mice were treated for an additional two weeks before the termination of treatment. The results are shown in Table 9. Individual mice began to show signs of disease 9 to 10 days after the induction of disease. By day 12 , most of the mice (4 to 6) in each group displayed various clinical stages of disease. Most mice experienced multiple bouts of remission followed by relapse. Remission in a few mice never occurred and the mice with high clinical scores eventually died. These events were generally asynchronous in the mice, although by day 32, many mice in all groups were in remission. This is reflected in the lower clinical scores at that time.

Oral anti-TNF treatment could significantly reduce the severity of disease and prolong the time of remission in the EAE model. By day 14, mice treated with anti-TNF IgY had a reduced clinical score compared to the preimmune control ($p<0.1$). An increased improvement of clinical score was noted in the anti-TNF IgY treated group compared to the preimmune control. The p values at days 45, 63, and 78 were significant at 0.01, <0.05 and <0.05, respectively. The clinical scores on average were lower in the untreated mice compared to the preimmune treated group although the values were not statistically different. The clinical scores of the anti-TNF IgY treated mice was statistically lower than the untreated mice at days 45, 63 and 78 with p-values of <0.1, <0.05 and 0.1, respectively.

The results also indicated that the effects of anti-TNF extended far beyond the treatment course. More than a week after the termination of treatment, at approximately day 23, 5 out of the 7 mice were in remission (a score of 0) and remained there until about day 50. Mice in the other treatment groups experienced relapses during this time.

The results also indicated that oral anti-IL-2 IgY treatment had a therapeutic benefit in the EAE model (Table 9). The therapeutic effects of anti-IL-2 were not as striking as anti-TNF, but resulted in an statistical improvement in clinical scores

compared to preimmune IgY. Average clinical scores were lower in the anti-IL-2 treated animals compared to scores of the preimmune treated mice. At day 73, the difference in scores were statistical at $p<0.1$. As with the anti-TNF treatment, the clinical effects of the anti-IL-2 IgY extended beyond the treatment period. The improvement of clinical score appears to result from a longer time of remission after anti-IL-2 treatment.

In contrast to both anti-TNF and anti-IL-2, oral treatment of mice with anti-IL-6 IgY had no benefical effect on the clinical course of disease (Table 9). The average clinical score throughout the disease process in the EAE model was very similar to that in the untreated and preimmune treated groups.

c) *treatment results using higher dosages of oral anti-TNF or anti-IL-12 IgY.*

Another experiment was performed using higher doses of oral anti-TNF IgY in the EAE model. The results supported the results of the previous experiment. Mice were treated twice per day (800 mg/kg) for 2 weeks starting 2 hours before the induction of disease with PLP. Treatment groups consisted of preimmune, anti-TNF and anti-IL-12 IgY. The results of the treatment study is shown in Table 10. The clinical onset of disease was similar to that in the previous experiment and started on about day 11. Oral anti-TNF IgY at 800 mg/kg significantly reduced the clinical score compared to the preimmune IgY treatment. The clinical scores were significantly lower (p values of <0.1 to 0.025) at most the times indicated in the Table 10, except for day 30 when remission was occurring in the preimmune treated mice. The higher dose of anti-TNF effectively reduced the clinical score to zero at most of the time points. The effect of oral anti-TNF was also long-lasting beyond the treatment period. Consequently, anti-TNF treatment prevented the onset of releapse which occurred on about day 41 in the preimmune treated mice. The results indicate that oral treatment with anti-TNF IgY is very effective in the murine EAE model.

At the treatment dose of 800 mg/kg, anti-IL-12 IgY was ineffective at reducing the clinical disease score in the EAE model. The average scores were very similar to those in the mice treated with preimmune IgY.

Overall, oral anti-TNF treatment in the murine EAE model was very effective at treating the clinical symptoms of disease. Results indicated that anti-TNF treatment could significantly reduce the severity of disease and extend the length of remission. In addition, oral anti-IL-2 treatment was also found to be effective at ameliorating the clinical indications of EAE.

Those skilled in the art will know, or be able to ascertain upon review of the above, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method of treatment, comprising:

a) providing:

i) a human patient with symptoms of autoimmune disease,

ii) a therapeutic formulation comprising one or more ligands directed to a proinflammatory cytokine, and;

b) administering said formulation to said patient.

2. The method of Claim 1, wherein said ligand is an antibody.

3. The method of Claim 1, wherein said ligand is a receptor to said cytokine.

4. The method of Claim 1, wherein said administering reduces said symptoms.

5. The method of Claim 1, wherein said administering is performed orally.

6. The method of Claim 1, wherein said administering is performed parenterally.

7. The method of Claim 1, wherein said administering is performed rectally.

8. The method of Claim 1, wherein said proinflammatory cytokine is selected from IL-2, TNF, IL-12 and IFN-gamma.

9. The method of Claim 2, wherein said antibody is avian polyclonal antibody.

10. The method of Claim 9, wherein said avian antibody is purified antibody.

11. The method of Claim 10, wherein said antibody is purified from eggs.

12. A method of treatment, comprising:

a) providing:

i) a human patient at risk of autoimmune disease,

ii) a therapeutic formulation comprising one or more ligands directed to a proinflammatory cytokine, and;

b) administering said formulation to said patient.

13. The method of Claim 12, wherein said ligand is an antibody.

14. The method of Claim 12, wherein said ligand is a receptor to said cytokine.

15. The method of Claim 12, wherein said administering delays the onset of autoimmune symptoms.

16. The method of Claim 12, wherein said administering is performed orally.

17. The method of Claim 12, wherein said administering is performed parenterally.

18. The method of Claim 12, wherein said administering is performed rectally.

19. The method of Claim 12, wherein said proinflammatory cytokine is selected from IL-2, TNF, IL-12 and IFN-gamma.

20. The method of Claim 13, wherein said antibody is avian polyclonal antibody.

21. The method of Claim 20, wherein said avian antibody is purified antibody.

22. The method of Claim 21, wherein said antibody is purified from eggs.

23. A method of treatment, comprising:

a) providing:

i) a human patient with symptoms of autoimmune disease,

ii) a therapeutic formulation comprising one or more ligands directed to a proinflammatory cytokine, and;

b) administering said formulation to the lumen of the intestines of said patient.

24. The method of Claim 23, wherein said ligand is an antibody.

25. The method of Claim 23, wherein said ligand is a receptor to said cytokine.

26. The method of Claim 23, wherein said administering reduces said symptoms.

27. The method of Claim 23, wherein said administering is performed orally.

28. The method of Claim 23, wherein said administering is performed rectally.

29. The method of Claim 23, wherein said proinflammatory cytokine is selected from IL-2, TNF, IL-12 and IFN-gamma.

30. The method of Claim 24, wherein said antibody is avian polyclonal antibody.

31. The method of Claim 30, wherein said avian antibody is purified antibody.

32. The method of Claim 31, wherein said antibody is purified from eggs.

33. A method of treatment, comprising:

a) providing:

i) a human patient at risk of autoimmune disease,

ii) a therapeutic formulation comprising one or more ligands directed to a proinflammatory cytokine, and;

b) administering said formulation to the lumen of the intestines of said patient.

34. The method of Claim 33, wherein said ligand is an antibody.

35. The method of Claim 33, wherein said ligand is a receptor to said cytokine.

36. The method of Claim 33, wherein said administering delays the onset of autoimmune symptoms.

37. The method of Claim 33, wherein said administering is performed orally.

38. The method of Claim 33, wherein said administering is performed rectally.

39. The method of Claim 33, wherein said proinflammatory cytokine is selected from IL-2, TNF, IL-12 and IFN-gamma.

40. The method of Claim 34, wherein said antibody is avian polyclonal antibody.

41. The method of Claim 40, wherein said avian antibody is purified antibody.

42. The method of Claim 41, wherein said antibody is purified from eggs.

Table 4.

Oral anti-cytokine therapy prevents the onset of prevents glucosuria In NOD mice

Treatment Group	% Diabetic Mice	
	Day 75	Day 115
Vehicle	67	83
Pre-immune	43	100
Anti-IL-2	14	43
Anti-IL-12	17	67

Table 5.

Oral anti-cytokine therapy prevents the onset of mortality In NOD mice

Treatment Group	% Mortality
Vehicle	67
Pre-immune	57
Anti-IL-2	0
Anti-IL-12	17

Table 6.

Oral anti-cytokine therapy prevents the onset of proteinuria in NZB/W mice

Treatment Group	% Proteinuria ≥30 mg/dl	
	Week 11	Week 28
Vehicle	100	86
Pre-immune	100	86
Anti-IL-2	0	28
Anti-IL-12	0	43

Table 7.

Oral anti-cytokine therapy reduces an Anti-ds DNA response in NZB/W mice

Treatment Group	Week 7	Week 13	Week 17	Week 24	Week 28
			Average Absorbance (450nm) +/- SEM		
Vehicle	0.224 +/- 0.04	0.612 +/- 0.23	0.73 +/- 0.19	0.836 +/- 0.17	1.1 +/- 0.12
Pre-immune	0.225 +/- 0.09	0.693 +/- 0.26	0.75 +/- 0.14	0.855 +/- 0.1	1.17 +/- 0.03
Anti-IL-2	0.158 +/- 0.02	0.236 +/- 0.06	0.384 +/- 0.32	0.494 +/- 0.13	0.577 +/- 0.14
Anti-IL-12	0.116 +/- 0.01	0.130 +/- 0.01	0.388 +/- 0.2	0.633 +/- 0.18	0.814 +/- 0.16

Table 8.

Oral anti-cytokine therapy prevents the onset of mortality in NZB/W mice

Treatment Group	Cumulative Mortality
Vehicle	57
Pre-immune	71
Anti-IL-2	0
Anti-IL-12	28

Table 9

Treatment with oral anti-TNF can reduce the severity of disease in the EAE model*

Mean Clinical Score**

Group	Untreated	Preimmune	Anti-TNF	Anti-IL-2	Anti-IL-6
Day 14	1.6 (0.8)	2.6 (0.6)	1.3 (0.6)	1.6 (0.6)	2.3 (0.6)
Day 32	1.4 (0.7)	1.7 (0.9)	0.7 (0.7)	1.4 (0.7)	1.1 (0.7)
Day 45	2.0 (0.7)	2.4 (0.7)	0.7 (0.7)	2.0 (0.8)	2.0 (0.6)
Day 63	2.1 (0.6)	2.6 (0.7)	0.9 (0.7)	1.6 (0.9)	2.1 (0.7)
Day 78	2.4 (0.6)	3.1 (0.7)	1.1 (0.7)	1.4 (0.9)	3.1 (0.7)

* Mice were dosed with 500 mg/kg /day.

** SEM's are shown in grams (in parenthesis).

Table 10

Treatment with oral anti-TNF can reduce the severity of disease in the EAE model*

Mean Clinical Score**

Group	Preimmune	Anti-TNF	Anti-IL-12
Day 13	0.6 (0.3)	0.0 (0.0)	0.1 (0.1)
Day 18	0.9 (0.1)	0.2 (0.6)	1.0 (0.5)
Day 30	0.3 (0.2)	0.0 (0.0)	0.1 (0.1)
Day 41	1.0 (0.5)	0.0 (0.0)	0.9 (0.5)
Day 48	1.4 (0.6)	0.0 (0.0)	1.3 (0.6)

* Mice were dosed with 800 mg/kg/day.

** The standard errors of the mean are shown in grams (in parenthesis).