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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/20, C07K 16/44, G01N 33/533, 33/94

A1

(11) International Publication Number: **WO 95/16026**

(43) International Publication Date: 15 June 1995 (15.06.95)

(21) International Application Number: PCT/US94/13558

(22) International Filing Date: 22 November 1994 (22.11.94)

(30) Priority Data:
08/165,121 10 December 1993 (10.12.93) US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: REAGENTS AND METHODS FOR THE DETECTION OF METHOTREXATE

(57) Abstract

A murine monoclonal IgG antibody is provided which is useful in the detection of methotrexate in body fluids. This monoclonal antibody, designated 4-351-178, may be employed in an assay for methotrexate, preferably a fluorescence polarization immunoassay (FPIA) of urine. Monoclonal antibody 4-351-178 is characterized by having no detectable cross-reactivity with 7-hydroxymethotrexate. Also disclosed is a hybridoma cell line, ATCC HB 11467, which produces the monoclonal antibody of the invention.

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REAGENTS AND METHODS FOR THE DETECTION OF METHOTREXATE

Field of the Invention

The present invention relates to the immunological detection of methotrexate. In particular, the invention relates to a cell line which secretes a monoclonal antibody specific for methotrexate and immunological methods for detecting the presence or amount of methotrexate in a test sample.

Background of the Invention

Methotrexate is an antineoplastic agent which is commonly used for treating patients having solid tumors and/or hematological malignancies. The dosages of methotrexate received by these patients can vary widely because the therapeutic effect of methotrexate varies with the dosage. Unfortunately, however, patients on high methotrexate regimens may experience toxic side effects.

In order to avoid the toxic side effects associated with high doses of methotrexate, immunoassays have been developed to monitor patients' methotrexate levels. These immunoassays commonly use antibodies, which are specific for methotrexate, to assist in the detection of the presence or amount of methotrexate in a patient's test sample. Results from these assays can be used to modify, if necessary, a patients methotrexate regimen to prevent the toxic side effects that may result from high doses of methotrexate or to optimize a patients methotrexate regimen.

Immunoassays designed to determine the presence or amount of methotrexate in a test sample require highly specific antibodies to avoid erroneous assay results which may lead to inappropriate dosage changes which, can in turn, lead to toxic side effects or the administration of less than optimal methotrexate doses. Erroneous assay results can occur when the antibody used to detect methotrexate cross reacts with other compounds. For example, 7-hydroxymethotrexate is a metabolite of methotrexate that is structurally similar to methotrexate and cross reacts with anti-methotrexate antibody.

Because 7-hydroxymethotrexate is a metabolite of methotrexate, nearly every test sample containing methotrexate will also contain 7-

hydroxymethotrexate, and, in some instances, the concentration of the metabolite can exceed the concentration of the parent compound. Thus, an assay designed to determine the presence or amount of methotrexate in a test sample should employ antibodies that have very little cross reactivity with 7-hydroxymethotrexate. Most desirably, such antibodies should have no detectable cross reactivity with 7-hydroxymethotrexate.

European Patent Application No. 0044441 generally describes the production of monoclonal antibodies specific for drugs, but it does not describe the production of a monoclonal antibody specific for methotrexate. Moreover, the disclosure does not describe an anti-methotrexate antibody with any particular specificity.

Assays which detect the presence or amount of methotrexate in a test sample and which are currently available, employ polyclonal antibodies to assist in the detection of methotrexate. However, according to the methods of determining cross reactivity used herein, such polyclonal antibodies can display cross reactivity with 7-hydroxymethotrexate.

Hence there is a need for an antibody that is more discriminating than those currently available which can be used in an immunoassay to accurately determine the presence or amount of methotrexate in a test sample.

Summary of the Invention

The present invention provides a monoclonal IgG antibody that is specific for methotrexate and has no detectable cross reactivity with 7-hydroxymethotrexate. Additionally, the invention provides a hybridoma continuous cell line which secretes the above monoclonal antibody and has been designated cell line number A.T.C.C. HB 11467.

The monoclonal antibody of present invention can be used in various immunoassay formats. According to one embodiment, a fluorescence polarization immunoassay (FPIA) for determining the presence or amount of methotrexate in a test sample is provided. The FPIA comprises the steps of (a) contacting the test sample with (i) the monoclonal IgG antibody provided herein and (ii) a fluorescent tracer molecule, to form a solution; (b) contacting the solution with plane polarized light; and (c) detecting a fluorescence polarization response. According to another embodiment, an improved immunoassay for

determining the presence or amount of methotrexate in a test sample is provided. The improved immunoassay is of the type having a step of contacting the test sample with antibodies specific for methotrexate, wherein the improvement comprises contacting the test sample with the monoclonal IgG antibody herein provided.

The present invention also provides a test kit for determining the presence or amount of methotrexate in a test sample. The test kit comprises (a) the monoclonal IgG antibody of the invention; and (b) a fluorescent tracer molecule.

Detailed Description of the Invention

The following definitions are applicable to the invention.

The term "analyte analog", as used herein, refers to a substance which cross-reacts with a methotrexate-specific antibody, although it may do so to a greater or lesser extent than does the analyte (methotrexate) itself. The analyte analog can include a modified analyte as well as a fragmented or synthetic portion of the analyte molecule, so long as the analyte analog has at least one epitope in common with the analyte of interest.

The term "immunogen", as used herein, refers to a substance which, when introduced into an animal having an immune system, is capable of stimulating an immune response. Certain low molecular weight substances which are not capable of stimulating an immune response are referred to as haptens. However, haptens can be made immunogenic by conjugating a hapten to a carrier molecule, which may itself be immunogenic. Thus, while generally not immunogenic, haptens conjugated to carrier molecules are immunogenic and can be considered immunogens.

The term "signal generating group" as used herein refers to a fluorescent compound (sometimes referred to as a fluorophore) which is capable of absorbing energy and emitting light or fluorescing. Examples of signal generating groups include, but are not intended to be limited to fluorescein, cascade blue, TEXAS RED™, p-phthallocyanines, cyanine dyes, thiazoles, dansyl, napthalene, p-toluidinyl napthalene sulfonic acid, coumarin, phycoerythrin, allophycocyanine and the like.

The term "test sample", as used herein, refers to a material suspected of containing methotrexate. The test sample can be used

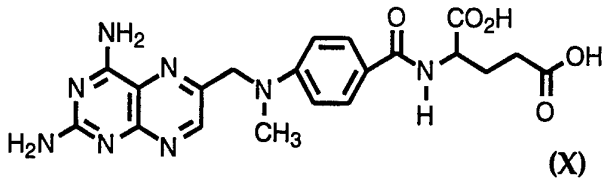
directly as obtained from the source or following a pretreatment to modify the character of the sample. The test sample can be obtained from any biological source, such as a physiological fluid, including, but not limited to blood, saliva, ocular lens fluid, cerebral spinal fluid, sweat, urine, milk, ascites fluid, mucous, synovial fluid, peritoneal fluid, amniotic fluid and the like; fermentation broths; cell cultures; chemical reaction mixtures and the like. The test sample can be pretreated prior to use, such as preparing plasma from blood, diluting viscous fluids, and the like. Methods of treatment can involve filtration, distillation, concentration, inactivation of interfering components, and the addition of reagents. In addition to biological or physiological fluids, other liquid samples can be used such as water, food products and the like for the performance of environmental or food production assays. In addition, a solid material suspected of containing the analyte can be used as the test sample. In some instances, it may be beneficial to treat a solid test sample to form a liquid medium or extract the analyte.

It has been unexpectedly discovered that according to the present invention, a cell line can be produced which secretes a monoclonal antibody which is specific for methotrexate and displays, surprisingly, no detectable cross-reactivity with 7-hydroxymethotrexate. Moreover, the antibody maintains its specificity over a range of temperatures. Consequently, the antibody herein provided can be employed in an immunoassay which is more discriminating than the methotrexate immunoassays currently available.

The monoclonal antibody of the instant invention can be produced following the techniques described generally in Kohler and Milstein, "Continuous Culture of Fused Cells Secreting Antibody of Predefined Specificity", Nature, 256, 495 (1975) and incorporating the modifications described herein. Briefly, an immunogen is created via the conjugation of methotrexate to an immunogenic carrier molecule to produce an immunogen. The immunogen can be used to immunize animals such as guinea pigs, sheep, rats and the like, preferably mice. Although virtually any animal capable of producing an immune response can be immunized with the above immunogen to form antibody producing cells, mice are preferred and will be used hereinafter for illustrative purposes. The immunization is periodically boosted to increase the titer of the mice antisera, and mice producing antisera specific for methotrexate are

identified. Splenocytes from the mice which produce antisera to methotrexate can then be removed and fused with a mouse myeloma cell line. The resulting hybrids are then screened for secretion of methotrexate specific antibody and positive hybrids are selected and expanded, or cloned, to produce a sufficient quantity of antibody for purification. Expansion of a positive clone can be accomplished using methodologies well known in the art such as, for example, in tissue culture or by injecting mice with the clone and collecting the ascites fluid from the mice after sufficient time has elapsed to allow the clone to replicate and produce antibody.

Immunogens which can be used to assist in the production of the antibody herein provided can generally be described as carrier-molecule-functionalized methotrexate. Structurally, the hapten methotrexate is shown below as the compound of the formula X.

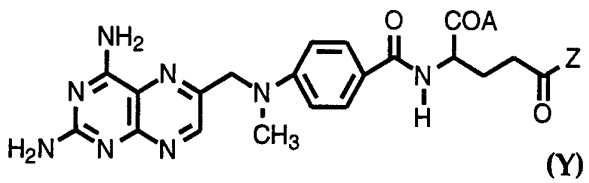


(X)

Carrier molecules that can be used to synthesize an immunogenic form of methotrexate can be selected from any of those conventionally used in the art, and in most instances will be a protein or polypeptide, although other materials such as carbohydrates, polysaccharides, lipopolysaccharides, poly(amino) acids, nucleic acids, and the like of sufficient size and immunogenicity can also be employed. Examples of carrier molecules include, but are not intended to be limited to thyroglobulin, keyhole limpet hemocyanin (KLH), and the like, preferably bovine serum albumin (BSA). As it will be understood, of course, some carrier molecules are not inherently reactive with methotrexate, however, carrier molecules can be made reactive through the introduction of an appropriate reactive moiety using methodologies well known in the art.

Methotrexate can be made immunogenic by functionalizing or conjugating it with at least one carrier molecule to synthesize a compound of the formula Y, shown below, wherein A and Z are independently -OH or a carrier molecule. Methods of synthesizing immunogens of the formula Y are well known in the art. For example, combining methotrexate and a carrier molecule in the presence of a

dehydrating agent will yield an immunogen of the formula Y. Suitable dehydrating agents include, but are not intended to be limited to dicyclohexyl carbodiimide (DCC), 3 ethyl 1-(3-dimethylaminopropyl) carbodiimide (EDAC) and the like. Heterobifunctional or homobifunctional linking agents, which are well known in the art, can also be used to synthesize an immunogenic methotrexate molecule. Such coupling agents include but are not intended to be limited to m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), sulfosuccinimidyl 4-(p-maleimidophenyl) butyrate (S-SMPB), m-maleimidobenzoylsulfosuccinimide ester (S-MBS), N-g-maleimidobutyryloxysuccinimide ester (GMBS) and the like. It will be understood, of course, that the instant invention is not limited by the methods employed to synthesize the immunogens taught herein.



(Y)

The present invention also provides improved immunoassays for determining the presence or amount of methotrexate in a test sample. An improved immunoassay includes or comprises a step of contacting a test sample, which may contain methotrexate, with the antibody herein provided. It is contemplated that any immunoassay for methotrexate utilizing the antibody herein provided is within the scope of the present invention. Examples of immunoassays include, but are not intended to be limited to, radioimmunoassays (RIAs), enzyme immunoassays (EIAs), enzyme linked immunosorbent assays (ELISAs), particle concentration fluorescence immunoassays (PCFIAs) and preferably fluorescent polarization immunoassays (FPIAs).

In general, the antibody herein provided can be utilized in competitive binding immunoassays to determine the presence and/or amount of an analyte in a test sample. Competitive binding immunoassays are typically used for measuring analytes in a test sample, and for purposes of the present invention, the analyte is methotrexate. The analyte and an analyte analog compete for the binding site on the antibody specific for the analyte and analyte analog.

The concentration of analyte in the test sample determines the amount of analyte analog which binds to the antibody, and the amount of analyte analog that will bind to the antibody is inversely proportional to the concentration of analyte in the test sample because the analyte and the analyte analog each bind to the antibody in proportion to their respective concentrations.

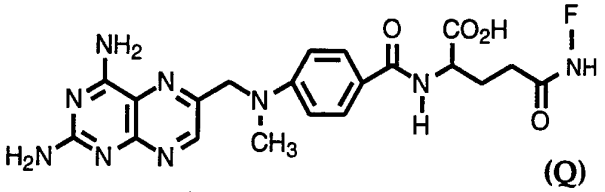
In one embodiment of the invention, FPIA techniques are utilized for determining the amount of tracer-antibody complex produced in a competitive binding immunoassay. Such procedures are based on the principle that a fluorescently labeled compound or tracer, when excited by plane polarized light, will emit fluorescence having a degree of polarization inversely related to its rate of rotation. Accordingly, when a tracer is bound by an antibody and the signal generating group associated with the tracer is excited with plane polarized light, the emitted light remains highly polarized because the fluorescent label is constrained from rotating between the time that light is absorbed and emitted. In contrast, when a signal generating group associated with an unbound tracer is excited by plane polarized light, its rotation is much faster than the corresponding tracer-antibody complex and the molecules become more randomly oriented. As a result, the light emitted from the unbound tracer molecules is depolarized.

A preferred FPIA method of the present invention for determining the presence or amount of methotrexate in a test sample comprises the steps of: (i) forming a solution by contacting a test sample with (a) the monoclonal antibody of the present invention and (b) a fluorescent tracer wherein the tracer is capable of producing a detectable fluorescence polarization in response to the presence of the antibody; (ii) contacting the solution formed in step (i) with polarized light to obtain a fluorescence polarization response; and (iii) detecting the fluorescence polarization response of the solution of step (ii) as a measure of the presence or amount of methotrexate in the test sample.

By adding a constant concentration of the antibody and tracer to a test sample, the ratio of methotrexate-antibody complexes to tracer-antibody complexes that are formed is directly proportional to the amount of methotrexate in the test sample. Upon exciting the mixture with linearly polarized light and measuring the polarization (in units of polarization) of the fluorescence emitted by the unbound tracer and

tracer-antibody complexes, one is able to quantitatively determine the amount or qualitatively determine the presence of methotrexate in a test sample. The results can be quantified in terms of net millipolarization units (mP) and span (in millipolarization units). The measurement of net millipolarization units indicates the maximum polarization when a maximum amount of the tracer is bound to the antibody (in the absence of any methotrexate) and the higher the net millipolarization units, the better the binding of the tracer to the antibody. Assay span is the difference between the net millipolarization values obtained when the maximum amount of tracer is bound in the absence of any methotrexate and the net millipolarization obtained when a specific amount of methotrexate is present in the test sample. A larger span allows for more millipolarization units to be placed between each of the calibrators of the standard curve generated for the assay, thereby providing better assay precision which, in turn, results in a better numerical analysis of the data obtained. It is important to note that the span varies depending on the sample size and the amounts of antibody and tracer can be altered accordingly.

Fluorescent tracer reagents (tracers) that can be used, in conjunction with the antibody of the instant invention, to perform FPIAs, substantially correspond to the aforementioned immunogens. However, instead of a carrier molecule, methotrexate molecules or analogs thereof are functionalized with signal generating groups. Methodologies well known in the art can be used to synthesize tracers using any of the signal generating groups previously defined. While any of the previously mentioned signal generating groups can be used to produce a tracer, fluorescein and derivatives thereof are preferred. Preferably, the tracer corresponds to a compound having the formula (Q) shown below wherein F is a signal generating group. Most preferably, F is 5-fluorescein. It will be understood, of course, that the invention herein provided is not limited by the signal generating group used to synthesize a tracer.



(Q)

As previously mentioned, an FPIA is the preferred method for using the antibody herein provided. Reagents that can be used for such an assay comprise: 1) a monoclonal antibody specific for methotrexate and 2) a fluorescent tracer reagent. Additionally, conventional reagents including a pretreatment solution, a dilution buffer, methotrexate calibrators and controls are desirably prepared. These reagents are commercially available in assay "kits" from Abbott Laboratories, Abbott Park, IL.

A test kit according to the present invention comprises all of the essential reagents required to perform a desired immunoassay for the quantification or qualitative determination of methotrexate in a test sample. The test kit is presented in a commercially packaged form as a combination of one or more containers holding the necessary reagents, as a composition or admixture where the compatibility of the reagents will allow. Particularly preferred is a test kit for a FPIA quantification of methotrexate in a test sample, comprising the antibody herein provided and a tracer having the formula Q shown above. It will be understood, of course, that the test kit can include other materials well known in the art and which may be desirable from a users standpoint, such as buffers, diluents, standards and the like.

The preferred procedure is particularly designed to be used in conjunction with the Abbott TDX® Clinical Analyzer, the Abbott TDXFLX™ or the Abbott ADX® Drugs of Abuse Systems, all of which are available from Abbott Laboratories. The calibrators, controls, or unknown samples, which do not require any prior preparation, are pipetted directly into the sample well of the TDX® sample cartridge and the remaining assay procedure is fully automated.

All references to patents or publications in this specification are incorporated herein by reference.

The following examples are provided to further illustrate embodiments of the invention and should not be construed as a limitation on the scope of the invention. All animals, media, reagents, and equipment used in the production of the cell line and monoclonal antibody of the invention are publicly available. Those skilled in the art will readily recognize that modifications and variations of the disclosed protocols and methods exist and are within the scope of the invention.

Example 1
IMMUNONOGEN SYNTHESIS

Materials:

1. (+)-Amethopterin (methotrexate) - available from Sigma Chemical Co. (Sigma); St. Louis, Missouri
2. 1-(3-Dimethylaminopropyl) carbodiimide·HCl (EDAC) - available from Sigma
3. Bovine serum albumin (BSA) - available from Intergen; Purchase, New York
4. SEPHADEX® G50-300 - available from Pharmacia-LKB; Piscataway, New Jersey
5. Phosphate buffered saline (PBS) - 0.01 M $NaPO_4$, 0.15 M NaCl, pH 7.2 to 7.5

Procedure:

Methotrexate (251.2 mg) is dissolved in 4.0 ml of distilled water and 1.0 ml of 2.0 N sodium hydroxide (NaOH) to form a solution. The pH of the resulting solution is adjusted to 7.5 with 0.1 N hydrochloric acid (HCl). The total volume of the solution is approximately 10.0 ml (approximately 25 mg/ml methotrexate). BSA (77.7 mg) is dissolved in 2.0 ml of distilled water to form a second solution. A third solution is prepared and comprises 250 mg of EDAC in 5.0 ml of distilled water.

1.5 ml of the methotrexate solution is added to the BSA solution and dissolved at room temperature. Over a three hour period, 0.7 ml of the EDAC solution is added to the methotrexate/BSA solution in 100 µl aliquots, and thereafter, 0.8 ml of the EDAC solution is added all at once for a total EDAC solution addition of 1.5 ml. This reaction mixture is allowed to mix overnight and after the mixing period the solution turns to a clear bright-yellow-solution.

A SEPHADEX® G50-300 column (2.5 mm x 400 mm) is equilibrated with PBS before the reaction mixture is applied to it and eluted with PBS. The eluant from the column is monitored for absorbance (A_{280}) and the immunogen (BSA-methotrexate complex) is contained in the first peak eluted from the column. The volume of the collected portion of the first peak is 44.5 ml with a protein concentration of 2.0 mg/ml which yields 89.0 mg of immunogen.

Example 2

Tracer Synthesis

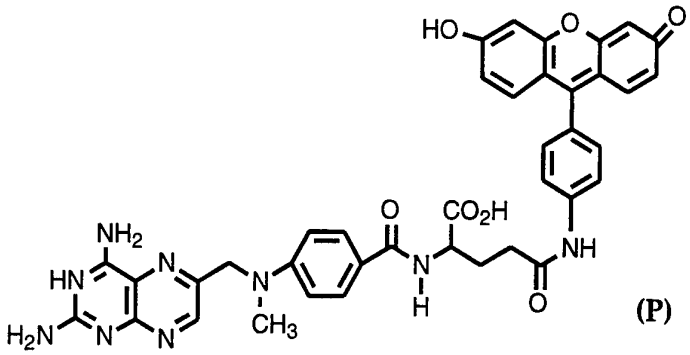
Materials:

1. Methotrexate - available from Sigma
2. 5-aminofluorescein - available from Aldrich Chemical Co. (Aldrich); Milwaukee, Wisconsin
3. SiO_2 Thin Layer Chromatography (TLC) plates - available from EM Reagents; Gibbstown, New Jersey
4. C_{18} TLC plates - available from Whatman Inc.; Clifton, New Jersey

Procedure:

The following solutions are prepared: 0.2305 g of methotrexate in enough dimethylsulfoxide (DMSO) to dissolve the methotrexate; 0.09 g of p-nitrophenol in enough DMSO to dissolve the p-nitrophenol; 0.11 g of dicyclohexyldicarbodiimide (DCC) in enough DMSO to dissolve the DCC; and 0.1747 g of 5-aminofluorescein in enough DMSO to dissolve the 5-aminofluorescein. The methotrexate (approximately 1.0 ml), DCC (approximately 1.0 ml), and p-nitrophenol solutions (approximately 1.0 ml) are combined to form a reaction mixture that is allowed to stir for 4 hours at room temperature. Then, the 5-aminofluorescein solution (approximately 1.0 ml) is added to the reaction mixture while it is stirring. The resulting reaction mixture is protected from light and allowed to stir overnight. After the mixing period, the reaction mixture is applied to eight, 2.0 mm SiO2 TLC plates. The full length of the plates are developed with (2:1) acetone:chloroform and allowed to dry prior to developing the plates half-way with (1:1) chloroform:methanol and allowing them to dry. The bands from one developed plate are separately collected and eluted from the SiO_2 plates with methanol before the bands from the plate are applied to a C_{18} TLC plate. The C_{18} plate is developed in 50% methanol with 0.05% sodium dodecyl sulfate (SDS) and 50% 0.2% KH_2PO_4 in distilled water and show that the product is in the region corresponding to R_f 0.05 to 0.38 of the SiO_2 plates. The remaining SiO_2 plates are scraped from R_f 0.05 to 0.38 and the product is eluted with methanol, concentrated to approximately 4.0 ml, and applied to ten 1.0 mm C_{18} TLC plates. The plates are developed as above and the brightest band, as determined by an ultraviolet lamp, is at R_f 0.69 to 0.76. The band is collected, eluted with ethanol and concentrated as before. The

concentrated product is applied to eight C_{18} TLC plates, developed, and the final product is collected as above and is shown below as the compound of the formula (P).



(P)

Example 3

Anti-methotrexate Monoclonal Antibody Production

Immunization strategy

A 2.0 mg/ml slurry, containing the methotrexate conjugate of Example 1 emulsified in Freund's Complete Adjuvant (Difco Inc., Detroit, Michigan) and 0.9% saline, is used to subcutaneously immunize ten RFB/DnJ mice (available from Jackson Laboratories; Bar Harbor, Maine) such that each mouse received a total of 100 µg of conjugate. Four weeks later each mouse is immunized a second time using the same procedure specified above except that the conjugate is emulsified in Incomplete Freund's Adjuvant (Difco Inc., Detroit, Michigan) and each mouse is immunized with 20 µg of conjugate. A third 20 µg/mouse immunization is performed after 8 weeks from the date of the original immunization with the Incomplete Freund's Adjuvant protocol and a final 20 µg/mouse immunization is completed 12 weeks after the original immunization. The mice are bled 6, 10 and 15 weeks after the initial immunization and sera from the bleeds is assayed for an immune response to methotrexate.

Serum Evaluation

Utilizing a competitive binding assay format, the mouse sera (from above) is assayed for anti-methotrexate activity. The samples are tested on a TDX® Clinical Analyzer (Abbott Laboratories) using a reagent configuration which includes reagents labeled S pot, T pot, and P pot which contain the following:

S pot: 0.1 M phosphate buffer, 2% ethylene glycol, 5% BSA, 0.1% sodium azide, 0.01% bovine gamma globulin (BGG)

T pot: 62 nM fluorescein tracer in 0.1 M tris, 0.7% sodium dodecyl sulfate (SDS), 0.5% lithium dodecyl sulfate (LDS), 0.01% (BGG), 0.1% sodium azide

P pot: 0.1 M tris, 0.7% SDS, 0.5% LDS, 0.01% BGG, 0.1% sodium azide

Before running the assay, the mouse sera is serially diluted in the S pot diluent which is dispensed in the sample cup of the sample cartridge. The dilutions range from a neat sample to a 1:32 dilution. Antibody displacement is tested by spiking 8.0 µl of the A and F calibrators in the predilute cup of the sample cartridge. The sample cartridges are placed on the carousel and the samples are tested using mode 11 pipetting sequence which has a 10 µl sample size. Displacement of the A-F (0-1.0 µmol/L) calibrators ranged from 28-65 mP. Because the serum sample from mouse designated 1-1A gave the best displacement, a curve is generated for the serum from this mouse and the serum is assayed for 7-hydroxymethotrexate, 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and aminopterin cross reactivity. The results from the assay show low cross reactivities for all three of the cross reactants and a decision is made to use splenocytes from mouse 1-1A for fusion.

Pre-Fusion Immunization

Three days prior to cell fusion and 12 weeks after the original immunization, mouse 1-1A is given a 20 µg injection of the methotrexate conjugate (from Example 1) diluted in 0.1 ml phosphate buffered saline (PBS - pH 7.2) via the tail vein.

Fusion

On the day of the fusion, mouse 1-1A is euthanized by cervical dislocation and the spleen is removed. The splenocytes are washed one time in Iscove's Modified Dulbecco's Medium (IMDM - GIBCO, Grand Island, New York) and centrifuged at 1000 RPM for 7 minutes. The supernatant is removed and the pelletted splenocytes are combined with SP2/0 myeloma cells (from the laboratory of Dr. Milstein, Cambridge, United Kingdom) at a 1:1 ratio, washed in IMDM, and centrifuged. The

supernatant is removed from the pelletted cells and 1.0 ml of 50% PEG (polyethylene glycol available from American Type Culture Collection, Rockville, Maryland) is added to the pellet and the pellet is gently dispersed for approximately 1 minute by tapping and swirling. 30.0 mls of IMDM is added to the mixture and centrifuged as previously described. The supernatant is decanted and the pellet is resuspended in IMDM with HAT (hypoxanthine, aminopterin, thymidine available from GIBCO), 10% Fetal Bovine Serum (FBS - Hyclone Laboratories, Logan, Utah) and 0.5% STM v/v (RIBI Immunochem Research, Inc., Hamilton, Montana). STM denotes Salmonella typhimurium mitogen, a B-cell specific mitogen, and is used to enhance fusion frequency. The fusion cell suspension is plated into 96-well tissue culture plates.

Fusion Screening

The primary screening of the 96-well fusion suspension supernatant occurred on day 10 of confluent cultures. A SCREEN MACHINE™, (IDEXX Laboratories, Westbrook, Maine) is used according to manufacturer's instructions for the primary fusion screen. Microparticles coated with goat anti-mouse IgG + M (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) and the monoclonal antibody fusion supernatant sample are added to the 96-well IDEXX FLUORICON® assay plates. Following a 15 minute incubation, the plates are washed and a methotrexate-fluorescein conjugate is added to each sample well. After a 10 minute incubation, the plates are washed a final time and the relative fluorescent intensity is read. Values with elevated signals indicate the presence of anti-methotrexate antibody in the culture. Cultures with a signal greater than 3 times the negative control are expanded to 24-well tissue culture plates for further evaluation.

Positive Hybrids from the IDEXX screen are then tested on the Abbott TDX® Clinical Analyzer according to a modified screening format which allows the monoclonal antibody supernatant to be tested in the sample wells. According to the modified assay format, the methotrexate reagent pack containing the S pot, T pot and P pot reagents (described above) and pipetting mode 11 are used. Additionally, the sample size is 10.0 µl and the sera from the hybrids is used as the sample. Polarization of the test samples is evaluated in the absence and

presence of free methotrexate and the cross reactivity of the antibody with 7-hydroxymethotrexate is also evaluated. Antibody from hybrids that had a millipolarization (mP) value for 7-hydroxymethotrexate and the A calibrator within 20 mP of each other are selected for cloning.

Hybrid cloning

Seventy hybrids are cloned by limiting dilutions starting at a 1-100 dilution, 10-fold down to 10^{-6}. The cloning media is IMDM with 10% v/v FBS and 1% v/v HT supplement (hypoxanthine, thymidine available from GIBCO). A 100 µl cell suspension is added to each of the 96 wells in the tissue culture plate and on day 7 the plates are fed with 200 µl/well of cloning media. The antibody from the resulting clones is assayed for anti-methotrexate activity and 7-hydroxymethotrexate cross reactivity using the same method used to screen the hybrids. Based on the clone screening, 23 clones are selected for antibody production and further screening.

Antibody Production

The selected clones are scaled up in ascites fluid by injecting approximately 10^6 cells into the peritoneal cavity of pristaned primed female BALB/c mice (Charles River Labs; Charles River, New York). Two weeks later the animals are euthanized and the ascites fluid is harvested. The antibody containing fluid is centrifuged at 1000 RPM for 10 minutes to remove the cells. The ascites fluid is then screened for specificity and temperature sensitivity.

Specificity and Temperature Sensitivity Screening

A modified screening format allows selection of a methotrexate clone based on temperature sensitivity (airset studies) and specificity (cross-reactivity studies). The antibody from the methotrexate clones is tested on the TDX® Clincal Analyzer (Abbott Laboratories) using pipetting mode 11, a sample size of 17.5 µl, 13.5 µl of altering A & F calibrator in the pre-dilute cup, and antibody straight or titered in the sample cup. The reagent pack configuration comprises the the S pot, T pot and P pot diluent and reagents previously described.

All antibodies from the anti-methotrexate producing clones are initially screened using the above procedure and the titer is selected to

have a Po value of between 170 and 190. When an antibody dilution gave a Po value between 170 and 190, the antibody is screened at that dilution for temperature sensitivity. The temperature sensitivity protocol requires three runs. All three runs follow the modified screening format previously described with the exception that, the A and F calibrators are run in duplicate on an instrument with a correct airset setting (34.0°C), a -2 airset setting (32.0°C) to lower reaction temperature, and +2 airset setting (36.0°C) to raise the reaction temperature. Actual temperatures of the liquid in the cuvette are measured with an external temperature probe in the last cuvette on the carousel at the completion of the run. The mPs are compared, looking for the least amount of change seen in the A and F calibrators for the 32.0°C and 36.0°C runs. For comparison, an unpurified antibody having undesirable temperature and cross reactivity characteristics (UP935) and the commercial polyclonal antibody (S pot) are used as positive and negative controls respectively. The data for the antibody from 23 clones assayed for the temperature sensitivity is shown in Table 1. Additionally, the values shown for the controls are an average taken from multiple runs.

Table 1

	34.0° Airset		-2 Airset (32.0 °C)			+2 Airset (36.0 °C)			Total Change		
Antibody	**A mP**	**F mP**	**A mP**	**F mP**	**°C**	**AmP**	**F mP**	**°C**	**AmP**	**FmP**	**°C**
4-263-310	173.3	37.9	175.1	37.8	32.8	168.7	36.7	35.0	6.4	1.1	2.2
4-422-182	194.2	102.7	201.9	102.6	32.8	186.1	104.1	35.0	15.8	1.5	2.2
4-512-169	178.3	68.0	180.7	63.2	32.8	171.6	69.1	35.0	9.1	5.9	2.2
4-618-107	182.9	115.8	189.1	121.7	32.9	170.7	114.9	35.2	18.4	6.8	2.3
4-681-225	172.8	41.6	175.9	39.9	32.9	165.2	39.5	35.2	10.7	0.4	2.3
4-757-121	***191.9***	***39.6***	***192.1***	***38.3***	***32.9***	***187.6***	***38.6***	***35.2***	***4.5***	***0.3***	***2.3***
4-843-216	178.5	112.7	184.2	112.2	32.7	169.8	110.2	35.2	14.4	2.0	2.5
4-1034-293	***190.7***	***47.7***	***188.3***	***45.0***	***32.7***	***185.8***	***46.5***	***35.2***	***2.5***	***1.5***	***2.5***
4-1083-171	177.4	92.2	179.9	89.8	32.7	169.6	89.1	35.2	10.3	0.7	2.5
UP935(+ctrl)			189.5	89.6	32.7	184.3	95.3	35.2	5.2	5.7	2.5
S pot(-ctrl)			185.9	62.5	32.7	181.9	63.2	35.2	4.0	0.7	2.5
4-351-178	***177.6***	***36.7***	***176.4***	***37.3***	***32.4***	***177.3***	***37.0***	***35.2***	***0.9***	***0.3***	***2.1***
4-424-125	185.9	37.3	189.9	36.9	32.4	182.3	36.4	35.0	7.6	0.5	2.6
4-606-194	176.0	36.0	182.2	36.8	32.4	176.2	36.4	35.2	6.0	0.4	2.8
4-704-151	***186.5***	***35.1***	***187.2***	***35.7***	***32.5***	***186.3***	***34.9***	***35.2***	***0.9***	***0.8***	***2.7***
4-768-178	***178.0***	***37.7***	***179.9***	***37.3***	***32.5***	***176.4***	***37.9***	***35.2***	***3.5***	***0.6***	***2.7***
4-803-251	184.7	51.4	187.6	51.1	32.5	179.5	56.6	35.2	8.1	5.5	2.7
4-957-150	***182.7***	***36.2***	***184.5***	***36.8***	***32.7***	***181.2***	***36.1***	***35.0***	***3.3***	***0.7***	***2.3***
4-1058-117	184.4	45.6	189.2	45.2	32.9	180.6	47.2	35.0	8.6	2.0	2.1
UP935(+ctrl)			188.7	87.5	32.9	184.5	95.4	35.0	4.2	7.9	2.1
S pot (-ctrl)			186.3	61.9	32.9	182.5	63.6	35.0	3.8	1.7	2.1
4-175-150	***179.4***	***37.2***	***181.4***	***37.1***	***32.6***	***176.0***	***36.7***	***35.3***	***5.5***	***0.4***	***2.7***
4-192-109	184.7	122.1	195.0	125.3	32.6	174.7	119.0	35.2	20.3	6.2	2.6
4-240-248	180.8	66.0	188.6	68.0	32.6	174.9	67.2	35.3	13.7	0.8	2.7
4-363-113	***173.8***	***36.5***	***178.0***	***37.4***	***32.6***	***173.7***	***36.4***	***35.2***	***4.2***	***1.0***	***2.6***
4-496-289	181.0	63.3	190.5	62.0	32.6	174.6	62.3	35.3	15.9	0.3	2.7
4-529-149	181.2	67.5	185.6	63.3	32.6	177.6	68.2	35.3	8.0	4.9	2.7
S pot (-ctrl)	180.4	62.1	185.2	62.3	32.6	179.1	63.0	35.3	6.1	0.7	2.7
UP935 (+ctrl)	179.4	88.4	184.4	84.5	32.6	178.0	93.0	35.3	6.4	8.4	2.7

Antibody from clones designated 4-757-121, 4-1034-293, 4-351-178, 4-704-151, 4-768-178, 4-957-150, 4-175-150, and 4-363-113 are selected as possible candidates from the airset experiment. The antibody from the eight clones is further screened for cross-reactivity.

Initial cross-reactivity testing is performed on the antibody from the eight clones using the TDX® Clinical Analyzer (Abbott Laboratories) and the cross-reactants are made in recalcified human plasma without the presence of methotrexate. The cross reactants (shown in Table 2) are added to the antibody solutions such that their concentration in the sample is as shown in Table 2. The respective cross-reactivities associated with the antibody form the eight clones are also shown in Table 2.

Table 2

Cross-Reactant	4-175-150	4-363-113	4-351-178	4-704-151
Aminopterin @ 39.5 µmol/L	1.4%	2.2%	>2.5%	1.9%
DAMPA @ 6 µmol/L	5.5%	16.0%	4.8%	7.8%
7-hydroxymethotrexate @ 100 µmol/L	0.04%	0.06%	0.05%	0.06%

Cross-Reactant	4-757-121	4-768-178	4-957-150	4-1034-293
Aminopterin @ 39.5 µmol/L	1.9%	1.9%	>2.5%	2.1%
DAMPA @ 6 µmol/L	3.5%	3.8%	5.0%	4.2%
7-hydroxymethotrexate @ 100 µmol/L	0.04%	0.04%	0.05%	0.05%

Additional airset studies and cross-reactivity testing are performed on the remaining seven antibodies using the S pot, T pot, and P pot reagents as previously described. New calibration curves are generated using pipetting mode 11 and sample volumes between 3.5 µl and 10.0 µl in order to optimize the calibration curves. The calibrators are run in duplicate at the optimal airset (34°C). The airset studies are then repeated by running the calibrators as samples (in duplicate) at ± 2 airset units to change the reaction temperature in the cuvettes to approximately 32°C and 36°C. The mP values of the calibrators from the two airset runs are evaluated for their degree of change. Cross-reactivity is then repeated by running the aforementioned cross-reactants as samples (in duplicate) using the above reagents on the new calibration curve at the optimal airset setting of 34°C. The commercial methotrexate

assay (Abbott) is also repeated as a control. Results from the second set of testing on the seven antibodies, are shown in Table 3. As shown in Table 3, the AmP, FmP and ^{o}C describe the total changes between 32°C and 36°C reaction temperatures. The A-F, A-B, and E-F columns represent the spans seen with each antibody reagent configuration.

Table 3

Antibody	AmP	FmP	°C Effect	Amin-opterin	DAMPA	7-hydroxy metho-trexate	A-F	A-B	E-F
Commercial	5.0	0.7	2.5	16.8%	55.2%	0.18%	120	10.5	13.3
4-351-178	**1.0**	**1.9**	**2.7**	**8.6%**	**7.8%**	**0.06%**	**130**	**5.5**	**34.1**
4-704-151	1.3	0.3	2.8	4.7%	13.3%	0.07%	139	8.2	21.5
4-768-178	4.9	1.5	2.9	7.3%	6.3%	0.06%	135	5.2	40.0
4-757-121	4.5	3.3	2.6	6.6%	6.0%	0.05%	131	6.0	44.0
4-957-150	4.8	1.9	2.9	14.7%	7.7%	0.06%	132	5.4	41.2
4-175-150	7.9	4.0	3.0	6.6%	6.3%	0.05%	132	6.1	42.2
4-1034-293	6.3	5.5	2.7	2.1%	6.3%	0.06%	139	8.6	18.1

Clone Selection

Based on the the results shown in Table 3, Clone 4-351-178 is selected for antibody production because the antibody that it secretes has a potential for a larger curve and has low cross reactivity values.

Isotype

The isotype of the monoclonal antibody secreted from the cell line 4-351-178 is determined to be an IgG^1 with a kappa light chain. The isotype was identified with a Mouse Monoclonal Antibody Isotyping Kit (Amersham Searle Corporation; Arlington Heights, Illinois) according to manufacturer's instructions.

Electrophoretic evaluation

The isoelectric point (pl) of the monoclonal antibody secreted from the cell line identified as 4-351-178 and purified using a protein A column is determined on an isoelectric focusing apparatus (Bio Rad, Richmond, California). The gel is cast and run according to vendor recommendations and the results indicate a pI of 6.7 ± 0.2.

According to the manufacturer's instruction, SDS-PAGE evaluation is performed with the S&S PROFILE™ Mini-Electrophoretic System (Schleicher & Schuell, Keene, New Hampshire). The antibody is run under 2-mercaptoethanol reduced conditions. Coomassie staining of the SDS-PAGE profile identifies the typical antibody banding pattern of a single light chain band at 24 kD and a single heavy chain band at 56kD.

Cell Line Deposit

In accordance with the Budapest Treaty, the hybridoma cell line, designated as hybrid 4-351-178, is deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville MD, 20852, United States of America. The deposit date is October 19, 1993, and the ATCC number assigned to the cell line is HB 11467.

Example 4

Specificity and Cross Reactivity

This example is intended to compare a prior art commercial assay (TDX® methotrexate assay - Abbott Laboratories), which uses a polyclonal antibody, to an assay using the antibody from the cell line designated 4-351-178 and commercial reagents. The cross-reactants were made in recalcified human plasma, as above, without the presence of methotrexate. The commercial assay is run according to the manufactures instructions and the assay using the antibody from clone 4-351-178 is run according to the commercial assay except that the pipetting mode is mode 43, and the antibody from cell line 4-351-178 (diluted in 0.001 M phosphate buffer, 0.15 M NaCl, 10% glycerol, 5% BSA, 0.01% BGG, and 0.1% sodium azide) is substituted for the antibody reagent (S pot). The T pot comprises 250 nM methotrexate fluorescein tracer in 0.1 M tris, 0.7% sodium dodecyl sulfate (SDS), 0.5% lithium dodecyl sulfate (LDS) and 0.1%sodium azide; and the P pot comprises 0.1 M tris, 0.7% SDS, 0.5% LDS, 0.35% lithium diiodosalicylate (LIS), and 0.1% sodium azide.

The results from the two assays are shown in Table 4 which shows the increased specificity of the monoclonal antibody secreted by the cell line designated 4-351-178 and the possibility of the commercial assay antibody cross reacting with 7-hydroxymethotrexate.

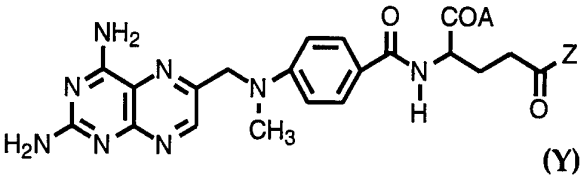
Table 4

Cross-Reactant	Commercial	4-351-178
DAMPA @ 1.0 µmol/L	47%	38%
DAMPA @ 6.0 µmol/L	>16.7%	15%
7-hydroxymethotrexate @ 1000 µmol/L	<1%	None detected

CLAIMS

1. A monoclonal IgG antibody which specifically binds to methotrexate having less than 0.01% cross reactivity with 7-hydroxymethotrexate.

2. The antibody of Claim 1, wherein the antibody is raised in response to an immunogen of the formula (Y)



(Y)

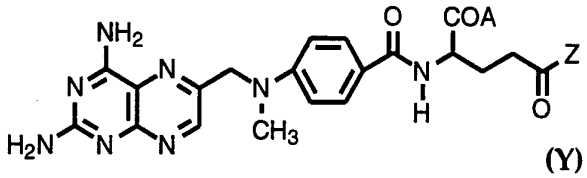
wherein one of A and Z is -OH and the other is a carrier molecule selected from the group consisting of bovine serum albumin, keyhole limpet hemocyanin, and thyroglobulin.

3. The antibody of Claim 2, wherein the carrier molecule is bovine serum albumin.

4. The antibody of Claim 1, wherein the antibody is secreted by a hybridoma cell line designated A.T.C.C. HB 11467.

5. A continuous hybridoma cell line which secretes IgG antibody which specifically binds to methotrexate wherein the antibody has less than 0.01% cross reactivity with 7-hydroxymethotrexate.

6. The cell line of Claim 5, wherein the antibody is raised in response to an immunogen of the formula (Y)



(Y)

wherein one of A and Z is -OH and the other is a carrier molecule selected from the group consisting of bovine serum albumin, keyhole limpet hemocyanin, and thyroglobulin.

7. The cell line of Claim 6, wherein the carrier molecule is bovine serum albumin.

8. The cell line of Claim 5 which is designated A.T.C.C. HB 11467.

9. A monoclonal IgG antibody secreted by the hybridoma cell line designated as A.T.C.C. HB 11467 which specifically binds to methotrexate.

10. The hybridoma cell line designated as A.T.C.C. HB 11467.

11. A fluorescence polarization immunoassay for determining the presence or amount of methotrexate in a test sample comprising the steps of:

a) contacting the test sample with

i. a monoclonal IgG antibody specific for methotrexate wherein the antibody has no detectable cross reactivity with 7-hydroxymethotrexate, and

ii. a fluorescent tracer molecule,

to form a solution;

b) contacting the solution with plane polarized light; and

c) detecting a fluorescence polarization response.

12. A test kit for determining the presence or amount of methotrexate in a test sample comprising:

a) a monoclonal IgG antibody specific for methotrexate having no detectable cross reactivity with 7-hydroxymethotrexate; and

b) a fluorescent tracer molecule.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/13558

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/20 C07K16/44 G01N33/533 G01N33/94

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 C12N C07K G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HYBRIDOMA, vol.6, no.1, February 1987, NEW YORK NY, USA pages 87 - 95 M. COT ET AL. 'Production and characterization of highly specific anti-methotrexate monoclonal antibodies.' see the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1995	20 -03- 1995

Name and mailing address of the ISA	Authorized officer
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	Nooij, F

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGICAL METHODS, vol.67, no.2, 1984, AMSTERDAM, THE NETHERLANDS pages 321 - 336 Y. KATO ET AL. 'Monoclonal antibodies to the chemotherapeutic agent methotrexate: Production, properties and comparison with polyclonal antibodies.' see abstract see page 329, line 18 - page 332, line 8 see page 335, line 6 - line 10 see figures 4-6 ---	1-10
A	ZEITSCHRIFT FüR MEDIZINISCHE LABORATORIUMSDIAGNOSTIK, vol.29, no.1, 1988, BERLIN, GERMANY pages 44 - 48 G. KRAUSE ET AL. 'Enzyme immunoassay for the determination of methotrexate in serum.' see the whole document ---	1-10
A	GAN TO KAGAKU RYOHO (JAPANESE JOURNAL OF CANCER AND CHEMOTHERAPY), vol.18, no.7, June 1991, TOKYO, JAPAN pages 1119 - 1125 S. SAKAMOTO ET AL. 'Serum monitoring of methotrexate (MTX) and 7-hydroxymethotrexate concentrations in patients treated with MTX using high-pressure liquid chromatography (HPLC) and comparison of serum MTX levels between HPLC method and fluorescence polarization immunoassay (FPIA).' see abstract ---	11,12
A	THERAPEUTIC DRUG MONITORING, vol.8, no.1, 1986, NEW YORK NY, USA pages 115 - 121 M. PESCE ET AL. 'Evaluation of a fluorescence polarization immunoassay procedure for quantitation of methotrexate.' see the whole document -----	11,12

1