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(54) Titre : PROCEDE DE CRIBLAGE UTILISANT L'ADSORPTION D'ECHANTILLON SUR UN PAPIER FILTRE
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(57) Abrégé/Abstract:
The present invention discloses a diagnostic test and method comprising mixing blood or another biological fluid sample with' a test compound and spotting the blood or the biological fluid on filter paper for subsequent analysis of the effect of the test compound on the blood or on the biological fluid. The biological fluid can be a cerebrospinal fluid, a peritoneal fluid, a cyst fluid, an amniotic fluid, a lavage fluid, a saliva, a. cell extract or a tissue extract. The test compound is chosen among an amino acid, a peptide, a protein, a carbohydrate, an oligosaccharide, a polysaccharide, a glycoprotein, a lipid, a lipoprotein, a glycosa- minoglycan, a hormone, a steroid, a vitamin, a low molecular weight synthetic or natural compound which influences the blood or the biological fluid to cause an alteration of its composition e.g. a toxin, allergen, autoantigen, bacterial protein or polysaccharide, viral protein, fungal protein or polysaccharide, parasitic protein or polysaccharide, bacterial lipopolysaccharide or any other compound relevant to diseases.

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(54) Title: SCREENING METHOD USING SAMPLE ADSORPTION ON FILTER PAPER

(57) Abstract: The present invention discloses a diagnostic test and method comprising mixing blood or another biological fluid sample with' a test compound and spotting the blood or the biological fluid on filter paper for subsequent analysis of the effect of the test compound on the blood or on the biological fluid. The biological fluid can be a cerebrospinal fluid, a peritoneal fluid, a cyst fluid, an amniotic fluid, a lavage fluid, a saliva, a. cell extract or a tissue extract. The test compound is chosen among an amino acid, a peptide, a protein, a carbohydrate, an oligosaccharide, a polysaccharide, a glycoprotein, a lipid, a lipoprotein, a glycosa- minoglycan, a hormone, a steroid, a vitamin, a low molecular weight synthetic or natural compound which influences the blood or the biological fluid to cause an alteration of its composition e.g. a toxin, allergen, autoantigen, bacterial protein or polysaccharide, viral protein, fungal protein or polysaccharide, parasitic protein or polysaccharide, bacterial lipopolysaccharide or any other compound relevant to diseases.

SCREENING METHOD USING SAMPLE ADSORPTION ON FILTER PAPER

Field of invention

The present invention discloses a diagnostic test and method for mixing blood or another biological fluid or sample with a test compound and spotting the blood on filter paper for subsequent analysis of the effect of the test compound on the blood or fluid sample.

General background

Blood is a complex mixture composed of plasma and cells (1-3). The plasma can be separated from the cells by centrifugation and other techniques. If the plasma is allowed to stand it will clot by coagulation and serum may be separated from the blood clot. The coagulation may be inhibited by addition of various anticoagulants, including EDTA, EGTA, heparin, citrate and others. The cells of the blood include dendritic cells, macrophages, monocytes, neutrophils, T lymphocytes, B lymphocytes, natural killer cells, red blood cells and various stem cells including hemopoietic stem cells. In addition megakaryocyte-derived platelets are present in large numbers. The plasma contains thousands of proteins, in principle any protein of the human proteome (4, 5). Some of the proteins are involved in transport, blood clotting or immune defence, while others function as signalling molecules between cells of the blood and cells of the tissues. In particular, the activity of the cells of the immune system (dendritic cells, macrophages, T cells, B cells, natural killer cells) is regulated by a complex network of signalling molecules (e.g. interleukins, chemokines, growth factors), tissue antigens and receptors (3, 6-9). The activity and specificity of immune system cells can be investigated and quantitated by several methods and assays. T cells, B cells and other cells can be quantitated by fluorescence-activated cell sorting using antibodies to cell surface marker molecules (10, 11). Specific T cells can be measured by cytotoxicity assays, chromium release assays and cytokine release assays (e.g. ELISPOT) (12-16) and by using various peptide-major histocompatibility complex (MHC) protein constructs (17, 18). The activ-

ity of B cells can be measured by determining the levels of specific antibodies released from the B cells (19, 20).

A major problem in measuring signalling molecules released from blood cells is that of storage and transport in relation to quantitation. Many blood constituents (e.g. cytokines) are labile and short lived, resulting in degradation during incubation, storage and transport. For this reason, comparative analyses and diagnostic tests have to be carried out immediately upon blood collection and incubation in central laboratories. Ideally, all samples to be compared should be analysed consecutively using a calibrated instrument.

This is not always practical, e.g. when taking blood samples in remote areas, when doing in vitro and in vivo time-studies or when comparing samples from many different individuals. One solution to this problem is to freeze samples for transport and storage. This, however, does not guarantee preservation of constituents, requires large freezing, transport and storage capacity, requires thawing each time an analysis is performed, and is vulnerable with regard to shortage of electric power supply. For this reason, there is a need for reliable methods of blood and biological sample preservation and a need for diagnostic tests employing reliable sample preservation in combination with sample manipulation.

The use of filter paper for spotting blood for subsequent analysis is well known, e.g. for analysis of blood samples of newborn babies for inherited metabolic diseases (21). The advantages of this are good preservation of blood constituents, easy transport and facile long term storage. However, the use of filter paper and similar methods for drying and storing blood samples after incubation with test compounds has not been used or described before, possibly because this has been anticipated to be impossible or impractical.

Summary of the invention

This invention discloses diagnostic tests and methods for mixing blood or another biological fluid with a test compound and spotting the blood on filter paper for dry-

ing, preservation and subsequent analysis of the effect of the test compound on the blood at any time hereafter.

Detailed disclosure of the invention

The present invention discloses a diagnostic test and method comprising mixing blood or another biological fluid sample with a test compound and spotting the blood on filter paper for subsequent analysis of the effect of the test compound on the blood. The biological fluid can be a cerebrospinal fluid, a peritoneal fluid, a cyst fluid, an amniotic fluid, a lavage fluid, a saliva, a cell extract or a tissue extract. The compound is chosen among an amino acid, a peptide, a protein, a carbohydrate, an oligosaccharide, a polysaccharide, a glycoprotein, a lipid, a lipoprotein, a glycosaminoglycan, a hormone, a steroid, a vitamin, a low molecular weight synthetic or natural compound which influences the blood to cause an alteration of its composition e.g. a toxin, allergen, autoantigen, bacterial protein or polysaccharide, viral protein, fungal protein or polysaccharide, parasitic protein or polysaccharide, bacterial lipopolysaccharide or any other compound relevant to diseases.

The diagnostic test and method according to the invention analyses the sample for the content of cytokines, chemokines and growth factors and/or neurotransmitters and other polypeptides and proteins e.g. CRP, IgG, IgA, IgM, IgD, IgE, specific (i.e. antigen specific) antibodies, transferrin, albumin and/or transthyretin.

In the diagnostic test the effect of the test compound is analysed by immunoassay, bioassay, mass spectrometry, HPLC, GC, GC-MS e.g. ELISA assays, FLISA assays, DELFIA assays, Luminex assays, luminescence assays, electrochemiluminescence assays, scintillation proximity assays, radioimmunoassays, MALDI-MS, ESI-MS and ambient-MS (e.g. DESI-MS).

This invention also discloses a method of mixing blood or another biological fluid or sample with a test compound and spotting the mixture on filter paper for storage, transport and/or handling before subsequent analysis of the effect of the test compound on the blood, biological fluid or sample.

Definitions :

Analyte means any compound which can be detected or quantitated by analytic means.

By the effect of test compound is understood that it interacts with constituents of the blood or any other biological fluid or sample to cause an alteration in the composition of the blood of any kind.

Drying means removal of water.

Filterpaper means any piece of paper, cloth or other material suitable for collecting, drying and storing blood.

PKU paper means paper/filter paper used for screening of blood samples from newborn babies for phenyl ketonuria syndrome.

Spotting means the application of a blood sample or any other biological fluid or extract or sample to a piece of standardised paper suitable for accurate blood sampling. The spotting is done by applying a fixed volume of blood to a piece of paper or by applying blood to the paper until a defined area is covered with blood. Subsequently, the paper is allowed to dry completely and either stored immediately at low humidity conditions or transported to a storage place for subsequent analysis.

Test compound means any chemical, biological or physical compound or substance which can be mixed with or added to blood or any other biological fluid or sample.

Test sample means any formulation or mixture of test compounds.

The following abbrevitions are used:

BCG means Bacillus Calmette-Guerin

BDNF means brain-derived neurotrophic factor

BSA means bovine serum albumin

CRP means C-reactive protein

DBSS means dried blood spot sample

EGF means epidermal growth factor

ELISA means enzyme-linked immunosorbent assay

ELISPOT means enzyme-linket immunospot assay

ESI means electrospray ionization

FLISA means fluorescence-linked immunosorbent assay

GC means gas chromatography

G-CSF means granulocyte-colony-stimulating factor

GM-CSF means granulocyte-macrophage colony stimulating factor

HPLC means high performance liquid chromatography

IFN means interferon

Ig means immunoglobulin

IGF means insulin-like growth factor

Il means interleukin

LPS means lipopolysaccharide

MALDI means matrix-assisted laser desorption/ionization

M-CSF means macrophage-colony-stimulating factor

MCP means monocyte chemoattractant protein

MHC means major histocompatibility complex

MIF means macrophage migration inhibitory factor

MIP means macrophage inflammatory/inhibitory protein

MMP means matrix metallo protease

MS means mass spectrometry

NT means neurotrophin

PBS means phoaphate-buffered saline

PCR means polymerase chain reaction

PKU means phenyl ketoneuria

PPD means purified protein derivative

TGF means transforming growth factor

TNF means tumor necrosis factor

TREM means triggering receptor expressed on myeloid cells

VEGF means vascular endothelial growth factor

The present invention discloses a diagnostic method where the reaction between a test compound and a blood sample or any other biological fluid or sample is initiated, allowed to proceed for a certain time and then stopped by spotting and/or drying the test sample on a filter paper which is then used subsequent for analysis of the effect of the test compound on the blood, fluid or sample and any of their constituents.

In a preferred embodiment a blood sample (e.g. 10 ml) is drawn from a person using standard anti-coagulation EDTA, heparin or citrate blood containers or glasses. The blood sample is divided in two aliquots and a test compound is added to one aliquot of blood, while the other aliquot is used as a control reference to which only the buffer/solution in which the test compound is dissolved is added. The test compound may also be added as a solid powder to be dissolved directly in the blood. The blood samples are incubated at ambient room temperature or a defined temperature (e.g. 5 °C, 20 °C, 37 °C) with or without mixing or agitation At certain time intervals (e.g. 0, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, 10 h, 15 h, 20 h, 24 h, 48 h) aliquots are drawn from the blood samples and spotted on filter paper (e.g. PKU paper) and allowed to dry as rapid as possible. After drying, the filter paper may be used immediately for analysis or stored for subsequent analysis. In a particular embodiment the filter paper may be transported (e.g. by ordinary mail) over a distance before storage or analysis in a laboratory.

Spotting, drying and storage of blood are carried out as follows: Blood is spotted on filter paper with a capillary tube, pipette or similar in one layer and dried at room temperature, e.g. in a well ventilated hood or in an ambient place. For storage, the filter papers may be kept in paper envelopes, plastic bags or similar containers, preferably air tight containers to keep the humidity as low as possible. A storage temperature of -20°C or lower is preferable, but room temperature is also possible as long as the paper is kept dry. However, the storage may take place at ambient temperature or at a temperature below 0 °C (e.g. -20 °C, - 50 °C, -80 °C, -180 °C) provided that the humidity is kept low to avoid deterioration of the samples. Samples can be stored for extended periods of time (e.g. months - years).

The test compounds may be any compound (e.g. an amino acid, a peptide, a protein, a carbohydrate, an oligosaccharide, a polysaccharide, a glycoprotein, a lipid, a

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lipoprotein, a glycosaminoglycan, a hormone, a steroid, a vitamin, a low molecular weight synthetic or natural compound), which influences blood to cause a measurable alteration of its composition.

Particularly useful test compounds are toxins, allergens, autoantigens, bacterial proteins and polysaccharides, viral proteins, fungal proteins and polysaccharides, parasitic proteins and polysaccharides, bacterial lipopolysaccharides, and any other compound relevant to diseases. Use of these test compounds will lead to important knowledge about how a certain compound affects the cells and the signalling between the cells.

In one embodiment, the diagnostic test and method is used for determining the effect of toxic compounds on blood, eg as part of a toxicological test programme or a preclinical test programme.

Analysis of the dried blood samples may be carried out by a number of different techniques (e.g. immunoassay, bioassay, mass spectrometry, HPLC, GC, GC-MS). Preferred methods of analysis are ELISA assays, FLISA assays, DELFIA assays, Luminex assays, luminescence assays, electrochemiluminescence assays, scintillation proximity assays, radioimmunoassays, MALDI-MS, ESI-MS, PCR.

Extraction of DBSS may be carried out by the use of any suitable buffer or solvent. In a preferred embodiment, filter paper disks, e.g. 3.2mm in diameter, are punched out from DBSS or standards on filter paper and placed together in microtiter wells. 140µl or 180 µl (for double-or triple-measurements, respectively) extraction buffer, PBS containing "Complete protease inhibitor cocktail with ethylenediamine tetraacetic acid (EDTA)" (Roche, Germany) 1 tablet dissolved per 25 ml assay buffer (PBS containing 0.5% Tween 20 and 1% BSA), are added to each well and the analytes are extracted protected from light at room temperature on a plate shaker set at 600 rpm for 60 minutes.

In one embodiment of the invention, analytes are measured by a Luminex assay as follows: coupling of capture antibodies to carboxylated beads (Luminex corp., Austin Texas, US) are performed according to the manufacturer's instruction: 2.5 X 10^6 beads are washed twice with activation buffer (0.1 mol/l sodium phosphate, pH 6.2),

re-suspended in 80µl activation buffer and sonicated until a homogenous distribution of the beads are observed. 10µl of solutions of N-hydroxysulfosuccinimide (sulfo-NHS from Pierce, Rockford US) and 10µl 1-ethyl-3(3-dimethylaminopropyl)-carbodiimidhydrochlorid (EDC from Pierce), both diluted in activation buffer to 50 mg/ml, are added to stabilize the reaction and activate the beads. After mixing, the beads are incubated for 20 min rotating in the dark at room temperature. The activated beads are subsequently washed with coupling buffer (mmol/l 2(N-morpholino ethanesulfonic acid, MES), pH 5.0), added 500µl azide-free solution of capture antibody (100 µg/ml) and incubated rotating for 2 hours or overnight. Azide is removed from antibodies by dialysis (Slide-A-Lyzer® dialysis cassette, MWCO = 10 000 from Pierce) in 3l PBS overnight at 4° C. After incubation, the beads are washed with washing buffer (PBS containing 0.05% Tween 20) and re-suspended in 75µl blocking/storage buffer (PBS containing 1% Bovine serum albumine (BSA) and 0.05% sodium azide).

The beads are counted with a hemocytometer, adjusted to a concentration of 20 X 10^6 beads/ml with blocking/storage buffer and stored protected from light at 2-8° C.

The assay procedure is performed as follows: A filter plate (MultiScreen MABVN 1.2µm 96-well, Millipore, Burlington US) are prepared by pre-wetting it with assay buffer (PBS containing 0.5% Tween 20 and 1% BSA). To each well are added 50µl of sample pipetted from the microtiter wells after extraction (100µl divided in duplicates or 150µl divided in triplicates) and a 50µl suspension of capture-antibody-conjugated beads, 1500 beads per analyte in assay buffer containing 1% guinea pig/pig serum (1:1). The capture-antibodies react with their corresponding antigens during 1½ hour of incubation and unbound material is removed from the beads by filtering it through the wells using a MultiScreen Vacuum Manifold (Millipore). The beads are washed twice using 200µl washing buffer (PBS containing 0.5% Tween) per well. The now captured antigens are reacted for 1½ hour with a mixture (50µl) of biotinylated detection antibodies each diluted 1:1000 in assay buffer. 50µl of streptavidin-phycoerythrin 20 µg/ml in assay buffer (Molecular Probes, The Netherlands) are added to the wells and the incubation continues for additional 30 min. The beads are finally washed twice with 200µl washing buffer and re-suspended in

125µl washing buffer. After 15 min of shaking, the samples are analyzed on the Luminex 100™ according to manufacturer's instructions.

In a preferred embodiment, the samples are analysed for content of cytokines, chemokines and growth factors (e.g. interleukins such as Il-1, Il-2, Il-3, Il-4, Il-5, Il-6, Il-7, Il-8, Il-9, Il-10, Il-11, Il-12, Il-13, Il-14, Il-15, Il-16, Il-17, Il-18, Il-19, Il-20, Il-21, Il-22, Il-23, Il-24, Il-25, Il-26, IFN, TNF, MCP, MIP, MMP-9, TREM, M-CSF, G-CSF, GM-CSF, chemokines such as CC, CXC, growth factors such as TGFα, TGFβ, EGF, VEGF, IGF I, IGF II, insulin, inflammatory mediators such as histamine, prostaglandins, leukotrienes, thromboxanes) and/or neurotransmitters.

In another embodiment the samples are analysed for standard and specific clinical parameters such as CRP, IgG, IgA, IgM, IgD, IgE, specific (i.e. antigen specific) antibodies, transferrin, albumin, transthyretin, etc.

In another embodiment the biological fluid to be analysed is a cerebrospinal fluid, a peritoneal fluid, a cyst fluid, an amniotic fluid, a lavage fluid, a saliva, a cell extract or a tissue extract.

In still another embodiment the source of cells are cell lines or isolated blood cells, manipulated cells, transgenic cells, transfected cells, or any cell type or altered or manipulated cell type.

The invention may be applied to blood and other body fluids and tissue extracts from any species and type of animal (e.g. human, monkey, mouse, rat, cow, dog, horse, cat, bird, fish and any other species) including transgenic animals.

In a special embodiment the test compound is immobilised on a solid surface (e.g. filter paper) and then incubated with a blood sample or a biological fluid or extract. After incubation, the filter paper is allowed to dry or the blood is spotted on filter paper and dried.

In a particular use of the invention, the volume of blood, biological fluid or extract is adjusted to allow interaction with an immobilised compound or a compound in solution for a fixed time while at the same time drying on the filter paper.

In one use of the invention a live test person or patient is infused with a test compound and blood samples are drawn from the person at certain time intervals, spotted on filter paper, dried and subsequently analysed.

The blood samples may be drawn from test individuals using standard needles and equipment and carried out by trained personal. However, the drawing of blood may also be carried out using devices allowing local individual sampling.

Examples

Example 1. Drawing, incubation, spotting, drying and storage of blood.
From a test person 10 ml blood is drawn into an anticoagulant test tube using a sterile needle and syringe. The blood is divided in aliquots of 1 ml using sterile anticoagulant test tubes. From one sample a blood sample is spotted directly on paper until the marked circle is full (the volume used is approximately 0.2 ml). To the other test tubes samples to be tested are added in predetermined concentrations and the test tubes are allowed to incubate at 37 °C or at ambient temperature for 1 h. Samples of 0.2 ml are then spotted from each on filter paper. The blood samples are spotted on filter paper with a capillary tube, pipette or similar in one layer and dried at room temperature, e.g. in a well ventilated hood or in an ambient place. Sampels are then stored at -20 °C or room temperature under low humidity, so that the paper is kept dry. For this purpose, ordinary freezers may be used and the papers may be kept in envelopes or in desiccators.

Example 2. Extraction of filter paper and analysis.
Two filter paper disks, 3.2mm in diameter, are punched out from DBSS or standards on filter paper and placed together in microtiter wells. 140µl or 180 µl (for double-or triple-measurements, respectively) extraction buffer, PBS containing "Complete protease inhibitor cocktail with ethylenediamine tetra-acetic acid (EDTA)" (Roche, Germany) 1 tablet dissolved per 25 ml assay buffer (PBS containing 0.5% Tween 20 and 1% BSA), are added to each well and the analytes are extracted protected from light at room temperature on a plate shaker set at 600 rpm for 60 minutes.

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Example 3. Luminex assay

Coupling of antibodies to beads:

Coupling of capture antibodies to carboxylated beads (Luminex corp., Austin Texas, US) are performed according to the manufacturer's instruction: 2.5 X 10^6 beads are washed twice with activation buffer (0.1 mol/l sodium phosphate, pH 6.2), re-suspended in 80µl activation buffer and sonicated until a homogenous distribution of the beads are observed. 10µl of solutions of N-hydroxysulfosuccinimide (sulfo-NHS from Pierce, Rockford US) and 10µl 1-ethyl-3(3-dimethylaminopropyl)-carbodiimidhydrochlorid (EDC from Pierce), both diluted in activation buffer to 50 mg/ml, are added to stabilize the reaction and activate the beads. After mixing, the beads are incubated for 20 min rotating in the dark at room temperature. The activated beads are subsequently washed with coupling buffer (mmol/l 2(N-morpholino ethanesulfonic acid, MES), pH 5.0), added 500µl azide-free solution of capture antibody (100 µg/ml) and incubated rotating for 2 hours or overnight. Azide is removed from antibodies by dialysis (Slide-A-Lyzer® dialysis cassette, MWCO = 10 000 from Pierce) in 3 l PBS overnight at 4° C. After incubation, the beads are washed with washing buffer (PBS containing 0.05% Tween 20) and re-suspended in 75µl blocking/storage buffer (PBS containing 1% Bovine serum albumine (BSA) and 0.05% sodium azide).

The beads are counted with a hemocytometer, adjusted to a concentration of 20 X 10^6 beads/ml with blocking/storage buffer and stored protected from light at 2-8° C.

Example 4. Assay procedure:

A filter plate (MultiScreen MABVN 1.2µm 96-well, Millipore, Burlington US) are prepared by pre-wetting it with assay buffer (PBS containing 0.5% Tween 20 and 1% BSA). To each well are added 50µl of sample pipetted from the microtiter wells after extraction (100µl divided in duplicates or 150µl divided in triplicates) and a 50µl suspension of capture-antibody-conjugated beads, 1500 beads per analyte in assay buffer containing 1% guinea pig/pig serum (1:1). The capture-antibodies react with their corresponding antigens during 1½ hour of incubation and unbound material is removed from the beads by filtering it through the wells using a MultiScreen Vacuum Manifold (Millipore). The beads are washed twice using 200µl washing buffer (PBS containing 0.5% Tween) per well. The now captured antigens are reacted for 1½ hour with a mixture (50µl) of biotinylated detection antibodies each

diluted 1:1000 in assay buffer. 50µl of streptavidin-phycoerythrin 20 µg/ml in assay buffer (Molecular Probes, The Netherlands) are added to the wells and the incubation continues for additional 30 min. The beads are finally washed twice with 200µl washing buffer and re-suspended in 125µl washing buffer. After 15 min of shaking, the samples are analyzed on the Luminex 100™ according to manufacturer's instructions.

Example 5. Test of Gc globulin, diphtheria toxoid, tetanus toxoid and lipopolysaccharide (LPS) for cytokine release

The following 8 solutions are mixed with blood from different persons and incubated at 37 °C:

1) 1 ml EDTA-blood (person X) + 30 µl Gc batch 11
2) 1 ml EDTA-blood (person X) + 30 µl Gc batch 13
3) 1 ml EDTA-blood (person Y) + 30 µl Gc batch 11
4) 1 ml EDTA-blood (person Y) + 30 µl Gc batch 13
5) 1 ml EDTA-blood (person X) + 30 µl PBS
6) 1 ml EDTA-blood (person Y) + 30 µl PBS
7) 1 ml EDTA-blood (person X) + 30 µl Gc batch 11 + 50 µl LPS from *Klebsiella pneumoniae* (5 mg/ml)
8) 1 ml EDTA-blood (person X) + 50 µl LPS from *Klebsiella pneumoniae* (5 mg/ml)
9) 1 ml EDTA-blood (person Z) + 30 µl diphtheria toxoid (5.78 mg/ml)
10) 1 ml EDTA-blood (person Z) + 30 µl tetanus toxoid (993 Lf/ml)
11) 1 ml EDTA-blood (person Z) + 30 µl LPS from *Klebsiella pneumoniae* (5 mg/ml)
12) 1 ml EDTA-blood (person Z) + 30 µl LPS from *Salmonella typhimurium* (5 mg/ml)
13) 1 ml EDTA-blood (person Z) + 30 µl milliQ water

After 1 min (A), 2 h (B), 24 h (C) and 48 h(D) 180 µl of each of the 8 solutions are spotted on filter paper and allowed to dry. Samples are subsequently (after 14 days

storage at -20 °C) analysed for content of cytokines using Luminex technology (22). The results are shown in Table 1. It can be seen from the table that LPS induces large increases in IL-1b, IL-6, IL-8, MIP-1a, MIP-1b, while smaller but statistically significant changes are seen for other analytes. Diphteria toxoid induces an increase in MIP-1b.

Table 1. Test of Gc globulin, diphtheria toxoid, tetanus toxoid and lipopolysaccharide (LPS) for cytokine release (see example 5 for details). All results are in pg/ml unless otherwise stated.

Analyte	IL-1b	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12	IL-17	IL-18	sIL-6ra ng/ml	IFN-g	TNF-a	TNF-b	MCP-1	TGF-b
1A	120	33	18	57	62	155	297	104	188	3674	1269,4	137	137	470	1795	828
1B	71	26	14	55	52	148	289	91	138	2985	970,3	96	105	427	1462	594
1C	111	31	24	48	35	7592	289	89	191	2357	695,2	36	85	519	1339	616
1D	101	17	20	60	42	5680	279	89	156	1444	620,4	75	88	355	1296	1007
2A	10	30	11	47	35	124	232	71	140	2818	900,9	39	69	503	1442	560
2B	47	21	18	49	44	210	232	80	163	2637	902,8	60	78	427	1296	700
2C	73	25	22	61	46	8471	258	93	161	2802	874,9	119	90	503	1391	937
2D	104	34	21	45	35	7588	262	78	115	1758	799,5	84	76	412	800	789
3A	<3	9	6	20	4	25	45	12	101	1502	516,9	24	17	213	889	91
3B	45	12	8	35	36	50	258	73	147	1600	498,1	28	70	434	903	430
3C	38	17	7	30	18	1956	80	25	101	1050	395,5	11	48	293	597	282
3D	55	15	6	40	43	2882	129	53	135	732	402,8	39	72	149	737	458
4A	69	24	7	44	38	77	272	102	138	2308	769,9	107	115	358	1472	557
4B	38	16	13	39	29	69	120	17	117	2003	613,8	28	56	332	1569	255
4C	43	25	18	36	47	2456	117	64	182	1317	537,8	30	102	351	903	477
4D	8	7	16	37	17	3346	43	39	94	727	430,0	15	70	165	597	414
5A	32	21	16	19	20	84	80	60	133	2535	876,4	39	95	427	1616	490
5B	25	22	11	29	18	110	67	25	122	2581	870,3	46	65	383	1391	446
5C	1	4	12	26	<3	3695	23	<3	70	1874	636,0	<3	26	261	889	273
5D	69	19	21	43	16	6488	166	44	122	1543	735,2	25	74	204	1540	870
6A	20	15	7	25	22	94	155	69	133	1980	651,5	74	105	284	1401	479
6B	45	24	16	37	44	68	82	80	152	1980	600,3	60	86	289	1124	506
6C	30	8	8	36	20	3055	61	2	67	1058	411,7	29	48	104	889	417
6D	<3	<3	<3	<3	<3	1781	<3	<3	<3	155	138,1	<3	<3	<6	<6	<50
7A	<3	24	15	24	26	81	115	27	124	2213	802,3	11	38	332	1472	183
7B	1223	25	24	28	578	2982	84	78	165	2234	712,3	14	724	371	1172	446
7C	14429	20	23	36	14077	13606	204	115	244	2686	706,2	964	1399	418	1422	529
7D	9413	15	15	39	11048	15148	117	108	228	1567	540,9	468	1020	342	860	594
8A	901	23	15	28	40	237	103	29	152	2627	915,1	41	52	341	1597	450
8B	1584	19	19	40	789	3494	101	66	251	2380	820,7	80	997	518	1328	344

8C	11466	18	26	33	12878	13903	345	119	320	1809	620,1	339	1512	468	1391	705
8D	7402	16	12	23	11902	14428	222	95	219	878	562,9	190	1471	268	753	438
9B	148	33	20	20	21	1785	46	12	88	1058	833,4	<3	81	350	875	624
9C	138	28	23	31	17	10091	3	12	149	1272	724,8	1	95	316	652	894
10B	25	37	22	15	14	383	92	7	106	1042	820,7	4	44	303	931	357
10C	69	28	22	23	11	9921	39	<3	110	987	664,7	6	61	268	56	578
11B	2948	24	43	21	1697	58145	103	78	183	1456	442,1	68	1205	222	489	623
11C	16953	37	24	66	4215	757774	299	73	264	1070	374,1	73	983	274	91	528
12B	1665	26	19	46	1539	16428	25	28	148	1153	382,8	129	844	388	247	479
12C	12805	32	21	39	3715	541309	100	70	268	1450	339,3	80	750	274	680	907
13B	83	17	14	37	<3	252	7	51	70	1017	351,4	27	19	328	389	554
13C	60	29	17	21	<3	67105	2	44	74	1384	361,4	225	40	326	273	508

Analyte	MIP-1a	MIP-1b	MMP-9 µg/ml	TREM-1	BDNF ng/ml	GM-CSF	NT-4	CRP µg/ml	RANTES ng/ml	int.st.dev .%
1A	286	1313	1,40	3509	16,2	63	85	0,68	188,0	32
1B	174	1285	1,19	3917	11,2	52	84	0,53	136,8	10
1C	221	1091	1,36	2609	14,2	62	43	0,43	158,3	-7
1D	210	549	1,05	2848	14,2	112	105	0,40	118,8	21
2A	254	1387	1,20	2848	11,9	65	90	0,49	153,0	-4
2B	192	1305	1,12	3001	11,6	88	57	0,53	116,7	7
2C	200	1055	1,33	2925	17,7	88	72	0,52	191,1	20
2D	219	677	1,16	3223	19,1	170	110	0,44	160,8	12
3A	73	852	1,15	2609	6,2	25	35	0,38	55,6	-8
3B	106	1067	1,15	2443	6,9	86	52	0,57	50,2	15
3C	154	627	1,26	2181	8,9	45	40	0,36	57,9	-5
3D	137	193	1,13	2690	8,9	82	47	0,38	51,7	13
4A	192	1124	1,59	3983	10,8	51	100	0,93	97,3	37
4B	112	996	1,51	2609	9,7	60	60	0,48	92,1	9
4C	201	716	1,37	2925	11,0	83	70	0,51	88,8	6
4D	96	199	1,32	1696	11,0	85	53	0,37	82,7	1
5A	192	1327	1,11	1358	11,0	41	43	0,48	127,2	-4
5B	167	1196	1,02	1800	10,9	22	57	0,44	119,8	-7
5C	88	940	1,03	2690	11,8	15	47	0,30	115,6	-6
5D	219	702	1,30	3001	17,4	76	85	0,37	176,5	3
6A	187	919	1,37	3223	9,5	68	74	0,63	84,2	18
6B	214	1087	1,31	3438	8,3	59	82	0,58	70,6	15
6C	118	495	1,19	2090	8,0	<6	42	0,36	59,9	4
6D	<6	<6	0,72	<313	2,9	<6	<3	0,03	20,3	-53
7A	51	1097	0,97	1231	9,2	48	45	0,38	118,4	-41
7B	4706	21819	1,05	<313	10,4	100	55	0,34	129,4	-1
7C	15802	32037	1,05	3076	15,6	76	67	0,34	159,3	-1
7D	15112	29767	0,74	2443	12,6	114	74	0,31	116,9	-4

8A	257	1349	1,09	3438	11,2	70	77	0,42	130,7	-3
8B	5333	24582	1,13	3368	11,8	91	105	0,42	112,4	10
8C	14303	31580	1,04	3296	10,9	100	63	0,36	144,6	2
8D	14937	28500	0,89	1476	13,4	106	30	0,28	106,3	-3
9B	591	6076	1,42	1996	22,8	59	35	0,60	161,3	-1
9C	643	5472	1,28	1476	26,2	83	55	0,55	141,6	-9
10B	96	1099	1,43	2090	20,0	82	53	0,60	154,9	-8
10C	141	950	1,26	1899	23,7	59	55	0,50	137,7	-6
11B	5992	22797	2,10	2920	30,1	14	51	1,08	240,6	10
11C	11035	26838	1,51	2087	32,8	15	63	0,83	183,0	2
12B	4055	19656	1,81	2920	18,8	10	24	0,92	163,0	5
12C	8644	26995	1,45	3257	26,2	23	15	0,76	175,0	-9
13B	142	1351	1,50	2747	16,7	12	35	0,86	159,8	-10
13C	77	1087	1,67	2920	22,9	2	13	0,76	164,7	-2

Example 6. Test of diphtheria toxoid, tetanus toxoid, tuberculin PPD and BCG for cytokine release.

The following 6 solutions are mixed and incubated at 37 °C:

1) 1 ml EDTA-blood (person Y) + 30 µl diphtheria toxoid (5.78 mg/ml)
2) 1 ml EDTA-blood (person Y) + 30 µl tetanus toxoid (993 Lf/ml)
3) 1 ml EDTA-blood (person Y) + 30 µl BCG (4-16 x 10^6 cfu/ml)
4) 1 ml EDTA-blood (person Y) + 30 µl tuberculin PPD (0.4 µg/ml)
5) 1 ml EDTA-blood (person Y) + 30 µl milliQ water
6) 1 ml EDTA-blood (person Y) + 30 µl BCG vaccine solvent (control)

After 1 min (A), 2 h (B), 4 h (C) 6 h (D), and 24 h (E) 180 µl of each of the 6 solutions are spotted on filter paper and allowed to dry. Samples are subsequently (after storage at -20 °C for 30 days) analysed for content of cytokines using Luminex technology (22). The results are shown in Table 2. From the table it can be seen that BCG induces a large increase in IL-8 and MIP-1b compared with the control. Similarly, diphtheria toxoid, tetanus toxoid and PPD induces increases in IL-8 and MIP-1b, while smaller but statistically significant changes are seen for other analytes.

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Table 2. Test of diphtheria toxoid, tetanus toxoid, tuberculin PPD and BCG for cytokine release (see example 6 for details). All results are in pg/ml unless otherwise stated.

analyte	IL-1b	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12	IL-17	IL-18
1A	47	42	9	11	161	99	477	160	73	2609
1B	54	76	11	14	130	284	394	201	69	3761
1C	90	146	27	8	115	588	637	226	87	2564
1D	97	124	26	22	217	783	706	267	111	3282
1E	106	153	17	20	135	7290	981	336	83	1745
2A	29	8	4	11	73	95	61	132	43	2233
2B	44	77	13	7	93	135	410	217	47	2202
2C	43	82	14	10	103	187	493	233	68	2726
2D	57	113	16	12	110	234	468	139	86	3265
2E	54	120	8	10	143	6335	811	185	63	1707
3A	33	26	26	33	75	113	240	213	78	2934
3B	77	93	14	2	97	268	268	158	56	2556
3C	160	155	11	18	127	998	533	252	54	2763
3D	145	<3	24	13	49	1439	378	76	54	3104
3E	113	92	3	10	67	27779	202	120	75	1597
4A	152	96	2	4	125	149	323	183	47	2461
4B	43	268	16	20	64	160	286	137	53	2512
4C	31	79	14	20	60	189	376	140	77	2567
4D	665	71	568	687	493	465	1812	746	1355	3170
4E	70	<3	8	13	81	7011	386	202	59	2432
5A	48	68	14	9	26	136	259	77	34	2660
5B	44	220	14	3	46	117	168	109	38	2461
5C	23	195	2	8	63	114	444	76	64	2221
5D	25	179	9	11	100	174	350	133	60	2982
5E	30	218	13	8	25	3558	417	146	79	1478
6A	27	121	16	10	46	126	461	154	51	2406
6B	32	95	10	3	34	128	400	149	41	2353
6C	19	101	13	24	9	131	256	91	71	2413
6D	50	102	7	22	70	163	281	183	51	2876
6E	27	97	10	11	87	5038	404	155	69	1474

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analyte	sIL-6ra ng/ml	IFN-g	TNF-a	TNF-b	MCP-1	TGF-b
1A	1016,9	78	61	1392	2869	816
1B	1218,9	118	39	1474	2288	210
1C	822,0	209	109	1569	2126	556
1D	1091,7	165	150	1872	3048	791
1E	863,0	155	136	1724	2213	852
2A	857,1	72	158	1329	1600	276
2B	839,0	57	7	1865	1760	554
2C	955,2	52	90	1390	1794	515

2D	1095,0	109	77	1599	2838	582
2E	835,5	91	34	1308	1990	551
3A	981,4	<3	69	1187	1506	671
3B	851,1	59	60	1291	2041	471
3C	985,7	153	59	1454	2543	405
3D	1045,6	67	48	1351	1500	542
3E	787,5	9	112	1183	1240	508
4A	941,4	71	60	1807	2800	623
4B	833,1	107	72	1310	1766	527
4C	937,6	165	112	1563	1211	384
4D	1186,3	93	247	2142	6253	113
4E	966,0	6	59	1012	1234	579
5A	885,7	5	35	1283	1473	305
5B	887,3	62	21	1593	2341	284
5C	735,4	229	65	1522	1262	520
5D	1042,6	154	129	1535	2441	426
5E	802,9	172	69	1602	107	256
6A	852,2	122	45	1674	1996	627
6B	758,5	41	97	1664	1189	372
6C	695,6	30	62	1492	884	378
6D	943,3	177	16	1516	736	473
6E	808,6	60	91	1305	1256	619

analyte	MIP-1a	MIP-1b	MMP-9 µg/ml	TREM-1	BDNF ng/ml	GM-CSF	NT-4	CRP µg/ml	RAN-TES ng/ml	int,st,dev,%
1A	85	1307	11,3	5676	8,0	21	23	0,46	56,2	6
1B	182	2534	15,6	6485	12,2	22	24	0,55	62,8	-1
1C	210	2883	14,1	6254	10,9	22	17	0,49	59,0	5
1D	224	3102	17,2	6562	13,0	26	33	0,57	71,8	9
1E	205	1053	14,3	5792	10,6	45	41	0,48	65,3	0
2A	75	1308	7,4	4821	7,1	19	16	0,38	50,0	-1
2B	116	1345	13,1	5599	8,3	24	13	0,41	55,1	3
2C	99	1163	17,7	5521	10,7	18	24	0,50	63,2	0
2D	70	1234	19,1	5521	10,7	25	20	0,53	59,3	1
2E	125	375	27,0	5985	11,4	41	23	0,45	56,9	0
3A	112	1189	12,0	7747	8,3	17	28	0,48	45,3	-4
3B	235	3717	8,1	5289	9,0	20	14	0,39	41,8	4
3C	410	7922	15,3	4860	9,2	20	26	0,45	41,5	2
3D	296	8706	10,8	7289	11,0	23	20	0,44	47,3	1
3E	281	6280	14,2	4232	12,3	33	17	0,38	42,5	-12
4A	128	1397	11,0	5715	9,1	20	20	0,47	39,6	4
4B	110	1159	10,7	5483	8,5	17	24	0,46	46,3	-1
4C	45	1122	10,0	3356	9,5	18	22	0,45	46,0	-4

4D	866	1301	15,4	21919	12,4	36	447	0,44	43,3	-5
4E	64	183	19,6	5521	8,3	37	15	0,45	47,3	-11
5A	69	993	15,3	4938	9,4	19	16	0,46	44,6	-5
5B	4	1087	10,4	4743	8,9	19	15	0,39	43,2	2
5C	60	1248	8,1	5521	8,3	23	15	0,45	45,6	2
5D	41	1296	21,3	4035	12,5	28	20	0,52	50,5	3
5E	35	388	8,7	4272	10,7	42	5	0,40	46,1	-17
6A	57	1200	11,8	6331	8,2	19	13	0,45	49,1	1
6B	98	1117	13,5	7251	8,5	21	18	0,42	48,6	0
6C	85	1228	10,1	5016	7,5	22	33	0,40	46,4	-7
6D	66	1118	20,6	6101	9,6	27	24	0,47	49,5	4
6E	83	256	11,9	4469	10,7	39	14	0,39	50,8	-9

Example 7. Storage of samples for extended periods of time.

Dried blood spot samples (DBSS) should be stored dried and preferable at about -20° C. Room temperature can also be used as long as the samples are protected from moisture.

In Denmark all residual DBSS have since 1982 been stored in a biological specimen bank at – 24° C, in accordance with regulations from the Ministry of Health (23). For stability studies, DBSS stored for 23 years, 3 years and 1 month respectively, were taken anonymously from the Danish DBSS specimen bank. The mean concentrations of each analyte from each period were calculated from 10 samples and compared to routinely collected anonymous DBSS that were stored in the laboratory for 2 weeks at -20C° (Table 3). It can be seen that within experimental error, there is no deterioration of the samples even upon 23 years of storage.

Table 3. Analysis of samples stored for short (1 month), long (3 years) and extended (23 years) periods of time. Results are expressed as per cent of concentration detectable in 2 week old DBSS not yet put to storage in the PKU-biobank. Samples were extracted and analyzed as described in examples 2-4.

	23 years	3 years	1 month
IL-1β	44	43	93
IL-2	116	115	113
IL-4	91	91	107
IL-5	105	116	122
IL-6	95	101	108
IL-8	28	38	64
IL-10	124	103	129
IL-12	95	108	107
IL-17	94	100	107
IL-18	138	113	129
TNF-α	92	101	109
TNF-β	88	94	93
IFN-γ	117	119	121
RANTES	87	89	90
MCP-1	94	112	112
GM-CSF	102	107	108
MIP-1α	85	88	98
MIP-1β	59	76	79
sIL-6ra	48	101	113
TGF-β	111	100	95
MMP-9	57	49	93
TREM-1	68	84	129
CRP	73	123	110
BDNF	22	54	58
NT-4	54	63	111

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CLAIMS:

1. A method for preparing a sample of blood or another biological fluid for analysis, said method comprising

(a) initiating a reaction by mixing the sample with a test compound, wherein the test compound interacts with constituents of the sample to cause an alteration in the composition of the sample;

(b) stopping the reaction by spotting the sample and test compound mixture on filter paper;

wherein the test compound is a toxin, allergen, autoantigen, bacterial protein or polysaccharide, viral protein, fungal protein or polysaccharide, parasitic protein or polysaccharide, or bacterial lipopolysaccharide.

2. The method according to claim 1, wherein the other biological fluid is a cerebrospinal fluid, a peritoneal fluid, a cyst fluid, an amniotic fluid, a lavage fluid, a saliva, a cell extract or a tissue extract.

3. The method according to claim 2, wherein the cell extract is derived from cell lines, isolated blood cells, manipulated cells, transgenic cells, transfected cells or any cell type or an altered or manipulated cell type.

4. The method according to any one of claims 1-3, wherein the sample is analysed for the content of cytokines, chemokines and growth factors or neurotransmitters or other polypeptides and proteins.

5. The method according to any one of claims 1-4, wherein the sample is analysed for the content of at least one of the clinical parameters CRP, IgG, IgA, IgM, IgD, IgE, specific (i.e. antigen specific) antibodies, transferrin, albumin and transthyretin.

6. The method according to any one of claims 1-5, wherein the effect of the test compound is analysed by at least one immunoassay, bioassay, mass spectrometry, HPLC, GC, or GC-MS.

7. The method according to claim 6, wherein the methods of analysis are ELISA assays, FLISA assays, DELFIA assays, luminescence assays, electrochemiluminescence assays, scintillation proximity assays, radioimmunoassays, MALDI-MS, ESI-MS or ambient-MS (DESI-MS).

8. The method according to any one of claims 1-7, wherein the test compound is immobilized on a solid surface and incubated with the sample.

9. A method according to any one of claims 1-8, further comprising the step of:

(c) analysing the effect of the test compound on the sample.