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(54) **Title:** PROCESS FOR PRODUCTION OF A VACCINE COMPOSITION

(57) **Abstract:** The present invention relates to a process for production of a vaccine composition, comprising the process steps: i) provision of a volume V_1 of an aqueous phase P_1 comprising an aluminium compound whose surface has been brought into contact with phosphate ions, ii) provision of a volume V_2 of an aqueous phase P_2 of an antigen, iii) addition of the volume V_2 of the aqueous phase P_2 to the volume V_1 of the aqueous phase P_1 with stirring of the volume V_1, whereby at least one, preferably each of the following conditions is fulfilled a) the aluminium compound was heated after the bringing into contact with the phosphate ions and before process step iii); b) the aqueous phase P_1 comprises less than 10 mg/ml aluminium; c) the aqueous phase P_2 comprises less than 1 mg/ml of the antigen; d) the volume V_2 is added with a rate of at most 10 % of the amount of the Volume V_1/minute to volume V_1. The invention also relates to the vaccine composition obtainable by this process, a vaccine composition comprising a substrate and antigen as well as the use of a vaccine composition.

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Process for production of a vaccine composition

The present invention relates to a process for production of a vaccine composition, the vaccine composition obtainable by this process, a vaccine composition comprising a substrate and an antigen, and the use of a vaccine composition.

Vaccine compositions for prevention of viral illnesses, for example for prevention of a hepatitis B infection, are known from the prior art.

Thus, EP-A-0 576 478 describes a vaccine composition based on aluminium salt particles, on whose surface an antigen as well as 3-O-deacetylated monophosphoryl lipid A (3D-MPL), have been absorbed. EP-A-0 689 454 and EP-A-0 633 784 describe a process for production of a vaccine composition, in which first an antigen and then a 3D-MPL as adjuvant are absorbed on the surface of an aluminium salt particle. The vaccine compositions described in these European patent applications are characterised in that the adjuvant and the antigen are at least partially immobilised in the same aluminium salt particles.

WO-A-00/23105 describes an adjuvant composition comprising immune stimulants, which are absorbed at the surface of a metal salt particle, whereby the metal salt particle is substantially free from other antigens. The adjuvant compositions described in this state of the art are obtained in that first the adjuvant is immobilised at the surface of an aluminium salt particle and, separately, the antigen is immobilised on the surface of another aluminium salt particle. In order to complete the vaccine composition, the aluminium salt particles are then combined which each other. The vaccine composition described in this international patent application is, accordingly, characterised in that the adjuvant and antigen are immobilised on different aluminium salt particles.

The disadvantage of the vaccine compositions known from the state of the art consists in that, *inter alia*, they are characterised by a poor stability. This poor stability of the vaccine compositions consisting of the three components aluminium salt particle, antigen and adjuvant expresses itself, *inter alia*, in that often a view minutes after the production of the vaccine composition, individual components sediment in the generally liquid preparations.

Furthermore, in particular those vaccine compositions, in which adjuvant and antigen are immobilised on different aluminium salt particles, such as, for example, the composition which is described in WO-A-00/23105, only have a low immune stimulatory effect. Furthermore, in particular the process described in WO-A-00/23105 requires the use of at least two separate stirring vessels, in which the aluminium salt particle-antigen composition, as well as the aluminium salt particle-adjuvant composition is prepared.

A further disadvantage of the vaccine compositions known from the prior art are the often pronounced variations in the immune stimulatory effect of the vaccine compositions between individual production batches.

The present invention had the object of overcoming the disadvantages of vaccine compositions known from the prior art, in particular of vaccine compositions for prevention of a hepatitis B illness.

In particular, the present invention had the object of providing a process for production of a vaccine composition, which enables the production of a vaccine composition with, to the greatest extent possible, more constant and batch-independent immune stimulatory effect. It should be possible to carry out this process as much as possible with a single stirring vessel, into which the individual components can be introduced, and still enable the production of vaccine composition with a high immune stimulatory effect.

The present invention also had the object of providing a process for production of a vaccine composition, which enables the production of vaccine compositions which are as storage-stable as possible.

Furthermore, the vaccine composition obtainable by the process according to the invention should, in particular if it is a vaccine composition for prevention of a HBV-illness, provide a sufficient vaccination protection as far as possible already after two administrations of the vaccine composition (= 2-dose vaccine).

A contribution to the solution of the above-mentioned objects is made by a process for production of a vaccine composition, comprising as process steps:

i) provision of a volume V_1 of an aqueous phase P_1 comprising an aluminium compound whose surface has been brought into contact with phosphate ions,

ii) provision of a volume V_2 of an aqueous phase P_2 of an antigen,

iii) addition of the volume V_2 of the aqueous phase P_2 to the volume V_1 of the aqueous phase P_1 with stirring of the volume V_1,

whereby at least one, preferably all of the following conditions are fulfilled:

a) the aluminium compound was heated after the bringing into contact with the phosphate ions and before process step iii);

b) the aqueous phase P_1 comprises, preferably before process step iii), less than 10 mg/ml aluminium;

c) the aqueous phase P_2 comprises less than 1 mg/ml of the antigen;

d) the volume V_2 is added with a rate of at most 10% of the amount of the volume V_1/minute to volume V_1.

Preferred embodiments according to the invention of the process according to the invention are those processes in which the following properties or combinations of properties are fulfilled: a), b), c), d), a)b), a)c), a)d), b)c), b)d), c)d), a)b)c), a)b)d), a)c)d), b)c)d) and a)b)c)d), whereby a), b)c)d) and a)b)c)d) are particularly preferred and a)b)c)d) is most preferred.

In process step i) of the process according to the invention, first a volume V_1 of an aqueous phase P_1 comprising an aluminium compound whose surface has to brought into contact with phosphate ions is provided. This aqueous phase P_1 can be, depending on the aluminium compound comprised, an aqueous solution, an aqueous dispersion, an aqueous emulsion or an aqueous suspension. The term "aqueous", as used herein, should merely mean that the corresponding phase comprises water as solvent or as part of a solvent mixture.

Preferred aluminium compounds comprise compounds selected from the group consisting of aluminium sulphate, aluminium chloride, aluminium hydroxide, in particular aluminium orthohydroxides, such as, for example, hydrargillite (□-$Al(OH)_3$, bayerite (□-$Al(OH)_3$) or Nordstrandite (□-$Al(OH)_3$), aluminium metahydroxides, such as, for example, bohmite (□-Al(O)OH), which is commercially obtainable, for example under the name Alhydrogel® or Rehydragel® or diaspor (□-Al(O)OH), aluminium phosphates, such as, for example, $AlPO_4$ or amorphous aluminium hydroxyphosphate gel ($Al(OH)_m(PO_4)_n$), such as is commercially obtainable, for example under the name AdjuPhos® or RehydraPhos®, as well as mixed oxides between aluminium and further metals, in particular between aluminium and titanium, such as, for example aluminium titanate or aluminium titanium oxide, whereby aluminium hydroxide-comprising aluminium compositions, in particular the aluminium metahydroxides obtainable under the name Alhydrogel® are most preferred. These aluminium metal hydroxides are obtainable

commercially in crystalline form. Furthermore, aluminium compounds comprising mixtures of at least two of the above-mentioned compounds conceivable, for example, mixtures comprising aluminium phosphate and aluminium hydroxide, in particular mixtures comprising aluminium hydroxyphosphate gel and Al(O)OH.

It is further preferred according to the invention that the aluminium compound in the vaccine composition according to the invention is preferably present in particulate form, whereby it is particularly preferred in this connection that at least 75 %, yet more preferably at least 85 % and most preferably at least 95 % of the particles, respectively based on the number of particles, are smaller than 2 μm. It is furthermore preferred in this context that the particles of the aluminium compound preferably have a size in a range from 0.5 to 15 μm, particularly preferably from 1 to 10 μm.

The surface of the aluminium compound provided in process step i) has been brought into contact with phosphate ions. This preferably occurs in that the aluminium compound is dissolved or dispersed, preferably dispersed, in an aqueous phase P_1, which comprises a preferably water-soluble salt of a phosphate, whereby the salt of a phosphate is preferably selected from the group comprising sodium phosphate, sodium dihydrogenphosphate, disodium hydrogenphosphate, potassium phosphate, dipotassium hydrogenphosphate, potassium dihydrogenphosphate or mixtures of at least two of these phosphates, whereby mixtures of disodium hydrogenphosphate and sodium dihydrogenphosphate are most preferred. Particularly suitable according to the invention is the use of a so-called PBS buffer (PBS = phosphate buffered saline) as aqueous phase for the aluminium compound. This PBS buffer generally comprises, in its normal concentration (= 1×PBS)

- disodium or dipotassium hydrogenphosphate, which is preferably present in a concentration in a range from 1 to 10 mM, particularly preferably from 3 to 8 mM and most preferably of about 5 mM,

- sodium or potassium dihydrogenphosphate, which is preferably present in a concentration in a range from 1 to 10 mM, particularly preferably from 3 to 8 mM, and most preferably of about 5 mM, as well as

- NaCl, which is preferably present in a concentration in a range from 125 to 175 mM, particularly preferably from 140 to 160 mM and most preferably in a concentration of about 150 mM.

The given amounts for the individual components of the PBS buffer corresponds to those amounts in the singly concentrated PBS buffer (= 1×PBS). The pH-value of the aqueous phase P_1, in particular of the PBS buffer, lies, at 25°C, preferably in a range from 6 to 8, particularly preferably from 6.5 to 7.5.

According to a particular preferred embodiment of the process according to the invention for production of a vaccine composition, as aqueous phase P_1, in which the aluminium compound is dissolved or dispersed, preferably dispersed, a 1.5× to 6×PBS buffer, particularly preferably a 1.75× to 5×PBS buffer and most preferably a 2× to 4×PBS buffer is used, whereby a 2× to 4×PBS buffer comprises the above-mentioned concentrations of sodium chloride, disodium or dipotassium hydrogenphosphate and sodium or potassium dihydrogenphosphate in two times to four times the amount of those concentrations which where given above as preferred, particularly preferred and most preferred concentrations for the individual components. Thus, for example, a 1.5×PBS buffer comprises

- disodium or dipotassium hydrogenphosphate preferably in a concentration in a range from 1.5 to 15 mM, particularly preferably from 4.5 to 12 mM and most preferably of about 7.5 mM,

- sodium or potassium dihydrogenphosphate preferably in a concentration in a range from 1.5 to 15 mM, particularly preferably from 4.5 to 12 mM and most preferably of about 7.5 mM, and

- NaCl preferably in a concentration in a range from 187.5 to 262.5 mM, particularly preferably from 210 to 240 mM and most preferably in a concentration of about 225 mM.

According to the most preferred embodiment of the process according to the invention, in process step i), a 2× to 4×PBS buffer is provided, in which an aluminium metal hydroxide, in particular the aluminium compound obtainable under the name Alhydrogel®, is dispersed.

If, in the process according to the invention for production of a vaccine composition, the condition a) is fulfilled, the aluminium compound is heated after the bringing into contact with the phosphate ions and before process step iii), whereby this heating preferably occurs to a temperature of at least 100°C, preferably of at least 110°C, and most preferably of at least 120°C, preferably to a temperature in a range from 100 to 150°C, particularly preferably to a temperature in a range from 110 to 140°C and most preferably to a temperature in a range from 120 to 130°C. The duration of the heating to a temperature within the above-mentioned temperature ranges is generally at least 10 minutes, particularly preferably at least 15 minutes and most preferably at least 20 minutes, whereby, advantageously, a duration of 4 hours, particularly preferably of 2 hours, is not exceeded. It can, furthermore, be advantageous to stir the aqueous phase P_1 during the heating, in order to ensure as homogeneous as possible a bringing into contact of the surface of the aluminium compound with the phosphate ions. Subsequently to the heating, the hot aqueous phase P_1 is allowed to cool, preferably to a temperature in a range from 15 to 40°C, particularly preferably to a temperature in a range from 18 to 24°C.

If, as aqueous phase P_1, in which the aluminium compound is dissolved and/or dispersed, a concentrated PBS solution, as described above, has been used, a dilution step can follow the heating of this PBS solution, in which the concentrated PBS solution is optionally diluted with deionised or distilled water, preferably until the concentration of the PBS buffer reaches the concentration of 1× bis 2×PBS buffer, particularly preferably 1,3× bis 1,7×PBS buffer.

If, in the process according to the invention for production of a vaccine composition, the condition b) is fulfilled, the aqueous phase P_1 comprises, after the heating, if this is carried out, and particularly after an optionally carried out dilution step, less than 10 mg/ml aluminium, particularly preferably less than 5 mg/ml, yet more preferably less than 2.5 mg/ml and most preferably less than 1 mg/ml aluminium.

In process step ii) of the process according to the invention, which can be carried out before, during or after process step i), a volume V_2 of an aqueous phase P_2 comprising an antigen is provided. The aqueous phase P_2 can also be, depending on the antigen comprised, an aqueous solution, an aqueous dispersion, an aqueous emulsion or an aqueous suspension.

Preferred antigens are selected from the group comprising antigens or antigen compositions from the HIV-1-virus, for example, tat, nef, gp120 or gp160 antigens or antigen compositions from the from humane herpes virus, for example, gD or derivatives thereof, so-called Immediate Early Proteins such as, for example, ICP27 from HSV-1 or HSV-2, antigens or antigen compositions from the cytomegalo-virus, such as, gB or derivatives thereof, antigens or antigen compositions from the rota-virus, antigens or antigen compositions from the Epstein-Barr-Virus, such as, for example, gp350 or derivatives thereof, antigens or antigen compositions from the Varicella-Zoster-Virus, such as, for example, gpI, gpII or IE63, antigen or antigen compositions from hepatitis-virus, in particular from the Hepatitis B-Virus,such as the hepatitis B surfaces antigens (HBsAg), hepatitis B

core antigen (HBcAg) or derivates of these antigens, from the hepatitis A-Virus, from the hepatitis C-virus or from the hepatitis E-virus, antigens or antigen compositions from other viral pathogens, such as Paramyxoviren, Respiratory-Syncytial-Virus, such as the F- or G-Proteins or their derivatives, the Parainfluenza-Virus, the measles-virus, the mumps-virus, the human Papilloma-Viruses, for example HPV6, HPV11, HPV16 or HPV18, the Flavi-Viruses, for example the yellow fever-virus, the Dengue-Virus, the Tick-borne-Encephalitis-Virus or the Japanese Encephalitis-Virus, or the Influenza-Virus, antigens or antigen compositions or antigen compositions of bacterial pathogens, for example *Neisseria spp.*, in particular *N. gonorrhoea* and *N. meningitidis*, *Streptococcus spp.*, in particular *S. pneumonie*, *S. pyogenes*, *S. agalactiae*, *S. mutans*, *Haemophilus spp.*, for example *H. influenzae npe B*, non-typable *H. influenzae*, *H. ducreyi*, *Moraxella spp.*, in particular *M. catarrhalis*, also known as *Branhamella catarrhalis*, *Bordetella spp.*, in particular *B. pertussis* (for example Pertactin, Pertussis-Toxin or derivatives thereof), *B. parapertussis* and *B. bronchiseptica*, *Mycobacterium spp.*, in particular *M. tuberculosis*, *M. bonis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M. smegmatis*, *Legionella spp.*, in particular *L. pneumophila*, *Escherichia spp.*, in particular enterotoxic *E. coli*, enterohemorrhagic *E. coli* or enteropathogenic *E. coli*, *Vibrio spp.*, in particular *V. cholera* (for example the Cholera-Toxin or derivatives thereof) *Shigella spp.*, in particular *S. sonnei*, *S. dysenteriae* oder *S. flexnerii*, *Yersinia spp.*, in particular *Y. enterocolitica*, *Y. pestis*, *Y. pseudotuberculosis*, *Campylobacter spp.*, in particular *C. jejuni* or *C. coli*, *Salmonella spp.*, in particular *S. typhi*, *S. paratyphi*, *S. choleraesuis* or *S. enteritidis*, *Listeria spp.*, in particular *L. monocytogenes*, *Helicobacter spp.*, in particular *H. pylori*, *Pseudomonas spp.*, in particular *P. aeruginosa*, *Staphylococcus spp.*, in particular *S. aureus* or *S. epidermidis*, *Enterococcus spp.*, in particular *E. faecalis* or *E. faecium*, *Clostridium spp.*, in particular *C. tetani* (for example the *Tetanus-Toxin* and derivatives thereof), *C. botulinum* (for example the *Botulinum-Toxin* or derivatives thereof), *C. difficile* (for example the Clostridium-Toxin A or B or derivatives thereof), *Bacillus spp.*, in particular *B. anthracis*, *Corynebacterium spp.*, in particular *C. diphtheriae* (for example the Diphtherie-Toxin and derivatives thereof), *Borrelia*

spp., in particular *B. burgdorferi*, *B. garinii*, *B. afzelii*, *B. andersonii* or *B. hermsii*, *Ehrlichia spp.*, in particular *E. equi*, *Rickettsia spp.*, in particular *R. rickettsii*, *Chlamydia spp.*, in particular *C. trachomatis*, *C. pneumoniae* or *C. psittaci*, *Leptospira spp.*, in particular *L. interrogans*, *Treponema spp.*, in particular *T. pallidum*, *T. denticola* or *T. hyodysenteriae*, antigens or antigen compositions from parasites such as *Plasmodium spp.*, in particular *P. falciparum*, *Toxoplasma spp.*, in particular *T. gondii*, *Entamoeba spp.*, in particular *E. histolytica*, *Babesia spp.*, in particular *B. microti*, *Trypanosoma spp.*, in particular *T. cruzi*, *Giardia spp.*, in particular *G. lamblia*, *Leshmania spp.*, in particular *L. major*, *Pneumocystis spp.*, in particular *P. carinii*, *Trichomonas spp.*, in particular *T. vaginalis*, *Schisostoma spp.*, in particular *S. mansoni*, or antigens or antigen compositions from yeasts, for example *Candida spp.*, in particular *C. albicans*, or *Cryptococcus spp.*, in particular *C. neoformans*.

According to a particularly preferred process according to the invention for production of a vaccine composition, the antigen is the HBsAg, a fragment of this antigen, a variant of this antigen or of the fragment of this antigen or a mixture of at least two thereof. By the term "fragment" of a HBsAg is preferably understood a polypeptide, in which a section of at least 25, preferably at least 50 and particularly preferably at least 100 amino acids is identical with a corresponding section of the HBsAg, while, by the term "variant" of the HBsAg or of the fragment of the HBsAg, preferably is understood a polypeptide, in which at most 10, preferably at most 5 amino acids, particularly preferably at most 2 amino acids and most preferably at most 1 amino acid of the HBsAg or the fragment of the HBsAg is, or are deleted, inserted, substituted or attached at the C- and/or N-terminal ends. The term "variant" also comprises fusion polypeptides, in particular fusions with HBcAg.

HBsAg is a 226 amino acid protein (p24/gp27 or S-protein), which self-assembles in 20-30 nm lipoprotein particles, in which approximately 100-150 sub-units are cross-networked by means of multiple inter-and intra-molecular disulfide bonds.

The HBsAg's can be derived from eight presently known HBV-standard genotypes A, B, C, D, E, F, G and H. These eight standard genotypes differ in approximately 8 % of their nucleotide sequence (see Bartholomeusz, Rev. Med. Virol. 13 (2003), 1-14), whereby the variation concerns above all the HBsAg-Gen. Preferably, the HBV genotype A has the reference nucleic acid sequence Genbank X02763 or the HBV genotype A_{afr} has the reference nucleic acid sequence according to Genbank AF297621. For the HBV genotype B_a, the reference nucleic acid sequence is Genbank D00330, for the genotype B_j, the reference nucleic acid sequence AB073858. For the HBV genotype C, the reference nucleic acid sequence is Genbank AY206389, for the genotype C_{aus}, the reference nucleic acid sequence according to Genbank AB048704. For the genotype D, the reference nucleic acid sequence is Genbank X02496, for the genotype E Genbank X75657, for the genotype F Genbank X69798, for the genotype G Genbank AF160501 and for the genotype H Genbank AY090454.

The term "HBsAg" further comprises, in addition to the 226 amino acid protein, also the two M- and L-proteins, which further comprise, in addition to the HBsAg, the 55 amino acid-long pre-S2-peptide (M-protein) or the 55 amino acid-long pre-S2-peptide and the 120 amino acid long pre-S1-peptide (L-protein).

According to a preferred embodiment of the process according to the invention, the aqueous phase P_2 comprises not only a HBsAg, but also, as described in WO-A-2005/02397, at least two HBsAg's or fragments or variants thereof, whereby the HBsAg differ in the HBV genotype in the S-region and/or in the pre-S1-region.

The HBsAg and/or the HBsAg's can be obtained either from the serum of patients with chronic hepatitis B, in synthetic ways, or as recombinant polypeptides by means of processes known to the skilled person.

A possibility for the production of HBsAg as recombinant polypeptide consists in amplifying the core or pre-core reading frame by means of polymerase chain reaction (PCR) of a virus isolate. It is also conceivable to produce the corresponding DNA-sequence in a synthetic way and then to amplify by means of PCR. The obtained PCR-fragment is then cloned in a plasmid vector. By means of further PCR-clonings, the fragment can then be inserted into an expression vector, for example into an expression vector for *E. coli*. The insertion preferably occurs in such a way that the gene coding for the HBsAg or for the fragment of the variant is under the control of an active promoter. The expression vector can at the same time comprise induction elements for increasing the expression rate. After the transformation of the expression vector in *E. coli*, individual clones are obtained, which express the HBsAg or the fragment or the variant. In order to obtain the polypeptides which are to serve as antigens, an individual clone is injected in culture medium and cultivated over night at 37°C in the shaking flask. The *E. coli* culture is then first harvested by centrifugation. The bacteria pellet is resuspended in buffer solution and then disrupted by ultrasound treatment or by high pressure homogenisation. The HBsAg or HBsAg variants respectively located in the homogenisat is concentrated by precipitation with ammonium sulphate and then purified of bacterial host components by gel filtration.

In principal, the HBsAg or respectively fragments thereof or variants thereof comprised in the composition according to the invention can also be obtained in a synthetic way.

Further information concerning the techniques of recombinant or synthetic production of polypeptides can be found in

- Sambrook, "*Molecular Cloning: A Laboratory Manual*", second edition (1989);
- "*DNA Cloning*", Volumes I and II (D. N. Glover, editor., 1985);
- "*Oligonucleotide Synthesis*" (M. J. Gait, editor, 1986);

- "*Nucleic Acid Hybridization*" (B. D. Hames & S. J. Higgins, editors, 1984);
- "*Transcription and Translation*" (B. D. Hames & S. J. Higgins, editors, 1984);
- "*Animal Cell Culture*" (R. I. Freshney, editors, 1986);
- "*Immobilized Cells and Enzymes*" (IRL Press, 1986);
- B. Perbal, "*A Practical Guide to Molecular Cloning*" (1984);
- "*The Methods in Enzymology series*" (Academic Press, Inc.), in particular Volumes 154 and 155;
- "*Gene Transfer Vectors for Mammalian Cells*" (J. H. Miller und M. P. Calos, Herausgeber, 1987, Cold Spring Harbor Laboratory);
- Mayer und Walker, editors, (1987), "*Immunochemical Methods in Cell and Molecular Biology*" (Academic Press, London);
- Scopes, (1987), "*Protein Purification: Principles and Practice*", second edition (Springer-Verlag, N.Y.) and
- „*Handbook of Experimental Immunology*", Volumes I-IV (D. M. Weir and C. C. Blackwell, editors, 1986)

The production of HBsAg is also described in detail in: Brocke P, Schaefer S, Melber K, Jenzelewski V, Müller F, Dahlems U., Bartelsen O., Park KN, Janowicz ZA, Gellissen G: „*Recombinant hepatitis B vaccines: disease characterization and vaccine production*" (2005) in Gellissen G (editor): „*Production of recombinant proteins – novel microbial and eucaryontic expression systems*", pages 319-360, Wiley-VCH, Weinheim).

Finally, HBsAg can also be commercially obtained, for example, from the company Rhein Biotech GmbH, Düsseldorf.

It is further preferred according to the invention that also the aqueous phase P_2 is buffered by means of a suitable buffer system, for example, by means of the PBS buffer described in connection with the aqueous phase P_1, whereby, contrary to

the aqueous phase P_1, the aqueous P_2 comprising the antigen is preferably present as 1×PBS buffer.

For production of the aqueous phase P_2, preferably, first an antigen stock solution is prepared, which is then diluted with a buffer solution, preferably with a 1×PBS buffer.

The thus-obtained solution can then be sterilised, before it is added to aqueous phase P_1 in process step iii), whereby this sterilisation preferably occurs by means of sterile filtration.

If, in the process according to the invention for production of a vaccine composition, the condition c) is fulfilled, the aqueous phase P_2 comprises less than 2 mg/ml, preferably less than 1 mg/ml, particularly preferably less than 0.5 mg/ml, yet more preferably less than 0.3 mg/ml and most preferably less than 0.2 mg/ml of the antigen.

In process step iii) of the process according to the invention for production of a vaccine composition, the volume V_2 of the aqueous phase P_2 is now added to the volume V_1 of the aqueous phase P_1 with stirring of the volume V_1, whereby, preferably, first the volume V_1 of the aqueous phase P_1 is provided with stirring, and then, successively the volume V_2 of the aqueous phase P_2 is stirred into the volume V_1 of the aqueous phase P_1. According to a particularly preferred embodiment of the process according to the invention for production of vaccine composition, in which aluminium metahydroxide is used as aluminium compound, first, as described above, the aluminium metahydroxide, in a 1.5× to 6×PBS buffer, particularly preferably in a 1.75× to 5×PBS buffer and most preferably in a 2× to 4×PBS buffer is provided, with stirring, then heated, with stirring, at the above-described temperature and for the above-described duration, afterwards cooled to the above-described temperature, optionally diluted with water, preferably until the concentration of the PBS buffer reaches the concentration of 1× bis 2×PBS

buffer, particularly preferably 1,3× bis 1,7×PBS buffer, and the volume V_2 of the aqueous phase P_2 stirred into the thus-obtained volume V_1 of the aqueous phase P_1.

If, in the process according to the invention for production of a vaccine composition, the condition d) is fulfilled, volume V_2, with a rate from at most 10 %, particularly preferably of at most 5 %, yet more preferably of at most 2.5 % and most preferably of most 1 % of the amount of volume V_1-minute, is added to volume V_1. For example, if an aqueous phase P_1 with a volume of 20 litre is provided, the volume V_2 is added with a rate of at most 2 litre/minute, particularly preferably of at most 1 litre/minute, yet more preferably of at most 0.5 litre/minute and most preferably of at most 0.2 litre/minute, whereby the addition of the volume V_2 to the volume V_1 preferably occurs with continuous stirring of the volume V_1.

It is furthermore preferred according to the invention that the amount of the volume V_2 is about 10 to 50 %, preferably about 15 to 40 % and most preferably about 20 to 30 % of the amount of the volume V_1.

Through the use of very dilute solutions of the aluminium compound and of the antigen and the through the deliberately slow addition of the aqueous phase P_2 of the antigen to the aqueous phase P_1 of the aluminium compound, a very homogeneous immobilisation of the antigen on the surface of the aluminium compound is achieved. This homogeneous distribution is *inter alia* responsible for the advantageous properties of the vaccine composition according to the invention, in particular for the good stability as well as the constancy of the immune stimulatory effect in different batches of the vaccine composition.

According to a particular embodiment of the process according to the invention for production of a vaccine composition, the process additionally comprises the following process step:

iv) provision of a volume V_3 of an aqueous phase P_3 comprising an adjuvant different to the aluminium compound,

v) addition of the volume V_3 of the aqueous phase P_3 to the volume V_1 of the aqueous phase P_1, to the volume V_2 of the aqueous phase P_2 or to the mixture of the volumes V_1 and V_2 obtained in process step iii), preferably, however, to the mixture of the volumes V_1 and V_2 obtained in process step iii),

whereby at least one, preferably each of the following conditions is fulfilled:

e) the aqueous phase P_3 comprises less than 1 mg/ml of the adjuvant;

f) the volume V_3 is added with a rate of at most 10 % of the amount of the volume V_1/minute to the volume V_1, of at most 10 % of the amount of volume V_2/minute to the volume V_2 or respectively of at most 10 % of the amount of the volume of the mixture of the volumes V_1 and V_2/minutes to the mixture of the volumes V_1 and V_2, preferably, however, of at most 10 % of the amount of the volume of the mixture of the volumes V_1 and V_2/minute, to the mixture of the volumes V_1 and V_2.

Embodiments preferred according to the invention of this in turn preferred embodiment of the process according to the invention are those processes, in which the following properties or combinations of properties are fulfilled: a)e), a)f), b)e), b)f), c)e), c)f), d)e), d)f), a)e)f), b)c)d)e)f) and a)b)c)d)e)f), whereby a), b)c)d)e)f) and a)b)c)d)e)f) are particularly preferred and a)b)c)d)e)f) is most preferred.

In process step iv) of this particular embodiment of the process according to the invention for production of a vaccine composition, first a volume V_3 of an aqueous phase P_3 of an adjuvant different to the aluminium compound is prepared, whereby the aqueous phase P_3, just as with the aqueous phases P_1 and P_2, can be

an aqueous solution, an aqueous dispersion, an aqueous emulsion or an aqueous suspension.

The adjuvant which is comprised in the aqueous solution can be any adjuvant known to the skilled person which is commonly used in vaccine compositions. In general, by the term "adjuvant" is understood a compound which enables the induction of an immune response, preferably the induction of a cellular immune response to the antigen comprised in the aqueous phase P_2.

Adjuvants preferred according to the invention and different from aluminium compounds are selected in particular from the group comprising aluminium-free, particulate adjuvants, such as, for example, sub-micron-oil-in-water emulsion, to which belong, for example, MF59 (5 % Squalene, 0,5 % Tween® 80 and 0,5 % Span® 85), SAF (10 % Squalene, 0,4 % Tween® 80, 5 % pluron-blocked polymer as well as thr-muramyldipeptide (thr-MDP)), incomplete Freundsches adjuvant, lipopolysaccharides, N-Acetylglucosaminyl-N-acetylmuramyl-L-alanyl-dipeptide (GMDP) or muramyldipeptide (MDP), Gal-ceramide, dimethyldioctadecylammonium bromide (DDAB), liposomes, preferably comprising phospholipid und cholesterol, microparticles from biodegradable polymers such as polylactide-co-glycolide (PLGA), oligodesoxyribonucleotides with or without CpG-motif, N,N-Di-(β-stearoylethyl)-N,N-dimethylammonium chloride (EQ1), glycopeptides, components of the cell wall of mycobacteria, quaternary amines such as, for example, cetylpyridinium chloride und benzalkonium chloride, zwitterionic amines such as CHAPS (3-(3-cholamidopropyl)-dimethyl-ammonio)-1-propanesulfonate), dextransulfate, granulocytes-macrophages-colonies stimulating factor (GM-CSF), tumor necrosis factor (TNF), block copolymers such as, for example, P1205 or Poloxamer 401, dimyristoylphosphatidylcholine (DMPC), 3β-hydroxy-5-androsten-17-one (DHEA), dimyristoylphosphatidyl-glycerol (DMPG), desoxycholic acid sodium salt, Imiquimod, DTP-GDP, 7-Allyl-8-oxoguanosine, Montanide® ISA 51, Montanide® ISA 720, murametide, murapalmitin, dicetylphosphate, polymethylmethacrylate (PMMA), PEG-sorbitan fatty

acid esters such as polysorbate 20 oder 80 (TWEEN® 20,80), detoxified mutants of bacterial ADP-ribosylating toxins such as cholera-toxin or pertussis-toxin or mixtures of at least two of these adjuvants. Particularly preferred as adjuvants are lipopolysaccharides, Gal-ceramides, oligodesoxyribonucleotides with CpG-motif, PEG-sorbitan fatty acid esters, a Saponin- or a Saponin derivative-comprising adjuvant, such as, for example, the commercially obtainable products AbISCO®-100, AbISCO®-200, AbISCO®-300 (ISCONOVA, Uppsala, Sweden) or Stimulon®QS-21 (ANTIGENICS, Woburn, USA), as well as 3-O-deacetylated monophosphoryl-lipid A or a derivative of a 3-O-deacetylated monophosphoryl-lipid A.

An overview of compounds suitable as adjuvant is given in Cox, J.C. and Coulter, A.R. *"Adjuvants-a classification and review of their models of action"* in Vaccine 15:248-256 (Feb. 1997) or also in Frederick R. Vogel et al., *"A Compendium of Vaccine Adjuvants and Excipients"*, 2nd edition. The disclosure of these publications with respect to possible adjuvants in vaccine compositions is hereby introduced as reference and forms a part of the disclosure of the present invention.

Particularly preferred adjuvants according to the invention are those derivatives of a 3-O-deacetylated monophosphoryl-lipid A, which are described in WO-A-98/50399, in particular in example 20, as well in WO-A-01/34617. The disclosure of WO-A-98/50399 as well of WO-A-01/34617 concerning the therein-described derivatives of the 3-O-deactylated monophosphoryl-lipid A is herewith introduced as reference and forms a part of the disclosure of the present invention.

Also in connection with the aqueous phase P_3 comprising the adjuvant, it can be advantageous according to the invention that this is buffered by means of a suitable buffer system, for example by means of the PBS buffer described in connection with the aqueous phases P_1 and P_2, whereby, contrary to the aqueous phase P_1 and in agreement with the aqueous phase P_2, the aqueous P_3 comprising the adjuvant preferably is present as a 1×PBS buffer. In particular, in the use of the de-

rivatives of a 3-O-deacetylated monophosphoryl-lipid A as adjuvant, it is, however, preferred to use deionised or distilled water as solvent.

In the production of the aqueous phase P_3, preferably, first, an adjuvant stock solution is provided, which is then diluted with a buffer solution or with deionised or distilled water, preferably with deionised or distilled water. If the derivatives preferred according to the invention of a 3-O-deacetylated monophosphoryl-lipid A are used as adjuvants, it is advantageous if, as adjuvant stock solution, first a suspension, particularly preferably a nanosuspension, comprising the adjuvant, water and a lipid, preferably a lipid selected from the group consisting of 1,2-dipalmitoylphosphatidylcholine, 1,2-dipalmitoylphosphatidylethanolamine, 1,2-dipalmitoylphosphatidylserine and 1,2-dipalmitoylphosphatidyldimethylaminoethanol, particularly preferably 1,2-dipalmitoylphosphatidylcholine (DPPC), is prepared, whereby the preparation of this suspension preferably occurs by means of ultrasound over a time period in a range from 10 minutes to 10 hours, particularly preferably from 60 minutes to 5 hours and most preferably from 1.5 hours to 3 hours. The mole ratio of adjuvant : lipid preferably lies in a range from 1 : 1 to 1 : 20, particularly preferably in a range from 1 : 2 to 1 : 10 and most preferably in a range from 1 : 3 to 1 : 5, while the mole ratio of lipid : water preferably lies in a range from 1 : 100 to 1 : 10,000, particularly preferably in a range from 1 : 500 to 1 : 5,000 and most preferably in a range from 1 : 2,000 to 1 : 3,000. Subsequently, the adjuvant stock solution obtained by means of ultrasound treatment can be further diluted with deionised or distilled water. After the dilution, it can furthermore be advantageous to further homogenise the suspension, preferably nanosuspension, obtained in this way, for example, by means of a high pressure homogeniser, in order to obtain as stable a suspension, preferably nanosuspension, of the adjuvant as possible.

The thus-obtained aqueous phase P_3 can then be sterilised, whereby this sterilisation preferably occurs by means of sterile filtration.

If, in this particularly preferred embodiment of the process according to the invention for production of a vaccine composition, the condition e) is fulfilled, the aqueous phase P_3 comprises less than 1 mg/ml, preferably less than 0.75 mg/ml, particularly preferably less than 0.5 mg/ml, yet more preferably less than 0.3 mg/ml and most preferably less than 0.15 mg/ml of the adjuvant.

In process step v) of the particular embodiment of the process according to the invention for production of a vaccine composition, the volume V_3 of the aqueous phase P_3 is now added to the volume V_1 of the aqueous phase P_1, to the volume V_2 of the aqueous phase P_2 or to the mixture of the volumes V_1 and V_2 obtained in process step iii), preferably, however, to the mixture of volumes V_1 and V_2 obtained in process step iii).

If, in this particular embodiment of the process according to the invention for production of a vaccine composition, the condition f) is fulfilled, the volume V_3 is added, with a rate of at most 10 %, particularly preferably of at most 5 %, yet more preferably of at most 2.5 % and most preferably of at most 1 % of the amount of the volume V_1, of the amount of the volume V_2 or of the amount of the mixture of volumes V_1 and V_2/minute to the volume V_1, to the volume V_2 or respectively to the mixture of volumes V_1 and V_2.

It is furthermore preferred in this particular embodiment of the process according to the invention that the volume V_3 of the aqueous phase P_3 is about 20 to 70 %, particularly preferably about 30 to 60 % and most preferably about 40 to 50 % of the volume V_1 of the aqueous phase P_1.

The process according to the invention can also comprise further process steps, in particular a process step vi), in which to the volume V_1, to the volume V_2, to the volume V_3, to the mixture of volumes V_1 and V_2, to the mixture of the volumes V_1 and V_3, to the mixture of the volumes V_2 and V_3, to the mixture of the volumes V_1, V_2 and V_3, particularly preferably, however, to the mixture of the volumes V_1,

V_2 and V_3, a further volume V_4 of a preferably aqueous and preferably sterile phase P_4 comprising one or more additives, for example, antioxidants, buffer components, colouring agents, viscosity regulators or preservatives is added.

A contribution to the solution of the above-mentioned objects is also made by a vaccine composition obtainable by the above-described process according to the invention.

A further contribution to the solution of the above-mentioned objects is also made by a vaccine composition, comprising

I) a substrate comprising an aluminium compound, whose surface has been brought into contact with phosphate anions, and

II) an antigen, which is immobilised on the substrate,

whereby the antigen is immobilised homogeneously on the substrate.

As aluminium compound and as antigen, those compounds and components respectively are preferred which have already been mentioned above in connection with the process according to the invention for production of a vaccine composition as preferred antigens and aluminium compounds respectively.

By a "homogeneous" immobilisation of the antigen on the substrate is preferably understood an immobilisation which, with an immunohistochemical colouring of the vaccine composition according to the invention by means of FITC- or TRITC-marked monoclonal antibodies which are directed against to the respective antigen, allow to recognise a homogeneous colouration of the substrate surface.

According to a particular embodiment of the vaccine composition according to the invention, this additionally comprises

III) an adjuvant, which is likewise immobilised on the substrate,

whereby the adjuvant is likewise immobilised homogeneously on the substrate.

As adjuvant are likewise preferred those compounds or components which have already been described as preferred adjuvants in connection with the process according to the invention for production of a vaccine composition.

A further contribution to the solution of the above-mentioned objects is also made by the use of the vaccine composition obtainable by the process according to the invention or of the vaccine composition according to the invention for the production of a pharmaceutical for prophylaxis or therapy, preferably prophylaxis, of viral illnesses, in particular the use of the vaccine composition comprising HBsAg as antigen as vaccine for the prophylaxis or therapy, preferably prophylaxis, of a hepatitis B illness in humans.

The term "prophylaxis" in connection with HBV-infection in humans preferably comprises

- the treatment of a person who has not yet had any contact with HBV, with the aim of preventing an infection with HBV from occurring,

- the treatment of a person who has already had contact with HBV and who is in the immunotolerant chronic phase, with the aim of preventing this person from entering into the immunoactive chronic phase, as well as

- the treatment of a person who has already had contract with HBV and who is in the inactive carrier stage, with the aim of preventing that this person enters again into the immunoactive phase,

whereby, most preferably, by the term "prophylaxis" is understood the treatment of a person who has not yet any contact with HBV, with the aim of preventing that an infection with HBV occurs.

Amounts which reach the above-mentioned aims can be achieved are defined as "therapeutically effective doses". The therapeutically effective dose for the respective application case depends, for example, on the exact composition of the vaccine composition according to the invention or respectively of the pharmaceutical according to the invention, the method of administration as well as the weight and the general state of health of the patient, but generally lies preferably in a range from about 0.1 to 2,000 µg (total amount of aluminium compound, antigen and optionally adjuvant) for a 70 kg patient, particularly preferably in a range from 0.5 to 1,500 µg, yet more preferably in a range from 1 to 1,000 µg and most preferably in a range from 10 to 500 µg, whereby the vaccine composition according to the invention preferably is administered in the form of merely 2 doses, preferably administered at a separation of 2 weeks to 2 months, particularly preferably at a separation of about one month.

The vaccine composition according to the invention or respectively the pharmaceutical according to the invention can, in principal, be administered orally, intranasally, subcutaneously, intramuscularly or intravenously.

A further contribution to the solution of the above-mentioned object is made by a process for immunisation of a human, whereby the vaccine composition obtainable by the process according to the invention, the vaccine composition according to the invention or the pharmaceutical is administered to a patient, preferably to a human, preferably in the form of a first and second doses, whereby the separation between the first and second dose is preferably 2 weeks to months, preferably about one month.

The invention is now more closely illustrated by means of a non-limiting example.

Example

The following phases are provided:

1. In an autoclavable vessel, which is equipped with a stirring device, about 1.5 kg aluminium metahydroxide gel (Alhydrogel®, Brenntag Biosector, Denmark) in 3×PBS (comprises about 2.94 mg/ml disodium hydrogenphosphate-dihydrate, 2.13 mg/ml sodium dihydrogenphosphate-dihydrate and 27 mg/ml sodium chloride) are provided and heated with stirring for at least 20 minutes at a temperature of 121°C. After cooling to room temperature dilution is carried out with distilled water to a 1.5×PBS-concentration. The concentration of Alhydrogel® after the dilution is about 1 mg/ml. An aqueous phase P_1 comprises Alhydrogel® as aluminium compound is obtained.

2. HBsAg (Rhein Biotech GmbH, Düsseldorf) is diluted with PBS (comprises about 0.98 mg/ml disodium hydrogenphosphate dihydrate, 0.71 mg/ml sodium dihydrogenphosphate dihydrate and 9 mg/ml sodium chloride) to a concentration of about 0.1 mg/ml. An aqueous phase P_2 comprising HBsAg as antigen is obtained.

3. About 1.5 g of the adjuvant RC529 (Corixa, USA), about 2.6 g DPPC and about 900 g distilled water are processed to a nanosuspension by means of ultrasound. To this nano suspension are added a further 300 g distilled water and the thus-obtained, diluted solution further homogenised by means of a high pressure homogeniser. An aqueous phase P_3 comprising RC-529 in a concentration of about 0.1 mg/ml as adjuvant is obtained.

4. To the aqueous phase P_1 (total volume about 18 litres) in the autoclavable vessel is added, with stirring, the aqueous phase P_2 (total volume about 4.4 litres) with a rate of about 0.1 litres/minute. The aqueous phase P_3 (total volume about 7.5 litres) is then added to the thus-obtained mixture with a rate of likewise about 0.1 litres/minute.

The thus-obtained vaccine composition was characterised by an excellent immunogenity and stability.

Claims:

1. A process for production of a vaccine composition, comprising the process steps:

i) provision of a volume V_1 of an aqueous phase P_1 comprising an aluminium compound whose surface has been brought into contact with phosphate ions,

ii) provision of a volume V_2 of an aqueous phase P_2 of an antigen,

iii) addition of the volume V_2 of the aqueous phase P_2 to the volume V_1 of the aqueous phase P_1 with stirring of the volume V_1,

wherein at least one, preferably all of the following conditions is fulfilled:

a) the aluminium compound was heated after the bringing into contact with the phosphate ions and before process step iii);

b) the aqueous phase P_1 comprises less than 10 mg/ml aluminium;

c) the aqueous phase P_2 comprises less than 1 mg/ml of the antigen;

d) the volume V_2 is added with a rate of at most 10% of the amount of the volume V_1/minute to volume V_1.

2. The process according to claim 1, wherein the aluminium compound comprises aluminium hydroxide.

3. The process according to claim 1 or claim 2, wherein the aqueous phase P_1 comprises, in addition to the aluminium compound, the salt of a phosphate.

4. The process according to claim 3, wherein the provision of the volume V_1 of the aqueous phase P_1 also comprises the heating of the volume V_1 of the aqueous phase P_1 to a temperature of at least 100 °C.

5. The process according to any one of the preceding claims, wherein the aqueous phase P_1 comprises less than 1 mg/ml aluminium.

6. The process according to any one of the preceding claims, wherein the antigen is a HBsAg.

7. The process according to any one of the preceding claims, wherein the aqueous phase P_2 comprises less than 0.2 mg/ml antigen.

8. The process according to any one of the preceding claims, wherein the volume V_2 is added with a rate of at most 1% of the amount of volume V_1/minute to volume V_1.

9. The process according to any one of the preceding claims, wherein the amount of the volume V_2 is about 10 to 50% of the amount of the volume V_1.

10. The process according to any one of the preceding claims, wherein the process additionally comprises the following process steps:

iv) provision of a volume V_3 of an aqueous phase P_3 comprising an adjuvant different to the aluminium compound,

v) addition of the volume V_3 of the aqueous phase P_3 to the volume V_1 of the aqueous phase P_1, to the volume V_2 of the aqueous phase P_2 or to the mixture of the volumes V_1 and V_2 obtained in process step iii),

wherein at least one, preferably all of the following conditions are fulfilled:

e) the aqueous phase P_3 comprises less than 1 mg/ml of the adjuvant;

f) the volume V_3 is added with a rate of at most 10% of the amount of the volume V_1/minute to the volume V_1, of at most 10% of the amount of the volume V_2/minute to the volume V_2 or of at most 10% of the amount of the volume of the mixture of the volumes V_1 and V_2/minute to the mixture of the volumes V_1 and V_2.

11. Process according to claim 10, wherein the aqueous phase P_3 comprises less than 0.15 mg/ml adjuvant.

12. Process according to claim 10 or claim 11, wherein the adjuvant is a derivative of a 3-O-deacetylated monophosphoryl lipid A.

13. Process according to any one of the preceding claims, wherein the volume V_3 is about 30 to 60% of the volume V_1.

14. A vaccine composition obtainable by the process according to any one of the preceding claims.

15. A vaccine composition, comprising

I) a substrate comprising an aluminium compound whose surface has been brought into contact with phosphate anions, and

II) an antigen, which is immobilised on the substrate,

wherein the antigen is homogeneously immobilised on the substrate.

16. The vaccine composition according to claim 15, wherein the aluminium compound comprises aluminium hydroxide.

17. The vaccine composition according to claim 15 or claim 16, wherein the antigen is a HBsAg.

18. The vaccine composition according to any one of the preceding claims, wherein the composition additionally comprises

III) an adjuvant, which is likewise immobilised on the substrate,

and wherein the adjuvant is likewise homogeneously immobilised on the substrate.

19. The vaccine composition according to claim 18, wherein the adjuvant is a derivative of a 3-O-deacetylated monophosphoryl lipid A.

20. Use of the vaccine composition according to any one of claims 14 to 19 for production of a pharmaceutical for prophylaxis of hepatitis B.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/004818

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/39 A61K47/02 C07F1/00 A61K39/29

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)
A61K

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)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 August 2007	11. 09. 2007
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Irion, Andrea

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International application No
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