Fig. 1

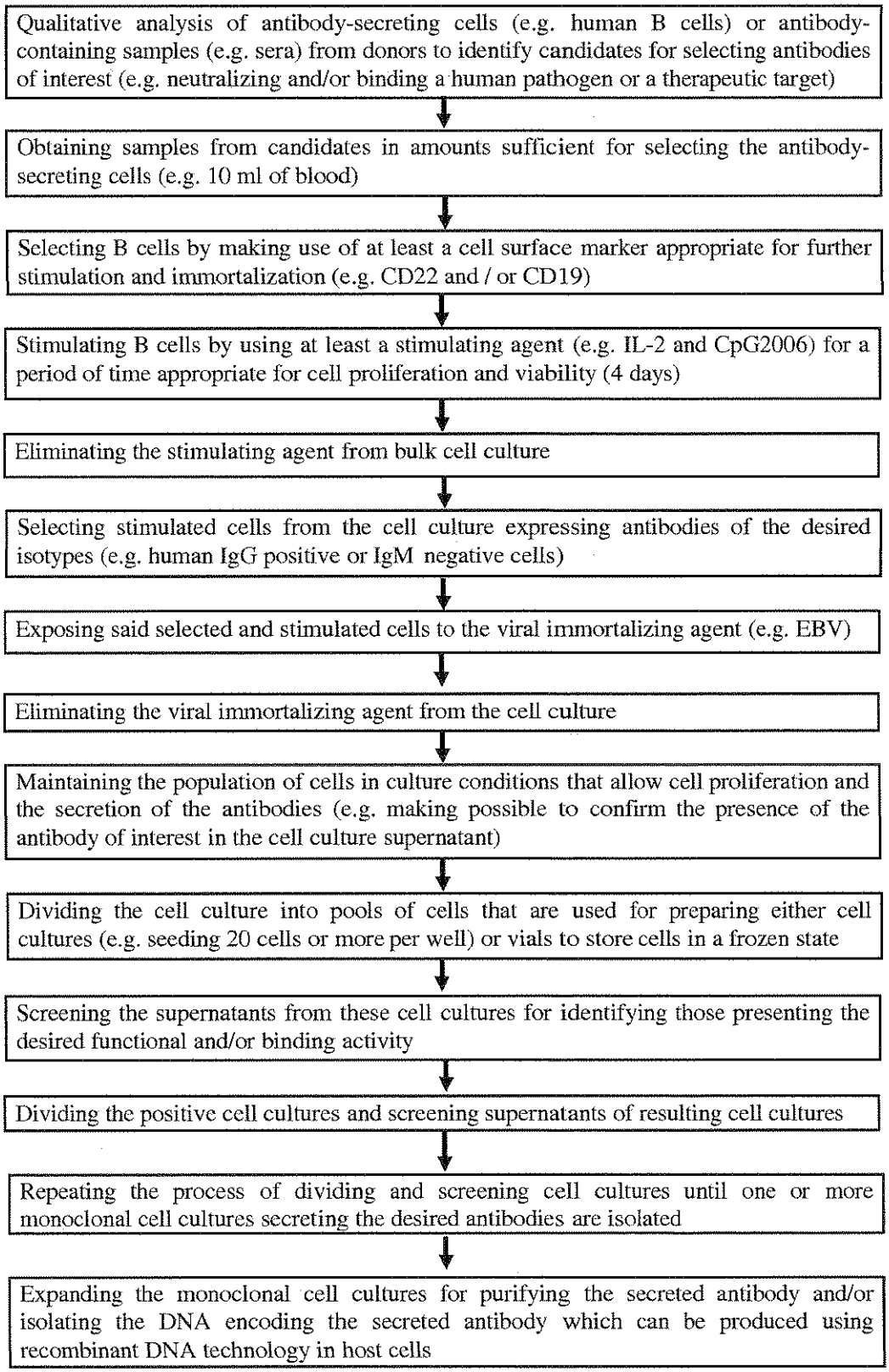

Qualitative analysis of antibody-secreting cells (e.g. human B cells) or antibody-containing samples (e.g. sera) from donors to identify candidates for selecting antibodies of interest (e.g. neutralizing and/or binding a human pathogen or a therapeutic target)
Obtaining samples from candidates in amounts sufficient for selecting the antibody-secreting cells (e.g. 10 ml of blood)
Selecting B cells by making use of at least a cell surface marker appropriate for further stimulation and immortalization (e.g. CD22 and / or CD19)
Stimulating B cells by using at least a stimulating agent (e.g. IL-2 and CpG2006) for a period of time appropriate for cell proliferation and viability (4 days)
Eliminating the stimulating agent from bulk cell culture
Selecting stimulated cells from the cell culture expressing antibodies of the desired isotypes (e.g. human IgG positive or IgM negative cells)
Exposing said selected and stimulated cells to the viral immortalizing agent (e.g. EBV)
Eliminating the viral immortalizing agent from the cell culture
Maintaining the population of cells in culture conditions that allow cell proliferation and the secretion of the antibodies (e.g. making possible to confirm the presence of the antibody of interest in the cell culture supernatant)
Dividing the cell culture into pools of cells that are used for preparing either cell cultures (e.g. seeding 20 cells or more per well) or vials to store cells in a frozen state
Screening the supernatants from these cell cultures for identifying those presenting the desired functional and/or binding activity
Dividing the positive cell cultures and screening supernatants of resulting cell cultures
Repeating the process of dividing and screening cell cultures until one or more monoclonal cell cultures secreting the desired antibodies are isolated
Expanding the monoclonal cell cultures for purifying the secreted antibody and/or isolating the DNA encoding the secreted antibody which can be produced using recombinant DNA technology in host cells

Fig. 2

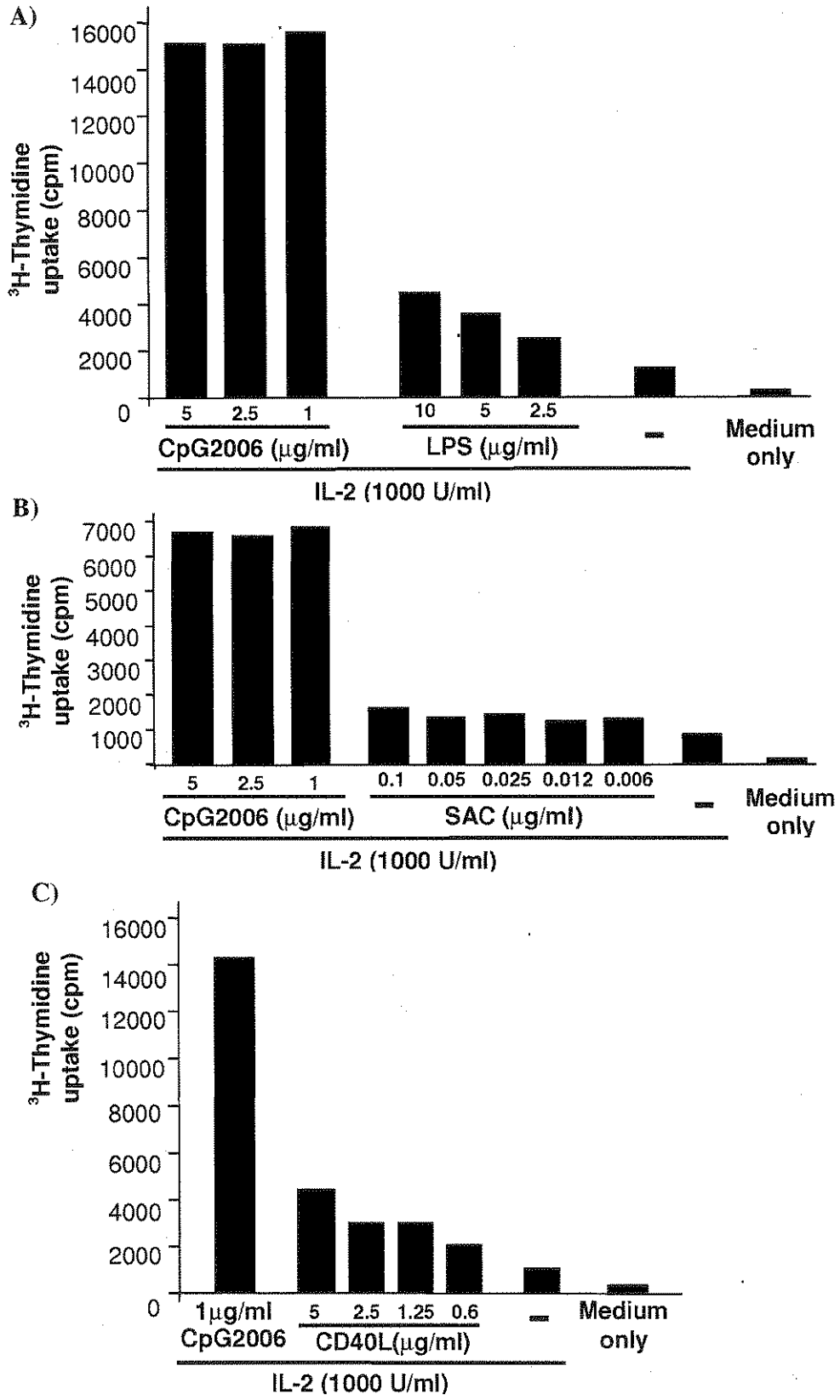
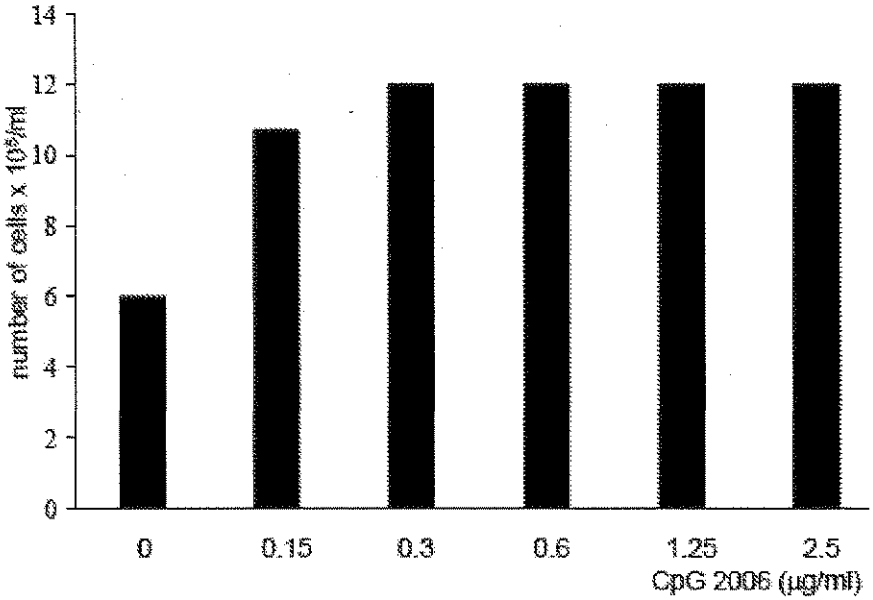

A)
16000
14000
12000
10000
8000
6000
4000
2000
0
^{3}H-Thymidine uptake (cpm)
5 2.5 1
10 5 2.5
CpG2006 (µg/ml)
LPS (µg/ml)
Medium only
IL-2 (1000 U/ml)
B)
7000
6000
5000
4000
3000
2000
1000
^{3}H-Thymidine uptake (cpm)
5 2.5 1
0.1 0.05 0.025 0.012 0.006
CpG2006 (µg/ml)
SAC (µg/ml)
Medium only
IL-2 (1000 U/ml)
C)
16000
14000
12000
10000
8000
6000
4000
2000
0
^{3}H-Thymidine uptake (cpm)
1µg/ml CpG2006
5 2.5 1.25 0.6
CD40L(µg/ml)
Medium only
IL-2 (1000 U/ml)

Fig. 3

A)


14
12
10
8
6
4
2
0
number of cells x 10⁵/ml
0
0.15
0.3
0.6
1.25
2.5
CpG 2006 (µg/ml)

B)

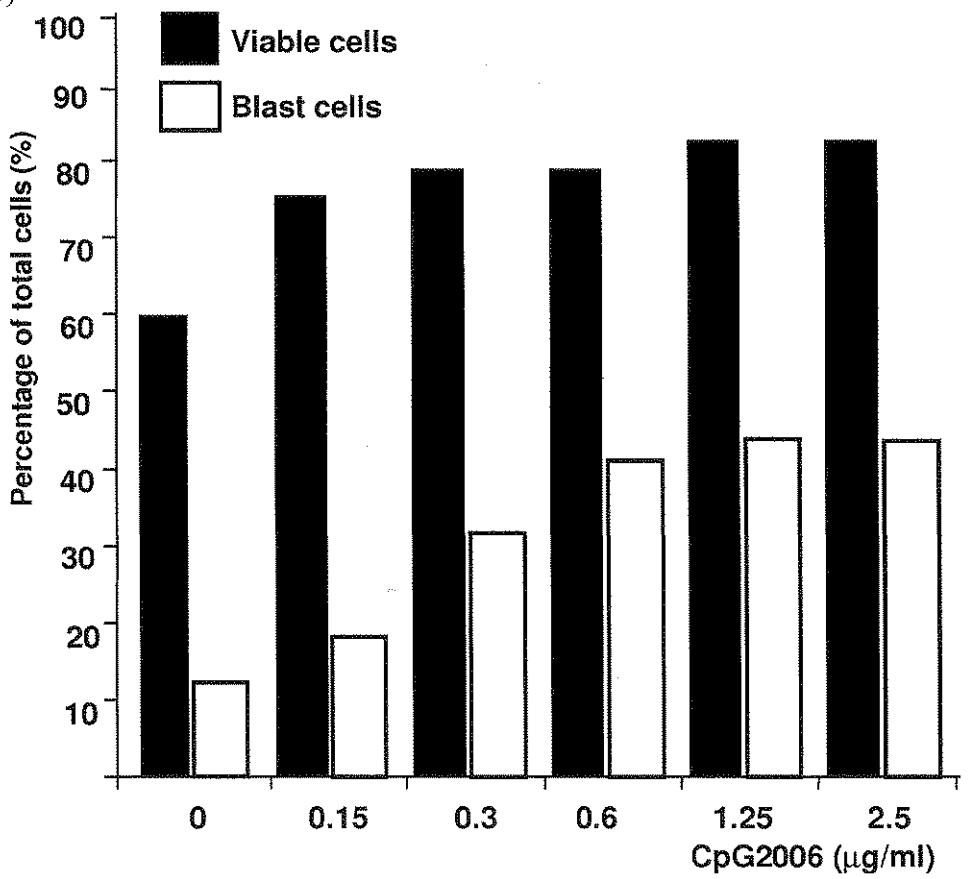
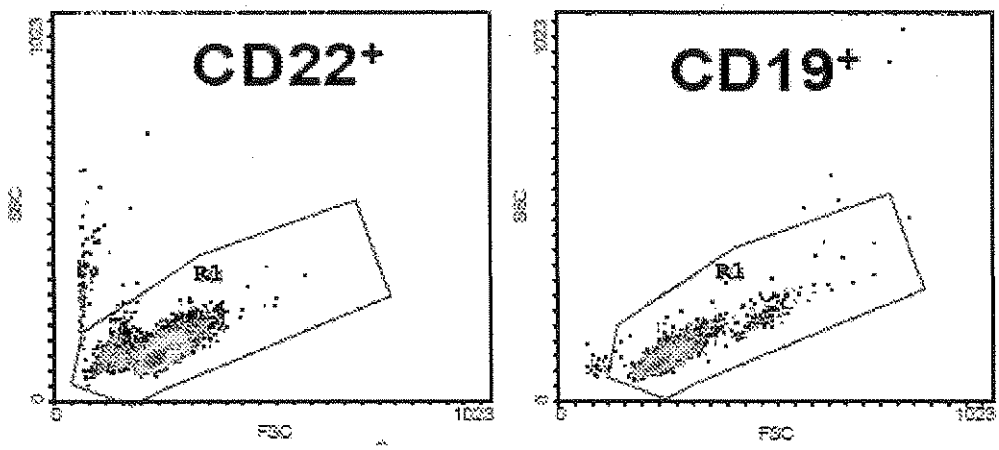

Viable cells
Blast cells
100
90
80
70
60
50
40
30
20
10
Percentage of total cells (%)
0
0.15
0.3
0.6
1.25
2.5
CpG2006 (µg/ml)

Fig. 4

A)


CD22+
R1
SSC
FSC
0
1023
CD19+
R1
SSC
FSC
0
1023

B)

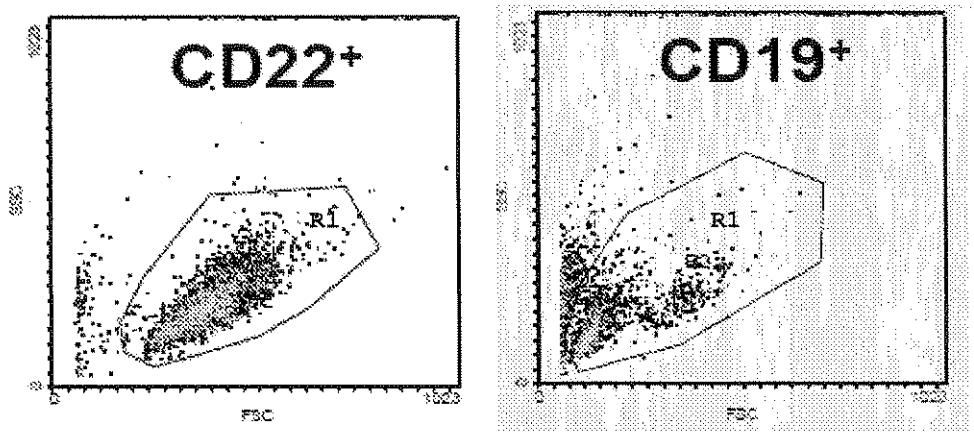

CD22+
R1
SSC
FSC
0
1023
CD19+
R1
SSC
FSC
0
1023

Fig. 5

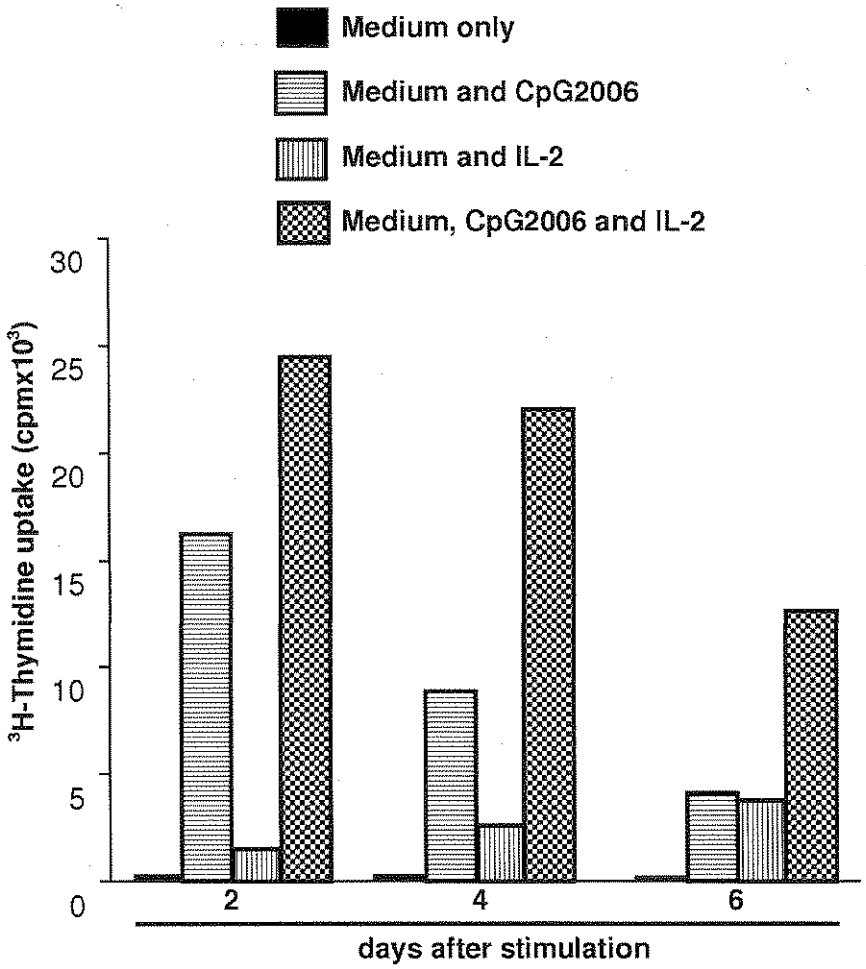
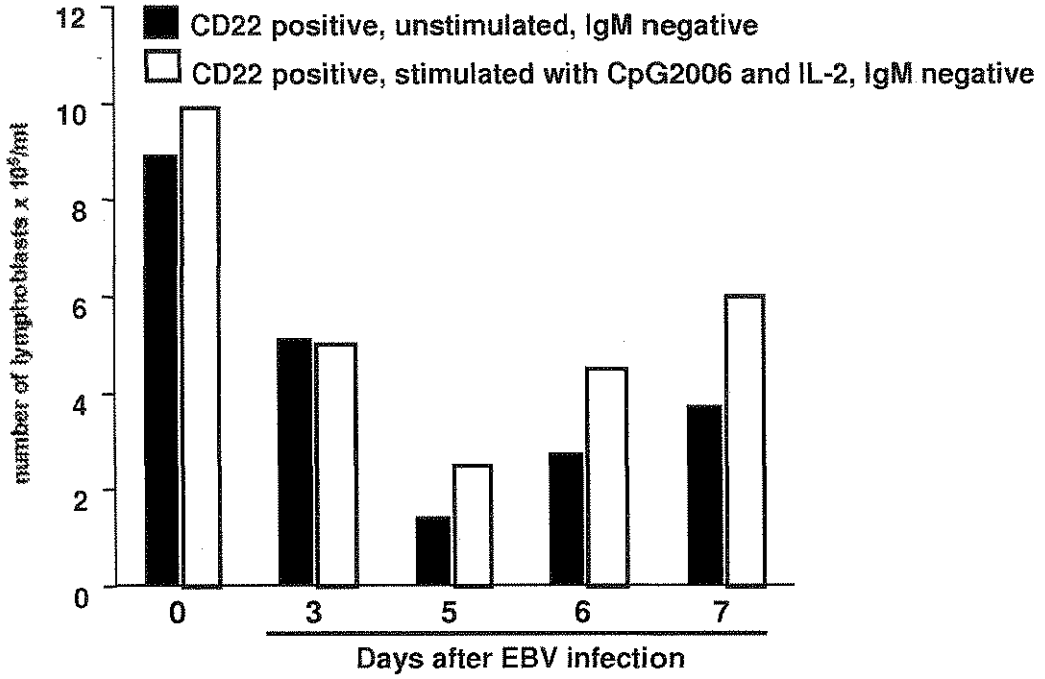

Medium only
Medium and CpG2006
Medium and IL-2
Medium, CpG2006 and IL-2
^{3}H-Thymidine uptake (cpmx10^3)
30
25
20
15
10
5
0
2
4
6
days after stimulation

Fig. 6

A)


CD22 positive, unstimulated, IgM negative
CD22 positive, stimulated with CpG2006 and IL-2, IgM negative
number of lymphoblasts x 10⁵/ml
12
10
8
6
4
2
0
0
3
5
6
7
Days after EBV infection

B)

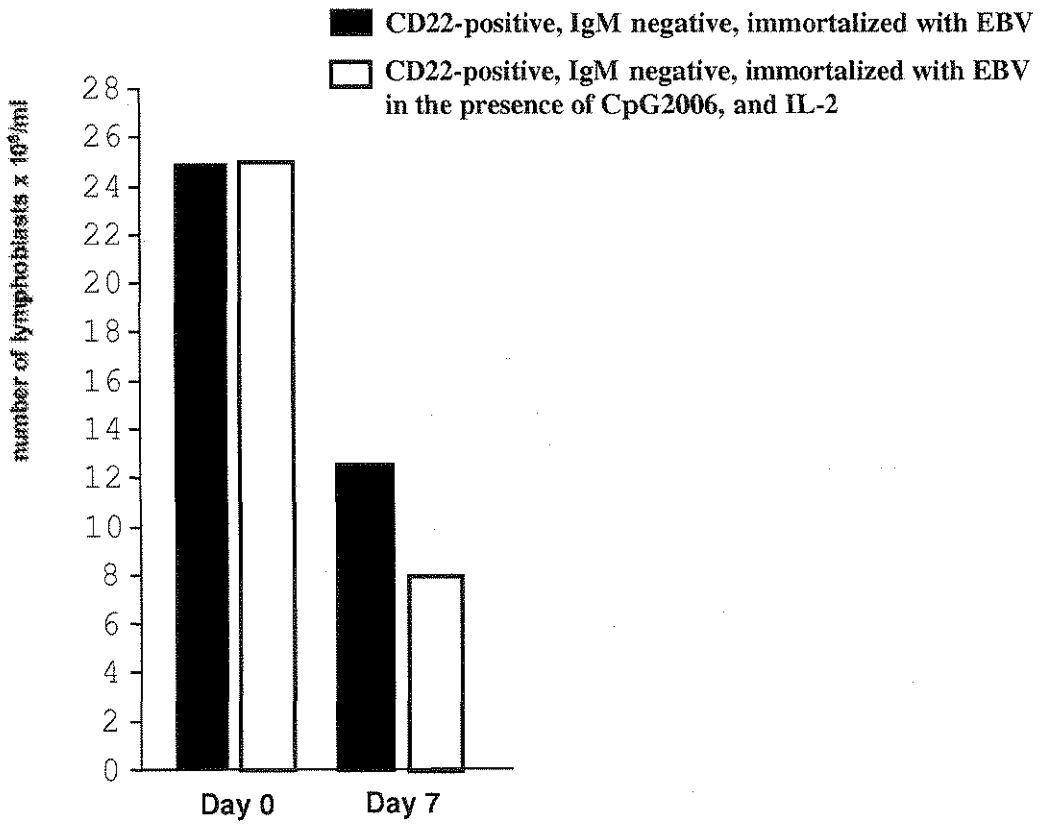
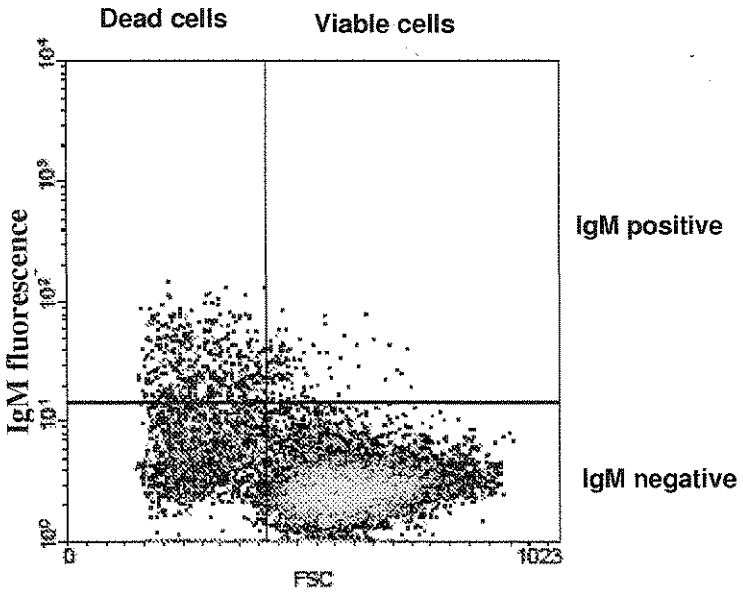

CD22-positive, IgM negative, immortalized with EBV
CD22-positive, IgM negative, immortalized with EBV in the presence of CpG2006, and IL-2
number of lymphoblasts x 10⁵/ml
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
Day 0
Day 7

Fig. 7

A)


Dead cells
Viable cells
IgM fluorescence
IgM positive
IgM negative
FSC
0
1023

B)

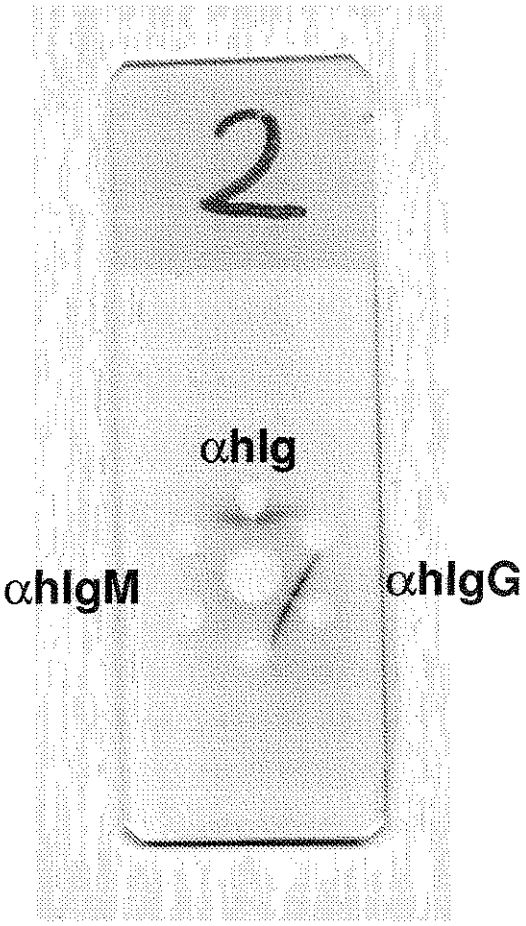
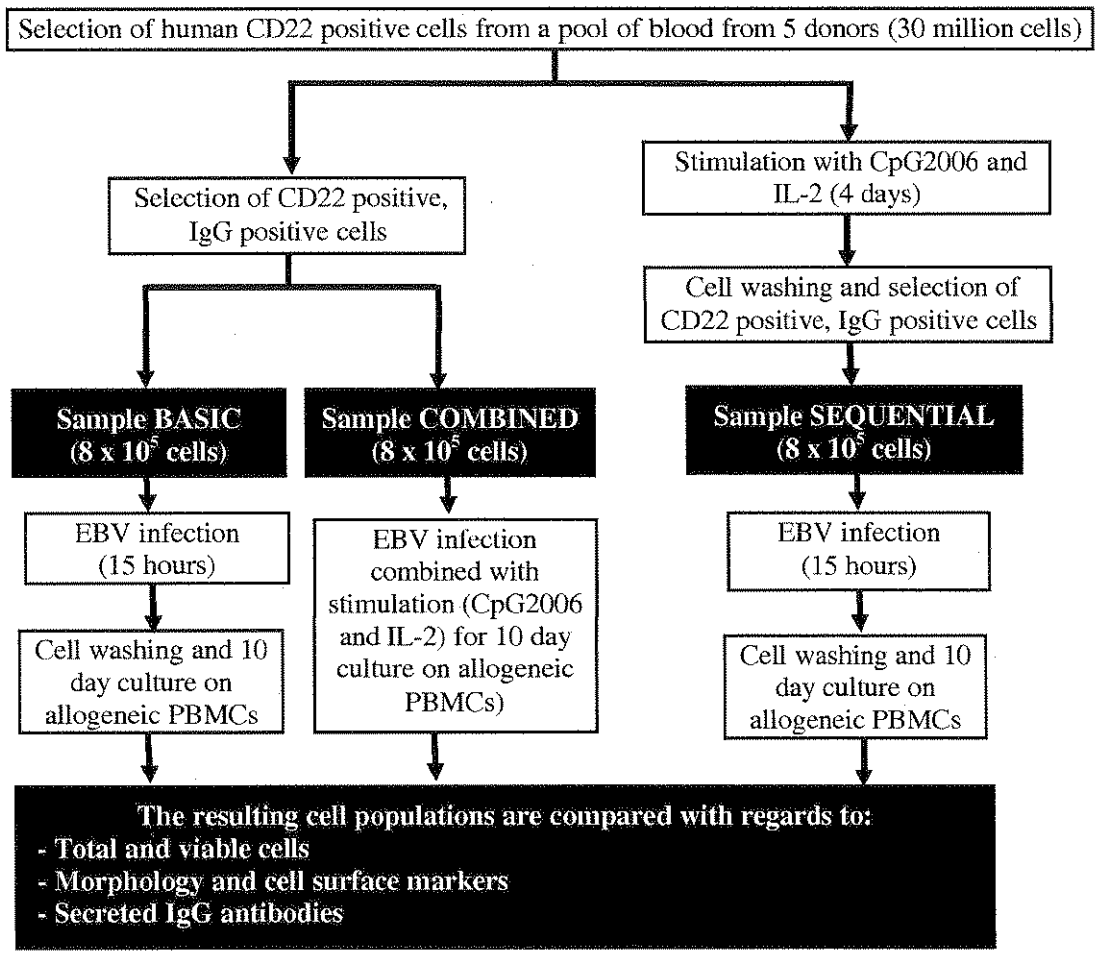

2
αhIg
αhIgM
αhIgG

Fig. 8

A)


Selection of human CD22 positive cells from a pool of blood from 5 donors (30 million cells)
Selection of CD22 positive, IgG positive cells
Stimulation with CpG2006 and IL-2 (4 days)
Cell washing and selection of CD22 positive, IgG positive cells
Sample BASIC (8 x 10^5 cells)
Sample COMBINED (8 x 10^5 cells)
Sample SEQUENTIAL (8 x 10^5 cells)
EBV infection (15 hours)
EBV infection combined with stimulation (CpG2006 and IL-2) for 10 day culture on allogeneic PBMCs)
EBV infection (15 hours)
Cell washing and 10 day culture on allogeneic PBMCs
Cell washing and 10 day culture on allogeneic PBMCs
The resulting cell populations are compared with regards to:
- Total and viable cells
- Morphology and cell surface markers
- Secreted IgG antibodies

B)

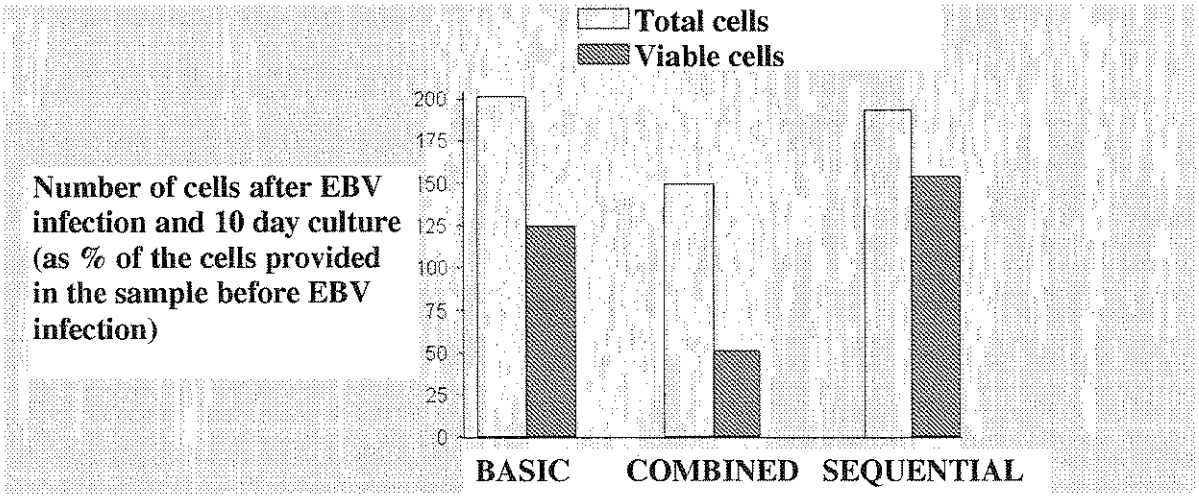

Total cells
Viable cells
Number of cells after EBV infection and 10 day culture (as % of the cells provided in the sample before EBV infection)
200
175
150
125
100
75
50
25
0
BASIC
COMBINED
SEQUENTIAL

Fig. 9

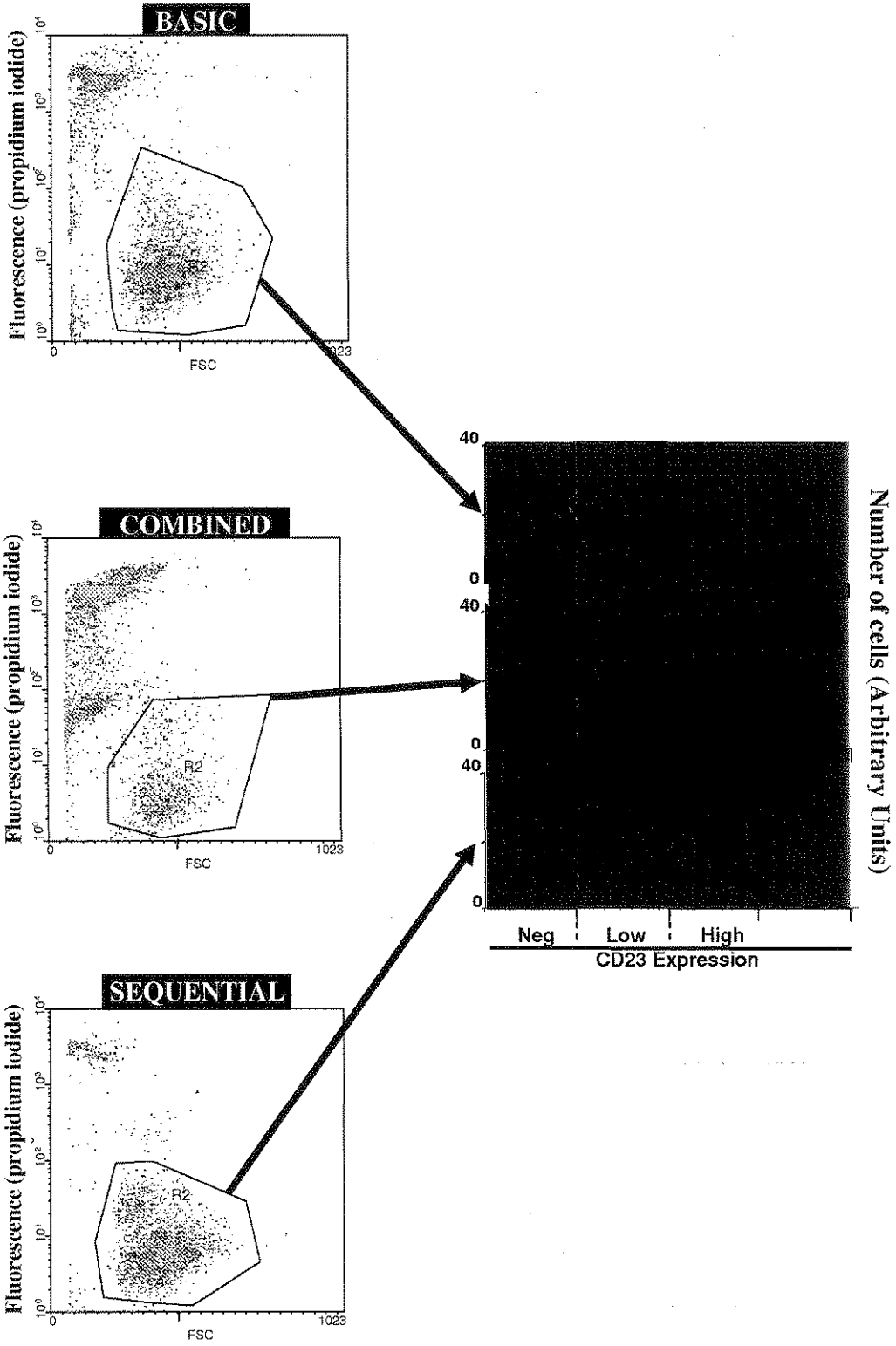

BASIC
COMBINED
SEQUENTIAL
Fluorescence (propidium iodide)
FSC
R2
0
1023
40
Neg
Low
High
CD23 Expression
Number of cells (Arbitrary Units)

Fig. 10

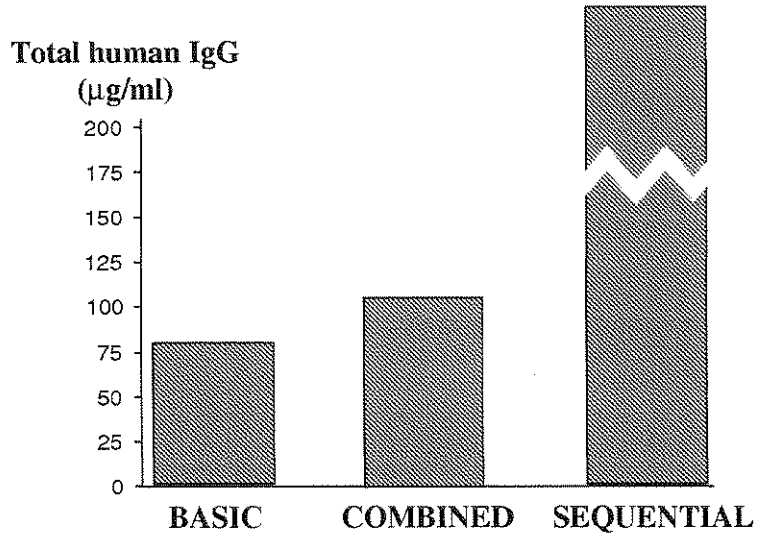

Total human IgG
(µg/ml)
200
175
150
125
100
75
50
25
0
BASIC
COMBINED
SEQUENTIAL

Fig. 11

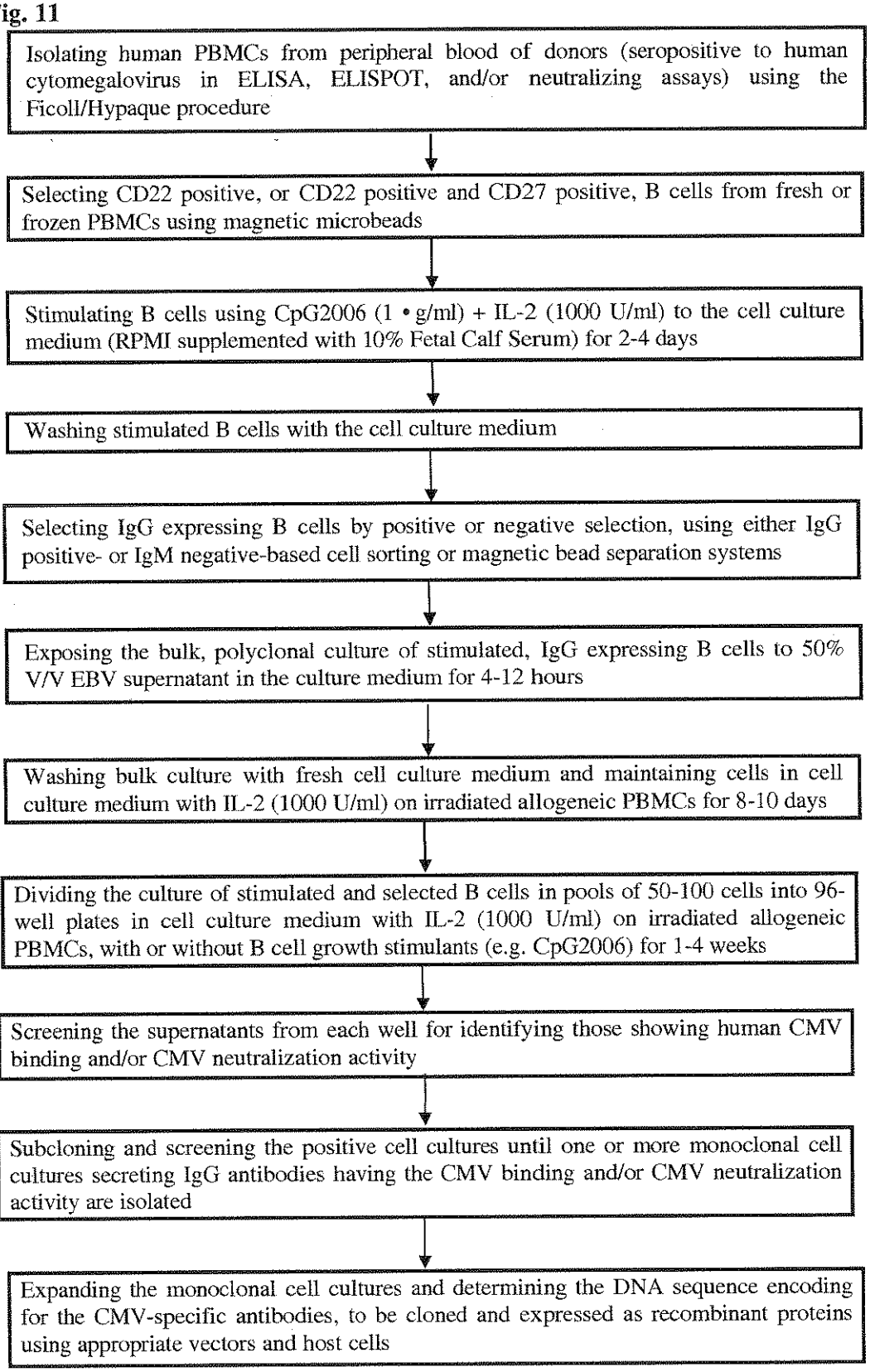
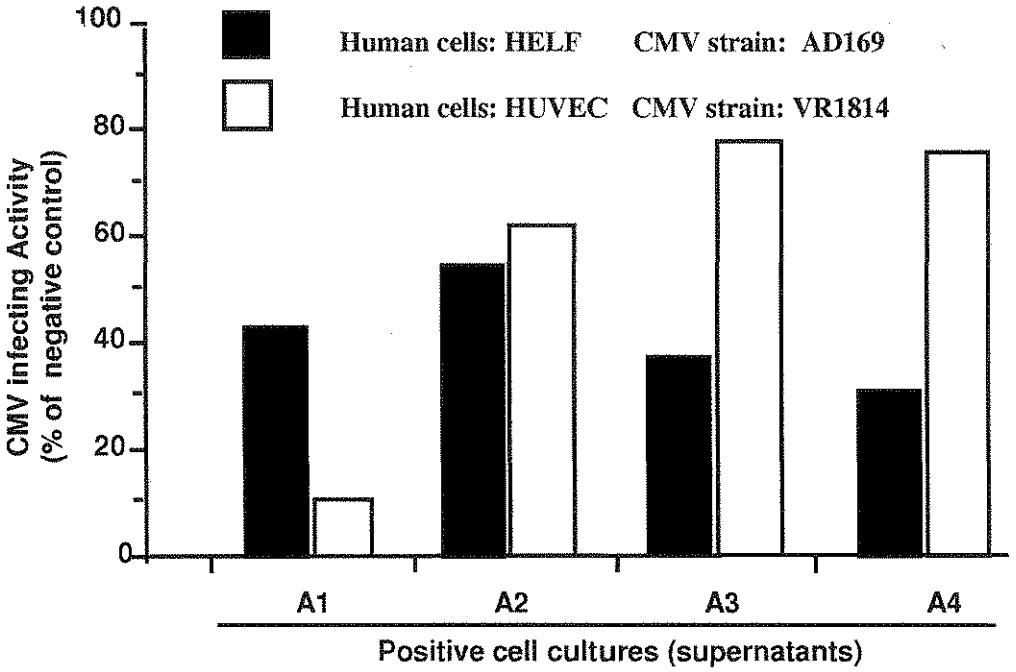

Isolating human PBMCs from peripheral blood of donors (seropositive to human cytomegalovirus in ELISA, ELISPOT, and/or neutralizing assays) using the Ficoll/Hypaque procedure
Selecting CD22 positive, or CD22 positive and CD27 positive, B cells from fresh or frozen PBMCs using magnetic microbeads
Stimulating B cells using CpG2006 (1 • g/ml) + IL-2 (1000 U/ml) to the cell culture medium (RPMI supplemented with 10% Fetal Calf Serum) for 2-4 days
Washing stimulated B cells with the cell culture medium
Selecting IgG expressing B cells by positive or negative selection, using either IgG positive- or IgM negative-based cell sorting or magnetic bead separation systems
Exposing the bulk, polyclonal culture of stimulated, IgG expressing B cells to 50% V/V EBV supernatant in the culture medium for 4-12 hours
Washing bulk culture with fresh cell culture medium and maintaining cells in cell culture medium with IL-2 (1000 U/ml) on irradiated allogeneic PBMCs for 8-10 days
Dividing the culture of stimulated and selected B cells in pools of 50-100 cells into 96-well plates in cell culture medium with IL-2 (1000 U/ml) on irradiated allogeneic PBMCs, with or without B cell growth stimulants (e.g. CpG2006) for 1-4 weeks
Screening the supernatants from each well for identifying those showing human CMV binding and/or CMV neutralization activity
Subcloning and screening the positive cell cultures until one or more monoclonal cell cultures secreting IgG antibodies having the CMV binding and/or CMV neutralization activity are isolated
Expanding the monoclonal cell cultures and determining the DNA sequence encoding for the CMV-specific antibodies, to be cloned and expressed as recombinant proteins using appropriate vectors and host cells

Fig. 12

A)


Human cells: HELF CMV strain: AD169
Human cells: HUVEC CMV strain: VR1814
CMV infecting Activity (% of negative control)
100
80
60
40
20
0
A1
A2
A3
A4
Positive cell cultures (supernatants)

B)

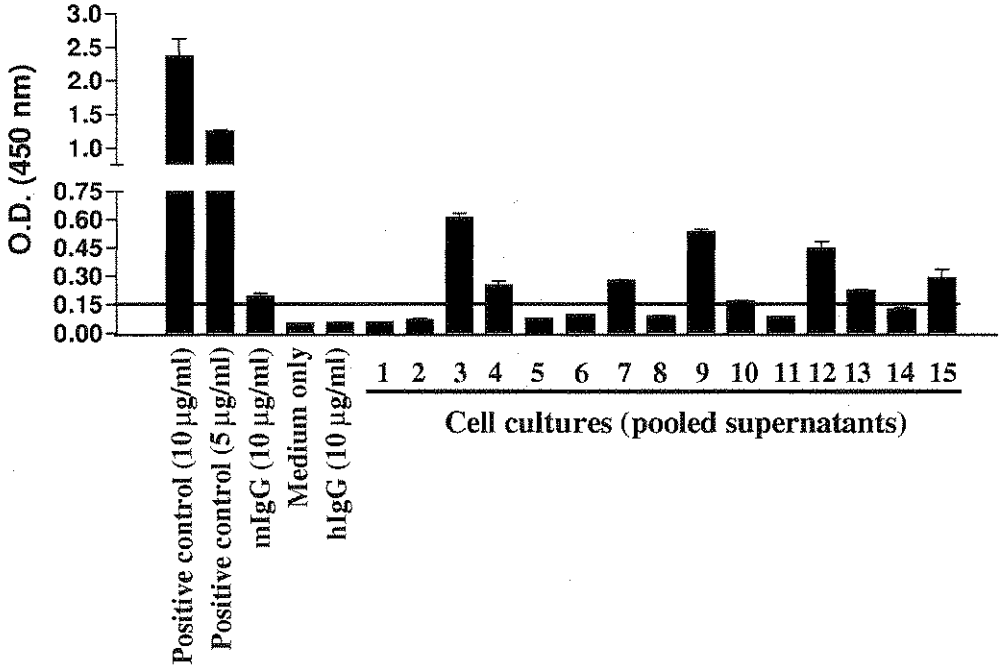

O.D. (450 nm)
3.0
2.5
2.0
1.5
1.0
0.75
0.60
0.45
0.30
0.15
0.00
Positive control (10 µg/ml)
Positive control (5 µg/ml)
mIgG (10 µg/ml)
Medium only
hIgG (10 µg/ml)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Cell cultures (pooled supernatants)

Fig. 13

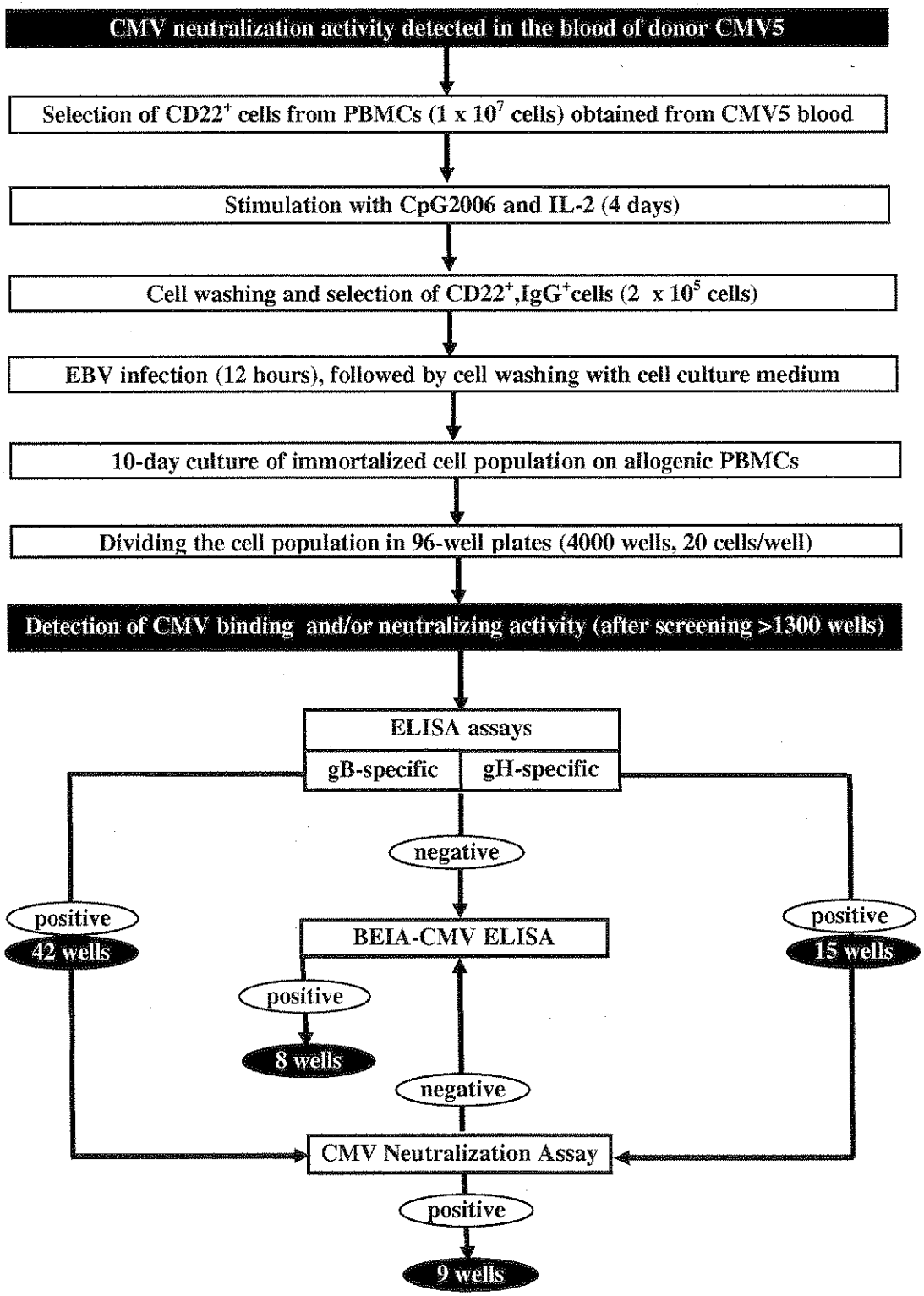

CMV neutralization activity detected in the blood of donor CMV5
Selection of CD22+ cells from PBMCs (1 x 10^7 cells) obtained from CMV5 blood
Stimulation with CpG2006 and IL-2 (4 days)
Cell washing and selection of CD22+,IgG+cells (2 x 10^5 cells)
EBV infection (12 hours), followed by cell washing with cell culture medium
10-day culture of immortalized cell population on allogenic PBMCs
Dividing the cell population in 96-well plates (4000 wells, 20 cells/well)
Detection of CMV binding and/or neutralizing activity (after screening >1300 wells)
ELISA assays
gB-specific
gH-specific
negative
positive
42 wells
BEIA-CMV ELISA
positive
8 wells
positive
15 wells
negative
CMV Neutralization Assay
positive
9 wells

Fig. 14

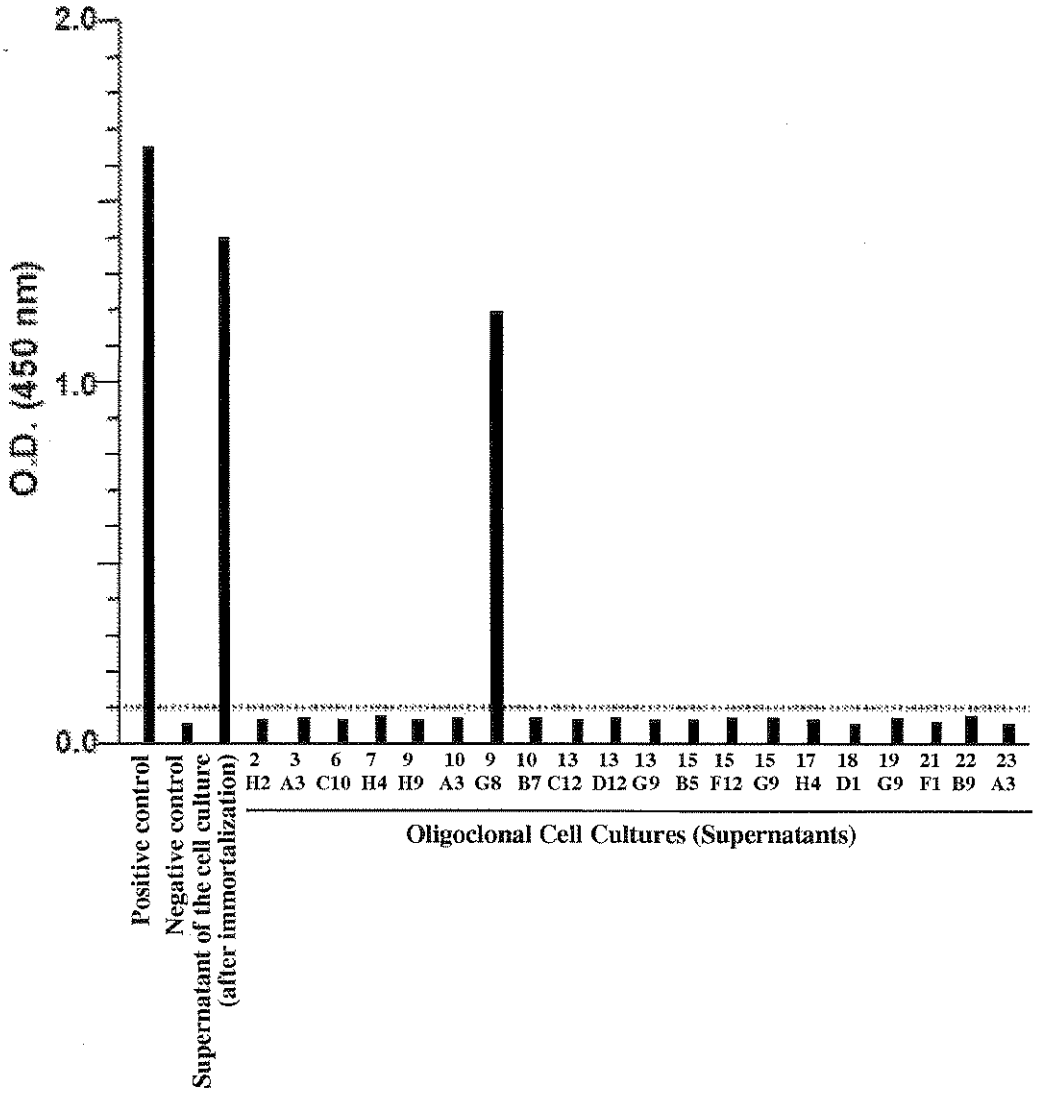

2.0
1.0
0.0
O.D. (450 nm)
Positive control
Negative control
Supernatant of the cell culture (after immortalization)
2 H2
3 A3
6 C10
7 H4
9 H9
10 A3
9 G8
10 B7
13 C12
13 D12
13 G9
15 B5
15 F12
15 G9
17 H4
18 D1
19 G9
21 F1
22 B9
23 A3
Oligoclonal Cell Cultures (Supernatants)

Fig. 15

A)

```
                10        20        30        40        50        60
        .........|.........|.........|.........|.........|.........|
HC 9G8  MGSTAILALLLAVLQGVCAEVQLVQSGAEVKKPGESLKISCKGSGYTFDSYWIGWVRQMP
                                                      HCDR1

                70        80        90       100       110       120
        .........|.........|.........|.........|.........|.........|
HC 9G8  GKGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTASLQWSSLRASDTAMYYCARHTY
                     HCDR2                                      HCDR3

               130       140       150       160       170       180
        .........|.........|.........|.........|.........|.........|
HC 9G8  PGPNSGYDYFEYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF
```

B)

```
                10        20        30        40        50        60
        .........|.........|.........|.........|.........|.........|
VL 9G8  FLLLLWLPDTTGEIVLTQSPATLSLSPGERVTLSCRASQSVYNYLAWYQQKPGQAPRLLI
                                              LCDR1

                70        80        90       100       110       120
        .........|.........|.........|.........|.........|.........|
VL 9G8  YDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQLRRGTFGQGTKVEIKRTVA
          LCDR2                                       LCDR3

               130       140       150
        .........|.........|.........|
VL 9G8  APSVFIFPPSDEQLKSGTASV
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SEQUENCE LISTING

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<120> METHODS FOR OBTAINING IMMORTALIZED ANTIBODY-SECRETING CELLS

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35 40 45

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50 55 60

Glu Trp Met Gly Ile Ile Tyr Pro Gly Asp Ser Asp Thr Arg Tyr Ser
65 70 75 80

Pro Ser Phe Gln Gly Gln Val Thr Ile Ser Ala Asp Lys Ser Ile Ser
85 90 95

Thr Ala Ser Leu Gln Trp Ser Ser Leu Arg Ala Ser Asp Thr Ala Met
100 105 110

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