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FINAL REPORT
February 1993-June 1994

PROJECT CRP/CUB91-01: "Modification of Therapeutic Monoclonal Antibodies by Recombinant DNA Techniques"

1. INTRODUCTION

As described in the Project outline, and our first Progress Report (February 1992-February 1993), the main objective of this work was to generate a mouse/human chimeric antibody specific for the CD6 leukocyte antigen. The mouse anti-CD6 monoclonal antibody (MAb) IOR-T1 (García et al. 1992), shown in pilot human studies to have potential usefulness in the treatment of T cell lymphomas (García et al. 1990), was taken as starting point.

1.1. Strategy.-

To achieve such objective, the following strategy was designed:

1.1.1. First year.-
(a) To amplify by PCR and sequence the IOR-T1 heavy (VH) and light (VL) chain variable region genes.
(b) To clone the IOR-T1 VH into an expression vector bearing the human gamma 1 domains, a mouse Ig promoter, the human Ig enhancer and selective markers.
(c) To select heavy chain heavy-chain mutant (HC) hybridoma lines, isolated from the IOR-T1 hybridoma by extensive cloning, transfect them with the chimeric heavy chain gene, and identify transfectomas.

These three tasks were accomplished as planned in the first year of the project, and were detailed in our First Progress Report.

1.1.2. Second year.-
(d) To clone the IOR-T1 VH and VL genes into expression vectors bearing the human gamma 1, or the human Ck domains, respectively. To transfect both vectors into host myeloma cells, and screen for mouse/human specific chimeric antibodies.
(e) To construct with the IOR-T1 VH and VL genes chimeric mouse/human heavy and light chain inserts, and subclone into a set of expression vectors bearing different

promoter, transcription, selection and amplification genetic elements. To transfect these vectors into host CHO and myeloma cells, and screen for mouse/human specific chimeric antibodies.
(f) To characterize the chimeric IOR-T1 antibodies in terms of effector functions (ADCC and CDC), with respect to the original mMAb.

1.2. Networking.-

From the point of view of networking and collaborative links, tasks (a), (b), (c), and (d) would be carried out in the Center for Genetic Engineering and Biotechnology (CGEBT) of Havana. Task (e) would be developed by a Cuban scientist from the CGEBT in Dr. Oscar Burrone's laboratory at the International Center for Genetic Engineering and Biotechnology (ICGEBT, Trieste). Task (f) would be developed in collaboration with Dr. Carlos García, head of the Immunology Group of the Cuban Institute of Oncology and Radiobiology, and one of the original co-developers of the IOR-T1 murine mAb (García et al. 1990).

Apart from the networking scheme envisaged for tasks (a)-(e) (see 2.1 for detailed information), new links were established in 1993 with Prof. Fabio Malavasi, from the University of Torino, who has done some collateral work in the characterization of the murine IOR-T1. Networking with Dr. Carlos García (task f) is still pending, as the chimeric antibodies for this study will be delivered in the second half of 1994 to his laboratory (see below).

2. DEVELOPMENT OF THE PROJECT

2.1. Administrative and Planning Aspects.-

2.1.1. Our First Progress Report was approved by Vienna in February 1993, and the final pending amount of USD 9,000.00 was received. Orders for expendables were placed, including plastic cultureware, reagents, and other laboratory miscellaneous items. Everything arrived in time and was used for the project.

2.1.2. Dr. Jorge V. Gavilondo, the project leader, visited Dr. Burrone's laboratory at the ICGEBT for a week in May 1992, and two days in September 1993. In both occasions Dr. Gavilondo delivered seminars for the staff of the ICGEBT, describing the structure

and projects of the Division of Immunotechnology and Diagnostics of Havana's CGEBT, and discussing in detail the results obtained by the Division concerning the modification of antibodies by genetic engineering.

Dr. Burrone visited Havana in September 1993, as part of the teaching staff of the International Course "Modification of Antibodies by Genetic Engineering: Antibody Fragments in Bacteria" sponsored by the ICGEBT and the Latinamerican Biotechnology Program of UNESCO/UNIDO, and organized by Dr. Gavilondo. Dr. Burrone delivered a lecture on the subject of chimeric antibodies expressed in mammalian cells, and made reference to the collaborative work in course between Havana and Trieste.

Two final actions involving the head counterparts will take place in 1994:
(a) Dr. Gavilondo will visit Trieste in September this year, in order to check the development of the project, examine new collaborative possibilities, and supervise the work to be done in Trieste until late 1994 by Dr. Alicia Pedraza (see below).
(b) Dr. Burrone will attend the International Meeting "Biotecnología Habana'94" as invited speaker for the Symposium "Immunotechnology and Diagnostics".

2.1.3. Dr. Alicia Pedraza was awarded a two-year fellowship by the ICGEBT, and travelled to Dr. Burrone's laboratory in January 1993. She has been working in task (e), and will prepare and deliver samples of the chimeric IOR-T1 for the development of task (f) before leaving Trieste (due beginning of 1995).

2.2. Scientific Aspects.-

The work developed during the period February 1993-June 1994 is described below in detail, followed by general comments. Details of the work performed during the period February 1992-February 1993 were included in our first Progress Report.

(A) Expression of the chimeric mouse/human IOR-T1 using the pAH4604 V_H and pAG4622 V_L vectors.-

INTRODUCTION

As planned, the work at the CGEBT of Havana was directed to the expression of the chimeric mouse/human IOR-T1 using the vector system provided by S.L. Morrison

(Coloma et al. 1992). These vectors were specifically designed for easy cloning of PCR-derived mouse immunoglobulin V-regions, and the secretion of mouse/human gamma1/Ck chimeric antibodies. The mouse V-regions to be cloned have to include the original leader sequences. The vectors bear mouse immunoglobulin promoters and human immunoglobulin enhancers, and are to be used exclusively with myeloma cells as expression hosts. The vector pAG4235, used by us during the first year of work for the expression of the chimeric heavy chain gene (see our first Progress Report), was changed now to the pAH4604, that replaces the selective marker from mycophenolic acid to histidinol. In every other aspect this vector is identical to pAG4235.

MATERIALS AND METHODS

Hybridoma.
The mouse hybridoma cell line IOR-T1 (García et al. 1992) was supplied by the Institute of Oncology and Radiobiology of Havana. Cells were grown in RPMI1640 with 10% newborn bovine serum supplemented with 2 mM glutamine, 50 µM 2-mercaptoethanol, 0.48 mM sodium pyruvate, 0.17 µM bovine insulin, 1.3 mM cis-oxaloacetic acid, and antibiotics.

RNA and cDNA synthesis.
RNA was extracted from 10^5 hybridoma cells, and first strand DNA synthesis performed using oligo $(dT)_{15}$ as primer, and AMV reverse transcriptase, for 60 minutes at 42°C, as described previously (Gavilondo et al. 1990).

Oligonucleotide primer design.
PCR primers were designed using the database of Kabat et al.(1991), and sequences available from GenBank™. These primers were: GCGGGATATCCACCATG(AG)A(CG)TT (CG)(TG)GG(CT)T(AC)A(AG)CT(TG)G(AG)TT and GGGCGCTAGCTGAGGAGACTGTGAGAGTGGTGCC for VH, and GGGGATATCCACCATG(GT)CCCC (AT)(GA)CTCAG(CT)T(CT)CT(TG) and AGCGTCGACTTACGTTT(TG)ATTTCCA(GA)CTT(GT)GTCCC for VL. The primers annealed with the mouse heavy and light chain 5' leader sequences, and the 3' end of framework 4 (FR4) regions. All primers had adequate restriction sites for cloning in Bluescript, and in the expression vectors. Oligonucleotides were made on a Gene Assembler Plus DNA synthesizer (Pharmacia-LKB), with no purification prior to use.

PCR.
Eighty µl of PCR mix will be added to the 10 µl of first strand cDNA. The PCR mix

was made following the instructions of the Perkin-Elmer Cetus PCR kit. Five µl of each primer were added to give a final primer concentration of 1 µM and the mixture subjected to PCR amplification using a Hybaid thermal cycler set, for 30 cycles. The temperatures and times for PCR were: melting at 94°C, 1 minute; primer annealing at 55°C, 1 minute; primer extension at 72°C, 1 minute. One minute ramp times were used between these temperatures. Ethidium bromide stained 2% agarose gels were used to visualize PCR fragments.

Sequencing.

The PCR bands were isolated using NA45 nitrocellulose paper, digested with EcoRV and NheI (VH), or EcoRV and SalI (VL), purified using low melting point agarose, and cloned into digested Bluescript modified vectors, provided by Dr. Sherie L. Morrison (University of California, Los Angeles, UCLA). Positive colonies were identified by hybridization (Sambrook et al. 1989) with radioactive primers. Dideoxynucleotide chain termination sequencing was carried out using the Sequenase 2.0 kit from USB, according to the manufacturer's protocol, with ^{35}S-αATP.

Expression vectors.

VH and VL were cloned into the expression vectors pAH4604 and pAG4622 (Coloma et al. 1992, Figure 1), respectively. The pAH4604 vector has the human gamma 1 constant region, uses histidinol as selective marker for eucaryotes, and has EcoRV and NheI as cloning sites for PCR-isolated VH regions. The pAG4622 vector has the human kappa constant region, uses mycophenolic acid as selective marker for eukaryotes, and has EcoRV and SalI cloning sites for PCR-isolated VL regions. Both vectors are designed to be used with myeloma cells as hosts. DNA from the Bluescript plasmids carrying the coding sequence for the IOR-T1 VH and VL was prepared, digested with appropriate restriction enzymes, purified, and cloned into the pAH4604 and pAG4622 vectors, respectively. Positive bacterial colonies were identified using radioactive hybridization procedures, and vector DNA checked by size and restriction analysis.

Transfection.

Sp2/0-Ag14 and P3/x63-Ag8-653 cells were used for transfection. The cells were maintained in RPMI 1640, with 10% fetal calf serum, and antibiotics. Vector DNA of

both constructions (10-20 μg) was prepared by conventional methods (Sambrook et al. 1989), linearized, mixed, and the cells transfected by electroporation (220 V, 950 μF), as reported (Coloma et al. 1992). The electroporated cells were plated in 96-well plates and selection started after 24 hours, using the maintenance medium, plus 5 mM histidinol, and endothelial cell supernatant, as growth factor mixture. After 10-14 days, the supernatants of resistant cultures were screened for the presence of human immunoglobulin heavy and light chain constant regions (see below). Selected cultures were transferred to 24-well plates, grown, and retested for human Ig production. Positive cultures were finally cloned by limiting dilution.

ELISA for human gamma immunoglobulins.

The culture supernatants were screened with a "sandwich" ELISA highly sensitive and specific for human gamma immunoglobulins. Nunc EIA plates were coated with rabbit anti-human gamma chains (DAKO). Bound hIgG was detected using peroxidase-conjugated anti human kappa chains immunoglobulins (DAKO). Purified hIgG was used as standard.

Indirect immunofluorescence.

The Hut78 $CD6^+$ human leukemia culture cells were grown in suspension culture, washed with PBS, and incubated at 4°C with 20x concentrated culture supernatant of a clone secreting chimeric antibodies. Purified IOR-T1 was used as positive control, and metabolized, antibody-free culture medium as negative control. Incubations were done at 4°C for 30 minutes. FITC-conjugated anti-human rabbit antibodies were used to develop the reaction.

RESULTS

Sequences of variable region genes of IOR-T1..

The sequence for VL, from leader to FR', was elucidated (see Figure 2). The VH sequence had been included already in our first Progress Report, and is depicted again in Figure 3). A comparison with the VL and VH sequences of IOR-T1 derived independently in Trieste, had 100% coincidence with these (see "B").

Transfectomas and expression.

Twelve different electroporation experiments, using two different host cell lines, were performed. Histidinol-resistant cultures were obtained in all instances, but only 12/876 cultures (wells) were shown by ELISA to secrete assembled human immunoglobulin constant heavy and light chains. Changes in host cell from P3/x63-Ag8-653 to Sp2/0-Ag14 brought forth, under similar transfection conditions, an increase in drug resistant cultures (35 to 67%) indicating that, for reasons not determined, the latter cell line apparently behaved better during this process. Most transfection experiments (8/12) were thus conducted with the Sp2/0-Ag14 as host. Of the total 12 cultures showing some level of human immunoglobulin secretion, only 5 were found stable for this property after cloning. All of these secreted very low amounts of chimeric immunoglobulin (i.e. < 500 ng/10^6 cells/day), probably due to single copy integrations.

Specificity.

Indirect immunofluorescence was used to show specificty. Hut78 human leukemia cells were positively identified by the chimeric antibodies secreted by one of the aforementioned clones (denominated MF-67).

(B) Expression of the chimeric IOR-T1 using the pCMV2 and pCBk vectors.-

INTRODUCTION

To complement the work performed in Havana, different vectors, and hosts cells (CHO cells), were used in experiments conducted in Trieste by the CGEBT scientist. CHO cells are particularly interesting for the production of recombinant proteins, due to the possibility of scaling up production using serum-free medium (Newman et al. 1992; Reff 1993). The expression cassettes (i.e. leader/murine V-region/human constant regions) were assembled in a series of intermediate vectors of pUC origin, to be finally transferred to the expression pCMV2 or pCBk vectors. This meant that the leader sequences used were not the original ones, and the IOR-T1 V-regions were essentially "PCRrd" from FR1 to FR4. The vectors used also the CMV promoter, vs. the mouse immunoglobulin employed in the vectors reported in (A). Finally, in the case of several pCMV2 vector constructions, the use of an amplifiable selection marker (DHFR),

opened the possibility of selecting transfectomas with multiple gene copies, and potential higher antibody production.

MATERIALS AND METHODS

Hybridoma.
The mouse hybridoma cell line IOR-T1 (García et al. 1992) was supplied by the Institute of Oncology and Radiobiology of Havana. Cells were grown in RPMI1640 medium with 10% fetal calf serum, and antibiotics.

RNA and cDNA synthesis.
RNA was extracted with the technique by Gough (1988); 10^7 hybridoma cells were used as starting material. First strand DNA synthesis was performed using the Boehringer-Mannheim cDNA synthesis kit. RNA was resuspended in 10 µl of DEPC-treated water and heated at 65°C for 5-10 min. For reverse transcription of mRNA, 18 µl of RT reaction mix: [1µl MMLV reverse transcriptase (50 U/µl), 1µl RNasin (20 U/µl), 2 µl 10x PCR buffer II (250 mM Tris-HCl, pH 8.3, 300 mM KCl and 50 mM DTT), 4 µl MgCl2 solution (25 mM), 1µl oligo dT 16-mer (20 µM) and 2 µl of 10 mM dNTP] was added to 2 µl of RNA and incubated for 60 min at 42°C.

Oligonucleotide primer design.
A first set of oligonucleotide primers was designed for PCR amplification of the VH and VL, from signal peptide to constant region, using the data base of Kabat et al. (1991). These were: AGGT(CG)(AC)A(AG)CTGCAG(CG)AGTC(AT)GG and GGCCAGTGGATAGAC for VH, and GATATTGTGATGACCCAGTCTCCA and GTTAGATCTCCAGCTTGGTCCC for VL. A second set of oligonucleotide primers were designed taking into account the first aminoacids of frameworks 1 and 4 (FR1 and FR4) of VH and VL with additional bases in the 5' portion, corresponding tc restriction enzyme sites. These primers were: GTGTGCACTCTGAGGTGCAGCTG and TGGTCGACGCTGAGGAGACGGT for VH, and GTGTGCACTC(GT)GATATTGTGATG and GGTGCAGCCACAGTCCGTTTTATTTC for VL.

PCR.
For PCR, 78 µl of PCR Master Mix: [2 µl MgCl2 solution (25 mM), 8 µl 10x PCR buffer II, 65.5 µl sterile distilled water and 0.5 µl AmpliTaq DNA Polymerase (5

U/μl)] were prepared for each DNA. Five μl of each primer were added to give a final concentration of 1 μM. The mixture was subjected to PCR amplification using the Perkin-Elmer thermal cycler set for 25 cycles. The temperatures and times used for PCR were: melting at 94°C, 1 min; primer annealing at 50 or 55°C, 1 min; primer extension at 72°C, 1 or 2 min. Ethidium bromide-stained 1% agarose gels were used to visualize PCR fragments.

Sequencing.
The VH and VL region genes amplified with the first set of oligonucleotide primers were cloned into pUC18 for sequencing (Sambrook et al. 1989).

Intermediate vectors.
The pUC18 plasmids carrying sequence-checked VH and VL were used as templates for new amplifications and modification of these regions, using the second set of oligonucleotide primers, for expression purposes. In the case of the heavy chain, the fragments obtained by PCR were recloned in pUC18 using the SmaI site. The plasmid was then digested with ApaLI (site within variable region) and XbaI (site on pUC18), and the digestion product was cloned in pUT-Sec (pUC18 containing a signal sequence for protein secretion with ApaLI restriction site on 3' end). One of the recombinant plasmids was digested with SalI and the resulting fragment cloned in a version of pUC19 already containing the human constant region of IgG1, keeping the correct reading frame (Figure 4).

In the case of the light chain, the VL obtained after the second amplification was filled in with Klenow and cloned in the EcoR V restriction site on the 5' end of the human constant region kappa gene of pUC/hCk (a version of pUC18), keeping the correct reading frame (Figure 5). The correct assembly and reading frame of all fragments were checked by sequence.

Expression vectors.
Different versions of the pCMV2 vector (ICGEBT, Trieste), carrying the Cytomegalovirus (CMV) promoter, ampicillin resistance, and BGH polyadenilation sites (poly A), were used for expression. The pCBk vector, with Hygromycin B as selective marker, and the BK T antigen, was also employed in some experiments. For

the sake of a better understanding, these different vectors and constructions will be described in detail in "Results".

Transfection.

CHO and Sp2/0-Ag14 cells were used for transfection experiments. CHO cells were grown in alpha MEM medium, and Sp2/0-Ag14 in RPMI 1640, both with 10% fetal bovine serum, supplemented with 2 mM L-glutamine, and 1 mM gentamicin. The cells were harvested from culture flasks and washed twice in PBS (137 mM NaCl, 1.47 mM KH_2PO_4, 6.46 mM Na_2HPO_4, 2.7 mM KCl). CHO cells were suspended at 10^6 cells/500µl in HeBS buffer (20 mM Hepes-NaOH pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) and transferred to an electroporation cuvette to which 50 µg of each plasmid (circular or linear) and 50 µg of sonicated herring sperm DNA, as carrier. The cuvette was placed on ice for 10 minutes. The electric pulse was 960 µF and 290 V. The Sp2/0-Ag14 cells were suspended at 10^7 cells/500 µl in HeBS buffer and transferred to an electroporation cuvette to which 10 µg of each plasmid (circular or linear) was added and the cuvette was placed 10 minutes on ice. The electric pulse was 450 µF and 250 V.

After the pulse the cuvettes were placed on ice for 10 minutes. Cells were diluted in culture medium, and allowed to recover for 48 h prior to selection. Stable antibody-producing cells lines were selected in G418, Methotrexate (MTX) or Hygromycin B (Hyg B), depending on the vector. To determine the stability of relevant transfectomas, cells were cloned at limiting dilution (0.5-1 cells/well), and periodically tested for chimeric antibody production.

ELISA for human gamma immunoglobulins.

Human IgG1 (hIgG1) present in cell supernatants was detected by ELISA. EIA 96 well plates were coated with rabbit anti-human gamma chains (DAKO) (final concentration 0.65µg/ml). Bound hIgG was detected using peroxidase-conjugated anti human kappa chains immunoglobulins (DAKO). Purified hIgG was used as standard.

Indirect immunofluorescence.

The Hut78 human leukemia culture cells, with high cell surface expression of the CD6 antigen, were grown in suspension culture, washed with PBS, and incubated at 4°C

with 100 µl of cutlure supernatant of clone TCCM1. Purified murine IOR-T1 (10 µg/ml) as positive control, and metabolized, antibody free culture medium as negative control, were included in the test. Incubations were done at 4ºC for 30 minutes. A FITC conjugated anti-human immunoglobulins antiserum was used to develop the reaction. Washes with PBS were used between incubations.

Immunoprecipitation.

Hut78 human leukemia cells were washed twice with PBS and labelled with ^{35}S-methionine (100 µCi) during 4 hours at 37ºC and under a 5% CO2-95% air atmosphere. Labelled cells were lysed in 50 mM Tris pH 8.0, 250 mM NaCl, 0.5% NP-40, 1 mM PMSF. Aliquots of the lysates were incubated 1 hour at 4ºC with each sample (100 µl of culture supernatant of the TCCM1 clone, 10 µg of purified murine IOR-T1, and two negative control antibodies). Sepharose-Protein A was added to each sample and incubated at 4ºC for 1 hour.The immunoprecipitates were washed with 5 ml of the same lysis buffer, with and without BSA (1 mg/ml), and loaded into 7.5% polyacrylamide gels, under reducing conditions. Following electrophoresis, the gels were dried and autoradiographed.

RESULTS

Sequencing of variable region genes of IOR-T1.

After the first amplification by PCR, positive colonies were detected by colony hybridization. Plasmid DNA was isolated from these colonies and analyzed by restriction enzyme digestion to determine the size of the fragments cloned in pUC18. Positives clones had 400-500 bp or 500-600 bp inserts for VL and VH respectively. Three of these clones were sequenced for each variable region. Checking the sequences against the Genbank and Kabat databases showed that they coded for complete variable regions. A checkout with the sequences obtained independently in the other networking laboratory (CGEBT, Havana) showed total coincidence. One clone of each variable region was chosen for the second amplification and the construction of chimeric genes, as described in Materials and Methods.

Expression.

pCMV2DtHC and pCMV2delta/LC.-

The chimeric heavy chain gene (Figure 4) was digested and cloned into pCMV2Dt. This vector bears the dihydrofolate reductase (DHFR) gene under SV40 promoter (Simian Virus 40) and SV40 poly A, and a transcription terminator that enhances transcription efficiency of upstream and downstream genes. The resulting construction was denominated pCMV2DtHC (Figure 6a). The chimeric light chain gene (Figure 5) was cloned in another version of the pCMV2 vector (pCMV2delta), without selectable marker nor transcriptional terminator. The resulting construction was denominated pCMV2delta/LC (Figure 6b). This strategy is in line with the fact that heavy chain expression alone is highly toxic to CHO and myeloma cells. Hence, the cotransfection of the heavy and light chain vectors only requires a selectable marker in the heavy chain construction.

The two expression vectors were cotransfected in DHFR$^-$ CHO cells. Stable transfectoma selection began 48 hours after transfection using alpha (-) medium (alpha MEM medium without ribonucleotides) and was changed to alpha (-) medium + 0.1 μM MTX after another 48 hours in half of the dishes. This MTX concentration is 10 times higher than the minimum concentration necessary for the selection of resistant clones in DHFR$^-$ CHO cells. The MTX concentration was increased to 0.4 μM, in the rest of the dishes. The number of MTX resistant clones in 0.4 μM was lower than when we used 0.1 μM, and were not good producers of chimeric antibodies.

The best transfectoma derived from the first transfection at 0.1 μM MTX, denominated TCCM1, produced 4 μg/ml in the first 24 hours and 30 μg/ml at 96 hours, with an initial seeding of 5 x 10^5cells, as determined by ELISA.The MTX concentration was gradually increased from 0.1 μM to 2 μM in two different ways: (a) with 5 days between each change of medium, and (b) conditioning each change of medium to the full recovery of the cells. No noticeable differences were observed among both protocols. We continued increasing the MTX concentration to 4 μM, 8 μM and 16 μM. However, the resistant cells did not produce more antibody that the initial clone.

We also cotransfected the pCMV2DtHC and pCMV2delta/LC vectors in CHO cells. The pCMV2delta/LC was previously modified so that it included a neomycin gene

cassette. 48 hours after transfection the cells were grown in alpha + mem with G418 (500 µg/ml). A large amount of resistant clones were obtained, but only two of them were good producers. The best producer (TICG58) cloned, and the best culture produced less than 1 µg/ml at 96 hours, with an initial seeding of 5 x 10^5cells.

Finally, Sp2/0-Ag14 cells were transfected with the pCMVdelta/LC, and a variant of the pCMV2DtHC heavy chain expression vector. The latter was modified so that the DHFR gene was substituted by a neomycin gene cassette. The selection of resistant clones was done with G418 (500 µg/ml) in RPMI 1640. There were many resistant clones but only 16 were good producers. All clones were inferior to TCCM1.

pCMV2DtHC/LC.-

A vector was constructed with the transcriptional cassettes for the chimeric heavy and light chains in tandem, each of them with CMV promoter and BGH poly A. This vector had the same terminator and DHFR gene as the pCMV2Dt (Figure 7). Resistant clones were selected as described above. The best producer clone was denominated THCM41, that secreted 3.2 µg/ml at 96 hours, with initial seeding of 5 x 10^5 cells. The production behavior of this clone was evidently inferior to TCCM1.

pcBkHC/LC.-

A replicative expression vector, derived from the pRBk vector, containing in tandem the transcriptional cassettes for expression for the chimeric heavy and light chains, under the control of the CMV promoter, and with BGH poly A was prepared. This vector also carries a Hyg B selection cassette and BK T antigen (Figure 8). The plasmid was transfected to CHO and Sp2/0-Ag14 cells. The selection began 48 hours after transfection with 200 µg/ml of Hygromycin B in alpha MEM (+) or RPMI 1640, respectively.

In CHO cells we obtained 40 resistant cultures and 9 good producers (denominated generically TFCH). All had a recombinant antibody production between 8 and 16 µg/ml at 96 hours, with an initial seeding of 5 x 10^5 cells. In the Sp2/0-Ag14 myeloma, we were able to identify many resistant cultures. However, only 8 were producers and the best of these, denominated TJSH43.II, was subcloned. The best subclone did not produce more recombinant antibody than the TFCH clones.

Specificity.

Two types of experiments were conducted in order to test for specificity of the TCCM1 chimeric antibodies: indirect immunofluorescence, and radioimmunoprecipitation. The immunofluorescence showed that the Hut78 human leukemia cells, with high number of cell surface CD6, were positively identified by the chimeric antibody. To further demonstrate that the TCCM1 antibodies were identifying the CD6 antigen, a radioimmunoprecipitation experiment was conducted. The molecular weight of the precipitated antigen (90-130 kDa), corresponds to the one shown to be precipitated by the murine IOR-T1 MAb.

3. FINAL REMARKS AND CONCLUSIONS

The effectiveness and safety of mouse MAbs in human therapeutics (Larrick and Bourla, 1986) can be affected by the fact that these murine proteins induce a human immune response (HAMA) in many patients (Shawler et al., 1985; Schroff et al. 1985). With the availability of new genetic engineering techniques, efforts have been directed to the construction of chimeric mouse/human antibodies in the hope that replacement of all but the murine variable regions or complementarity determining regions (CDRs) with human sequences will reduce the HAMA response (Liu et al., 1987; Steplewski et al., 1988; Sun et al., 1987; Beidler et al., 1988; Riechmann et al., 1988; LoBuglio et al., 1989; Hale et al., 1988; Wright et al. 1992).

IOR-T1 is a mouse monoclonal antibody that recognizes an epitope within the CD6 human leukocyte differentiation antigen (García et al. 1992). This antibody has shown potential as a therapeutic agent for in cutaneous T cell lymphomas in human pilot studies (García et al. 1990), and recently finished its Phase I clinical trial in Havana (scaling up dose to 800 mg/patient), with low collateral toxicity, and several complete remissions lasting for months. The ultimate goal of this project and our efforts to continue is the clinical comparison of this promising therapeutic mouse mAb, and its chimeric mouse/human version. The chimeric version is intended to avoid the HAMA response that already has been seen in more than 50% of the patients treated with IOR-T1, and that seems directed to isotype domains. On the other hand, the chimerization

should potentially enhance the effector functions of the murine antibody, by providing a human Fc fragment.

There is a limit to the amount of protein (including recombinant ones) that a mammalian cell can synthesize and secrete, while still replicating. For immunoglobulins, these levels seem to be around 60 to 100 pg $cell^{-1}$ day^{-1} (Reff 1993). As there is no evidence indicating that a given type of mammalian cell will secrete more immunoglobulin than other, the selection of a host cell line depends more on practical and regulatory aspects related to transfection and production (i.e. the characteristics of the available expression vectors, cell culture capacity, downstream processing, etc.). Expression vectors bearing immunoglobulin promoter/enhancer sequences have to be used with myeloma and myeloma-derived host cells, while those using strong viral promoters, as CMV, can be employed with success both with lymphoid and non-lymphoid cells (i.e. CHO).

From the production point of view, CHO cells are preferred by many, due to their ability to be adapted to grow in serum-free conditions (hence simplifying the downstream purification), and because of the less stringent regulatory aspects for the final product (due to the absence of animal retroviruses in these cells, when compared to murine lymphoid cells). $DHFR^-$ CHO cells (Newman et al. 1992, Wright et al. 1992) provide the adequate substrate to exploit an additional mechanism for the selection of multicopy integration, using plasmids with the DHFR gene, and susceptible to rounds of selection with increased amount of Methotrexate.

In the Project several of these variants were tested: vectors with either immunoglobulin or viral promoters, and lymphoid and non-lymphoid cells as hosts. As shown from our experiments, the best results, in terms of secretion levels of immunolgobulin, and clonal stability, were obtained with $DHFR^-$ CHO cells and the CMV promoter vectors.

If host cell type selection is not necessarily the reason for the best results with CHO cells, versus lymphoid cells, we could explain argue that these are probably caused by the use of CMV promoter vectors that allow the Methotrexate selection for multicopy gene integration. Vectors of this kind have the potential to lead to transfectomas with increased synthesis of the relevant recombinant protein.

The chimeric immunoglobulin production of the TCCM1 clone (i.e. around 40 μg/10^6 cells/day) is within to the levels reported for single copy immunoglobulin gene production of a good hybridoma (20-30 μg/10^6 cells/day). The initial high MTX concentration in the medium (0.1 μM) allows only the survival and growth of the initial transfectoma clones with high plasmid copy number. This is why further increases in the drug brought forth no additional enhancement of secretion.

The lymphoid vectors used in experiments described under (A), while have been shown to work successfully in other several occasions (Coloma et al. 1992; Wright et al. 1993; S.L. Morrison, personal communication), did not produce in our case transfectomas secreting amounts of chimeric immunoglobulins comparable with the viral promoter ones. Because all these methodologies depend upon random integration, position effects have to be taken in mind for initial cell clonal levels. It has been reported that for immunoglobulins the range of initial expression from single-copy integrations can be from 2.5 ng ml^{-1} to 10 μg ml^{-1}. A high initial level could also depend on integration in or close to genome areas that, while still unidentified, are operatively denominated "hot spots". Hence, multicopy integration increases also the probability to "hit" one of the hot spots. In any case, a high number of transfection experiments are still required to be able to select a "good producer".

To the moment of its official ending, the project has produced several transfectoma clones that secrete a mouse/human chimeric antibodies that identify the CD6 antigen in immunofluorescence assays, and that precipitate the CD6 antigen. The work to be continued this year will lead to the testing of such antibodies vs. the murine MAb in a series of in vitro assays that will indicate their potential, with respect to the putatively added human effector functions. This last task of the project will not be accomplished within the official project time due to the fact that several technical factors slightly delayed the production of the needed transfectomas.

However, the project has fulfilled successfully all the initial objectives, technical and scientific. New technologies and procedures have been assimilated by both networking laboratories, strong collaborative links have been created, young scientific personnel has been trained, and, probably the most important, chimeric mouse/human antibodies have been obtained, and will be tested. Some of the results of this work will be

assembled as publications and submitted to international journals this year, and part of it will be presented in the 1994 edition of the International Meeting "Biotecnologia Habana".

As head of the project, I feel very much rewarded by this opportunity of sharing scientific tasks, and by the support received from the ICGEBT of Trieste, my center, in Havana, and co-workers in Cuba and Italy.

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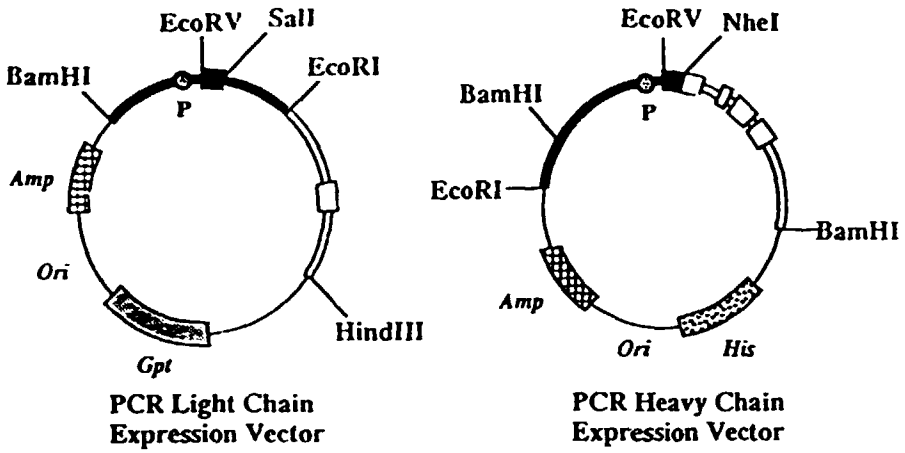

Figure 1 Diagrams of PCR light- and heavy-chain expression vectors. The light-chain expression vector contains the unique sites (EcoRV and SalI) of restriction endonucleases for PCR cloning of the V region gene of the light chain. The heavy-chain expression vector is shown with the unique cloning sites EcoRV and NheI for the PCR-cloned, V-region gene of the heavy chain. The blank boxes represent the human constant-region exons for Cκ and Cγ, and the black boxes the murine variable region exons. The blank thick lines are from the human Ig gene and the black thick lines from the murine Ig gene. "P" designates the promoter; *Amp* is the β-lactamase gene for prokaryotic selection; *ori* is the origin of replication of pBR322; and *gpt* and *his* are the eukaryotic selective markers.

Figure 2. Sequence for the Immunoglobulin Light Chain Variable Region of Monoclonal Antibody IOR-T1 (anti-CD6)

```
-19<------------------------ SIGNAL PEPTIDE ---------------------------- -1
ATG GCC CCA GCT CAG TTT CTT GGG ATC TTG TTG CTC TGG TTT CCA GGT ATC AGA TGT

1<---------------------------------FR1-------------------------------------
GAC ATC AAG ATG ACC CAG TCT CCA TCC TCC ATG TAT GCA TCG CTG GGA GAG AGA GTC
               Thr Gln                                          Gly

------------>23  24<--------------CDR1-------  ----------->34  35<-----------
ACC ATC ACT TGC AAG GCG AGT CGG GAC ATT AGA AGC TAT TTA ACC TGG TAC CAG CAG
            Cys      Ala Ser                                  Trp      Gln

--------------FR2------------------------>49  50<----------CDR2------>56  57
AAA CCA TGG AAA TCT CCT AAG ACC CTG ATC TAT TAT GCA ACA AGC TTG GCA GAT GGG
                                 Leu                                    Gly

<-----------------------------FR3-------------------------------------------
GTC CCG TCA AGA TTC AGT GGC AGT GGA TCT GGG CAA GAT TAT TCT CTC ACC ATC AGC
    Pro      Arg Phe     Gly Ser      Ser Gly                          Ile

--------------FR3---------------------------->88  89<----------CDR3---------
AGC CTG GAG TCT GAC GAT ACA GCA ACT TAT TAC TGT CTA CAA CAT GGT GAG AGT CCA
                    Asp              Tyr      Cys

---->97  98<--------------FR4---------------------->109
TTC ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA AAA CGG GCT
    Thr Phe Gly      Gly Thr Lys Leu Glu      Lys Arg Lys
```

Figure 3. Sequence for the Immunoglobulin Heavy Chain Variable Region of Monoclonal Antibody IOR-T1 (anti-CD6)

```
-19<----------------------SIGNAL PEPTIDE------------------------------ -1
ATG GAC TTC GGG CTC AGC TGG GTT TTC CTT GCC CTC ATT TTA AAA GGT GTC CAG TGT


1<-----------------------------------FR1---------------------------------
GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA
     Leu                                                 Pro

----------------------------------------->30  31<--CDR1------>35  36<-------
CTC TCC TGT GCA GCC TCT GGA TTC AAG TTT AGT AGA TAT GCC ATG TCT TGG GTT CGC
     Cys             Gly                                              Trp

----------FR2---------------------------->51  52<52a-----------CDR2---------
CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT TAC
Gln                  Leu                      Tyr

--------------------------------->65  66<-----------------FR3--------------
ATC TAC TAT CCA GAC AGT GTG AAG GGT CGA TTC ACC ATC TCC AGA GAC AAT GTC AAG
                                 Arg Phe

---------------------------82a-82b-82c-------------------FR3--------------
AAC ACC CTG TAT CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC ACG GCC ATG TAT TAC
                                 Leu          Asp     Ala     Tyr Tyr

--------->94  95<------CDR3---------100a-100b--->102 103<----------FR4------
TGT GCA AGA CGA GAT TAC GAC CTG GAC TAC TTT GAC TCC TGG GGC CAA GGC ACC ACT
Cys                                              Trp Gly     Gly Thr

---------------->113
CTC ACC GTC TCC TCA
    Thr Trp Ser
```

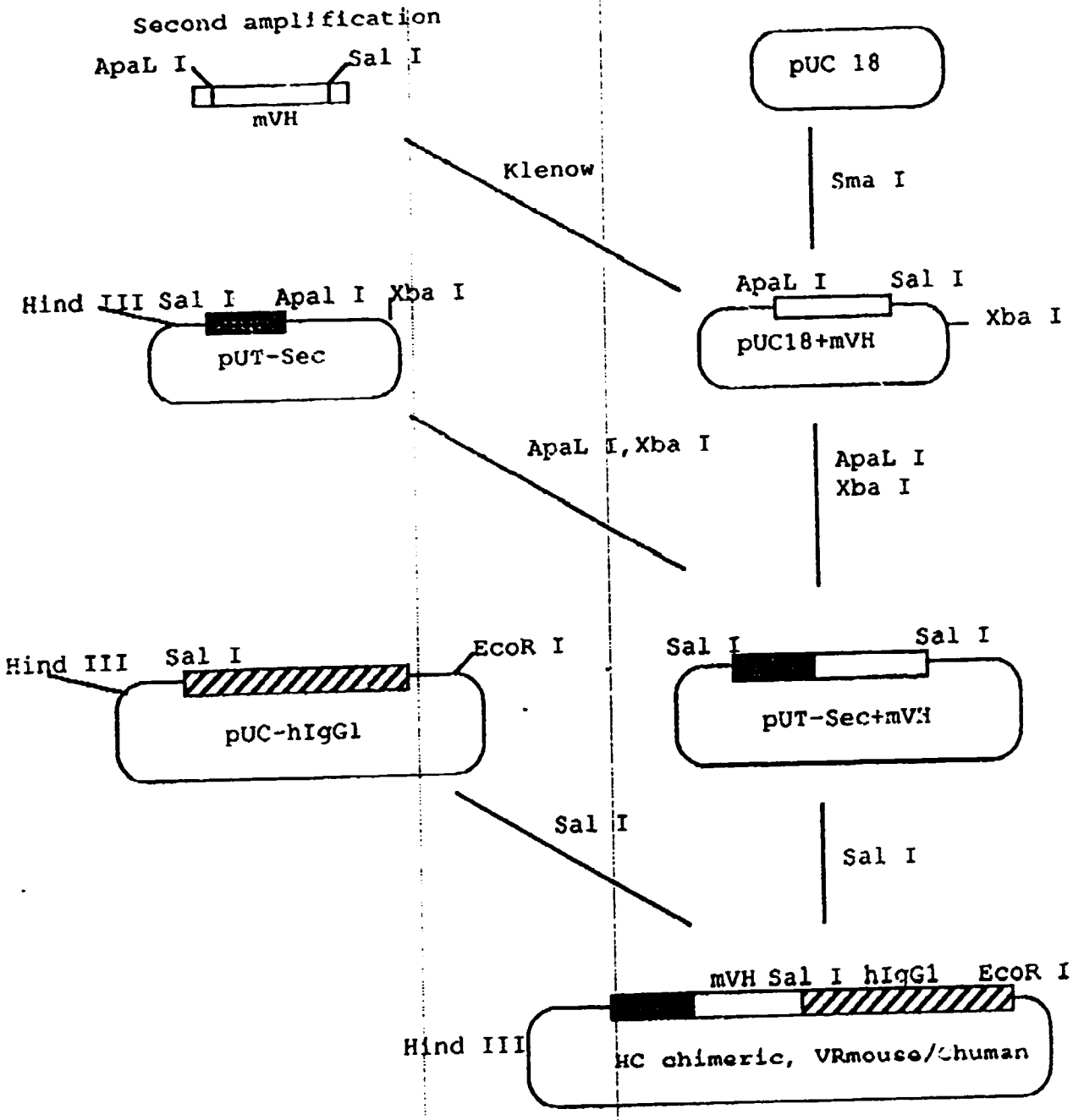
Second amplification
ApaL I
Sal I
mVH
pUC 18
Klenow
Sma I
Hind III Sal I
Apal I
Xba I
pUT-Sec
ApaL I
Sal I
Xba I
pUC18+mVH
ApaL I, Xba I
ApaL I
Xba I
Hind III
Sal I
EcoR I
pUC-hIgG1
Sal I
Sal I
pUT-Sec+mVH
Sal I
Sal I
mVH Sal I hIgG1
EcoR I
Hind III
HC chimeric, VRmouse/Chuman

Figure 4

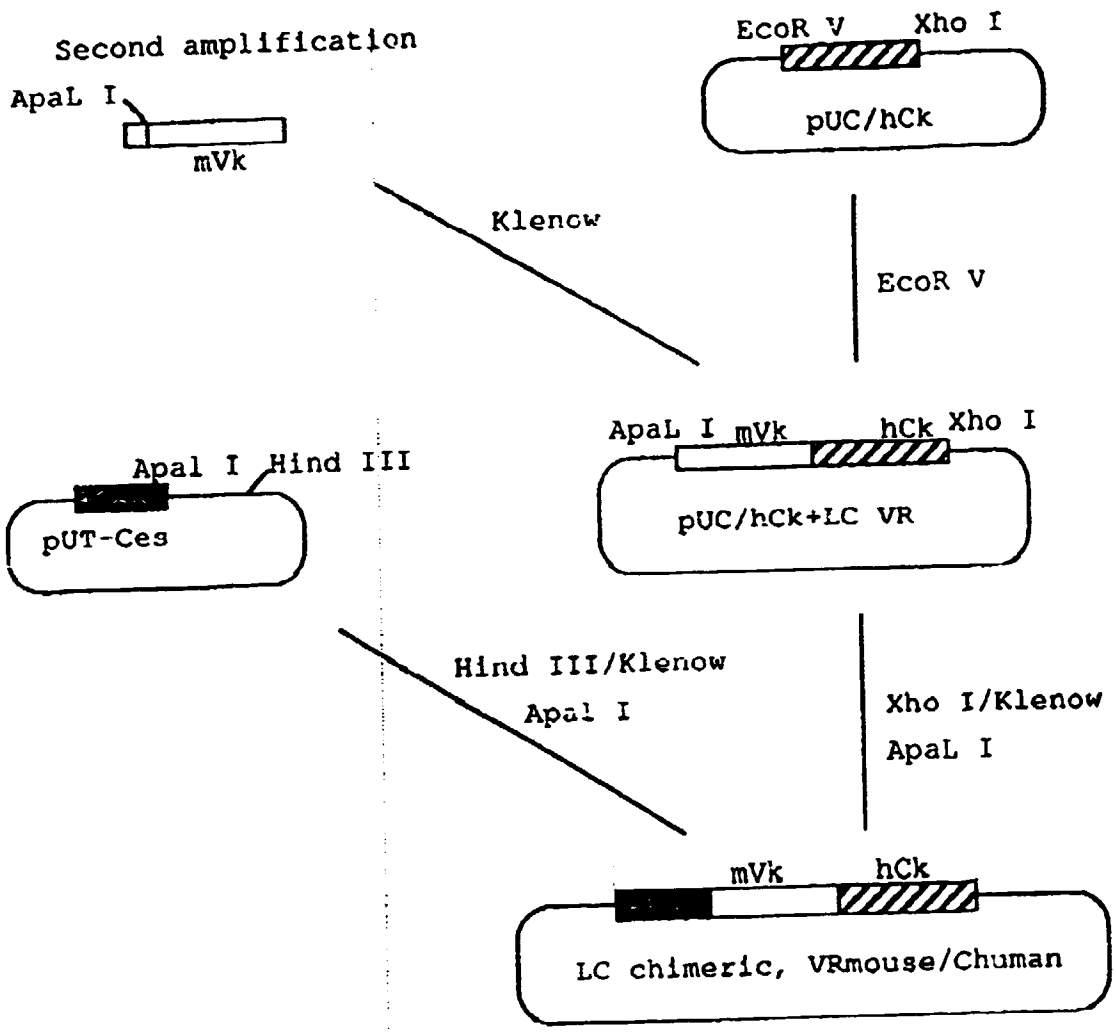
Second amplification
ApaL I
mVk
EcoR V
Xho I
pUC/hCk
Klenow
EcoR V
ApaL I mVk
hCk Xho I
pUC/hCk+LC VR
Apal I
Hind III
pUT-Ces
Hind III/Klenow
Apal I
Xho I/Klenow
ApaL I
mVk
hCk
LC chimeric, VRmouse/Chuman

Figure 5

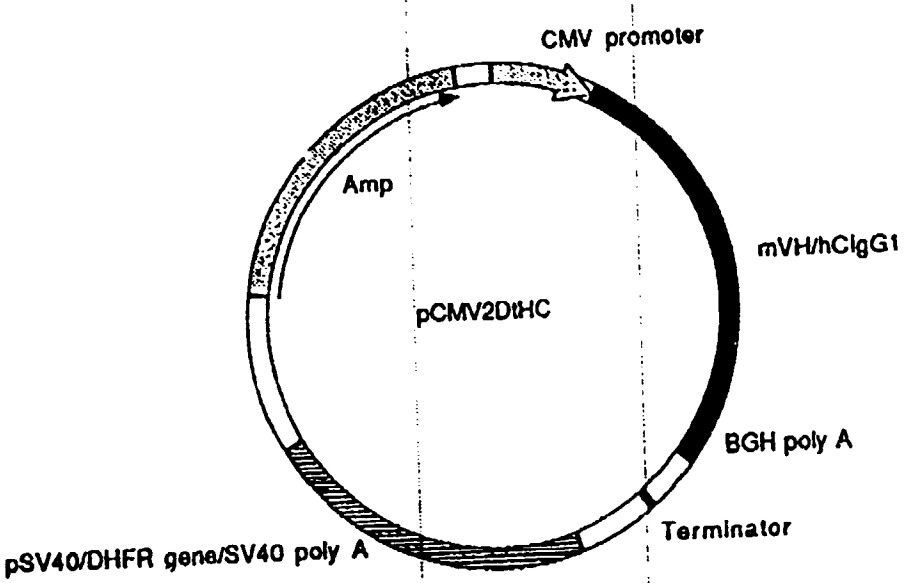

Figure 6a

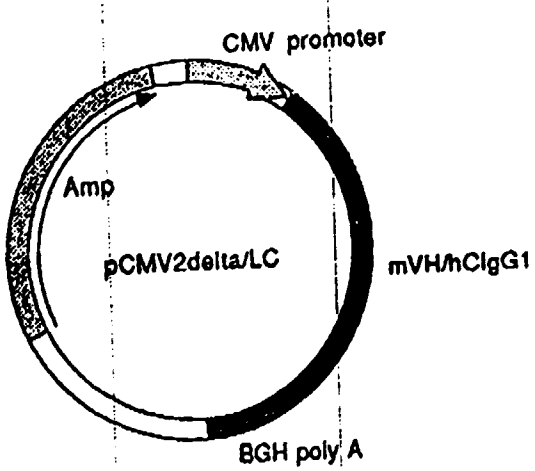

Figure 6b

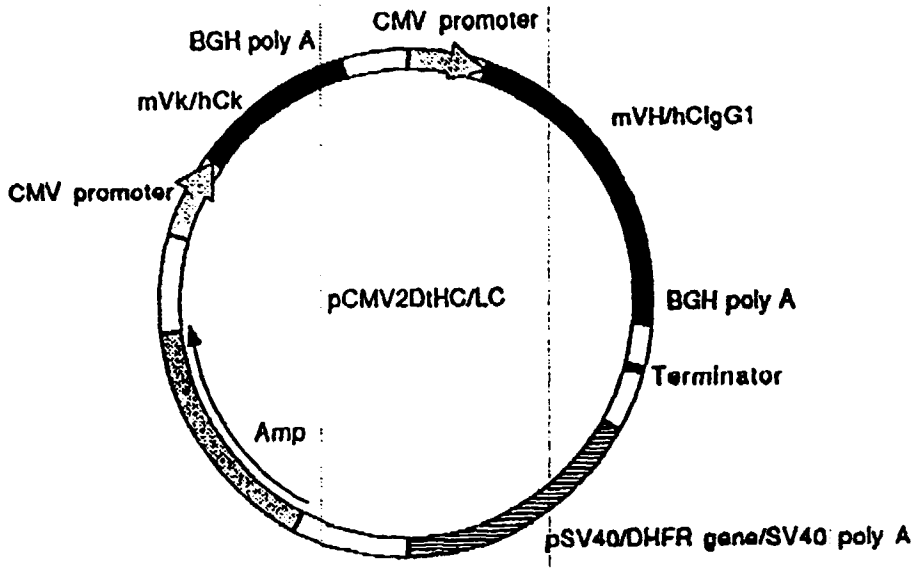
BGH poly A
CMV promoter
mVk/hCk
mVH/hCIgG1
CMV promoter
pCMV2DtHC/LC
BGH poly A
Terminator
Amp
pSV40/DHFR gene/SV40 poly A

Figure 7

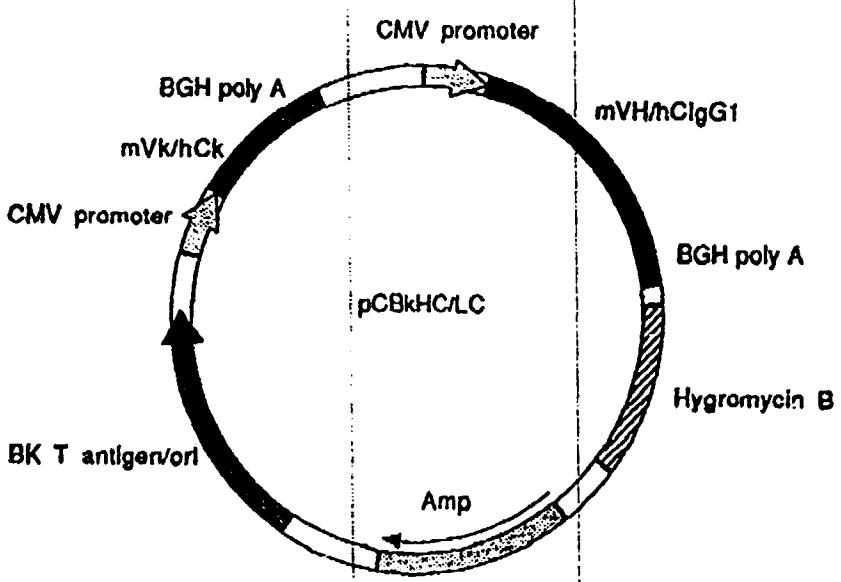
CMV promoter
BGH poly A
mVH/hCIgG1
mVk/hCk
CMV promoter
BGH poly A
pCBkHC/LC
Hygromycin B
BK T antigen/ori
Amp

Figure 8