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BIOTECHNICAL METHODS SECTION (BTS)

A novel approach to identify antigens recognized by CD4 T cells using complement-opsonized bacteria expressing a cDNA library

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In patients with hematological malignancies receiving HLA-matched stem cell transplantation, T cells specific for minor histocompatibility antigens play a major role in graft rejection, induction of graft-versus-host disease and beneficial graft-versus-leukemia reactivity. Several human minor histocompatibility antigens recognized by T cells have been identified, but only two are presented by HLA class II molecules. In search of an efficient approach to identify antigenic peptides processed through the HLA class II pathway, we constructed a cDNA library in bacteria that were induced to express proteins. Bacteria were opsonized with complement to enforce receptor-mediated uptake by Epstein–Barr virus immortalized B cells that were subsequently used as antigen-presenting cells. This approach was validated with an HLA class II-restricted antigen encoded by gene DBY. We were able to identify bacteria expressing DBY diluted into a 300-fold excess of bacteria expressing a nonrelevant gene. Screening of a bacterial library using a DBY-specific CD4 T cell clone resulted in the isolation of several DBY cDNAs. We propose this strategy for a rapid identification of HLA class II-restricted antigenic peptides recognized by CD4 T cells.
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Introduction

Polymorphic proteins encode peptides that can be recognized as minor histocompatibility antigens (mHags) by T cells from HLA-identical individuals.[1–7] Following HLA-matched allogeneic stem cell transplantation, mHags that differ between donor and recipient can elicit allogeneic CD8 and CD4 T cell responses.[8,9] These allogeneic T-cell responses are responsible for graft rejection[10–13] and induction of the detrimental graft-versus-host disease (GVHD).[2,6] Alternatively, donor-derived T cells can mediate a beneficial graft-versus-leukemia or graft-versus-lymphoma reactivity (GVL).[9,14–18] Identification of mHags may lead to cellular therapy protocols using *ex vivo* generated CD4 and CD8 T cells specific for these antigens. It allows also analysis of T-cell responses against these antigens during graft rejection, GVHD and GVL.

Peptides presented by HLA class I molecules are derived from cytosolic or nuclear proteins and most of them are generated by a multicatalytic proteinase complex, the proteasome. The peptides are translocated by the transporter associated with antigen processing into the lumen of the endoplasmic reticulum where they can bind to HLA class I molecules.[19] This HLA class I presentation pathway operates in almost all cell types. Several HLA class I-restricted mHags have been identified using T cells isolated from leukemia patients during clinical response following HLA-matched allogeneic stem cell transplantation. Various methods have been used: acid-elution of antigenic peptides from HLA class I molecules followed by purification and identification by mass spectrometry, screening of peptides predicted in polymorphic candidate proteins, or screening of cells transfected with cDNA libraries or candidate genes that were identified by genetic approaches.[1–4,12,13,20–26]

The methods used to characterize HLA class I-restricted antigens may not be suitable for the identification of HLA class II-restricted antigens. Peptides presented by class II molecules are heterogeneous in length and difficult to purify, because they do not elute in a single peak when fractionated by reversed phase high-performance liquid chromatography. Using linkage analysis, candidate genes have been cloned, transduced in antigen-presenting cells and tested for recognition by CD4 T cells.[27] The selection of candidate genes based on the presence of relevant HLA class II binding peptides is hampered by less accurate predictive methods of class II-binding peptides as compared to class I-binding ones.[28] Screening of cDNA libraries is performed after transfection in recipient cells, which are not professional antigen-presenting cells specialized in taking up exogenous antigens and do not express molecules essential for transport and processing of peptides such as HLA-DM and invariant chain (Ii).[29–31] Using adapted cell lines that were engineered to express HLA-DM, Ii, and the relevant class II molecules, a few tumor-antigens presented by HLA class II molecules on melanoma cells have been identified.[32–35] However, it is not clear whether all endogenously expressed proteins can enter the HLA class II presentation pathway.

We propose here a new sensitive and rapid approach using the exogenous pathway to take up and process proteins encoded by a cDNA library expressed in bacteria. We hypothesized that, after opsonization with complement, recombinant bacteria can be endocytosed via receptor-mediated uptake by Epstein–Barr virus (EBV) immortalized B cells (EBV-LCL) to allow protein processing and presentation. To validate this approach, we made use of an mHag encoded by the human male-specific

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gene DBY.[6,9] A recombinant bacteria library was constructed and screened with a DBY-specific CD4 T cell clone.

Materials and methods

T-cell clone and EBV-lymphoblastoid cell lines (EBV-LCL)

The DBY-specific HLA-DQ5-restricted CD4 T cell clone was derived by limiting dilution from peripheral blood mononuclear cells (PBMCs) of a male patient with chronic myeloid leukemia who developed acute GVH disease grade III–IV after transplantation of HLA genotypically identical female stem cells.[9] The CD4 T cell clone was maintained by stimulation with irradiated allogeneic PBMCs and patient-derived EBV-transformed B cells in Iscoves modified Dulbecco's medium (IMDM) (BioWhittaker, Verviers, Belgium) containing 10% human serum, 3 mM L-glutamine, and 100 U/ml recombinant interleukin-2 (Cetus, Emeryville, CA, USA). EBV-lymphoblastoid cell lines (EBV-LCL) derived from the patient or from the donor were maintained in IMDM containing 10% fetal bovine serum (BioWhittaker). The culture conditions of the MAGE-1-specific HLA-DR15-restricted CD4 T cell clone were previously described.[36]

Cloning of the full-length DBY gene and DBY minigenes

Total RNA was isolated from the male patient-derived EBV-LCL with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's procedure. cDNA was synthesized from RNA using M-MLV BRL reverse transcriptase (Invitrogen) for 60 min at 37°C. One fiftieth of the cDNA reaction was amplified using DBY specific primers; forward 5′-TATATA**CGGCCG**GATGAGT CATGTGGTGGTGAAA-3′ containing an *Eag*I site (bold) and reverse 5′-TATATA**GGCGCGCC**GTTGCCCCACCAGTCAACCC C-3′ containing an *Asc*I site. After amplification, DBY cDNA was visualized on an ethidium bromide stained agarose MP gel (Roche Diagnostics GmbH, Mannheim, Germany). cDNA was isolated from the agarose gel using the gel extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's procedure. Subsequently, DBY cDNA was digested with *Eag*I and *Asc*I, purified and ligated into the pKE-1 vector digested with *Eag*I and *Asc*I. The pKE-1 vector was kindly provided by Davis[37] (Department of Developmental Molecular Genetics, Ben Gurion University of the Negev, Beer Sheva, Israel). Minigenes coding for the DBY/DQ5 epitope (HIENFSDIDMGE) and the DBY epitope extended N-terminally with two amino acids and C-terminally with three amino acids (PPHIENFS-DIDMGEIIM) were prepared by annealing the following oligonucleotides: 5′-**GGCCG**ACATATTGAGAATTTTAGCGA-TATTGACATGGGAGAAA**GG**-3′ and 5′-**CGCGCC**TTTCTCCCA TGTCAATATCGCTAAAATTCTCAATATGT**C**-3′ for the first minigene, 5′-**GGCCG**TCCTCCACATATTGAGAATTTT;AGCGA TATTGACATGGGAGAAATTATCATGG**GG**-3′ and 5′-**CGCGC C**CCATGATAATTTCTCCCATGTCAATATCGCTAAAATTCTCAA TATGTGGAGGA**C**-3′ for the second extended minigene. After annealing, the minigenes were ligated into the pKE-1 vector digested with *Eag*I and *Asc*I. All constructs were checked by sequence analysis as described below before use. *Escherichia coli (E. coli)* XL1-blue were transformed by electroporation with the different recombinant plasmids, and selected with ampicillin (50 μg/ml) and kanamycin (7.5 μg/ml).

Construction of the recombinant E. coli *bacteria library*

A cDNA library was constructed as described by Davis and Benzer[37] using the cDNA synthesis kit from Invitrogen. Polyadenylated mRNA was isolated from the EBV-LCL derived from the patient, followed by single-strand cDNA synthesis using primers with the sequence 5′-GCTCGCCCTCGC**GG CGCGCC**NNNNNT-3′ containing an *Asc*I restriction site. Adaptors were prepared by annealing the oligonucleotides 5′-**GGCCG**AAGGGGTTCG-3′ and 5′-p-CGAACCCCTT**C**-3′. Synthesis of double-stranded cDNA was followed by ligation of adaptors containing an *Eag*I site and digestion with *Asc*I resulting in cDNAs with an *Eag*I site at the 5′ end and an *Asc*I site at the 3′ end, thus permitting directional cloning. After separation of the cDNAs according to size by column chromatography, the different cDNA fractions (A–E) were directionally ligated into the pKE-1 vector digested with *Eag*I and *Asc*I. This pKE-1 expression vector contains an inducible *tac* promoter and in addition to an ampicillin resistance gene, a kanamycin resistance gene. cDNAs inserted in the correct reading frame confer kanamycin resistance to the host, whereas the vector alone or cDNA inserts containing stop codons in the frame translated by the bacteria should not.[37] The ligation products were electroporated into *E. coli* XL1-blue bacteria. The recombinant *E. coli* bacteria representing each fraction of the cDNA library were plated out on large agar plates containing ampicillin (50 μg/ml) and kanamycin (7.5 μg/ml) and, after overnight incubation at 37°C, the bacteria corresponding to each fraction of the library were scraped in LB medium and frozen in LB/20% glycerol at −80°C.

The complement-opsonized bacteria assay

To obtain a standard amount of bacteria encoding cDNAs of interest, the different recombinant bacteria were amplified in deepwell 96-well plates (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) in 100 μl LB medium per well containing ampicillin (50 μg/ml) and kanamycin (7.5 μg/ml), at 37°C with agitation 320 rpm until an OD_{600} of 0.5 was reached. In total, 30 μl from each well were kept as back up and 30 μl LB medium containing isopropyl β-D-thiogalactoside (IPTG; 1 mM) were added to induce protein expression. Incubation was continued for 4 h at 320 rpm and 37°C. Bacteria were opsonized by adding 15 μl of human serum containing 17% (v/v) of complement (Sigma Chemical Co., St Louis, MO, USA) during 1 h at 320 rpm and 37°C. Thereafter, to eliminate the induction medium from the complement-opsonized bacteria, the bacterial cultures were transferred into U-bottom microplates and centrifuged at 2500 rpm for 15 min. The supernatant was discarded and the bacteria were resuspended in 150 μl IMDM/10% FCS with gentamycin (30 μg/ml) (Sigma Chemical Co.) to prevent further bacterial growth. Subsequently, 10 μl of complement-opsonized bacteria from each well were added to EBV-LCL expressing the relevant restriction molecules and plated out in 96-well U-bottom plates (3×10^4 cells/well) in 50 μl IMDM/10% FCS with gentamycin. After overnight incubation to allow uptake and processing of the bacteria expressing the cDNA encoded proteins, the relevant antigen-specific CD4 T cells (3000–5000 cells/well) were added in 100 μl IMDM/10% human serum with IL-2 (100 U/ml) and gentamycin (30 μg/ml) to the EBV-LCL. After coculture for 24 h, supernatants were harvested, and the amount of IFN-γ released by the specific CD4 clone was measured using a standard ELISA (Sanquin, Amsterdam, The Netherlands).

Bacteria expressing MAGE-1

The vector expressing a fusion protein of MBP with MAGE-1 was constructed by ligating a BamHI/ECORI fragment carrying the MAGE-1 coding region isolated from a pGBT9 plasmid into vector pMAL-C2 (New England Biolabs, Herts, UK). The vector was then electroporated in *E. coli* XL1-blue. The stimulation of the anti-MAGE-1 DR15-restricted CD4 clone was estimated by testing the capacity of the coculture supernatant to stimulate the proliferation of M-07e cells, which grow in the presence of any of several growth factors, including GM-CSF, IL-3, IL-6 and IL-15.[38,39] Briefly, the clone (3000 cells/well) was incubated in microwells (100 μl) with EBV-LCL stimulator cells (3×10^4 cells/well) in medium containing IL-2 (25 U/ml). After 24 h, 50 μl of medium were collected and added to M-07e cells (10^4 cells/microwell). After 24 h, [^{3}H]thymidine was added, and thymidine incorporation was measured after another 16 h.

Screening of the recombinant E. coli *bacteria library using the complement-opsonized recombinant bacteria assay*

One of the fractions of the library (fraction C), which contained cDNA inserts of average length, was selected for the screening. To determine the titer of bacteria from a frozen sample, a series of dilutions from the bacteria was performed, and subsequently plated on agar plates. After 18 h, the number of colony-forming units (CFU) growing on the plates were counted. The recombinant bacteria were diluted to obtain pools of $\sim$40 CFU and seeded in deep well 96-well plates at $\sim$40 CFU/well in 100 μl LB medium plus antibiotics. Simultaneously, 2×100 μl of this dilution was plated on agar plates to verify the number of CFU in one pool of bacteria by counting the CFU after 18 h of incubation. A total of 1500 bacterial pools were amplified as described above. From each well, 30 μl were withdrawn and replica transferred into a new microplate containing 100 μl LB medium plus antibiotics and stored at 4°C for future recovery of the antigen-coding cDNA clone from a pool identified as positive. As a positive control, after determination of the titer of bacterial suspensions, we prepared a suspension containing approximately 5 DBY-expressing bacteria and 40 000 bacteria coding for fraction C of the library. This suspension was subsequently divided into 100 pools of around 40 bacteria that were treated according to the procedure described above and screened with the CD4 clone.

DNA sequence analysis

To analyze the cDNA inserts of the positive bacterial colonies found after screening of the library, DNA sequencing was performed using the ABI PRISM® Big Dye™ Terminators v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the primers: forward 5′-CTGGCAAGCCACGTTTGGTG-3′ and reverse 5′-AGACGTTTCCCGTTGAATATGG-3′, corresponding to sequences of the pKE-1 vector.[37] Sequences were determined with an ABI PRISM® 310 Genetic analyzer (Applied Biosystems) and evaluated using the Sequencing Analysis Software™ v3.7 (Applied Biosystems) and compared to Genbank sequences with DNAman version 5.2.9 (Lynnon corporation, Vaudreuil-Dorion, Quebec, Canada).

Results

The choice of the antigen-presenting cells

Our goal was to produce proteins from pools of a bacterial library, to load these proteins on antigen-presenting cells and to test a CD4 clone for its ability to be stimulated by the protein-loaded antigen-presenting cells. As a first step, a MAGE-1 cDNA was cloned into an expression vector. After induction of protein synthesis by IPTG treatment, bacteria expressing MAGE-1 were incubated with different antigen-presenting cells that were subsequently used to stimulate an anti-MAGE-1 CD4 clone.[36] Dendritic cells were efficient antigen-presenting cells but often stimulated nonspecifically the CD4 clone, as we experienced previously with several CD8 T cell clones in contact with dendritic cells and bacterial products. Moreover, large numbers of autologous human dendritic cells are usually not available. In search of alternative antigen-presenting cells, we tested EBV-LCL that express complement receptors CR1 and CR2, allowing uptake of complement-coated antigens.[40,41] Provided that MAGE-1-expressing bacteria were first incubated with human serum and complement, EBV-LCL proved to be very efficient in stimulating specifically the anti-MAGE-1 CD4 clone. Titration experiments enabled us to estimate optimal concentrations of EBV-LCL, bacteria and complement (data not shown). Figure 1 shows an example of such an experiment, where EBV-LCL loaded with recombinant bacteria stimulated efficiently the CD4 clone even when the bacteria expressing MAGE-1 were diluted into a 100-fold excess of bacteria expressing nonrelevant proteins. We determined that EBV-LCL should be loaded with a small quantity of bacteria (Figure 1) and chose 10 μl of bacterial suspension for the screening of the cDNA library. Noteworthy, increasing the amount of bacteria resulted in decreased CD4 responses. This might be due to bacterial

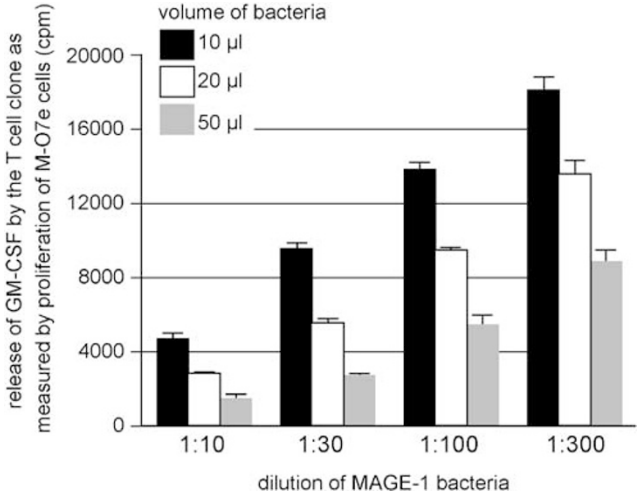

Figure 1 Titration of complement-opsonized bacteria for presentation by EBV-LCL to anti-MAGE-1 CD4 T cells. Recombinant bacteria expressing MAGE-1 were diluted into bacteria expressing nonrelevant proteins. After IPTG treatment, they were opsonized with complement and different amounts of bacteria were loaded on DR15 EBV-LCL. The antigen-presenting cells were cocultured overnight with a MAGE-1 specific DR15-restricted CD4 clone. The stimulation of the CD4 clone was estimated by measuring the release of GM-CSF in the supernatant after an overnight co-culture. The presence of GM-CSF was measured by testing the capacity of the supernatant to stimulate the proliferation of M-07e cells. The results shown represent an average of triplicate co-cultures.

endotoxins. Accordingly, we observed decreased CD4 responses to antigen-positive stimulators in the presence of increasing amounts of bacteria (data not shown).

The choice of the plasmid vector and validation of the approach with a minor histocompatibility antigen

To validate this approach with an mHag, the coding sequence of the male-specific DBY protein was cloned into expression vector pKE-1. We made use of vector pKE-1, where translation in the correct reading frame confers kanamycin resistance to the bacterial host.[37] The use of this type of vector can be very efficient for the construction of a cDNA library, which should produce mostly authentic proteins. Translation of library cDNAs is initiated by prokaryotic signals in the vector. Cloning cDNAs in an expression vector usually yields a majority of clones in either the wrong orientation, the wrong frame, or containing 5′ or 3′ untranslated sequences. In pKE-1, selection with kanamycin selectively eliminates nonrecombinants and clones containing stop codons in the cDNA reading frame translated by the bacteria.

The recombinant bacteria, containing the DBY coding sequence inserted in pKE-1, were amplified with constant agitation until an OD_{600} of 0.5 was reached, treated for 1 h with IPTG to induce the expression of proteins and incubated with human serum and complement to allow opsonization. After washing, the bacteria were incubated in the presence of antibiotics with the female EBV-LCL autologous to an anti-DBY CD4 clone. Importantly, the CD4 clone was not stimulated by female EBV-LCL, whether they were loaded or not with control bacteria, and therefore the female EBV-LCL could be used as antigen-presenting cells for the screening of the library. The CD4 clone secreted similarly high amounts of IFN-γ in response to autologous female EBV-LCL loaded with 10 μl of complement-opsonized bacteria expressing DBY, female EBV-LCL loaded with the DBY-derived peptide, or male EBV-LCL endogenously expressing DBY (Figure 2). Here again, a low amount of bacterial suspension (10 μl) seemed to be optimal.

Screening a library requires testing pools of recombinant bacteria. The size of these pools depends on the sensitivity of the screening procedure with a given clone. Bacteria expressing the DBY protein diluted at 1:300 into irrelevant bacteria could still induce the secretion of IFN-γ by the CD4 clone (Figure 3).

Any library contains truncated cDNAs and, therefore, small protein fragments are produced. During antigen processing by EBV-LCL, this could result in a rapid degradation of small protein fragments and, therefore, in the destruction of the antigenic peptide. We prepared minigenes encoding the DBY antigenic peptide, HIENFSDIDMGE, or an extended version with flanking amino acids, PPHIENFSDIDMGEIIM. Recombinant bacteria expressing either of these peptides were diluted into irrelevant bacteria, treated with IPTG, opsonized with complement, and loaded onto EBV-LCL. Positive bacteria expressing the extended antigenic peptide could still be detected by the CD4 clone at a dilution of 1:1000 (Figure 3). We concluded that this method can be safely used for the screening of bacteria producing small fragments of proteins. Moreover, using minigenes coding for protein fragments could be very useful for localizing the region encoding an antigenic peptide in a positive cDNA identified in a library. The observation that the bacteria expressing the extended DBY peptide were more efficient at stimulating the CD4 clone than bacteria expressing the minimal peptide is in accordance with previous reports, showing that residues flanking HLA class II-presented peptides are involved in their immunogenicity.[42,43]

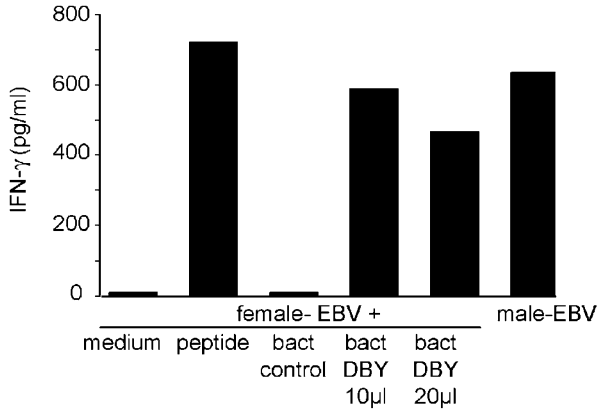

Figure 2 Recognition of EBV-LCL loaded with DBY-expressing bacteria by the DBY-specific CD4 clone. DBY-specific DQ5-restricted CD4 T cells (3000/well) were cocultured with antigen-presenting cells (30 000/well). The amount of IFN-γ secreted in the supernatant of the coculture was measured using a standard ELISA. The antigen-presenting cells were: autologous female-derived EBV-LCL, pulsed or not with peptide or loaded with complement-opsonized bacteria, which expressed either DBY or MAGE-3 as a negative control. The male-derived EBV-LCL were used as positive control. The results shown represent an average of triplicate co-cultures. Standard deviations were always lower than 10% of the average.

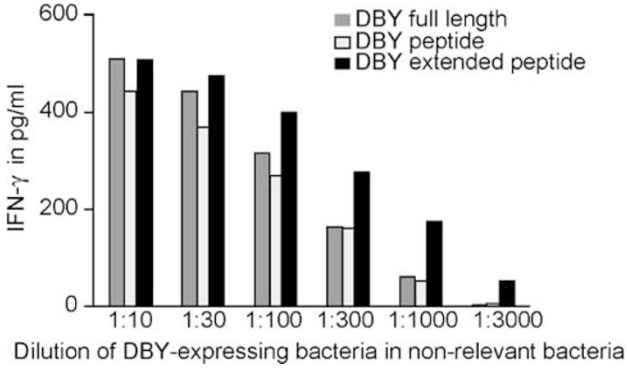

Figure 3 Titration of recombinant bacteria expressing the DBY antigen into nonrelevant bacteria. Recombinant bacteria contained either the full-length DBY cDNA or a cDNA encoding the antigenic peptide HIENFSDIDMGE or an extended peptide PP**HIENFSDIDMGE**IIM. The bacteria were titrated up to 1:3000 into nonrelevant bacteria containing a MAGE-3 coding sequence. After induction of protein expression by IPTG and opsonization with complement, the bacteria were added to female-derived EBV-LCL to allow antigen uptake and processing. Thereafter, the anti-DBY CD4 clone (3000 cells) was added to the autologous EBV-LCL (30 000 cells) and after 24 h of incubation supernatant was harvested. The amount of IFN-γ secreted by the CD4 clone in response to the different bacteria was measured in an ELISA. The results shown represent an average of triplicate co-cultures. Standard deviations were always lower than 10% of the average.

Screening of an expression library with the anti-DBY clone

We constructed a cDNA library with RNA extracted from the male EBV-LCL with HLA molecules identical to the sibling

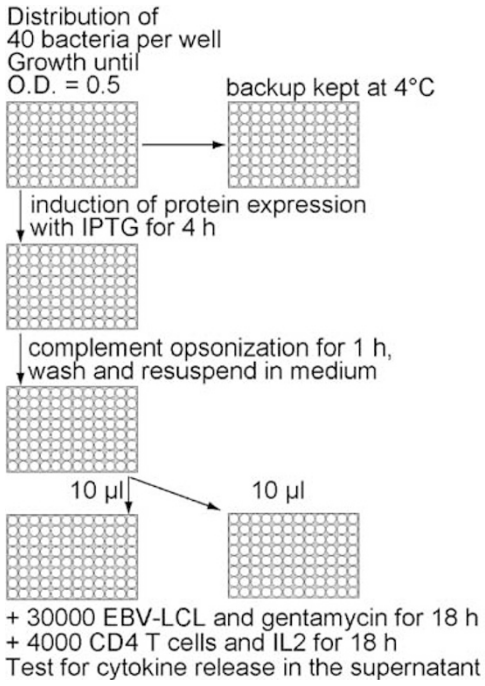

Figure 4 A schematic overview of the screening of the library. A cDNA library derived from antigen positive cells was constructed using the pKE-1 vector. *E. coli* XL1-blue bacteria are transformed by electroporation of the recombinant plasmids. Pools of ~40 bacteria are distributed in deepwell 96-well plates. Bacteria are grown under agitation until the suspension reaches an OD_{600} of 0.5. Part of the bacteria are transferred to new microplates and stored at 4°C for eventual recovery. IPTG is added to the bacteria in the mother plates to induce expression of proteins. The bacteria are then opsonized with complement, washed, and resuspended in cell culture medium. Only small amounts of these bacteria are then transferred in duplicate to new microplates in which antigen-negative EBV-LCL are seeded. After receptor-mediated uptake and processing of the bacteria, antigen-specific $CD4^+$ T cells are added. Secretion of IFN-γ by the T cells in response to the autologous EBV-LCL is measured in the supernatant of the coculture. The duplicate of a positive pool can be recovered from the backup plate kept at 4°C and subcloned. The procedure is repeated to identify a single bacterial colony containing the cDNA.

donor. The recombinant plasmids were electroporated into bacteria that were selected for recombinants with ampicillin and kanamycin. Although the DBY-expressing bacteria can be detected at a dilution of 300, we made pools of 40 bacteria to operate well above the detection limit. The pools of bacteria were amplified in deep 96-well plates under agitation, treated with IPTG, opsonized with complement, and loaded on female EBV-LCL. The antigen-loaded cells were subsequently screened with the anti-DBY CD4 clone for release of IFN-γ, according to a strategy schematically outlined in Figure 4. The screening of 1500 pools of the library with the CD4 clone identified four positive pools, the duplicates for which were consistent. Two positive pools, #295 and #615, were selected for further screening (Figure 5a and b). The bacteria were plated on agar, and 200 colonies from each pool were amplified, treated with IPTG, opsonized with complement and loaded on antigen-presenting cells. The CD4 clone was stimulated with several bacterial clones and results of one representative plate are shown in Figure 5c. Two colonies from each bacterial pool that showed the highest CD4 responses were selected for sequence analysis. Colonies isolated from pools #295 and #615 contained inserts of 745 and 1815 bp, respectively, corresponding to the

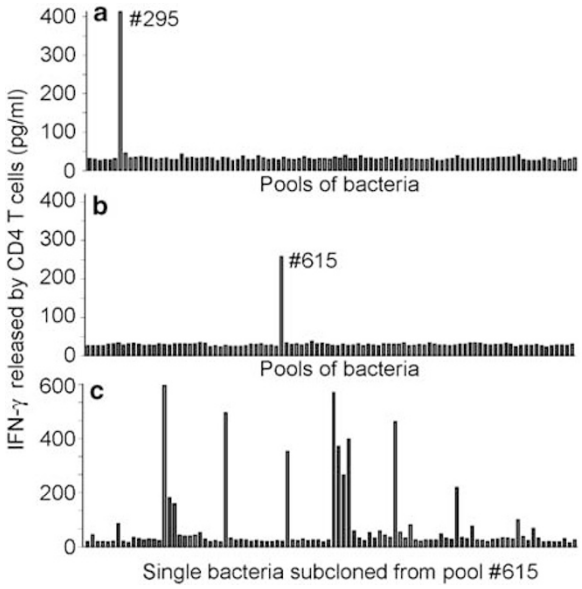

Figure 5 Screening of the cDNA library with the anti-DBY CD4 clone. In total, 3000–5000 anti-DBY CD4 T cells were cocultured overnight with autologous EBV-LCL loaded with bacteria pools (~40 bacteria) of the cDNA library. The IFN-γ released in the supernatant was measured by ELISA. The results shown represent the average of duplicate co-cultures. Results of two representative plates are shown: (a) one containing positive pool #295, (b) one containing positive pool #615, (c) a plate containing single colonies isolated from pool #615. Standard deviations were always lower than 10% of the average.

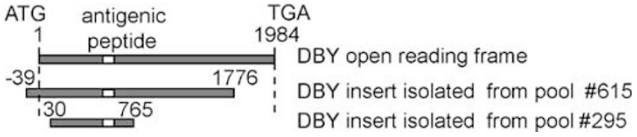

Figure 6 Schematic view of the two truncated DBY cDNA isolated from the library.

sequence of DBY (Figure 6). These two inserts contain the region of the antigenic peptide, but neither of them contains the entire coding sequence of DBY.

During the screening of the library, two positive controls were used to check for proper release of IFN-γ by the CD4 clone upon contact with a small amount of antigen, and to check for the sensitivity of the screening procedure. Male-derived EBV-LCL were titrated into female-derived EBV-LCL and cocultured with the anti-DBY clone. Male EBV-LCL were still detectable at a dilution of 1:30 (Figure 7a). We also performed a reconstruction experiment where a few DBY-expressing bacteria were mixed with nonrelevant bacteria. After determination of the titer of bacterial suspensions, we prepared a suspension containing approximately five DBY-expressing bacteria and 40 000 bacteria from the library. This suspension was subsequently divided into 100 pools of around 40 bacteria that were treated according to the procedure described above and screened with the CD4 clone. We identified seven positive pools, which stimulated the clone to release more than 100 pg/ml of IFN-γ (Figure 7b).

Discussion

We propose to use complement-opsonized bacteria, which are processed by EBV-LCL, as a robust and rapid approach for the

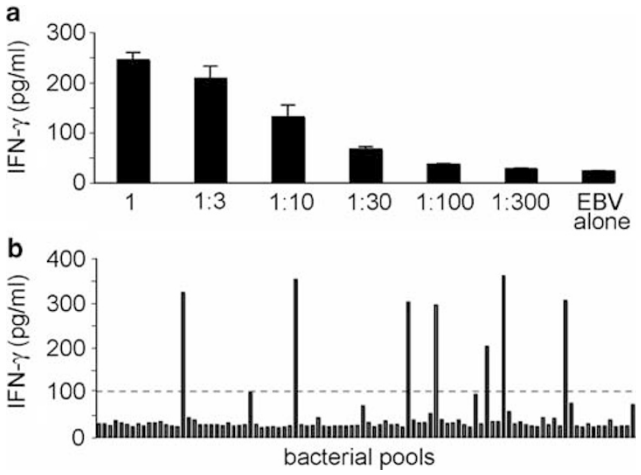

Figure 7 Release of IFN-γ by the anti-DBY CD4 clone upon contact with a small number of male cells or with female cells loaded with DBY-expressing bacteria. (a) To check the sensitivity of the T-cell clone during the screening of the library, male-derived EBV-LCL were titrated into female-derived EBV-LCL up to 1:300, and co-cultured with the anti-DBY DQ5-restricted CD4 clone. The results shown represent the average of triplicate co-cultures. (b) As a positive control for the screening procedure, we prepared a suspension containing approximately five DBY-expressing bacteria and 40 000 bacteria from the library. This suspension was subsequently divided into 100 pools of around 40 bacteria that were treated according to the procedure described in the Materials and methods and screened with the CD4 clone. The results shown represent the average of duplicate co-cultures. Standard deviations were always lower than 10% of the average.

screening of cDNA expression libraries with CD4 T cells. This approach can be applied broadly for the identification of HLA class II-restricted antigens, in particular for mHags as described in this report.

In human applications, using autologous antigen-presenting cells is a significant advantage. The HLA class II presenting molecules do not have to be identified. An unambiguous identification is sometimes difficult because multiple heterodimers are formed between the α and β subunits. It also obviates the need for cDNAs encoding the appropriate class II subunits in order to generate recipient cell lines expressing class II molecules, invariant chain, and possibly DM. The feasibility of screening with CD4 T cells a cDNA expression library, which is expressed in bacteria that are taken up by antigen-presenting cells, was previously shown by Alderson *et al*[44] for a mycobacterial antigen and by Sahara and Shastri[5] for a murine mHag. In both reports, the bacteria were taken up by dendritic cells. Human autologous dendritic cells are always available in limited numbers, in particular for cancer patients. We propose here to use EBV-LCL, which are matchless autologous antigen-presenting cells. They express high amounts of HLA class II molecules and large numbers of cells can be grown easily. We have shown here that EBV-LCL have a high capacity for phagocytosis of bacteria, provided that they are opsonized. We made use of the natural exogenous processing pathway of B cells, which can take up antigens coated with complement fragments.[40,41] B cells are relatively inefficient in fluid-phase pinocytosis compared with immature dendritic cells, but they can compensate for this weakness with the presence of antigen-specific cell surface receptors. In addition, they express other receptors to take up molecules that have been tagged as foreign by activation of the complement system, in particular by activation of C3 and the deposition of C3b on the antigen. EBV-LCL express two receptors for C3 fragments: CR1, a C3b/C4b-binding protein controlling complement activation, and CR2, an heterodimeric integrin involved in binding of C3 fragments.

Our approach with complement-opsonized bacteria is very sensitive. The anti-DBY T cells were only able to detect male EBV-LCL diluted at 1:30 in female EBV-LCL whereas they detected DBY-producing bacteria diluted at 1:300. This sensitivity can most probably be achieved with many CD4 clones available in our laboratories. As vector pKE-1 selectively eliminates nonrecombinants and clones containing stop codons in the cDNA reading frame translated by the bacteria, the size of the library to be screened is greatly reduced. Our approach can be carried out reliably with pools of 50 bacteria, and screening 150 000 recombinants necessitates only 30 96-well plates. This approach is also rapid: a cDNA library can be constructed in a few weeks and the amplification of the bacteria, the first screening, and the subcloning of a positive pool can take less than 2 weeks.

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