Phage display of a CTL epitope elicits a long-term *in vivo* cytotoxic response

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Received 20 September 2006; revised 18 December 2006; accepted 23 January 2007. First published online 28 March 2007.

DOI:10.1111/j.1574-695X.2007.00229.x

Editor: Willem van Eden

Keywords
cytotoxicity; bacteriophage; multiepitopic carrier.

Abstract

The Ovalbumin$_{257–264}$ CTL epitope on the major coat protein of the filamentous bacteriophage in different antigen formulations was displayed and the immune response in C57BL6/J mice studied. The display of single cytotoxic epitope on the surface of the virion is sufficient to induce priming and sustain long-term major histocompatibility complex class I restricted cytotoxic T lymphocytes response *in vivo*. The filamentous bacteriophage is a versatile carrier able to display simultaneously either single or multiple epitopes and can elicit a cellular response carrying very little peptide ($<1.5\,\mu g$).

Introduction

Induction of robust and long-lasting cytotoxic T lymphocyte (CTL) responses by a vaccine is an open and difficult challenge. In particular, protective immunity to viral infections is mediated by neutralizing antibodies and by $CD8^+$ CTLs. Both responses depend on antigen-specific $CD4^+$ T helper lymphocytes. It was originally proposed that peptides recognized by $CD8^+$ CTLs result from the processing of endogenous intracellular proteins and that peptides recognized by $CD4^+$ T helper cells derive from exogenous antigens. More recently, it has been shown that peptides derived from exogenous antigens can also be provided to major histocompatibility complex (MHC) class I molecules for presentation to $CD8^+$ CTLs. This 'cross-presentation' process involves both dendritic cells and macrophages such as antigen-presenting cells, priming a wide variety of $CD8^+$ T cell responses to different exogenous antigens (Ramirez & Sigal, 2002, 2004; Shen *et al.*, 2004). We have developed an antigen delivery system based on filamentous bacteriophage virions engineered to display foreign epitopes. *Fd* phages (di Marzo Veronese *et al.*, 1994; Veronese *et al.*, 1994; Malik & Perham, 1996, 1997; Malik *et al.*, 1996a) can display multiple copies of exogenous peptides inserted in the exposed N-terminal region of the 2700 copies of the major coat protein, pVIII, that form the capsid of virion. We have demonstrated that *fd* phages could be processed both by MHC class I and class II pathways to induce CTL and T helper responses (Gaubin *et al.*, 2003). In fact, double hybrid virions were used simultaneously displaying a CTL and a T helper epitope of the HIV-1 reverse transcriptase (RTAse) to prime specific CTL from human peripheral blood lymphocytes (PBL) and to perform *in vivo* immunization of HLA-A2 transgenic mice (De Berardinis *et al.*, 1999, 2000).

To test the possibility of using the phages as a polyepitopic carrier we first investigated the role of the T helper exogenous epitope during CTL induction in this system. We used the filamentous bacteriophage as a carrier of H2-K^b-restricted Ovalbumin$_{257–264}$ CTL epitope (SIINFEKL) and I-A^b-restricted hepatitis B virus core antigen$_{128–140}$ T helper epitope (TPPAYRPPNAPIL). The immunogenicity of the mix of two single hybrid phages, one carrying the CTL epitope and the other the T helper epitope, was compared with the double hybrid phage, displaying both epitopes on the surface of the same virion. The two different phage combinations were tested in C57BL6/J mice using either CFA/IFA or polyI:C as adjuvant. We found that the efficiency of the two antigen formulations on CTL activation is different. It is noteworthy that the single hybrid phage displaying CTL epitope is able to induce an efficient and long-lasting response even in the absence of the T helper exogenous epitope.

Materials and methods

Construction and purification of hybrid bacteriophages

Single display fdOVA (SIINFEKL) and fdHBVc (TPPAY-RPPNAPIL) bacteriophages were constructed by ligating a SacII-StyI oligonucleotide insert encoding peptide OVA (5′CCGCGGAGGGTTCCATCATCAACTTCGAAAAACTG GACGATCCCGCCAAGG-3′) or HBVc (5′-CCGCGGAGG GTACCCCGCCGGCTTACCGTCCGCCGAACGCTCCGATC CTGGACGATCCCGCCAAGG-3′) into SacII-StyI-digested fdAMPLAY88 phage genome. *Escherichia coli* XL1-Blue MRF' Kan cells transformed with recombinant bacteriophages produced single hybrid phages in the supernatant. Double display bacteriophage fdOVA/HBVc was constructed first by legating a SacII-StuI oligonucleotide insert encoding peptide OVA into SacII-StuI-digested plasmid pTfd8 and then infecting *E. coli* XL1-Blue MRF' Kan cells transformed with plasmid pTfd8-OVA by the bacteriophage fdHBVc. The bacteriophage fdOVA/HBVc produced, simultaneously displayed both OVA and HBVc peptides on the same virion particle.

The hybrid bacteriophage virions were harvested from *E. coli* supernatant, purified by caesium chloride gradient and analyzed by SDS-PAGE as described elsewhere (Malik *et al.*, 1996b). The single hybrid phage preparations carrying the OVA or HBVc peptides (fdOVA or fdHBVc) displayed 450–750 recombinant copies of major coat protein pVIII. The double hybrid virion fdOVA/HBVc, carrying both OVA and HBVc peptides on the same capsid displayed 200–400 recombinant copies of major coat protein pVIII. The percentages of recombinant pVIII have been estimated by N-terminal sequences analysis of the purified virions for each phage preparation.

Mice and immunization procedures

C57BL6/J mice were purchased from Harlan Nossan (Corezzana, Italy) and maintained at the IGB animal facility, according to the institutional guidelines. Sentinel mice were screened for seropositivity to Sendai virus, Rodent Coronavirus and *Mycoplasma pulmonis* by the Murine Immunocomb test (Charles River) and were negative.

Peptides derived from Ovalbumin ($OVA_{257–264}$) and hepatitis B virus core antigen ($HBVc_{128–140}$) were purchased from Primm (Milan, Italy). Purity was $> 95\%$, as analyzed by HPLC and mass spectrometry. On the basis of preliminary experiments, the following immunization schedule was followed: immunization (day 0), boost (day 14), sacrifice (day 28). The immunizations were performed using one of the following antigen formulations: 140 μg double hybrid phage fdOVA/HBVc carrying 1.4 μg OVA and 3.5 μg HBVc peptides; phage mix containing 50 μg fdOVA and 140 μg fdHBVc, displaying 1.4 μg OVA and 7 μg HBVc peptides, respectively; 30, 50 and 140 μg single hybrid phage fdOVA displaying, respectively 0.84, 1.4 and 3.8 μg peptide. C57BL6/J female mice were injected intraperitoneally with recombinant phages plus 150 μg polyI:C and boosted 2 weeks later using the same protocol. Alternatively, C57BL6/J female mice were injected subcutaneously with recombinant phages plus complete Freund's adjuvant (Sigma) and boosted 2 weeks later using the same amount of recombinant phage plus incomplete Freund adjuvant.

Cytotoxicity test

The cells purified from the spleens of control and immunized mice (responder cells) were tested for anti-$OVA_{257–264}$ peptide cytotoxic response using the JAM test (Matzinger, 1991; Hoves *et al.*, 2003). Briefly, responder cells (4×10^6 cells well^{-1}) were stimulated in 24-well plates with irradiated syngeneic female spleen cells (stimulator cells, 2×10^6 well^{-1}), which were prepulsed with $OVA_{257–264}$ peptide at 10 μg mL^{-1} in the presence of interleukin 2 (IL-2) at 20 U mL^{-1}. As a positive control, spleen cells from each mouse were stimulated in parallel cultures with irradiated BALB/c female spleen cells bearing the H-2^d alloantigen (antiallo response). After 6–7 days, cells were harvested and tested for cytotoxic activity against ^{3}H-TdR labeled targets in a 4-h assay. The anti-$OVA_{257–264}$ peptide responder cells were tested against EL4 target cells (H-2^b, syngeneic to C57BL6/J mice), which had been either prepulsed or not with $OVA_{257–264}$ peptide; the antiallo responder cells were tested against either P815 (H-2^d) or EL4 target cells.

ELISPOT

Single splenocyte suspensions were prepared by mechanical disruption from either control or immunized mice (responder cells). For the $CD8^+$ T cell response, incubated cells were harvested in anti-interferon-γ (IFN-γ) mAb precoated multiscreen plates with IL-2 at 20 U mL^{-1} and 500 000 irradiated syngeneic spleen cells, prepulsed with either $OVA_{257–264}$ peptide at 10 μg mL^{-1} or medium alone. There were 500 000 responder spleen cells per well and, as positive control, 100 000 responder spleen cells from each mouse were incubated with either medium alone or ConA at 10 μg mL^{-1}. After 40 h of incubation at 37 °C, cells were washed and the plates sequentially incubated with anti-IFN-γ biotinylated mAb, poly-HRP-streptavidin (Endogen, Woburn, MA) and AEC substrate (Sigma, Milan, Italy). IFN-γ transfected TSA cells and their parental untransfected line were used as controls in each ELISPOT plate after gamma irradiation (Parretta *et al.*, 2005). The spots were counted using the ELISPOT reader (AELVIS).

Pentamer staining

Cells were incubated with 24G2 mAb (Sigma) and stained with either PE labeled SSYSYSSL K^b pentamer (control peptide-K^b pentamer, ctrl-pent, ProImmune, Oxford, UK) or PE labelled SIINFEKL K^b pentamer ($OVA_{257–264}$ peptide pentamer, ProImmune) in ice for 45 min. Anti-CD8α-PE-Cy5 mAb was added and cells incubated for additional 15 min. After fixation with 30% methanol, 0.4% paraformaldehyde in phosphate-buffered saline, cells were analyzed by flow cytometry, gating on $CD8^+$ cells and acquiring 50 000 events per sample (Parretta *et al.*, 2005). Background staining with ctrl-pent was subtracted for each sample. Spleen cells from OT-1 transgenic mice (carrying an anti-$OVA_{257–264}$ T cell receptor) were used as positive controls of staining.

Statistics

Statistical analysis was performed using the unpaired Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Results

Immunogenicity of hybrid phages carrying CTL and T helper epitopes

To assess the antigen-specific $CD8^+$ T cell response induced by hybrid bacteriophages we carried out four groups of experiments using different adjuvants and phage formulations. For each group of experiments, six C57BL6/J mice were immunized according to the following protocols:

(**1**) 140 μg fdOVA/HBVc double hybrid phages plus Freund's adjuvant;

(**2**) single hybrid phage mix containing 50 μg fdOVA and 140 μg fdHBVc plus Freund's adjuvant;

(**3**) 140 μg fdOVA/HBVc double hybrid phages plus polyI:C adjuvant;

(**4**) single hybrid phage mix containing 50 μg fdOVA and 140 μg fdHBVc plus polyI:C adjuvant.

Control mice were either untreated or treated with 140 μg wild-type bacteriophages with adjuvant using the same immunization schedule. Two weeks after the second antigen administration, mice were sacrificed and the splenocytes

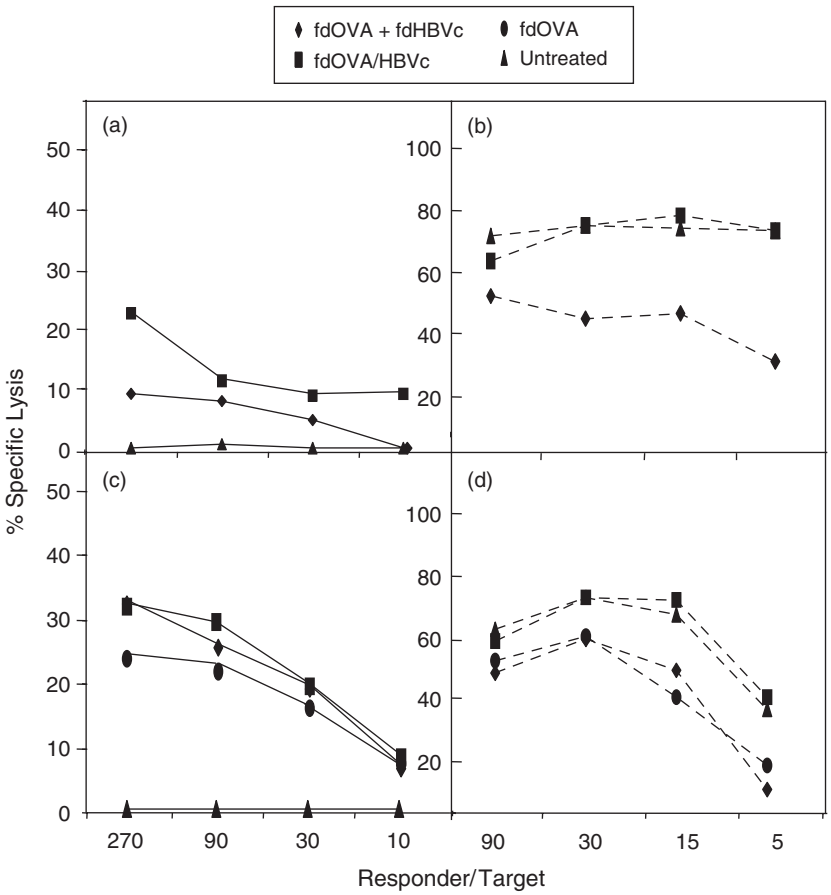

Fig. 1. Antigen-specific and allogenic cytotoxic $CD8^+$ cell response of mice immunized with recombinant phages. Mice were immunized with either fdOVA+fdHBVc or fdHBVc/OVA as indicated in the legend. Control mice were left untreated. We used either Freund's adjuvants (a and b) or polyI:C (c and d). Splenocytes from each mouse in parallel cultures for $OVA_{257–264}$ specific cytotoxicity response (a and c) and for allogenic response (b and d). The percentages of antigen-specific killing are shown on the *y*-axis, after subtraction of background killing of unpulsed targets. Responder/target ratios are shown on the *x*-axis.

tested for anti-$OVA_{257–264}$ peptide-specific CTL and IFN-γ responses.

Figure 1 shows representative examples for each of the four groups (1–4). The panels show the percentage of sacrifice at different responder-to-target ratio. Mice were immunized using Freund's adjuvant in (a) and (b) and polyI:C adjuvant in (c) and (d). (a) and (c) show the $OVA_{257–264}$ specific CTL responses and (b) and (d) the alloreactivity responses of the same mice, demonstrating the effectiveness of the mouse immune system.

Figure 2 summarizes the results obtained with the two groups of mice immunized subcutaneously using the Freund's adjuvants. Group (1) was immunized with the double hybrid phage fdOVA/HBVc and group (2) immunized with a mix of the two single hybrid phages fdOVA and fdHBVc. Panel (a) shows the percentage of specific lysis after subtraction of background lysis of the unpulsed target at the responder/target ratio 270:1. Immunization of mice with either the double hybrid phage or the mix of the two single hybrid phages resulted in a similar OVA-specific CTL activity, but no antigen-specific lysis was detected when mice were injected with wild-type phages. We further evaluated the effector cell populations by determining the frequency of IFN-γ producing splenocytes in response to *in vitro* stimulation with $OVA_{257–264}$ peptide. We set up a short-term culture with antigen-presenting cells that had either been pulsed or not with OVA peptide and IFN-γ producing cells were measured by ELISPOT after culture for 40 h. The results are shown in panel (b). The number of antigen-specific IFN-γ SFC per million cells is shown on the *y*-axis, and each symbol represents the spots obtained from a single mouse after subtraction of background killing achieved with unpulsed antigen-presenting cells. The mean values of the spots for each group are also indicated. In all mice analyzed, the mix of the two single hybrid phages was consistently better at inducing IFN-γ producing cells than the double hybrid phage.

Figure 3 summarizes the results obtained with the two groups of mice immunized intraperitoneally using polyI:C adjuvant: group c, immunized with the double hybrid phage

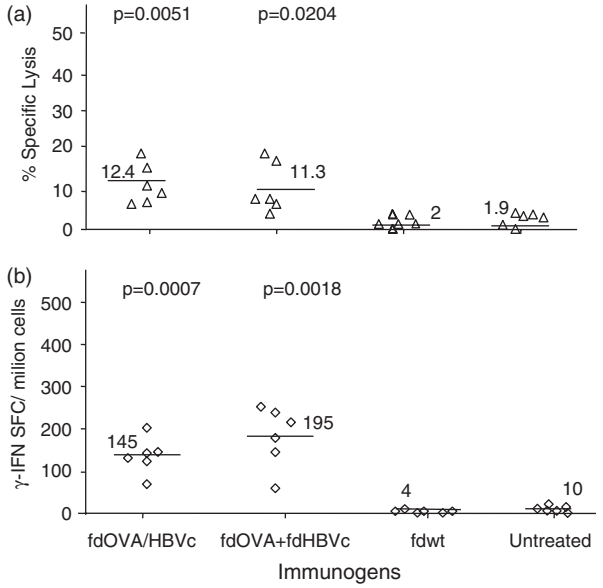

Fig. 2. Antigen-specific CD8 responses of mice immunized with recombinant phages plus Freund's adjuvants. Mice were immunized by two injections with either fdHBVc/OVA (group 1) or fdOVA+fdHBVc (group 2) plus Freund's adjuvants. Control mice were either left untreated or injected with wild-type phage. (a) Cytotoxicity test. The percentages of antigen-specific killing are shown on the y-axis, after subtraction of background killing of unpulsed targets. Responder/target ratios is 270:1. Each symbol represents the lysis obtained from a single mouse. The mean value is represented for each group. (b) ELISPOT assay. The number of antigen-specific IFN-γ SCF per million cells is shown on the *y*-axis. Each symbol represents the spots obtained from a single mouse after subtraction of background spots with unpulsed APC. The mean value is represented for each group. The *P* value was calculated by comparing each group of immunized mice with the group of untreated mice.

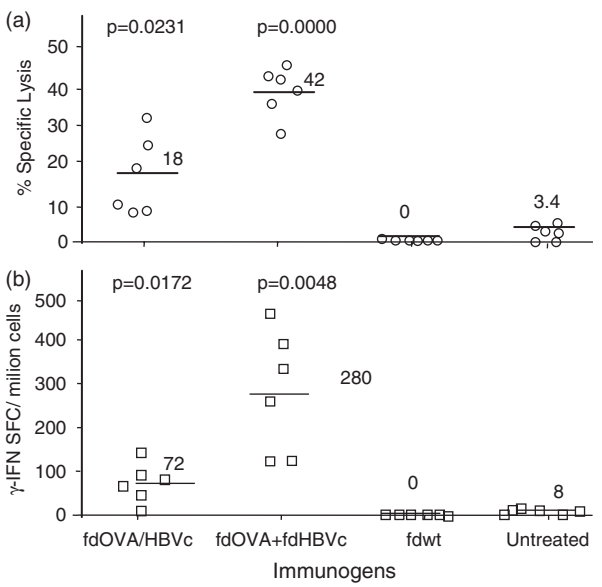

Fig. 3. Antigen-specific CD8 responses of mice immunized with recombinant phages and plus polyI:C adjuvant. Mice were immunized by two injections with either fdHBVc/OVA (group 3) or fdOVA+fdHBVc (group 4) plus polyI:C adjuvant. Control mice were either left untreated or injected with wild-type phage. (a) Cytotoxicity test. The percentages of antigen-specific killing are shown on the *y*-axis, after subtraction of background killing of unpulsed targets. Responder/target ratios is 270:1. Each symbol represents the lysis obtained from a single mouse. The mean value is represented for each group. (b) ELISPOT assay. The number of antigen-specific IFN-γ SCF per million cells is shown on the *y*-axis. Each symbol represents the spots obtained from a single mouse after subtraction of background spots with unpulsed APC. The mean value is represented for each group. The *P* value was calculated by comparing each group of immunized mice with the group of untreated mice.

fdOVA/HBVc, and group d, immunized with a mix of the two single hybrid phages fdOVA and fdHBVc. Panel (a) shows the results of the cytotoxicity test. The percentages of peptide-specific lysis were 18% and 42%, respectively, for groups c and d. (b) shows the ELISPOT results for the same groups. The number of antigen-specific IFN-γ produced confirms the results obtained in the cytotoxicity test. Comparing groups c and d, we found that the mix of the two single hybrid phages was more effective than the double hybrid phage in both assays ($P = 0.0019$ for CTL test and $P = 0.012$ for ELISPOT assay).

Our results show that the mix of two single hybrid phages works significantly better than the double hybrid phages in inducing peptide specific cytotoxicity when administered with polyI:C. This effect could be generated either by the higher amounts of HBVc peptide displayed or by the major amount of the carrier used. In conclusion, when using either Freund's and polyI:C adjuvant we obtained better results with the mix of phages, and when polyI:C was used, the results became significant.

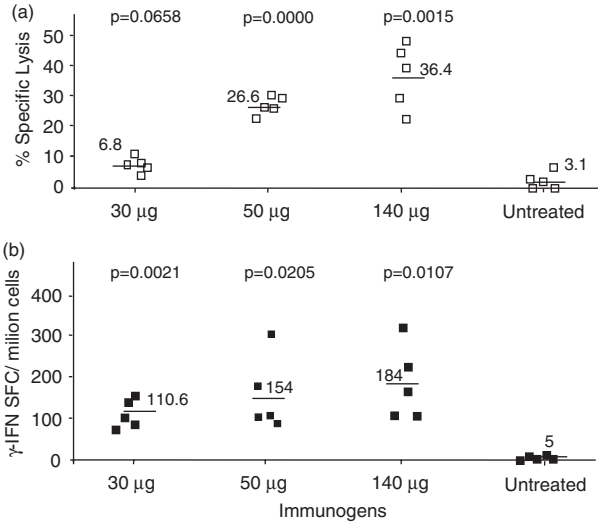

Fig. 4. Antigen-specific $CD8^+$ cell responses after immunization with fdOVA plus polyI:C. Mice were immunized by two injections of fdOVA plus polyI:C adjuvant. (a) shows the CTL test and the (b) the ELISPOT assay. Each group of mice received a different amount of fdOVA antigen, as shown on the *x*-axis. The *P* value was calculated by comparing each group of immunized mice with the group of untreated mice.

Use of single hybrid phage fdOVA to induce CTL response in the absence of exogenous T helper epitopes

We further explored the ability of hybrid phages to induce a CTL response by testing a single hybrid phage in the absence of exogenous T helper epitopes. We used polyI:C adjuvant, which in the previous experiments was very efficient in inducing CTL activity. C57BL6/J mice were immunized with two intraperitoneal injections of the single hybrid virion fdOVA plus polyI:C. We treated the animals with graded amounts of fdOVA, 30, 50 and 140 µg, carrying respectively 0.84, 1.4 and 3.8 µg of the OVA epitope. Two weeks after the second antigen boost, the mice were sacrificed and the splenocytes tested for anti-$OVA_{257–264}$ peptide-specific responses.

Figure 4 (panel a) shows the cytotoxicity test and panel b shows the ELISPOT assay for a total of 20 mice, five per group, analyzed in three experiments. In the CTL experiment, an antigen-specific CTL response was obtained using the single hybrid fdOVA in the absence of HBVc epitope (panel a). An increasing percentage of specific lysis was observed after injecting the mice with higher amounts of recombinant phage. These results are statistically significant compared with data from untreated mice. The number of IFN-γ-secreting cells for individual mice and the mean for each group of immunized mice are shown in panel b. As demonstrated for the CTL test, the number of spots correlated with the amount of phage administered.

Using pentamer staining, we further characterized the effector cell population induced by immunization with 50 µg fdOVA single hybrid phages or single hybrid phage mix fdOVA and fdHBVc from group d, both injected with polyI:C. Figure 5 shows the cytofluorimetric analysis of a representative mouse. Spleen cells were stained with anti-$CD8^+$α-PE-Cy5 mAb and either OVA-pent-PE or ctrl-pent-PE. The percentage of $OVA_{257–264}$ peptide-specific cells in the absence of *in vitro* restimulation was 0.1% in immunized mice (a) after subtraction of background killing. Following *in vitro* antigen restimulation, the percentage of $OVA_{257–264}$ peptide-specific cells was respectively 2.8% and 1.9% in immunized mice (b), after subtraction of background killing. We examined in parallel control samples in which we mixed splenocytes from untreated B6 mice and from OT-I mice at a given ratio. The percentages of OVA-pent-PE specific cells were found to be in the same range as those found in immunized mice (Fig. 5), confirming our results.

Finally, we observed that the ability of bacteriophages to induce cytotoxicity and IFN-γ production does not depend on the display of the T helper epitope HBVc, likely because the carrier bacteriophage contains CD4 epitopes or helper activity. In fact, the strength of the cytotoxic activity directly correlated also with the dose of recombinant phage administered.

Long-term antigen-specific $CD8^+$ T cell responses

To establish whether the recombinant phages induce long-term $CD8^+$ T cells, we immunized mice by intraperitoneal

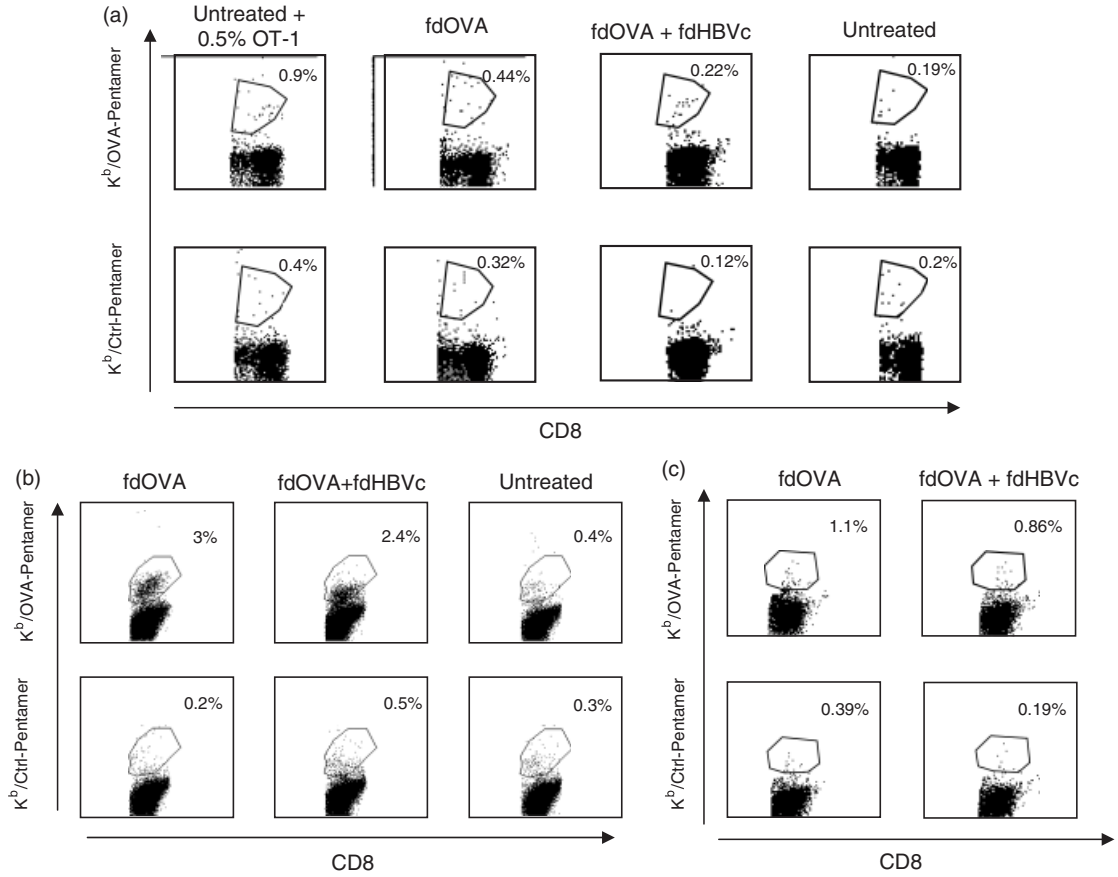

Fig. 5. Pentamer staining of spleen cells from immunized mice. The dot plots represent flow cytometric analysis of spleen cells stained with anti-$CD8^+\alpha$-PE-Cy5 mAb (*x*-axis) and either OVA-pent-PE or ctrl-pent-PE (*y*-axis). The scales on *x*- and *y*-axes are logarithmic, in arbitrary units. The numbers represent the percentages of cells in the indicated region, after gating on $CD8^+$ cells. (a) The positive control, in which we analyzed spleen cells from a B6 untreated mouse after mixing the cells with 0.5% splenocytes from OT-1 transgenic mice and short-term, nonrestimulated $OVA_{257–264}$ peptide-specific cells. (b) Short-term restimulated $OVA_{257–264}$ peptide-specific cells. (c) Pentamer staining of spleen cells from long-term immunized mice, after *in vitro* restimulation.

injections of either the mix of two single hybrid phages (50 μg fdOVA + 140 μg fdHBVc) or the single hybrid phage carrying OVA epitope alone (50 μg fdOVA) plus polyI:C adjuvant. Mice were injected twice, with a 2-week interval, and tested 2 months later to check long-term antigen-specific responses. Figure 6 shows the results of cytotoxicity test and demonstrates that both groups of immunized mice display $OVA_{257–264}$ specific lysis.

Using pentamer staining we confirmed the results obtained. Figure 5 (c) is a dot plot representative of the results obtained for one of four immunized mice, for each group. Spleen cells were restimulated *in vitro* with OVA peptide and stained with antiCD8α-PE-Cy5 mAb and either OVA-pent-PE or ctrl-pent-PE. The percentage of $OVA_{257–264}$ peptide-specific cells was, after subtraction of background killing, 0.71% for the mice immunized with single hybrid phages fdOVA and 0.67% for the mice immunized with single hybrid phages mix.

Our results indicate that antigen-specific CD8 T cells persist in the spleen of mice immunized by phages at least for 2 months after immunization and display a prompt response to the antigen after *in vitro* stimulation.

Discussion

In the search for new vaccines to replace older, ineffective or toxigenic formulations, peptides that are the target epitope of T and B cells represent attractive candidates. Peptides are usually poor immunogens and induce minimal $CD8^+$ T cell responses, a shortcoming that may be overcome by coupling them to various carrier molecules. Moreover, vaccine formulations based on soluble exogenous antigens have been shown to be inefficient for class I presentation. To find the best strategy to deliver exogenous antigens into the MHC class I pathway and induce potent MHC class I-restricted CTL responses, a broad panel of soluble and particulate

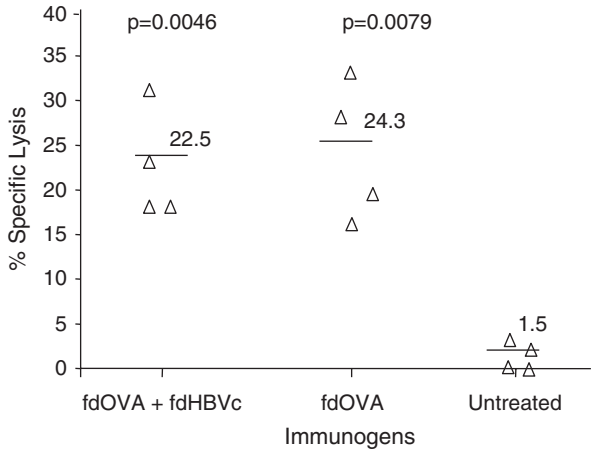

Fig. 6. Long-term antigen-specific cytotoxicity after immunization with recombinant phages plus poly I:C adjuvant. Mice were immunized by two injections of either the mix fdOVA+fdHBVc or fdOVA alone plus polyI:C adjuvant. Two months later, mice were sacrificed and tested for antigen-specific cytotoxicity. The percentages of antigen-specific killing are shown on the *y*-axis, after subtraction of background killing of unpulsed targets. Responder/target ratios is 270:1. Each symbol represents the lysis obtained from a single mouse. The mean value is represented for each group. The *P* value was calculated by comparing each group of immunized mice with the group of untreated mice.

vectors has been engineered (Moron *et al*., 2004) to take advantage of the capacity of antigen-presenting cell cross-presentation. Filamentous bacteriophage was used as a carrier of foreign peptides because it is an excellent antigen delivery system, a nonpathogenic and nonreplicating vehicle. It is simpler to handle and less expensive than other carriers (Guardiola *et al*., 2001). In previous papers we have demonstrated that antigenic peptides displayed on the phage capside are very immunogenic because these particles may be processed by class I and class II pathways (Gaubin *et al*., 2003). Other authors have also confirmed the mechanism of cross-presentation of phage particles that are initially degraded in the proteasome. Subsequently, the resulting peptides may be delivered back into the phagosome lumen and loaded onto MHC class I molecules with help from the TAP complex (Wan *et al*., 2005). A very peculiar characteristic of bacteriophages is their ability to display multiple copies of exogenous peptides on the capside. The viral assembly allows the incorporation of 10–30% recombinant pVIII in the capside structure during the single or double hybrid phages synthesis, depending on the biochemical characteristics of peptides (di Marzo Veronese *et al*., 1994). For this reason the concentration of specific peptides administered with carrier is very low (1–4 μg) and the peptides have to be established by N-terminal sequence for each phage preparation.

We used the bacteriophage antigen delivery system to test its ability to induce specific $CD8^+$ response in wild-type mice. This is the first time that they have been shown to induce a long-term specific cytotoxicity. Both phage formulations, the double hybrid phages fdOVA/HBVc displaying CTL and T helper epitopes on the same capside structure, and the mix of the two single hybrid phages fdOVA and fdHBVc, are able to induce peptide specific cytotoxicity and IFN-γ production. Our results were confirmed by antigen-specific tetramer staining. Although both phage formulations displayed 1.4 μg OVA peptide, the mix of single hybrid phages induced a higher CTL activity and IFN-γ production, probably because of the greater number of carrier phages used with a possible adjuvant role.

In fact, we demonstrated that the single hybrid phage fdOVA can induce specific cytotoxic response, independently from the display of a T helper exogenous epitope. Increasing the amount of peptide carried (0.84, 1.4 and 3.8 μg) influences the strength of the CD8 response. The capside structure probably provides enough help to sustain the CD8 response, in analogy with results obtained with antibody production (Perham *et al*., 1995).

Both in the dose-response experiment with fdOVA and in the experiment comparing the double hybrid phages with the mix of the two single hybrid phages, a more effective response was found when higher amounts of phage were used.

Moreover, we found that two boosts with 50 μg of single fdOVA are sufficient to induce an early (2 weeks) as well as late secondary response (2 months). The role of the adjuvant polyI:C and the kinetics in the establishment of the memory response remain to be investigated further (Salem *et al*., 2006). The establishment of a long-lived population of memory CD8 cells that can persist for the life of the immunized individual, and which can rapidly proliferate and assume effector functions upon re-exposure to antigen (secondary response), has very important implications for the design of successful vaccines (Williams *et al*., 2006).

In conclusion, we and other authors have previously demonstrated the *in vivo* immunogenicity of phage carriers (De Berardinis *et al*., 2000; Wan *et al*., 2001; Yang *et al*., 2005), their ability to induce CTL priming in *in vitro* experiments with human PBMC (De Berardinis *et al*., 1999), as well as their capacity to elicit the production of specific and protective antibody (Perham *et al*., 1995; Fang *et al*., 2005). Here, we demonstrate that recombinant bacteriophages induce a long-term antigen-specific CD8 response, a very important requirement for an effective carrier molecule. Our aim will be to use phages as polyepitopic immunogens and to design a mix of double hybrid phages carrying at least four to six different CTL epitopes.

Acknowledgements

This paper is dedicated to the memory of John Guardiola.

This work was supported by grants from European Union (Project No. QLK2-CT-2002-0062, EPI-PEP-VAC), the Italian government (Fondi Italiani per la Ricerca di Base 2001, Project No. RBNE01RB9B, RBNE01S29H006 and 2003 project No RBLA033WJX), Istituto Superiore di Sanità (V Research Project AIDS).

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