

Th2-type immune response induced by a phage clone displaying a CTLA4-binding domain mimic-motif

Research Article

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Abbreviations: Alkaline phosphatase, (AP); gene 3 proteins, (g3p); gene 8 proteins, (g8p); hen egg lysozyme, (HEL); HPLC-purified g3p, (F2-g3p); intraperitoneally, (i.p.); monoclonal antibody, (mAb); phosphate-buffered saline, (PBS); wild type, (wt)

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Summary

We have recently isolated a phage clone, F2, which displays the CTLA4-binding domain mimic from a phage display library. To investigate the *in vivo* effects of an F2 motif on the regulation of immune responses, we immunized Balb/c mice intraperitoneally with varying doses of an F2 phage in a phosphate buffered saline and followed the resulting antibody and cytokine responses. It was shown that the F2 phage enhanced the IgG antibody response to phage particles in comparison to control phages that were randomly selected from the library. When the antigen specificity of the induced antibody response was examined, the production of an anti-g3p antibody was preferentially increased while an anti-g8p antibody was slightly down-regulated by the immunization of F2 in comparison to control phage clones. The increase of an anti-g3p antibody response was found in the isotype of IgG1 but not in the IgM or IgG2a. When the cytokine production was examined by culturing spleen cells from these mice under stimulation with anti-CD3 mAb, IL-4 production was approximately twice higher in the F2-primed cells than in the L4-primed cells while IFN- γ production was higher in L4-primed cells than in the F2-primed cells. Thus, these results suggested that the F2 phage clone bearing g3p with the CTLA4-binding domain mimic-motif induced the Th2-type response when compared to control phage clones.

I. Introduction

T-cell co-stimulatory receptors CD28 and CTLA4 deliver opposite signals on T-cell activation, mediating augmentation and inhibition of T-cell responses, respectively (Linsley, 1995; Thompson, 1995; Bluestone, 1997; Thompson and Allison, 1997). These two receptors use the same ligands, CD80 (B7-1) and CD86 (B7-2), expressed on the antigen-presenting cells (Azuma et al, 1993; Freeman et al, 1993). The kinetic study on the expression of these molecules suggested that CD28 may be responsible for CD86, and CTLA4 is responsible for CD80, respectively, since CTLA4 and CD80 expression reaches maximal levels 2-3 days after antigenic stimulation (Hathcock et al, 1994; Schweitzer et al, 1997).

However, CTLA4 may have more of a role in regulating T-cell responses at earlier stages in the process than had initially been thought (Krummel and Allison, 1995).

In principle, the interactions of co-stimulatory molecules were possible with four kinds of combinations: CD28-CD80, CD28-CD86, CTLA4-CD80, and CTLA4-CD86. However, the functional difference between CD80 and CD86 is still obscure although considerable interest has focused on the possible role of the CD80 and CD86 co-stimulatory molecules expressed on antigen-presenting cells (APC) in skewing CD4⁺ T cells to either the Th1 or Th2 phenotype (Hathcock et al, 1994; Schweitzer et al, 1997; Manickasingham et al, 1998).

Recently, we selected phage clones from a phage display library by employing a CTLA4-conformation recognizing a monoclonal antibody (mAb) (Fukumoto et al, 1998). A phage clone, F2, is specifically recognized with the anti-CTLA4 mAb and able to bind to CD80. The F2 motif consists of the unique 15-amino-acid sequence with an internal disulfide bond and is inserted in gene 3 proteins (g3p), which display only three to five copies in contrast to approximately 2700 copies of gene 8 proteins (g8p) per fd phage. The HPLC-purified g3p (F2-g3p) is also recognized with anti-CTLA4 mAb but not anti-CD28 mAb and binds to CD80 but not to CD86. When hen egg lysozyme (HEL)-primed lymph node cells were stimulated with HEL in the presence of the F2-g3p *in vitro*, cell proliferation was highly potentiated (Fukumoto et al, 1998). In the absence of antigenic stimulation, the F2-g3p induced no T-cell proliferation, indicating the co-stimulatory nature of the F2-g3p. Thus, the F2 motif represents a peptide mimic of the CTLA4-binding domain.

In this study, we examined the effect of F2 phage as an immunogen on the anti-phage immune response *in vivo*. When the phages were administered intraperitoneally (i.p.) in mice in the form of a phosphate-buffered saline (PBS) solution, F2 phage induced an anti-phage antibody response two to three times higher than that of the control phage. In this augmented response, the anti-g3p antibody production was predominantly increased, whereas the anti-g8p antibody production was rather decreased. The isotype of the increased antibody was IgG1 but not IgM or IgG2a.

When the spleen cells were stimulated with the immobilized anti-CD3 mAb *in vitro*, cells derived from F2-immunized mice showed the augmented response in IL-4 production while a weaker response was shown in IFN- γ production in comparison to those of the control phage-primed mice.

Thus, an F2 motif that interferes with the interaction of CTLA4 with CD80 but not CD86 preferentially generated the Th2-type immune response *in vivo*, suggesting that the predominant interaction of CD86 with CD28 in the absence of CTLA4/CD80 signaling may skew the immune response to the Th2-type *in vivo*.

II. Results

A. *In vivo* antibody response of Balb/c mice immunized with fd phage clones

Balb/c mice were administered i.p. with a PBS solution containing varying doses of wild type (wt), F2 or a control phage (L4), which was randomly selected from the library. The anti-phage IgG antibody responses were followed by using control phage (K7)-coated plastic plates (K7-ELISA). As shown in **Figure 1**, 50 μ g of F2 phage induced a marked anti-phage antibody response when compared to those of L4 and wt clones. In the case of an L4 or wt clone, 50 and 5 μ g of phage induced almost comparable IgG antibody responses, while 0.5 μ g barely induced a response. In the next experiments, mice were

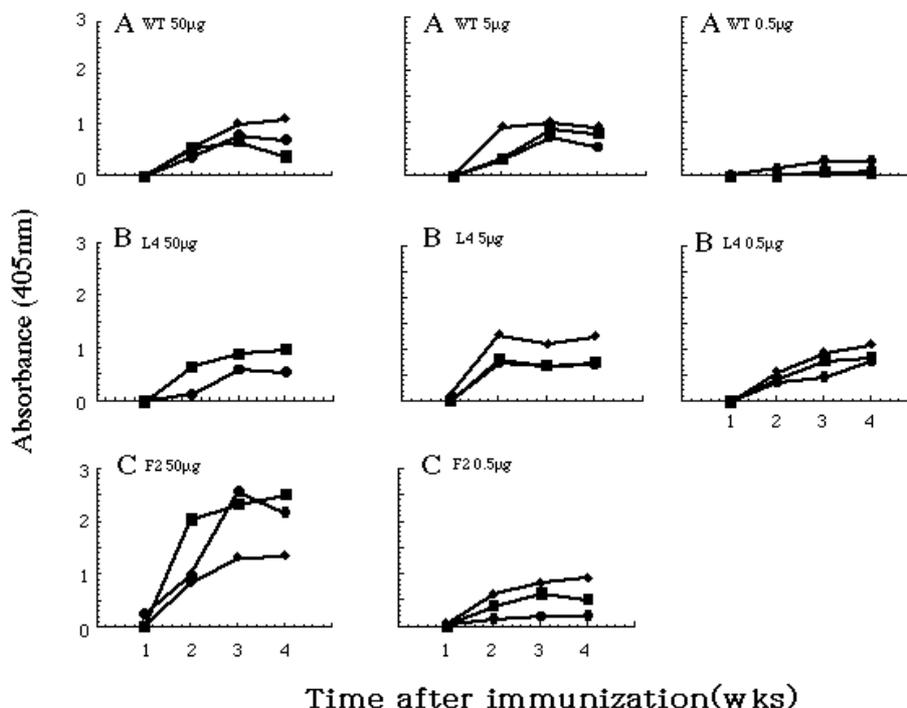


Figure 1. Antibody responses induced with intraperitoneal administration of PBS solution containing varying doses of phage clones: Balb/c mice were administered i.p. with 0.5, 5, and 50 mg of either F2, L4, or a wild-type (wt) phage clone, in PBS. An anti-phage IgG antibody was measured by a control phage (K7)-coated ELISA every week after the immunization. K7 was randomly selected from the phage library. Each line indicates a response pattern of a mouse. The serum was tested at a dilution of 1: 1080.

administered 5 μ g of a wt, L4, or F2 phage clone, and the antibody production was estimated by either the wt or K7 phage-coated ELISA plate. The wt-ELISA estimated the amount of anti-phage antibodies except for the anti-g3p antibody because the wt lacks g3p due to a frame shift, while K7-ELISA measured the amount of whole anti-phage antibodies including the anti-g3p antibody. When the antisera were assayed with K7-ELISA, F2 induced an IgG response that was approximately four times higher in comparison to those of the L4 or wt (**Figure 2A**). In contrast, when the same antisera were estimated by wt-ELISA, the magnitudes of antibody responses were almost at the same levels among these groups (**Figure 2B**). These results suggested that the amplified IgG response by F2-immunization might be directed to the specificity against g3p but not to the other constituents of phage in comparison to the wt or L4-immunization.

B. F2 motif enhanced the antibody response to g3p but not to g8p molecules

In order to determine the antigen specificity of antibodies amplified by the F2 phage clone, the antisera were assayed by using both phage clone- and HPLC-purified g3p/g8p-coated plastic plates. The sera from the L4-immunized mice showed a value by K7-ELISA that was as high as the value of the wt-ELISA (**Figure 3A**).

In contrast, the sera of the F2-immunized mice showed a value with the K7-ELISA that was approximately twice as high as that shown by the wt-ELISA. These sera were assayed by g3p or g8p-coated plates (**Figure 2B**). The sera from the L4-immunized mice reacted to both g3p and g8p with a slightly higher value of the anti-g8p antibody, while the sera of the F2-immunized mice exhibited a value to g3p that was approximately twice as high as that to g8p. The sera of the wt-immunized mice predominantly reacted to g8p but not to g3p (data not shown). Thus, the F2 motif influenced the immune response to its carrier protein molecule but not to other phage proteins associating with g3p.

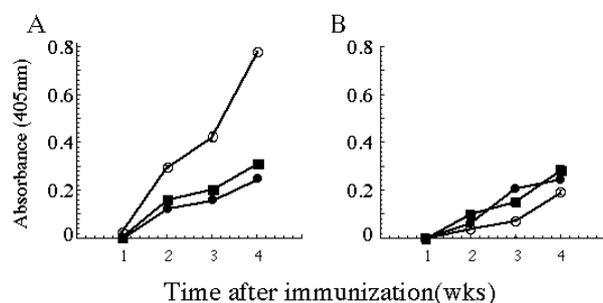


Figure 2. F2 induced the augmented anti-phage IgG antibody response relative to the control phage clones: Balb/c mice were immunized i.p. with 5 μ g of various phage clones (F2 \circ , L4 \bullet , WT \blacksquare) in PBS. Antibodies were measured by a control phage (K7)-coated ELISA (panel A) and wt phage-coated ELISA (panel B). The serum was tested at the dilution of 1: 1080.

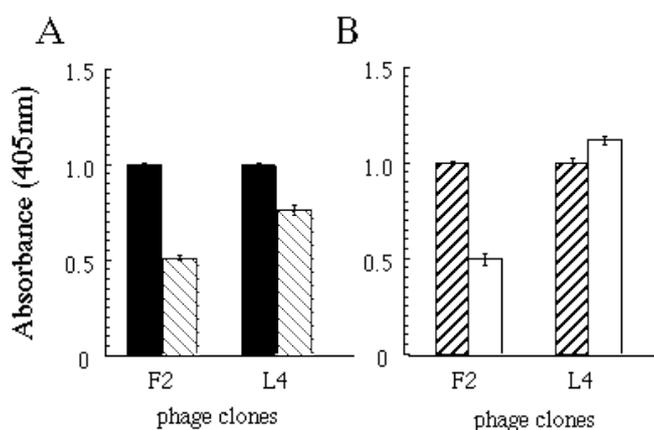


Figure 3. Preferential enhancement of an anti-g3p antibody response by the introduction of the F2 motif: Balb/c mice immunized with 50 μ g of various phage clones. Sera were collected four weeks after immunization. The amount of anti-phage IgG antibodies was measured by phage (K7 \blacksquare or wt hatched)-coated ELISA (panel A: serum dilution:1/1080) or purified g3p (hatched) or g8p (\square)-coated ELISA (panel B: serum dilution:1/500) plates. Each value represents the mean of three mice per group \pm S.E.

C. IgG1 production was preferentially augmented by the F2 motif

Next, we examined the immunoglobulin isotype on the amplified anti-g3p antibody response induced by the introduction of the F2 motif. As shown in **Figure 4**, L4 and F2 induced almost the same kinetic patterns of the IgM antibody responses. However, the IgG1 isotype was preferentially produced in the augmented anti-g3p antibody response by the immunization of F2. The IgG2a isotype was barely detected by the immunization of F2 or L4. These results suggested that the F2 motif might skew the immune response to the Th2-type. The F2 motif does not appear to accelerate the time course of the IgG antibody response because two weeks after the F2-immunization, only the IgM and not the IgG1 was significantly produced.

D. Cytokine production affected by the F2 motif in g3p

In order to examine the effect of the F2 motif on the T-cell activation, spleen cells derived from L4- or F2-immunized mice were stimulated with anti-CD3 mAb *in vitro*, and IL-4 and IFN- γ that were produced in supernatants were measured by ELISA. As shown in **Figure 5**, the control phage of L4-primed cells showed a significant amount of IFN- γ production and a very weak IL-4 production. However, in the case of the F2-primed cells, the IFN- γ production was rather decreased, and in contrast, the IL-4 production was augmented in comparison to the L4-primed cells. These results suggested that the anti-g3p antibody response might be skewed toward Th2-type immune responses by the insertion of the F2 motif. We carried out the same kind of experiments using L4 or F2 phage as an *in vitro*-stimulant instead of

anti-CD3 mAb. In these cases, we failed to detect a significant production of IFN- or IL-4 in culture supernatants (data not shown).

III. Discussion

A phage clone, F2, was isolated from a phage display library by using a CTLA4-conformation recognizing a monoclonal antibody (Fukumoto et al, 1998). The HPLC-purified F2-g3p exhibited the ability to bind to CD80 and inhibited the interaction of CTLA4 and CD80. These characteristics of F2-g3p appeared to result in the marked augmentation of the antigen-stimulated T-cell proliferation *in vitro* (Fukumoto et al, 1998).

In this study, we characterized the immune response to fd phage by simply administering the F2 phage intraperitoneally without an adjuvant to Balb/c mice. We demonstrated here that the F2 motif augmented the anti-phage antibody responses approximately two to three times higher than those of the control phage-primed mice, although the F2-mediated augmentation on T-cell proliferation *in vitro* was much more remarkable as described in previous studies (Fukumoto et al, 1998a, 1998b). The augmented response was shown in the production of the anti-g3p but not in the anti-g8p antibody (Figure 3). The anti-g8p antibody response was rather decreased by the introduction of the F2 motif in the g3p molecules. We have demonstrated in *in vitro* studies that the addition of F2-g3p augments the proliferation of the

hen egg lysozyme (HEL)-primed lymphocytes when cells are stimulated with HEL, indicating that the F2 motif is not necessarily fused with the antigen (Fukumoto et al, 1998). The F2-g3p inhibited the interaction of CD80/CTLA4 and did not inhibit the interaction of CD86/CTLA4 or CD86/CD28 (Fukumoto et al, 1998). It is, therefore, conceivable that the fusion of the F2 motif with the antigen, g3p, results in their efficient binding to the g3p-specific T lymphocytes *in vivo*.

The enhancement of this response was markedly demonstrated by the amount of IgG1 but not IgM or IgG2a isotypes (Figure 4). A characteristic cytokine profile of the F2-primed spleen cells was detected by stimulating the cells with an anti-CD3 mAb, but it was not with the F2 or L4 phage. These results may be attributed to the very small size of the F2-responding populations that were generated. The IL-4 produced from these F2-responding T cells may stimulate the generation of the bystander Th2-type T-cell populations. This amplifying process might enable us to detect IL-4 or IFN- in our cell culture system when cells were stimulated with anti-CD3 mAb.

Thus, these results suggested that the inhibition of the CTLA4/CD80 interaction with a peptide mimic of the CTLA4-binding domain may have skewed the response to the Th2 pathway, implying that the predominant interaction of CD86 with CD28, in the absence of CTLA4/CD80 signaling, preferentially induces the Th2 immune response *in vivo*.

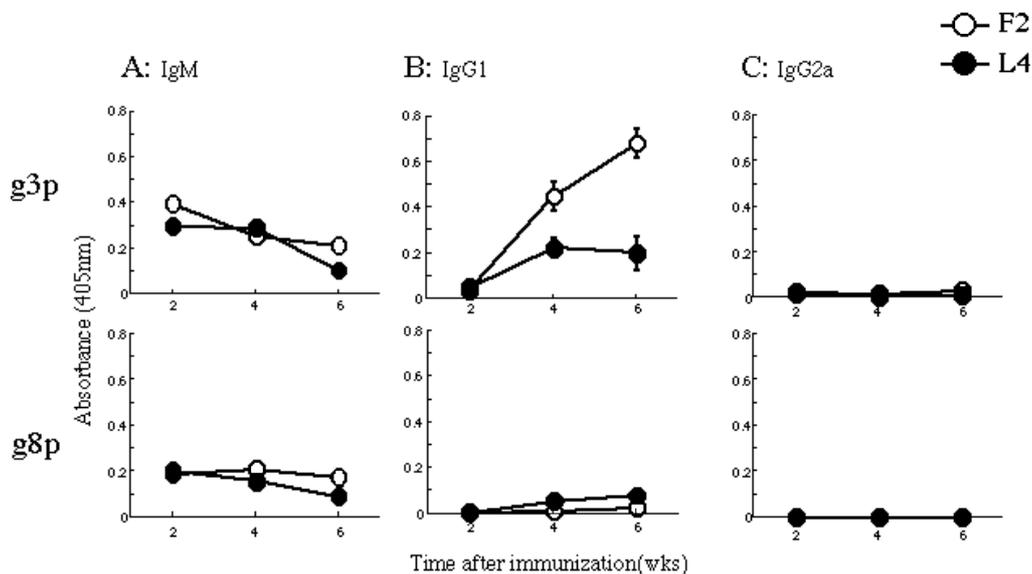


Figure 4. The F2 motif augmented the anti g3p IgG1 antibody response: Balb/c mice immunized with 50 μ g of F2 or L4 phage clones. Anti-g3p (upper panel) or -g8p antibodies (lower panel) were measured on the immunoglobulin subclasses, which were detected by AP-anti-mouse IgM (panel A), IgG1 (panel B), and IgG2a antibody (panel C). Sera were tested at a dilution of 1:500. Each value represents the mean of five mice per group \pm S.E.

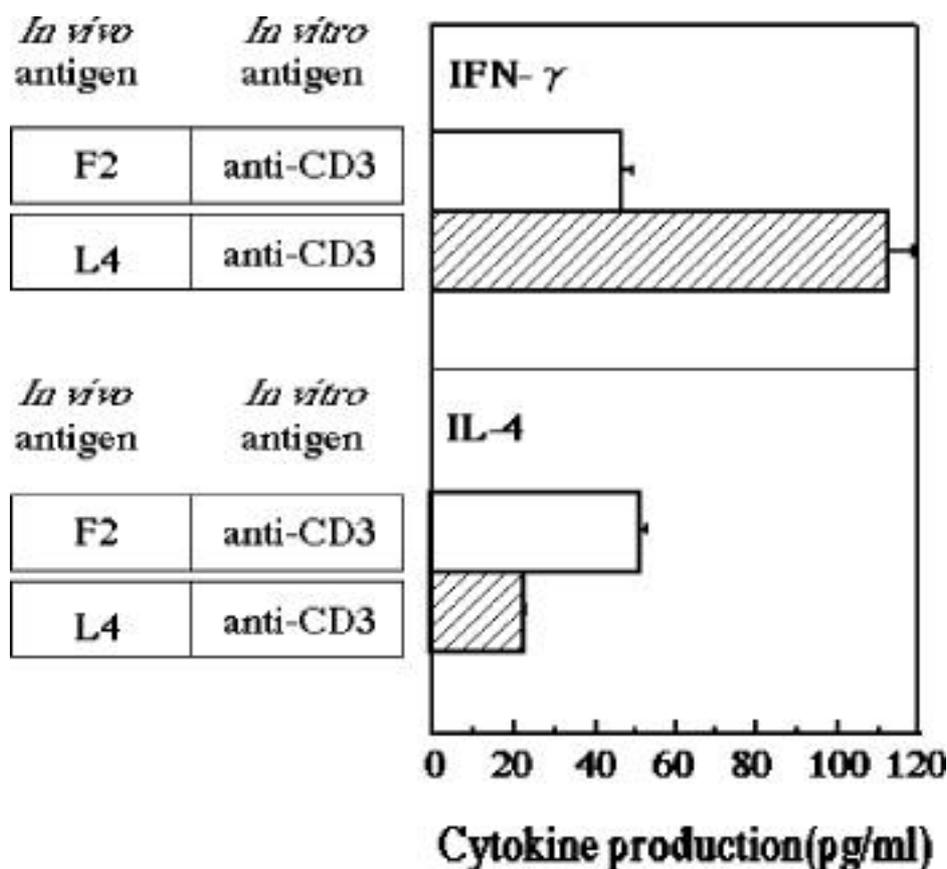


Figure 5. IL-4 production was augmented in F2-primed spleen cells relative to L4-primed spleen cells in vitro: Spleen cells were obtained from Balb/c mice, which had been immunized i.p. with 50 μ g F2 or L4 phage in PBS seven weeks before. The cells were cultured in the anti-CD3 Ab-immobilized culture plates (10 μ g/ well) for 48 hr (IL-4) or 72 hr (IFN- γ). Murine IFN- γ or IL-4 in supernatants was measured using sandwich ELISA. Each value represents the mean of five mice per group \pm S.E.

Regarding these results, studies by Freeman and coworkers indicated that CD86, but not CD80, co-stimulation during the anti-CD3 Ab-mediated CD4⁺ T-cell activation skewed the cells to produce IL-4, suggesting that CD80 and CD86 may provide distinct signals during the development of CD4⁺ T-cell responses (Freeman et al, 1995). Consistent with this, studies by Kuchroo and coworkers indicated that the administration of anti-CD86 Abs to mice during priming with proteolipid protein for induction of experimental autoimmune encephalomyelitis (EAE) skewed CD4⁺ T-cell development to the Th1 phenotype and exacerbated disease, whereas the administration of anti-CD80 Ab skewed CD4⁺ T-cell development to the Th2 phenotype and the induction of disease was blocked or decreased (Kuchroo et al, 1995). These results have suggested that CD4⁺ T-cell engagement of CD80 may direct development to the Th1 phenotype and engagement of CD86 may direct development to the Th2 phenotype.

A similar finding was reported by Khoury and Gallon et al, (1996) using a derivative of CTLA4, CTLA4IgY100F. This molecule binds to CD80 but not to CD86, which is the same characteristic as F2-g3p. These reagents are able to avoid the potential of signaling, which

is induced by the addition of anti-CTLA4 antibodies. Using the CTLA4IgY100F in the induction of experimental autoimmune encephalomyelitis (EAE), they showed that CD28-CD80 interaction may lead the response to the Th1 pathway. However, other studies have reported that the qualitative differences were not detected in the capacities of murine CD80 and CD86 to induce IL-4 production (Natesan et al, 1996). In the case of Schweitzer et al, (1997) they showed that CD86 has a more important role than CD80 in initiating antibody responses in the absence of an adjuvant and that CD86, and to a lesser extent CD80, makes significant contributions to the production of both IL-4 and IFN- γ .

CD80 and CD86 contribute to the magnitude of T-cell activation, but they do not appear to selectively regulate Th1 versus Th2 differentiation (Schweitzer et al, 1997; Schweitzer and Sharpe, 1998). A recent study by Anderson et al. shows that a CTLA4 blockade can enhance or inhibit the clonal expansion of different T cells that respond to the same antigen, depending on both the T-cell activation state and the strength of the T-cell receptor signal delivered during T-cell stimulation (Anderson et al, 2000).

Thus, it is still unclear if there is any difference in the role of CD80 as opposed to CD86 on the interaction with CTLA4. In our case, we have shown here that the selective inhibitor of CD80-binding, F2-g3p induced the skewing toward a Th2-type response when administered in mice *in vivo*. The influences of the F2-motif on the generation of cytotoxic T cells or delayed-type hypersensitivity-responsible T cells remains to be investigated.

As functional motifs of co-stimulatory molecules appear to manipulate the immune responses by introducing it into the target molecule, this strategy may lead us to novel approaches to gene therapy and DNA vaccine developments.

IV. Materials and Methods

A. Mice and antibodies

Balb/c mice (female) were purchased from Nihon SLC Co. (Fukuoka). Alkaline phosphatase (AP)-conjugated anti-mouse IgG, 1, 2a, and antibody were obtained from ZYMED (San Francisco, CA). AP-conjugated anti- μ antibody was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Anti-CD3 mAb (cat. # 01081D) was purchased from PharMingen (San Diego, CA).

B. Phage proteins

The fd (fUSE5) phage clones were isolated and the phage proteins were purified as described previously (Fukumoto et al, 1998; Nishi et al, 1996). Briefly, the fd phages (7mg/ml) were incubated with 1% sodium dodecyl sulfate (SDS) at 37°C for 20 min. The g3p and g8p were purified by size-exclusion chromatography on a HiLoad superdex 200 (26/60) column (Pharmacia, Uppsala, Sweden) as described. The F2 phage displays the motif of GFVCSGIFAVGVGGRC at the fifth position of the N-terminal of g3p molecule (Fukumoto et al, 1998).

C. ELISA

ELISA was performed as described previously (Fukumoto et al, 1998, 1998). Plastic plates (Nunc) were coated with phages (4×10^9 transducing unit [TU]/40 μ l/well) or phage proteins (30 ng / 40 μ l/well) in 50 mM Tris HCl, pH 7.5 and 150 mM NaCl (TBS) containing 0.02% NaN₃. Blocking was done by using 350 μ l of 1% bovine serum albumin (BSA, Sigma, St. Louis, MO). The plates were washed five times with TBS containing 0.05% Tween 20 (TBS/Tween) and once with TBS. After the incubation with varying concentrations of mouse antiserum for 1 hr at 4°C, AP-conjugated anti mouse IgG, 1, 2a, or μ antibody was added at a dilution of 1:250. The substrate (85 μ l) consisted of 1 mg / ml p-nitrophenolphosphate (Wako Co., Osaka), and 10% diethanolamine (Wako Co., Osaka) in TBS. Absorbance was read at 405 nm by a microplate photometer (InterMed NJ-2300, Tokyo). All assays were carried out after the dilution rates of sera were determined on their linearity for ELISA. The cytokine ELISA was performed using the IL-4 plate (cat. # M4000) and IFN- γ plate (cat. # MIF00) of R&D systems Co. (Minneapolis, MN), according to the manufacturer's description. Statistical analysis was carried out by a Student's t-test.

D. Cytokine production assay

The cell culture was carried out as described (Fukumoto et al, 1998a, 1998b). Briefly, cells (2×10^6 /1ml/well of 24 well-plate) were stimulated with varying doses of phage clones, anti-CD3 mAb (10 μ g/ml). The supernatants were harvested 72 hr later for the assay on the cytokine production. In parallel with these cultures, T-cell proliferation was monitored by culturing cells (1.5×10^5 /0.2ml/well) in flat-bottomed 96-well plates (Iwaki Glass, Tokyo) for three days and pulsing cells with 0.5 μ Ci ³H-thymidine (Amersham, St. Louis, MO) for the final 18 hr.

Acknowledgments

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