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A novel strategy for converting recombinant viral protein into high immunogenic antigen

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Interleukin 2 (IL-2) is a lymphokine promoting immune response and therefore has been investigated as an immunological adjuvant. In order to enhance the immunogenicity of recombinant viral protein, herpes simplex virus type 1 (HSV-1) glycoprotein D (gD), we gentically created a fusion protein consisting of gD and human IL-2. The fusion protein, without any other adjuvants, induced high antibody responses and cell-mediated immunity to HSV-1 in mice. Mice immunized with the fusion protein were protected against HSV-1 infection. The results indicate that IL-2-fusing can provide a means for converting a weak immunogenic protein into a high immunogenic antigen, and the strategy would be widely applicable to the other antigens for pathogens.

Interleukin 2; Fusion protein; Immunopotentiation; Glycoprotein D

1. INTRODUCTION

Recent technological advances have resulted in the production of safe subunit and synthetic peptide vaccines against infectious diseases. Unfortunately, these antigens are usually not, or weakly, immunogenic in the absence of an immunological adjuvant [1,2]. Since many of these adjuvants cause unacceptable side effects, only aluminium hydroxide (alum) is licensed for humans. However, alum is far from ideal; it only slightly increases cell-mediated immunity, and it requires refrigerated storage. In the studies to develop safe and effective adjuvants, interleukin 2 (IL-2) has been revealed to enhance immune responses when the lymphokine was administered with antigens in animals [3–5]. However, it shows only a marginal adjuvant effect because of its dispersion and short half-life in vivo. We assumed that IL-2 fused to proper antigens would be efficiently delivered together with the antigens to the site at which the immune response occurred, and would exhibit full adjuvant activity even in vivo. On the basis of this hypothesis, we genetically constructed a novel fusion protein (t-gD-IL-2) consisting of truncated herpes simplex virus type 1 (HSV-1) envelope glycoprotein D (t-gD) fused with human IL-2 and then investigated its immunogenicity in mice.

2. MATERIALS AND METHODS

2.1. *Construction of the plasmid for expressing t-gD-IL-2*

The *Bam*Hi fragment (6.6 kbp) containing gD gene which coded for a polypeptide consisting of 394 amino acid residues was cloned from HSV-1 strain Miyama (unpublished data). The *Nco*I–*Hin*fI fragment (0.83 kbp) was prepared from this fragment, following which a chemically synthesized *Eco*RI–*Nco*I adaptor (73 bp) was ligated to the *Nco*I site, and the *Hin*fI site was changed to a *Nhe*I site using a synthetic *Nhe*I linker (8 bp; Pharmacia). The resultant *Eco*RI–*Nhe*I fragment (0.9 kbp) included the truncated gD (t-gD) gene coding for amino acid residues 1 to 301 from the N-terminus. The *Hgi*AI–*Bam*HI fragment (0.43 kbp) including mature IL-2 gene which coded for 133 amino acid residues excluding a signal sequence was prepared from the plasmid pTB399 [6] (4.5 kbp) which expressed the IL-2 gene under the control of murine leukemia virus long terminal repeat (MuLV LTR). The *Hgi*AI site was then changed to a *Nhe*I site using the *Nhe*I linker. The two fragments containing t-gD and IL-2 gene were simultaneously inserted into the *Eco*RI and *Bgl*II site of pTB399 to give plasmid pHDL201 (5.4 kbp). An expression plasmid, pHDLdhfr1 (7.4 kbp), was consequently constructed as follows: the *Cla*I fragment (2.9 kbp) of pHDL201 was ligated with a *Cla*I-digested pTB348 [7] (4.5 kbp) which was the expression plasmid for the hamster dehydrofolate reductase (DHFR) gene under the control of SV40 early promoter. Another plasmid, pSHD-dhfr1 (7.0 kbp), for expressing the t-gD gene alone was similarly constructed as a control. All enzymes used for constructing plasmids and plasmic vectors were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan).

2.2. *Preparation of t-gD-IL-2 expressed in CHO cells*

The expression plasmid, pHDLdhfr1 or pHSDdhfr1, was introduced into DHFR$^-$ CHO cells, and then high-producer clones which secreted antigens at the level of several mg/liter in a serum-free medium, ASF104 (Ajinomoto, Japan), were established by amplification with 20 μM of methotrexate (Sigma). The culture supernatant (5 liter) was applied to a Butyl-Toyopeal 650M (Tosoh Co., Ltd., Japan) column. The antigens were fractionated with a linear gradient of saturated ammonium sulfate from 20 to 0%. t-gD and t-gD-IL-2 were eluted at 5 and 2%, respectively. Finally, each fraction was applied to a Sephacryl S-300 (Pharmacia) column, and then eluted with Dulbecco's phosphate-buffered saline (PBS).

2.3. *Measurement of anti-HSV antibody titers*

Anti-HSV antibody titer (IgG) was determined with a commercially available kit (Herpes Stat; Whittaker Bioproducts, USA) for detec-

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ting antibodies to HSV-1 and -2 with some modification. Microplates coated with inactivated HSV-1 and -2 were blocked with PBS containing 20% fetal calf serum (FCS) at room temperature for 2 h. Test sera diluted serially twofold with Tris-HCl buffer (40 mM Tris-HCl (pH7.5), 5% NaCl, 0.05% Tween 20, 20% FCS) were incubated on the plates at room temperature for 1 h. IgG bound to the plates were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed). Antibody titers were determined on the basis of the probit analysis [8]; a purified mouse monoclonal IgG antibody to gD, M42, was used as a reference. The titer of the reference (1.9 mg/ml) was arbitrary defined as 1.9 U/ml. Data represent mean values ± SD for 10 mice.

2.4. *Cytolytic assays*

Cell culture and analysis of killer activity were carried out according to the method reported previously [7]. Spleen cells were infected with 1×10^7 plaque-forming units (pfu) of HSV-1 strain Miyama. The HSV-1-infected or uninfected spleen cells were suspended at 2.5×10^6 cells/ml in complete RPMI 1640 medium containing 10% FCS (50 ml), and were cultured at 37°C for 5 days under humidified 5% CO_2 in air. After the cells were washed, viable cells were counted and adjusted to the proper concentration. A syngeneic macrophage cell line, P388 [9], was used. In order to prepare HSV-infected target cells, P388 cells (4×10^6) were infected with HSV-1 Miyama strain at the multiplicity of infectious dose of 1, and incubated at 37°C for 1 h. After the infection, these cells were immediately labelled with ^{51}Cr. Uninfected P388 cells were similarly labelled with ^{51}Cr. The effector spleen cells were mixed with the HSV-1-infected or uninfected target cells at the indicated ratio, and were incubated in microplates at 37°C for 4 h. Lysis of the target cells was determined from the amounts of ^{51}Cr released into the culture supernatant. Data represent mean values in duplicate assays.

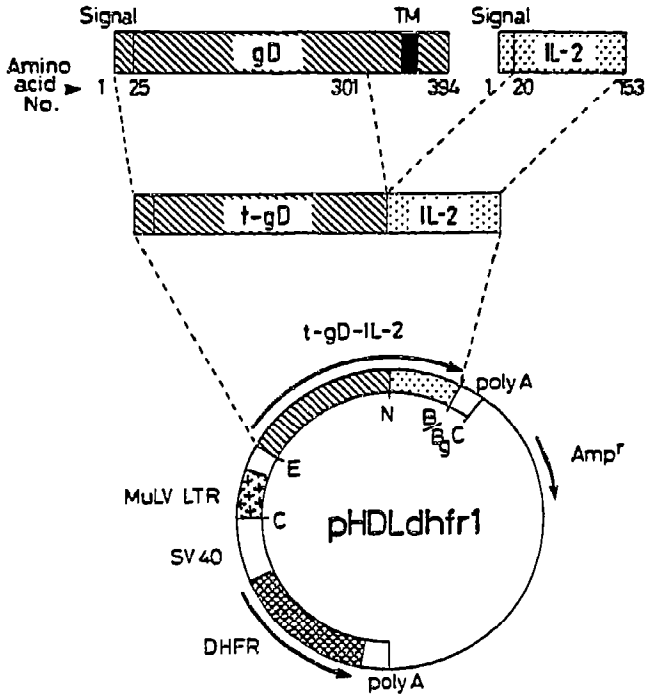

Fig. 1. Construction of t-gD-IL-2 expression plasmid. Abbreviations represent as follows; TM = transmembrane domain, E = EcoRI, N = NheI, B = BamHI, Bg = BglII, C = ClaI, Poly A = polyadenilation site.

3. RESULTS AND DISCUSSION

As shown in Fig. 1, we constructed a plasmid, pHDLdhfr1, to express the fusion protein composed of gD and human IL-2 in mammalian cells: the IL-2 gene excluding the signal sequence was fused to the truncated gD (t-gD) gene from which the coding sequence of the C-terminal region including the transmembrane (TM) domain had been removed. Another plasmid to express t-gD alone was similarly constructed as a control. These plasmids were introduced into Chinese hamster ovary (CHO) cells, and clones secreting t-gD or IL-2-fused t-gD (t-gD-IL-2) into the culture supernatant were established. These antigens were purified by the procedure consisting of hydrophobic column chromatography and gel-filtration. The purity of t-gD and t-gD-IL-2 was estimated to be 95 and 79%, respectively from densitometric analyses (Fig. 2). t-gD-IL-2 could support growth of IL-2-dependent cells in vitro, indicating that the fused IL-2 retained its biological activity (37% of specific activity compared with recombinant human IL-2).

We immunized BALB/c and C57BL/6 mice subcutaneously (s.c.) with t-gD, t-gD mixed with recombinant human IL-2 (rIL-2), t-gD-IL-2, or t-gD adsorbed on alum, and then evaluated serum anti-HSV antibody responses by enzyme immunoassay (EIA) (Table I). After a primary immunization, t-gD alone induced little anti-HSV antibody production in both strains. Although the mixture of t-gD and rIL-2 elicited several times higher response than t-gD alone in BALB/c mice, it only slightly induced the antibody production in C57BL/6 mice. In contrast, t-gD-IL-2 remarkably induced the antibody production in both strains: it was 27 to 77 times more potent than t-gD alone, and 17 to 20 times more potent than the mixture of t-gD and rIL-2 in

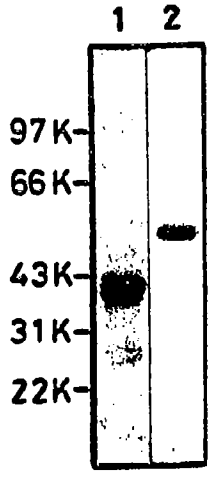

Fig. 2. Electrophoretic analysis of Purified t-gD-IL-2. The purified antigens were electrophoresed on a 12% polyacrylamide SDS gel [14], followed by Coomasie blue staining. Lanes correspond to 10 μg of purified (1)t-gD and (2)t-gD-IL-2. The molecular weight of t-gD and t-gD-IL-2 were about 40 000 and 54 000, respectively. Densitometric analyses were performed using a Video-densitometer (Bio-Rad).

Table I

Efficient production of anti-HSV antibody in mice immunized with t-gD-IL-2

Antigen[a]	Anti-HSV antibody titer (mU/ml)		
	Primary (dose/mouse)		Secondary (dose/mouse)
	1 μg	5 μg	1 μg
(BALB/c mice)			
Control	< 7	nd	nd
t-gD	< 15	9 ± 4	1,018 ± 1,833
Mixture of t-gD and rIL-2[b]	23 ± 29	35 ± 38	nd
t-gD-IL-2	400 ± 292	692 ± 442	46,183 ± 38,443
t-gD adsorbed on alum[c]	341 ± 267	481 ± 451	nd
(C57BL/6 mice)			
Control	< 14	nd	nd
t-gD	< 14	nd	nd
Mixture of t-gD and rIL-2	< 14	nd	nd
t-gD-IL-2	1,465 ± 802	nd	nd
t-gD adsorbed on alum	88 ± 120	nd	nd

[a]Eight-week-old female BALB/c or C57BL/6 mice (10/group) were s.c. immunized with the indicated antigens in 200 μl PBS. Control mice were injected with PBS alone. Taking the purity into consideration, the amounts of purified t-gD and t-gD-IL-2 were properly adjusted to give the indicated doses. For the primary response, mice were bled 5 weeks after the immunization. For the secondary response, mice were primarily immunized with t-gD or t-gD-IL-2 at the indicated dose, and 4 weeks later they received a secondary immunization of the same dose. Sample sera were prepared 2 weeks after the secondary immunization.
[b]rIL-2 was mixed with the indicated dose of t-gD in the molar ratio of 1 : 1, i.e. 0.25 μg and 1.25 μg of rIL-2 were added to 1 μg and 5 μg of t-gD, respectively.
[c]Alum (100 μg) was admixed with the indicated doses of t-gD. nd, not done.

Table II

Induction of killer cells in spleen cells immunized in vivo with t-gD-IL-2 after in vitro stimulation with HSV-1

Immunization in vivo	Stimulation with HSV-1 in vitro	HSV-1 infection of target cells	Lysis (%) Effector/target ratio		
			25	50	100
Control	–	–	< 1	< 1	< 1
	–	+	< 1	< 1	< 1
	+	–	< 1	< 1	< 1
	+	+	< 1	< 1	< 1
t-gD	–	–	< 1	< 1	nd
	–	+	< 1	< 1	< 1
	+	–	< 1	< 1	nd
	+	+	< 1	< 1	< 1
Mixture of t-gD and rIL-2	–	–	< 1	< 1	< 1
	–	+	< 1	< 1	< 1
	+	–	nd	nd	nd
	+	+	< 1	< 1	nd
t-gD-IL-2	–	–	< 1	< 1	< 1
	–	+	< 1	< 1	< 1
	+	–	14.7	19.7	24.8
	+	+	26.5	34.3	38.7

BALB/c mice 4/group) were immunized with t-gD, a mixture of t-gD and rIL-2, or t-gD-IL-2 at a dose of 5 μg/mouse in the same manner as described in the footnote of Table I. Control mice were immunized with PBS alone. Five weeks after the immunization, spleen cells were obtained from each group of mice and pooled. nd, not done.

BALB/c mice; furthermore, it was at least 100 times more potent than tgD alone or tgD mixed with rIL-2 in C57BL/6 mice. Upon a secondary immunization, t-gD-IL-2 was 45 times more potent than t-gD alone in BALB/c mice. These results demonstrated that the fused IL-2, but not the IL-2 mixed with the antigen, acts as an extremely potent adjuvant, although the responses differed somewhat between the two strains. In addition, the fused IL-2 was comparable to or slightly more potent than alum in BALB/c mice; however, it was 17 times more potent than alum in C57BL/6 mice. Although anti-HSV antibody titers were measured by the ELISA method, we observed that these antibody titers almost corresponded to significance of virus neutralizing activities (unpublished). The mechanism by which alum exhibits its adjuvant effect is generally thought to depend on creating a depot at the injection site, and it prolongs the release of antigens and stimulates antigen presenting cells [1,10]. However, the fused IL-2 is expected to act in a different way. In fact, we have observed that t-gD-IL-2 can elicite the specific antibody production upon intravenous administration. In addition, t-gD-IL-2 and alum synergistically enhanced the antibody production (unpublished).

We next examined whether t-gD-IL-2 could induce cell-mediated immunity. BALB/c mice were immunized with t-gD, a mixture of t-gD and rIL-2, or t-gD-IL-2. The primed spleen cells were then assayed for in vitro cytotoxity. As shown in Table II, spleen cells obtained from mice immunized with t-gD-IL-2, but not t-gD or the mixture of t-gD and rIL-2, showed apparent killer activity against both HSV-infected and uninfected P388 target cells when restimulated with HSV-1. The percentage of lysis in HSV-1-infected target cells was higher than that in uninfected target cells, indicating that both non-specific and HSV-1-specific killer cells were induced in the spleen cells immunized with t-gD-IL-2. Since the target used here was a macrophage cell line [9] and the killer cells failed to lyse HSV-1-infected syngenic fibroblast cells (unpublished), the specific killing was thought to be major histocompatibility complex class II-restricted. These results indicated that t-gD-IL-2 could efficiently induce cell-mediated immunity as well as humoral immunity.

Such a highly immunogenic property of t-gD-IL-2 was shown in response to live virus challenge. Fig. 3 shows that mice immunized with 1 μg of t-gD-IL-2 were protected from lethal HSV-1 infection. Most mice immunized with t-gD died within 6–13 days after virus inoculation. Immunization with the mixture of t-gD and rIL-2 could reduce the incidence of HSV-1 infection, while most mice immunized with t-gD-IL-2 survived during the experimental period, although one mouse died on day 11. IL-2 was shown to protect neonatal mice from lethal HSV infection [11], and treatment of HSV-infected guinea pigs with IL-2 reduced the frequency and severity of genital recurrences [12]. In addition, we preliminary observed that the mice administered with t-gD-IL-2, but not t-gD, after HSV-1 inoculation, were completely protected against viral infection even in drug-induced immunosuppressive state (unpublished). Thus, we think that t-gD-IL-2 may be a useful immunotherapeutic agent against recurrent HSV disease, because immunosuppression is thought to be a major trigger of recurrence of HSV [13].

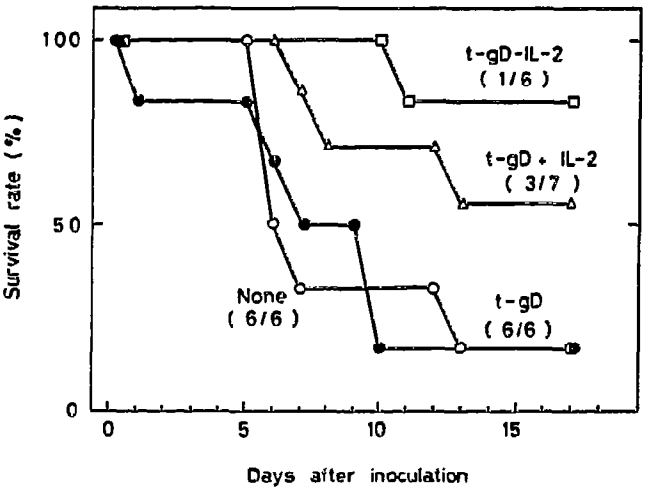

Fig. 3. Protection of BALB/c mice from HSV infection by immunization with t-gD-IL-2. BALB/c mice were s.c. immunized with t-gD (●), a mixture of t-gD and rIL-2 (△) or t-gD-IL-2 (□) at a dose of 1 μg/mouse in the same manner as described in the footnote of Table I. Control mice were injected with PBS alone (○). Five weeks after the immunization, the mice were intraperitoneally inoculated with 2×10^4 pfu of HSV-1 strain Miyama (100 LD50) and then monitored daily for a period of 17 days. A number of parenthesis represents the symptomatical and dead mice per total mice.

In this study we demonstrated that IL-2 fused to the proper antigen could exhibit very potent adjuvant activity in vivo. As a possible mechanism, the prolonged serum half-life of antigen-fused IL-2 might interpret the high immunogenicity of t-gD-IL-2. However, t-gD-IL-2 showed only marginal aduvant effect on antibody response to human serum albumin when the unrelated antigen was administered with t-gD-IL-2 (unpublished). Alternatively, IL-2 delivered together with t-gD to regional lymphoid tissues may efficiently activate the gD-specific lymphocytes. We think that the lymphokine fused with the antigen would be useful as a safe adjuvant to overcome the various drawbacks associated with the conventional adjuvant, alum. Our strategy reported here would be widely applicable to other antigens and lymphokines.

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