Journal of Microbiology and Biotechnology
Vol. 7. No. 6, 423-428, 1997

Preparation of a *Vibrio vulnificus* Vaccine with Immunogenicity and Protective Efficacy

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Vibrio vulnificus is a halophilic gram-negative human pathogen, which affects people with underlying liver diseases or a suppressed immune system, often leading to primary septicemia with a mortality rate of higher than 60%. In an effort to develop an oral vaccine against *V. vulnificus* infection, we prepared a whole cell killed vaccine of *V. vulnificus* on a large scale and compared the immunogenicity and protective efficacy of the vaccine administered in three formulation forms in rabbits. Since *V. vulnificus* O-antigen serotypes 1, 2, 3, 4, 5, and 7 account for more than 95% of clinical isolates, we prepared cell lysates from these six serotype strains and mixed in equal amounts for a vaccine. The vaccine was administered to rabbits intramuscularly (i.m.), orally as granules or as enteric-coated granules. In rabbits, all three formulation forms elicited a high level of serum IgG antibody reactive not only to the six strains but also to other O-antigen serotypes 6, 8 and 9, indicating cross-reactivities among the strains. Immunotherapeutic efficacy of the antisera was also evaluated by a passive immunization assay, which revealed that the orally immunized antisera as well as the i.m. immunized antisera was protective against a subsequent lethal challenge of *V. vulnificus*. These data demonstrate that oral immunization with a *V. vulnificus* whole cell lysate vaccine induced a systemic immune response and suggest the feasibility of development of this vaccine preparation as an oral vaccine.

Vibrio vulnificus is an estuarine gram-negative bacterium that has been recognized as a human pathogen. *V. vulnificus* infection is mediated primarily by consumption of raw fish, especially oysters, or by exposure of preexisting wounds to seawater (5, 15). *V. vulnificus* especially affects people with chronic hepatitis, alcoholic cirrhosis, diabetes mellitus or other underlying diseases (18). Even with an extensive antibiotic therapy, *V. vulnificus* infection often ends up with permanent damages, and the mortality rate of primary septicemia resulting from the infection is higher than 60% (17). Major virulence factors associated with *V. vulnificus* pathogenesis include capsular polysaccharide (CPS), lipopolysaccharide (LPS), an extracellular cytolysin and an elastase (1, 4, 10, 13, 19-22). Previous studies for vaccine development have been mainly focused on CPS and LPS. Kreger *et al.* (11) showed formalin-killed cells of virulent strains elicited strong immune response and conferred protection against the homologous strain and suggested that the heat labile surface polysaccharide is a major protective antigen. Devi *et al.* (2, 3) used CPS of carbotype 1 *V. vulnificus* as a conjugate with either tetanus toxoid, cytolysin or elastase. Among them, a CPS-tetanus toxoid conjugate, when used with Ribi-adjuvant, elicited a high titer of antibody which was protective against carbotype 1 *V. vulnificus* infection (2, 3, 11). LPS, a major cell surface component of gram-negative bacteria, is also known to be highly immunogenic and antibodies to LPS have been demonstrated to be protective against gram-negative infections. Toxicity, however, associated with this promising candidate limits its use as vaccines. In addition, serotype specificity of CPS and LPS makes it difficult to develop a vaccine with a wide protection range. Recently, Ju *et al.* (7) have shown that a whole cell killed vaccine of *V. vulnificus* conferred protection from a lethal challenge of the homologous strain and that IgG purified from hyperimmune sera was also protective, claiming that O-antigen in the preparation was responsible for the immunity. Surprisingly, however, the antisera also showed cross-protection against heterologous strains.

In an attempt to develop an oral vaccine against *V. vulnificus*, we prepared a *V. vulnificus* vaccine made of whole cell lysates on a large scale and investigated the immunogenicity and protective efficacy of the vaccine

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Key words: *Vibrio vulnificus*, vaccine preparation, immunization route, immunogenicity, protective efficacy

when administered in three different formulation forms.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

V. vulnificus strains, originally isolated from hospital patients, were kindly provided by Dr. S.D. Park of Wonkwang University, Iksan, Korea. The strains were identified by an API test using an api 20 NE kit (Bio Merieux S.A., France), characterized for their biochemical and serological properties and designated CJVV001-CJVV009 according to serotypes. Six O-antigen serotypes 1, 2, 3, 4, 5 and 7, were used to prepare a vaccine. *V. vulnificus* strains were grown on 3× YPS (0.9% yeast extract, 3% pepton, 1% NaCl) agar plates at 37°C and maintained at 20°C, or lyophilized and stored in glass ampules at 4°C.

To establish optimal growth conditions for *V. vulnificus* strains, *V. vulnificus* was cultured in 500 ml of liquid media in a fermenting jar at 37°C under various conditions. For a large scale fermentation, a 5-liter subculture of each *V. vulnificus* strain in a log phase was inoculated into 100 liters of fresh 3× YPS media containing 0.005% Neolin 400 (Korea Polyol, Korea) as an antifoaming agent in a 150-liter jar fermenter. The culture was grown with stirring at 250 rpm for 6~14 h, while being aerated at 0.25 v/v/min. Absorbance was measured spectrophotometrically at 600 nm at intervals of an hour or two.

Preparation of *V. vulnificus* Whole Cell Lysate

After cultivation, bacterial cells were boiled in the fermenter at 100°C for 2 h. The resulting cell lysate was transferred to the Sartocon II system (Sartorius, Germany) and concentrated to one-tenth of the original culture volume by filtration on a 0.2 μm filter. Then the lysate was washed extensively with 100 liters of 0.9% saline until the final volume decreased to 8 liters, which was lyophilized and stored at -20°C until used. Fifty grams of each *V. vulnificus* dried cell lysate was dissolved in 500 ml of distilled water, mixed and passed twice through M-110 EH Microfluidizer (Microfluidics Corp., U.S.A.) at 10,000 psi. The cell lysate suspension was directly used for intramuscular (i.m.) injection or for preparation of granular forms of the vaccine.

Preparation of *V. vulnificus* Vaccine Granules

V. vulnificus cell lysate was granulated using a granulator (SPIR-A-FLOW, Freund Industrial, Japan). Cell lysate suspended in 20% hydroxypropyl methylcellulose (Pharmacoat 9603, Pharmacia, U.S.A.) aqueous solution was sprayed at 8.5 ml/min to form granules with corn starch as nucleus. The inlet air temperature was 70~80°C; granule temperature, 25~30°C; and rotor speed, 300 rpm. Granules were enteric-coated with 6% hydroxypropyl methylcellulose phthalate 55 (Shin-Etsu Chemical Co., Japan) solution in the granulator, and coating conditions were as follows: liquid spray speed, 15 ml/min; inlet air temperature, 62°C; granule temperature, 35°C; and rotor speed, 300 rpm.

The granule size distributions were determined by passing granules through a series of sieves. Enteric-coated granules were also tested for gastric resistance and disintegration in simulated intestinal fluid. For this, 0.5 g of enteric-coated granules was suspended in 50 ml of simulated gastric fluid (0.2% NaCl, 0.24% HCl, pH 1.2) or simulated intestinal fluid (0.05 M KH_2PO_4, 0.23 N NaOH, pH 6.8) and stirred at room temperature for 2 h. Protein content released from the granules was determined using a Biorad protein assay kit (Biorad, U.S.A.). Gastric resistance of granules was expressed as the percentage of protein remaining in enteric-coated granules in simulated gastric fluid to that released in simulated intestinal fluid.

Immunization and Antibody Analysis

Japanese white rabbits, weighed 2.2~2.3 kg, purchased from Samyook Experimental Animals Co. (Korea), were immunized five times at four-day intervals (on days 0, 4, 8, 12 and 16) with the *V. vulnificus* vaccine corresponding to 10 mg cell lysate. For i.m. immunization, 0.2 ml of cell lysate suspension (50 mg/ml) were used. For oral administration, granules and enteric-coated granules were suspended in 3 ml of distilled water and 0.5% carboxy methylcellulose suspension (pH 2.5), respectively, and fed to rabbits using a tube. Blood samples were drawn from ear veins and kept at room temperature for 1 h before centrifugation. The sera were kept frozen at -70°C until used.

Antibody titers induced by the *V. vulnificus* vaccine were determined against *V. vulnificus* whole cells of various O-antigen serotypes by enzyme-linked immunosorbent assay (ELISA). A 96-well microtiter plate (Costar, U.S.A.) was coated with 100 μl of bacterial cell suspension, which had been grown in 3× YPS at 37°C for 6 h, heat-killed at 56°C for 2 h and adjusted to OD_{600}= 0.1 in phosphate-buffered saline (PBS). Wells were blocked with 300 μl of 1% bovine serum albumin in PBS for 2 h and washed three times with PBS containing 0.05% Tween 20 (PBST). One hundred microliter of twofold serial dilutions of rabbit antisera was added and incubated for 2 h at 37°C. After three rinses in PBST, bound IgG antibodies were detected with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Accurate, U.S.A.). *o*-Phenylenediamine dihydrochloride (Sigma, U.S.A.) was used as a chromogenic substrate, and absorbance was measured spectrophotometrically at 490 nm using a Wellscan ELISA reader (Denley, U.S.A.).

Protection Assays

Protective efficacies of antisera from immunized rabbits

were evaluated by passive immunization experiments. Five week-old ICR male mice were purchased from Charles River Japan Inc. Groups of 8 mice were given intraperitoneally (i.p.) 0.3 ml of antisera from immunized rabbits. The control groups were given i.p. 0.3 ml of saline or rabbit pre-immune sera. Two hours later, mice were challenged i.p. with 4.7×10^7 cfu of the CJVV004 strain and observed for 5 days postinfection. Results were expressed as the percentage of protection by comparing the number of survivors against the total number of mice challenged in each group.

RESULTS

Preparation of a *V. vulnificus* Vaccine

The prevalence of *V. vulnificus* strains have been reported to be different between natural environment and clinical infections (6, 9, 12, 16). In nature, antigen serotypes O1 and O4 were the most prevalent strains (17% and 18%, respectively), followed by serotypes O2, O7 and O8. Among hospital patients, however, serotype O4 was found to be a major type, accounting for more than 60% of clinical isolates, and 95% of clinical strains belonged to serotypes O1, O2, O3, O4, O5 or O7. In an attempt to maximize the protection range of a *V. vulnificus* vaccine, we prepared a vaccine with 6 strains together, each one strain from serotypes O1, O2, O3, O4, O5 and O7.

In order to optimize culture conditions before a main culture, we investigated growth characteristics of *V. vulnificus* strains in a 5-liter jar scale. Supplement of YPS media with 1% galactose, lactose or sucrose as a carbon source did not affect the growth, while fructose, glucose or maltose drastically reduced the growth rates and eventually led to cell lysis (Fig. 1). This seemed to be due to accumulation of organic acids in the culture media (data not shown). *V. vulnificus* strains showed similar growth rates under medium conditions at pH 5.0 to pH 9.0, and constant adjustment of pH to 7.0~7.2 by adding NH_4OH or NaOH did not promote the bacterial growth. Since *V. vulnificus* is halophilic (8), we tested an effect of NaCl concentration on the bacterial growth. NaCl concentration ranging from 1% to 4% promoted the bacterial growth but a concentration over 4% was inhibitory (data not shown). High speed stirring (350 rpm) or aeration greatly enhanced the cell growth (data not shown). Based on the results from small scale cultures, we set up large scale fermentations of *V. vulnificus* strains at 37°C in 3×YPS media in a Chemap fermenter (Switzerland) with a working volume of 100 liters. In this condition, the cultures of strains CJVV001, CJVV004 and CJVV007 reached a stationary phase at 6~7.5 h after inoculation and the final OD_{600} was as high as 9.2. CJVV005 was the slowest to reach a stationary phase (14.5 h after inoculation), and CJVV002 showed the lowest value in the final OD_{600} (7.6) (Table 1 & Fig. 2).

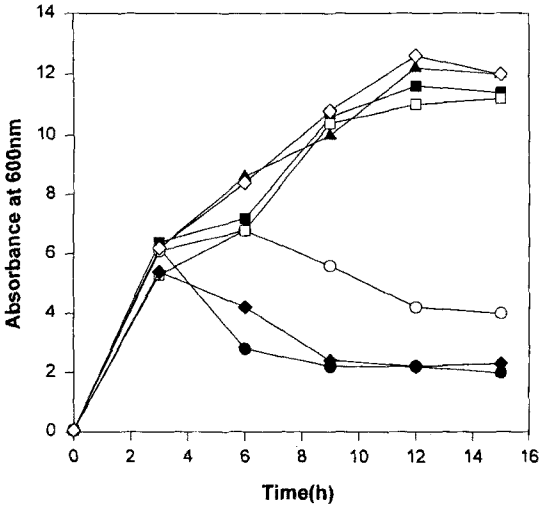

Fig. 1. Effects of various sugars on growth of *V. vulnificus*.
Five mililiters of *V. vulnificus* strain CJVV004 culture in a log phase was inoculated into 500 ml of 3×YPS media with or without various sugars (1%) and cultured at 37°C with stirring. Absorbance was measured at 600 nm every 3 h. ■, control; ◆, fructose; ▲, galactose; ●, glucose; □, lactose; ○, maltose; ◇, sucrose.

The fermentation culture was boiled at 100°C for 2 h to kill the bacteria and to inactivate cytolytic toxin secreted into the media, and subjected to concentration and extensive washing. After lyophilization, the final yield was between 360~540 g in dry weight, depending on strains (Table 1). Since the purpose of this study was to develop an oral vaccine, we prepared granular forms of the vaccine. After granulation, a majority of granules fell between 60~80 mesh in size comprising 61% of total granules. The granules were then enteric-coated to protect vaccine components from low pH in stomach. After coating, 81% of total coated granules was between 40~80 mesh in size, which was used for further experiments

Table 1. Large scale fermentation of *V. vulnificus* vaccine strains*.

Strain	Final absorbance (OD_{600})	Culture time (h)	Dry weight of cell lysate (g)
CJVV001	9.2	6.0	430
CJVV002	7.6	9.0	366
CJVV003	8.6	9.0	406
CJVV004	10.0	7.5	471
CJVV005	8.2	14.5	543
CJVV007	9.8	6.0	450

*Each strain of *V. vulnificus* was grown in 100 liters of 3×YPS media until it reached a stationary phase and processed to dry cell lysate as described in Materials and Methods.

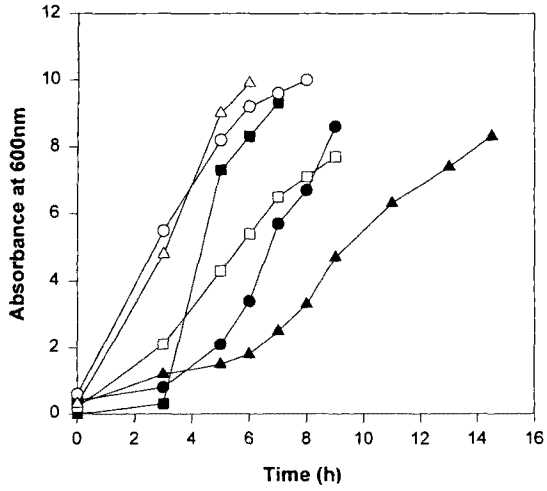

Fig. 2. Growth patterns of *V. vulnificus* strains in a large scale fermentation.
■, strain CJVV001; □, CJVV002; ●, CJVV003; ○, CJVV004; ▲, CJVV005; △, CJVV007.

(data not shown). The coated granules were tested for gastric resistance and disintegration rate. In simulated intestinal fluid, there was no particles visible after 2 h-stirring, indicating that the content of the granules would be completely released in intestine upon oral administration. The protein released from enteric-coated granules in simulated gastric fluid and simulated intestinal fluid was 350 μg and 28 mg per g granules, respectively. Thus, the gastric resistance index of the coated granules was 98.8%, which is considered to be an acceptable level in the actual application.

Immunogenicity and Protective Efficacy of the Vaccine

The cell lysates from six vaccine strains were mixed in equal amounts and used to immunize rabbits. General patterns of serum IgG antibody responses induced by the *V. vulnificus* vaccine were similar regardless of formulation forms of the vaccine, i.e., the antibody titer began to rise on day 8, reached a peak on day 25 and slightly decreased thereafter (Fig. 3). There were, however, differences in the actual antibody titer elicited by each formulation form, depending on strains used as ELISA antigens. As shown in Fig. 4, to strains CJVV001, CJVV002, CJVV004, CJVV005, CJVV008, and CJVV009, oral administration of enteric-coated granules induced high antibody responses comparable to that by i.m. immunization, but to strains CJVV006 and CJVV007 lower titers were induced, approximately 60% of those induced by i.m. immunization. Oral immunization with uncoated granules elicited serum IgG antibody levels corresponding to 60~90% of those elicited by enteric-coated granules. For all three vaccine forms, the induced antibody showed strong binding activity to whole cells

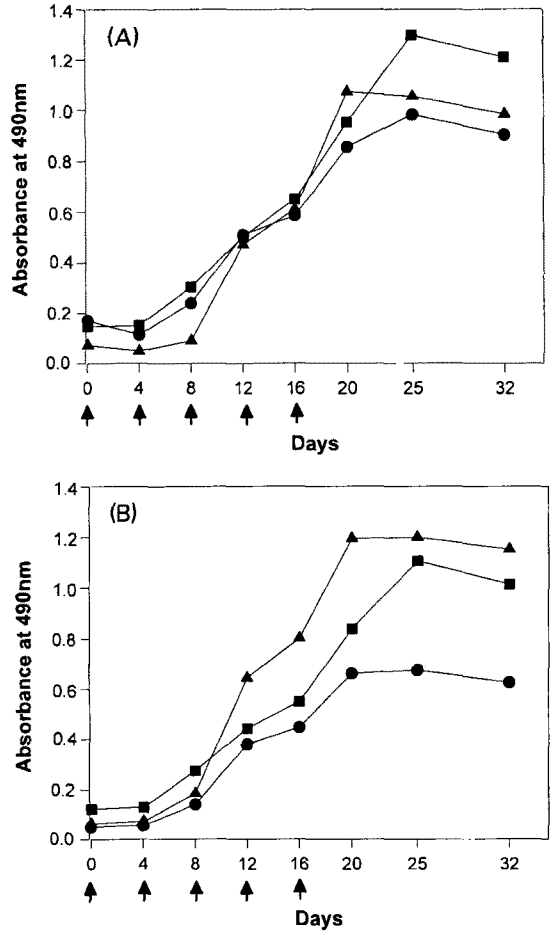

Fig. 3. Serum IgG titers of rabbits immunized with the *V. vulnificus* cell lysate in three formulation forms.
Ten miligrams of *V. vulnificus* cell lysate was administered five times at four-day intervals (marked as arrows) to rabbits by either i.m. injection of cell lysate suspension (▲), oral administration of granules (●) or of enteric-coated granules (■). Blood samples were collected on the day as noted on the x-axis and antibody titers in antisera were determined against *V. vulnificus* strains CJVV001 (A) and CJVV004 (B) by ELISA at a 1 : 1600 dilution.

of strains CJVV006, CJVV008 and CJVV009 as well as to those of the vaccine strains, indicating high cross-reactivities among the strains (Fig. 4).

To determine whether the antibody response induced by the immunization is correlated with protective efficacy against *V. vulnificus* infection, the immunized sera were also evaluated for immunotherapeutic efficacies. All the mice injected i.p. with i.m.-immunized antisera survived for 5 days after a lethal challenge of CJVV004, showing 100% protection rate, while 5 out of 8 mice (62.5%) injected with orally immunized antisera recovered from the infection (Table 2). All the mice of control groups pretreated with saline or rabbit pre-immune serum, on the other hand, succumbed the next day after

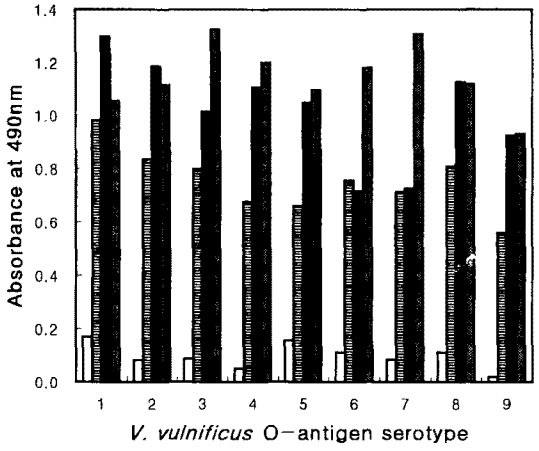

Fig. 4. Cross-reactivity of antibody elicited by the *V. vulnificus* vaccine to various *V. vulnificus* O-antigen serotypes.
Rabbits were immunized with the *V. vulnificus* lysate made of six strains, and blood samples were collected on day 25. Serum IgG antibody responses were measured against whole cells of various O-antigen serotypes by ELISA at a 1 : 1600 dilution. □, control; ▤, oral administration of granules; ■, oral administration of enteric-coated granules; ⊠, i.m. injection.

Table 2. Immunotherapeutic efficacy of antisera of rabbits immunized with the *V. vulnificus* vaccine in mice.

Rabbit antiserum	No. of mice (survived/challenged)*				% Survival
	Day 0	Day 1	Day 2	Day 5	
No serum (saline)	8/8	0/8	0/8	0/8	0.0
Pre-immune serum	8/8	0/8	0/8	0/8	0.0
Oral administration of granules	8/8	8/8	5/8	5/8	62.5
I.m. injection of cell lysate	8/8	8/8	8/8	8/8	100.0

*Groups of mice were passively immunized i.p. with 0.3 ml of antiserum or saline per mouse, and 2 h later challenged with *V. vulnificus* strain CJVV004. Mice were observed for death up to 5 days post-infection.

the infection. These results indicate that antibody induced by the vaccine is protective against an acute *V. vulnificus* infection.

DISCUSSION

In developing vaccines, safety and efficacy, i.e., immunogenicity and protective capacity, are the first priority to consider. Secondly, since a vaccination program is generally a procedure practiced to a mass population, convenience in storage and application as well as the cost for mass production is a factor that should not be ignored. From this point of view, an oral vaccine is an ideal form which is easier to manufacture than others and convenient to handle and administer. One major problem in developing an oral vaccine, is associated with findings that mucosal immunization does not elicit consistent immune responses. The aim of the present study was to establish a large scale manufacturing process for a *V. vulnificus* vaccine and to determine whether mucosal immunization with the *V. vulnificus* vaccine induce a systemic immune response. This study showed that *V. vulnificus*-specific antibody was detected in the antisera of orally immunized rabbits, and the antibody level was comparable to that induced by systemic immunization, suggesting the feasibility of oral vaccination. In addition, it was confirmed by a passive immunization assay that the antibody elicited by oral immunization was protective. In this study, the cost for producing enteric-coated granules was higher and the yield was approximately 50% of regular granules. The immune response induced by enteric-coated granules, however, was higher (up to 50%) than that by granules for most of strains (Fig. 4). Thus, it needs to consider both efficacy and production cost in order to determine which is the better formulation.

It has been demonstrated by Ju *et al.* (7) that *V. vulnificus* cell lysate elicited serum antibody that conferred protection from lethal infections as determined by active immunization of rabbits. Our study further showed that systemic immune response was induced by mucosal immunization with a *V. vulnificus* vaccine. It also appears that enteric-coating of granules enhanced immunogenicity of the vaccine by protecting vaccine components from harsh conditions in stomach. Ju *et al.* (7) suggested that the immune response and protection given by *V. vulnificus* whole-cell lysate were due to O-antigen on LPS. Cross-protective ability of the vaccine preparation, however, strongly suggest that a common antigen(s) other than O-antigen on LPS is responsible for the protection (7). This is also supported by our observation that the rabbit immune sera were highly reactive with whole cells of strains not used for preparation of the vaccine (Fig. 4). Our preliminary studies with a serotype O4 strain also revealed that oral immunization with serotype O4 lysate elicited antibody reactive to all serotypes, confirming the cross-reactivities among strains (data not shown). One possible explanation for this is that the immunogen in the lysate is outer membrane proteins, which have been shown, in our Pseudomonas vaccine, to be a good immunogen and to be able to confer protection against a subsequent bacterial infection (14). The nature of immunogens in the *V. vulnificus* vaccine, however, are to be confirmed. Whether this cross-reactivity among strains is correlated with cross-protection against heterologous strains is also to be further investigated.

Acknowledgement

We are grateful to Dr. S.D. Park of Wonkwang University for *V. vulnificus* strains and Dr. J.-W. Ju of Pusan

University for the standard antisera against *V. vulnificus* and helpful suggestions.

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(Received July 11, 1997)