Old Herborn University Seminar Monograph 11:
Immunomodulation of the gastrointestinal mucosa.
Editors: John C. Cebra, Peter J. Heidt, Volker Rusch, Dirk van der Waaij, and Richard I. Walker.
Herborn Litterae, Herborn-Dill, Germany: 102-111 (1998).

MODULATION OF IMMUNE RESPONSES TO BACTERIAL VACCINE ANTIGENS IN MICE: USE OF CYTOKINES AS ORAL MUCOSAL ADJUVANTS

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SUMMARY

Oral administration of exogenous cytokines may modulate immune responses, and hence may act as an adjuvant to enhance the efficacy of a co-delivered vaccine. The adjuvant capacity of IL-2 and IFN-γ for inactivated Campylobacter or cholera whole vaccines were investigated in mice. Cytokines delivered via oral route was not toxic, and was able to augment vaccine induced humoral, as well as cellular immune responses. This augmentation varied depending upon the organ system (local vs. systemic), and the type of immune response (cellular vs. humoral) induced, and was a function of the cytokine delivered. This observation suggests that, to some extent, these cytokines induce and regulate immune responses by distinct yet interdependent pathways.

INTRODUCTION

Appropriately prepared and orally delivered, vaccines consisting of dead bacterial cells are safe, but generally lack sufficient immunogenicity to stimulate long-lasting protection against disease. Some examples of these vaccine candidates currently being developed include, *Vibrio cholera* (*Sanchez* et al., 1994), enterotoxigenic *Escherichia coli* (*Wenneras* et al., 1994), and *Campylobacter jejuni* (*Baqar* et al., 1995). Recent attempts to produce improved mucosal vaccines against these bacterial diarrhoeal diseases has focused on the use of inactivated whole bacterial cells co-administered orally with potential mucosal adjuvants.

An effective vaccine against an infection must be capable of inducing the appropriate protective immune responses. It is now known that cytokines have an important role in inducing, regulating and augmenting protective immune responses following infection or vaccination. Therefore, the outcome of a subsequent infectious challenge after vaccination or prior infection with the same agent may be the result of limiting or preferentially producing one or more of these immunological mediators. This suggests that the use of exogenous recombinant cytokines as vaccine adjuvants may provide a mechanism(s) whereby the magnitude and the characteristics of vaccine-specific immune responses could be favourably modulated. Various cytokines administered parenterally or orally, have been shown to be effective immunological adjuvants in animal or human models augmenting protection induced by viral (*Cummins* and *Rosenquist*, 1980; *Schijns* et al., 1994), bacterial (*Miller* et al., 1996) or parasitic (reviewed in *Correlissen* and

Schetters, 1996) vaccines, as well as enhancing anti-tumour immunisation in clinical trials (*Kirchner* et al., 1995). The systemic use of cytokines as therapeutics or vaccine adjuvant has been limited primarily due to its associated toxicity at effective doses; however, this draw back may potentially be overcome by administering these molecules orally. Cytokines delivered orally have also been shown to alter the kinetics and the immune response to bacterial/viral infections in animals (*Chong* 1987; *Baqar* et al., 1993; *Cummins* and *Rosenquist*, 1980;) as well as acted as therapeutics in humans (*Koch* and *Obe*, 1990; *Jordan*, 1994). We have previously reported that selected cytokines administered orally have no apparent side effects, retain their biological activity (*Rollwagen* et al., 1997), and can alter the course of infection, as well as augment immunity to *C. jejuni* infection in mice (*Baqar* et al., 1993). Here we report the results of studies evaluating IL-2 and IFN-γ, both Th1 type cytokines (*Finkelman* et al., 1988; *Fiorentino* et al., 1989), as mucosal adjuvants for co-administered inactivated bacterial whole cell vaccines.

MATERIALS AND METHODS

Mice

BALB/c mice (females, 10-week old) were purchased from Jackson Laboratories Bar Harbor, ME, and housed in laminar flow cages for 10-12 days before being used in these experiments.

Vaccines and adjuvants

The formalin inactivated *C. jejuni* whole cell (CWC) vaccine, prepared as previously described (*Baqar* et al., 1995), was used at 10^8 vaccine particles per dose. The *V. cholerae* whole cells (VWC) vaccine (National Bacteriological Laboratory, Stockholm, Sweden, obtained from the U.S. Army under IND#3842) was delivered at 10^9 vaccine particles/dose. Murine recombinant Interleukin-2 (IL-2) and Interferon gamma (IFN-γ) were purchased from Genzyme Diagnostics, Cambridge, MA.

Enterotoxicity assays

All cytokines or CT was delivered at the indicated concentration using 0.5 ml PBS pH 7.4 supplemented with 0.1% bovine serum albumin (PBS-BSA). After neutralising stomach pH with 5% sodium bicarbonate buffer, 0.5 ml of PBS-BSA alone or containing 50 U to 500 U of IL-2, 40 ng to 160 ng of IFN-γ or 5 mg of *V. cholera* toxin (CT, Swiss Serum and Vaccine Institute, Berne, Switzerland) was delivered orally to 5-7 mice. The details of the oral feeding procedure are reported previously (*Baqar* et al., 1993). Six to 8 h after feeding, animals were euthanised and weighed. The weights of the GI tracts, and remaining carcasses from individual animals were then also determined. Enterotoxicity was directly related to the amount of fluid accumulated in the intestine of mice following oral dosing, and the amount of fluid was calculated as the ratio of the GI tract weight to the remaining carcass weight as:

$$\frac{\text{GI tract weight}}{\text{Body weight - GI tract weight}}$$

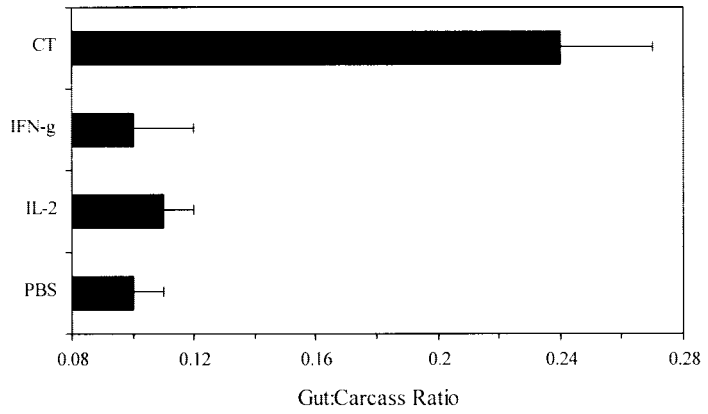

Figure 1: Comparative enterotoxicity of orally delivered cytokines and cholera toxin. Five mice per group were fed 0.5 ml of PBS-BSA alone or containing 500 units of IL-2, or 160 ng of IFN-γ, or 5 mg of cholera toxin. At 6-8 hrs after feeding, gut:carcass ratios for individual mice were determined. The figure presents mean and standard deviation values for these ratios derived from the indicated groups.

Vaccination

Mice (10-15 per group) received two oral doses at 8 day intervals, of 0.5 ml PBS-BSA only, or PBS-BSA containing 500 U of IL-2, or 10^8 CWC vaccine particles, or the same amount of vaccine delivered in combination with 500 U of IL-2. The VWC was tested using a similar vaccination regimen, except that 10^9 vaccine particles were used. The VWC vaccine was also delivered with 160 ng of IFN-γ.

Humoral immune responses

Campylobacter specific sIgA (intestinal lavage collected 7 days after vaccination; *Baqar* et al. 1993) were measured using an isotype-specific enzyme-linked immunosorbent assay (ELISA). Mononuclear cell suspensions prepared from Peyer's patches, lamina propria (*Hornqvist*, 1991), peripheral blood, spleen and mesenteric lymph nodes (*Baqar* et al., 1996), were used to enumerate vaccine antigen specific IgA and IgG antibody secreting cells (ASC). Details of the ELISA procedure (*Baqar* et al., 1993) and the methods employed to determine ASC in tissues (*Baqar* et al., 1996) are published.

Cell mediated immune responses

Mononuclear cells isolated from mesenteric lymph nodes and spleens were used to determine vaccine antigen specific *in vitro* lymphocyte proliferation 28 days after vaccination. Cells (10^5) were stimulated in triplicate in the presence of 10^5 inactivated bacterial cells for 7 days, at which time radioactive thymidine was added. After an additional 16 hrs incubation, tritium containing DNA was determined using standard liquid scintillation procedures. The procedures used for lymphocyte replication assays and data analysis are published elsewhere (*Murphy* et al., 1987, 1989).

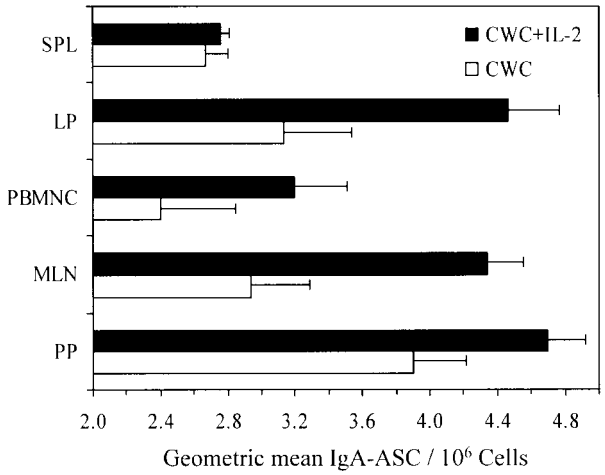

Figure 2: *Campylobacter* specific IgA-ASC responses after immunisation with inactivated CWC vaccine. Seven days after immunisation cells from 5-6 mice were separated individually from the indicated tissues and organs. The number of glycine extract antigen specific ASC/10^6 mononuclear cells was determined for each mouse. The figure presents geometric mean (loge) and standard deviations for the indicated groups. Abbreviations used; PP Peyer's patches; MLN mesenteric lymph nodes; PBMNC peripheral blood mononuclear cells; LP lamina propria; SPL spleen.

RESULTS

Enterotoxicity of orally delivered cytokines

The enterotoxicity of orally delivered cytokines were evaluated at a range of doses that were potentially thought or previously reported (*Baqar* et al., 1993) to have adjuvant action in mice. IFN-γ was tested across a 40 to 160 ng/mouse dosing range, and IL-2 at 50-500 units/mouse. In addition, a group of mice received 5 mg of CT as a positive control for enterotoxicity. The data presented in Figure 1 demonstrate that mice receiving only PBS-BSA exhibited no significant fluid accumulation as evidenced by a mean gut:carcass ratio of 0.10±0.01 (mean±standard deviation). CT stimulated a substantial amount of fluid secretion (ratio of 0.24±0.03), while the doses of IL-2 and IFN-γ examined failed to show any enterotoxicity giving gut:carcass ratios (IL-2: 0.11±0.01; IFN-γ: 0.10±0.02, data shown are for the highest dose only) which were essentially identical to those seen in control (PBS) mice.

C. jejuni vaccine

Inactivated CWC vaccine given alone or in combination with IL-2 was well tolerated with no apparent vaccine associated side effects being observed in mice.

Immune response to *Campylobacter* antigen

Separate groups of mice were immunised with PBS, IL-2, CWC or CWC+ IL2 and *Campylobacter* specific IgA-ASC were determined in tissues and peripheral blood samples collected 7-10 days after immunisation (Figure 2). No antigen specific ASC were detected in animals receiving PBS or IL-2. *Campylobacter* whole cell vaccine alone induced only a marginal ASC response, whereas, the response was augmented when the vaccine was delivered

Table 1: Murine IL-2 as an oral adjuvant for inactivated *Campylobacter* whole cell vaccine

Campylobacter specific[a] sIgA			*Campylobacter* colonisation[b]		
Immunisation	Titre	% Responder	log10/mg	Excretion (%)	Efficacy (%)
PBS	2.86±0.73	0	3.11±0.4	100	0
IL-2	3.06±0.88	0	2.40±0.8	100	0
CWC	3.44±0.89	14	2.04±0.5	57	43
CWC+IL2	4.75±0.48	71	1.47±0.5	28	72

[a]: *C. jejuni* 81-176 glycine extracted proteins (*Logan* and *Trust*, 1982) were used as antigens. Data are presented as geometric mean ($\log_e$) and standard deviation (stds). Responders are animals whose end-point titres were >4.32 (geometric mean + 2 stds of PBS mice = 4.32).
[b]: Four weeks after vaccination mice were orally challenged with $8x10^9$ CFU of *C. jejuni* 81-176, 6 days following challenge, faecal samples from individual mice were collected and CFU of *C. jejuni* / mg of faeces were determined. Vaccine efficacy was calculated as:

$$\frac{\text{\% control colonised - \% vaccinee colonised}}{\text{\% control colonised}} \times 100$$

with IL-2. The adjuvant effect of IL-2 was differentially expressed within the various tissues examined. The most pronounced immune enhancing effect of IL-2 was seen in mesenteric lymph nodes (2.9±0.35 vs. 4.3.4±0.21) and the lamina propria of vaccinated mice (compare the CWC vs. CWC+IL2 ASC responses in Figure 2). The detection of similar ASC response levels in the spleens of animals immunised with CWC or CWC+IL2 may be, in part, due to the less than optimal time (7-10 days) that these cells were collected to enumerate ASC.

Intestinal lavage fluid collected from PBS or IL-2 immunised mice had no detectable levels of *Campylobacter* specific sIgA (Table 1). When CWC was delivered alone, only 14% of vaccinated mice mounted a significant antigen specific sIgA response. In contrast, administering the same dose of CWC vaccine with IL-2 resulted in a significant enhancement in the number of sIgA responders (71%) to this vaccine.

Acquired resistance to *Campylobacter* infection

Vaccine efficacy was determined by orally challenging mice with *C. jejuni* 28 days after primary immunisation. Six days following challenge, 100% of PBS or IL-2 immunised animals were shedding the challenge organisms in their faeces, whereas, only 57% of CWC immunised and 28% of CWC+IL2 immunised animals remained colonised at this time. Mice which were positive for colonisation were excreting bacteria at much lower level (3.11±0.4 vs. 1.47±0.5 $\log_{10}$CFU/mg of faeces). Vaccine efficacy based on intestinal colonisation was calculated to be 43% and 72% for CW or CWC+IL2 immunised groups, respectively (Table 1).

V. cholerae vaccine

Inactivated VWC vaccine alone or in combination with IL-2 or IFN-γ was well tolerated with no apparent vaccination associated side effect being seen in mice.

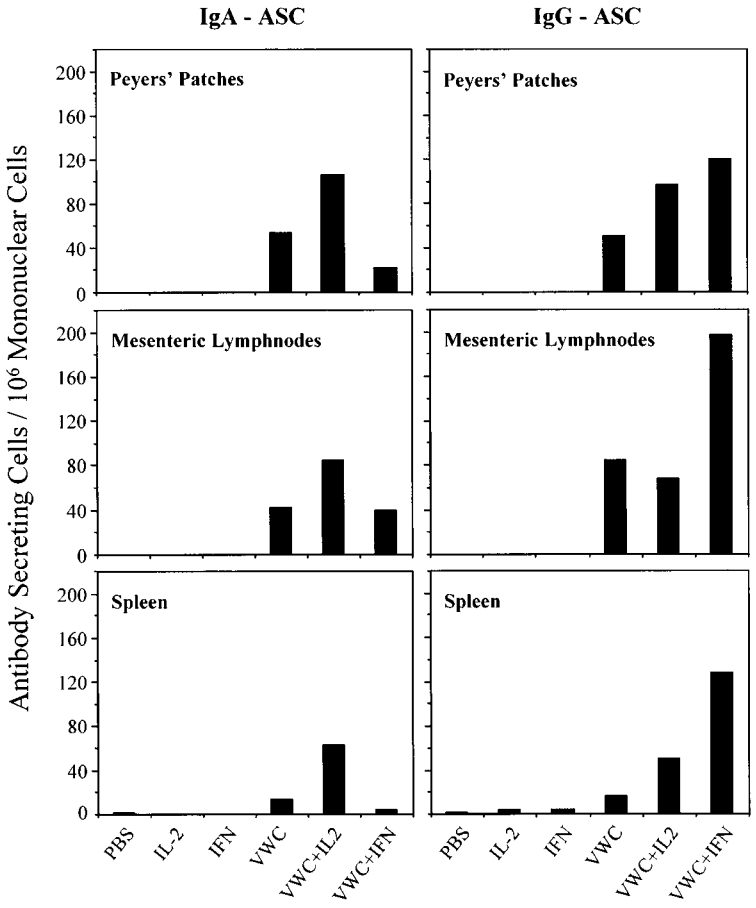

Figure 3: *V. cholerae* 01-LPS specific IgA- and IgG-ASC responses after immunisation with inactivated VWC vaccine. Seven days after immunisation cells from 5-6 mice were separated individually from the indicated tissues and organs. The number of 01-LPS specific ASC/10^6 mononuclear cells was determined for each mouse. The geometric mean (loge) and standard deviations for these values within the various group were determined. The figure presents these mean values after back-transformation to linear form. The standard deviations remained between 2-14%.

Humoral immune response to *Vibrio* antigen

Experiments were done to learn if humoral immune responses to cholera vaccine associated antigens could be enhanced by co-administering the VWC vaccine with IL-2 or IFN-γ, as oral mucosal adjuvants. Mononuclear cells isolated from Peyer's patches, mesenteric lymph nodes and spleens were used to detect cholera 01-LPS specific IgA- and IgG-ASC 7-10 days after vaccination (Figure 3). Oral administration of cholera vaccine alone resulted in a significant IgA-ASC response in Peyer's patches and mesenteric lymph nodes. These responses were further enhanced when the vaccine was delivered with IL-2, but not with IFN-γ. Compared to other tissues, the IgA-ASC response pattern in the spleen was different; VWC alone was a poor immunogen, but inclusion of IL-2 at the time of vaccination enhanced the response in this organ. In contrast to IgA responses, IL-2 had only a minimal adjuvant effect on

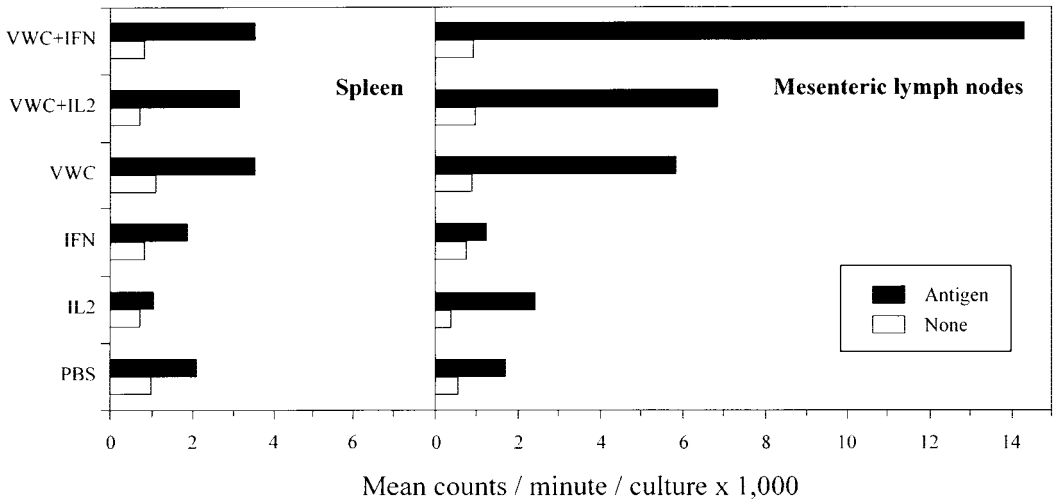

Figure 4: *Vibrio cholerae* whole cell antigen specific lymphocyte replicative responses after immunisation with inactivated cholera whole cell vaccine. The counts per minute for each culture well were loge transformed and geometric means were calculated. Data are presented as back transformed means, standard deviations were <13%.

vaccine specific IgG-ASC. However, IFN-γ substantially enhanced 01-LPS specific IgG-ASC in mesenteric lymph nodes and spleens.

Cellular immune response to *Vibrio* antigen

To determine the extent to which cell-mediated immune responses may develop following immunisation with the VWC vaccine alone or the IL-2 or IFN-γ supplemented formulations, spleen and mesenteric lymph nodes from individual (5 mice per group) animals were isolated and cultured in medium alone or medium containing VWC vaccine. Spleen and mesenteric lymph node cells from the various vaccination groups exhibited similar rates of basal lymphocyte replication (Figure 4: open bars). Cells from all the groups responded similarly to stimulation with Concanavalin A, thus vaccination did not cause non-specific stimulation or suppression of lymphocyte replication (data not shown). The proliferative response patterns differed substantially when lymphocytes from immunised and control (PBS, IL-2 or IFN-γ immunisation) mice were cultured *in vitro* in the presence of cholera whole cell antigens. Spleen and mesenteric lymph node lymphocytes from control mice showed low (mean CPM <3,000) and similar replication responses to vaccine antigens. Inclusion of either cytokine with VWC vaccine did not enhance the cholera antigen specific replicative responses of the splenocytes (mean CPM of 3,570; 3,170; 3,566 for VWC, VWC+IL2, VWC+IFN-γ respectively). In contrast, compared to spleen cells, mesenteric lymph node cells from VWC+IFN-γ vaccinated animals showed substantially enhanced proliferative responses to *Vibrio* antigens (mean CPM 14,3331 vs. 5,830 for VWC).

DISCUSSION

Recombinant IL-2 and IFN-γ retained their biological activity when orally delivered to mice. The doses used were sufficient to initiate a physiological/immunological cascade of action(s) to modulate protective immune responses without causing any detectable enterotoxicity. Some of these cytokines are known to be acid-stable and it has been suggested that recombinantly produced proteins that are glycosylated may resist proteolysis in the intestine (*Rollwagen* and *Baqar*, 1996). It has also been shown, that it takes at least 20 minutes in the presence of high concentration of trypsin or chymotrypsin to inactivate IL-6 *in vitro*. *In vivo* studies also showed that a longer incubation was necessary to inactivate IL-6 than the "normal" transit time through the gut. In this study where nonoglycosylated cytokines were used, administering these molecules with a carrier protein (BSA) after neutralising stomach acidity may have protected them from complete or partial digestion.

Previous work in our laboratory has shown the induction of various cytokines in response to *C. jejuni* infection in mice (*Baqar* et al., 1991). We have shown that exogenous orally delivered cytokines (IL-2, IL-5, IL-6) could modulate the course of *C. jejuni* infection and also enhanced protective immune responses in mice (*Baqar* et al., 1993). In the present study, we found that immune responses to cholera vaccine and protective immunity against *Campylobacter* colonisation in mice were induced only when inactivated bacterial whole cell vaccines were given with IL-2 or IFN-γ at the time of vaccination.

An adjuvant when delivered with an antigen, is capable of selectively enhancing specific immune responses *in vivo*. In mice immunised with vaccine alone (no cytokine added), immune responses and protection against infection were minimal, whereas, when the same vaccine was given with IL-2 or IFN-γ a distinct enhancement of vaccine-specific immune responses were observed. This augmentation varied depending upon the organ system (local vs. systemic), and the type of immune response (cellular vs. humoral) induced, and was a function of the cytokine delivered. This observation suggests that, to some extent, these cytokines induce and modulate immune responses by distinct yet interdependent pathways. We have previously (*Rollwagen* et al., 1997) shown, that ^{125}I-labelled IL-6 or IL-2 fed to mice have different distribution patterns *in vivo*; IL-6 remained in the gut for up to 6 hrs post administration whereas, IL-2 counts were more uniformly distributed among local and systemic compartments.

The mechanism(s) underlying the adjuvant effect of IL-2 or- IFN-γ remains to be determined. These T cell subset-specific cytokines are known to regulate and expand their own subset, although at times the activation of Th1 vs. Th2 subsets could be mutually exclusive (*Fiorentino* et al., 1989). Thus, administration of particular cytokines during immunisation might be expected to influence the physiological cytokine balance; and may serve to inhibit or stimulate antigen-specific immune reactions. The fact that these orally administered molecules can modulate humoral, as well as cellular immune responses both at the local site and systemically, suggests that they can also exert their adjuvant effect beyond the intestinal mucosa, the primary site of delivery. Although some biological activity is probably destroyed when cytokines are given by this route, a sufficient amount apparently remains to effect immunomodulatory functions which leads to enhanced protective immune responses to killed bacterial vaccines.

ACKNOWLEDGEMENTS

The opinion and assertions contained herein are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large. The experiments reported herein were conducted to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Research, National Research Council, National Academy Press (1996). This work was funded by Naval Medical Research and Development Command Work unit no. 62787A.001.01.EVX.1522. We thank Jason Castro and Shaun Wright for technical assistance.

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