INFECTION AND IMMUNITY, Sept. 1996, p. 3863–3869 Vol. 64, No. 9
0019-9567/96/$04.00+0
Copyright © 1996, American Society for Microbiology

A Live (Δ*aroA*) *Aeromonas salmonicida* Vaccine for Furunculosis Preferentially Stimulates T-Cell Responses Relative to B-Cell Responses in Rainbow Trout (*Oncorhynchus mykiss*)

MATTHEW J. MARSDEN,[1] LAURENCE M. VAUGHAN,[2] TIMOTHY J. FOSTER,[3] AND CHRISTOPHER J. SECOMBES[1]*

Department of Zoology, University of Aberdeen, Aberdeen AB9 2TN, United Kingdom,[1] and National Pharmaceutical Biotechnology Centre[2] and Department of Microbiology,[3] Moyne Institute, Trinity College, Dublin 2, Ireland

Received 29 March 1996/Returned for modification 13 May 1996/Accepted 26 June 1996

We have previously described (L. M. Vaughan, P. R. Smith, and T. J. Foster, Infect. Immun. 61:2172–2181, 1993) the construction of a kanamycin-resistant aromatic-dependent mutant of *Aeromonas salmonicida*, the causative agent of furunculosis, and its use as a live vaccine. Here we describe the construction of an unmarked *aroA* deletion mutant and examine the nature and magnitude of immune responses in rainbow trout (*Oncorhynchus mykiss*) to this vaccine strain. Good proliferative and antibody responses were elicited by using a range of vaccine doses from 2×10^6 to 2×10^9 live bacteria per fish, and a clear vaccine dose effect was apparent. A significant positive effect of using live bacteria to prime for lymphocyte proliferation and antibody production was apparent. However, the problem of directly comparing the vaccine doses of live and killed bacterial preparations is discussed, since some replication of live bacteria in vivo is expected. Most importantly, the live vaccine preferentially stimulated enhanced T-cell responsiveness, as evidenced by significantly greater increases in T-cell proliferation than in B-cell proliferation, compared with responses by the respective cell populations from fish given a killed vaccine. The manner in which live vaccines elicit strong cell-mediated immune responses and the relevance to fish vaccine design are discussed.

Live vaccines against fish bacterial pathogens have been used with success in several recent fish farm trials (13, 18, 19). Despite knowledge on the degree of protection afforded by these vaccines, very little information exists as to the nature of immune responses elicited by live vaccines in fish and how they compare with responses to conventional killed vaccines. In mammals, live vaccines have a number of advantages over dead vaccines (1) and are known to be effective stimulators of cell-mediated immune responses, responsible for elimination of intracellular microbial pathogens (9). Activation of antigen-specific T cells is a key event in such responses, resulting in the release of cytokines able to increase phagocyte activity. Several major bacterial diseases in aquaculture are considered to be caused by intracellular pathogens able to reside, in particular, within macrophages; these bacteria include *Aeromonas salmonicida* (5) and *Renibacterium salmoninarum* (4), the causative agents of furunculosis and bacterial kidney disease, respectively. Thus, vaccines that preferentially stimulate cell-mediated responses may be desirable in these cases.

We have previously described the construction of a kanamycin-resistant *aroA* mutant of *A. salmonicida* (Brivax) and its use as a live vaccine to protect brown trout (*Salmo trutta*) from laboratory infection with *A. salmonicida* (19). Here we describe the construction of an *aroA* deletion mutant (Brivax II) which is suitable for use as a field vaccine. Preliminary investigation has shown that this strain is substantially attenuated, with doses as high as 2×10^8 bacteria per fish having no observable effect on fish health (11). The Brivax II strain is therefore suitable for vaccine development, but studies on the magnitude and nature of the immune response elicited are warranted. In the present study, rainbow trout (*Oncorhynchus mykiss*) were immunized with this live vaccine, using several doses, and we examined the proliferative responses of blood leukocytes and antibody production at various times postvaccination. In addition, comparisons were made between the responses elicited with the live vaccine relative to those elicited with formalin-killed preparations of the same strain.

* Corresponding author. Mailing address: Department of Zoology, University of Aberdeen, Tillydrone Ave., Aberdeen AB9 2TN, United Kingdom.

MATERIALS AND METHODS

Fish. Rainbow trout (*O. mykiss*) specimens obtained from local fish farms were maintained in aerated fiberglass tanks supplied with a continuous flow of recirculating water at 16°C. Fish were fed twice daily with commercial trout food (EWOS Ltd.). Fish vaccinated with the live Brivax II bacterium were placed in the pathogen containment aquarium facility at the Department of Zoology, University of Aberdeen, where the outflow water was sterilized by contact with hypochlorite.

Construction of an *aroA* deletion mutant (Brivax II). The *aroA* deletion strain (Brivax II) was derived from the wild-type virulent *A. salmonicida* 644Rb (19) by using the suicide delivery plasmid pSUP202 (15) as described previously (19). Plasmid pSUP202ΔA2 (19), which contains a deletion of the entire *aroA* coding sequence with flanking sequences to promote homologous recombination, was introduced into *A. salmonicida* 644Rb by conjugation from the *Escherichia coli* donor strain S17-1 (15). Plasmid integrants were selected on tryptic soy agar (TSA; Gibco) plates containing chloramphenicol or tetracycline (Sigma) at 8 and 3 μg/ml, respectively. Donor *E. coli* cells were counterselected with nalidixic acid at 30 μg/ml. Strains containing integrated plasmids were single colony purified on selective agar (TSA plus 10 μg of tetracycline per ml) before inoculation into drug-free tryptic soy broth (TSB; Gibco). Plasmid excisants were identified on TSA plates by the loss of plasmid-encoded antibiotic resistance. Rare aromatic-dependent mutants were identified by cross-streaking colonies onto *A. salmonicida* minimal agar lacking aromatic supplements (19). Colonies which failed to grow were restreaked onto TSA plates and checked for antibiotic sensitivity and ability to grow on minimal agar plates to which aromatic supplements had been added. One strain which satisfied these criteria was identified, and chromosomal DNA was isolated as previously described (19). The structure of the deleted *aroA* locus was verified by Southern hybridization (14) by using the wild-type *Pst*I *aroA* fragment from plasmid pAA2 (19) labelled with [α-^{32}P]dATP as a probe. This strain was designated Brivax II (Δ*aroA*).

Bacterial passaging and antigen preparation. Brivax II bacteria stored at −70°C were thawed and inoculated into 3% TSB. Following an initial 24-h incubation of cultures at 20°C, bacteria were subcultured and grown for a further 24 to 48 h at this temperature prior to their use for vaccination. Some cultures, prepared as described above, were killed by the addition of 1% formalin (BDH) for 24 h. Killed cultures were then washed and adjusted to 5×10^{10} bacteria per ml in 0.15 M phosphate-buffered saline (PBS), pH 7.2, and stored in aliquots at −20°C until required. Live cultures were also washed in PBS prior to use and adjusted to an appropriate concentration. For use as in vitro antigens, other strains of *A. salmonicida* were incubated for an initial 24 h at 20°C and then subcultured and grown for a further 48 h, prior to being killed and stored as described above. These strains included the wild-type strain, 644Rb, from which Brivax was derived (i.e., without an *aroA* deletion) and strain MT004, an avirulent strain lacking an A layer (3). In some cases, the bacteria were sonicated instead of being killed with formalin. Following centrifugation ($13,000 \times g$) and three washes in PBS, suspensions were sonicated on ice until clear. Such preparations were stored at −20°C until used for antibody assays (see below). In addition, sonicated preparations were centrifuged at $13,000 \times g$ for 10 min to remove cellular debris, the supernatants were collected and filtered through a 0.22-μm-pore-size filter, and protein concentration was determined. These lysed cell supernatants were also stored at −20°C until use in proliferation assays.

Immunization protocols. Groups of 15 to 30 rainbow trout, weighing 50 g each and acclimated to 16°C, were anesthetized with ethyl-4-aminobenzoate (Benzocaine; BDH) at 25 μg/ml of water. The fish were then vaccinated intraperitoneally with live Brivax II in 200 μl of PBS at doses of 2×10^6, 2×10^7, 2×10^8, and 2×10^9 bacteria per fish (i.e., 4×10^4 to 4×10^7 bacteria per g of fish). Preliminary trials showed that doses higher than 2×10^9 bacteria per fish caused some mortalities and so were not used. Some fish were immunized with formalin-killed Brivax II at a dose of 2×10^8 bacteria per fish or given PBS as a control. Fish were sampled once (i.e., terminally) at week 2, 4, or 8 for proliferation studies or repeatedly at weeks 2, 4, 6, 8, and 11 for determination of serum antibody titers (see below).

Proliferation. Antigen-specific proliferation of rainbow trout peripheral blood leukocytes (PBL) was performed essentially by the method of Marsden et al. (12). PBL were isolated from five to seven fish per group at each time studied, resuspended in RPMI 1640 medium (Gibco) containing 25 mM $NaHCO_3$ (pH 7.2), and diluted to trout tonicity (312 mosmol). Aliquots (100 μl) of RPMI 1640 medium containing 2.5×10^5 leukocytes were added to 96-well tissue culture plates (Nunc), in triplicate per treatment examined, in the presence of a further 100 μl containing 10^5, 10^6, or 10^7 formalin-killed 644Rb (wild type) or MT004 *A. salmonicida* cells or in 100 μl containing 4 or 10 μg of lysed 644Rb or MT004 cells per ml (to achieve overall concentrations of 2 and 5 μg/ml, respectively). The cells were then cultured in a saturated humidity atmosphere of 5% CO_2–95% air at 18°C ± 1°C for 3 h prior to the addition of fetal calf serum to give an overall concentration of 10%. Leukocytes were incubated for a further 96 h prior to the addition of 0.5 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham) per well. Twenty-four hours later, the cells were harvested and samples were counted by the method of Marsden et al. (12). Data were calculated and expressed as mean (± standard error [SE]) counts per minute minus the counts per minute from respective controls cultured without antigen. While the specificity of this assay has been confirmed in a previous study using cells from fish immunized with killed *A. salmonicida* (10), in the present study controls were also carried out where cells from Brivax II-immunized fish were cultured with formalin-killed *Vibrio anguillarum* to determine if this was also the case using a live vaccine. Controls were also carried out in the absence of leukocytes but in the presence of the highest dose of bacterial antigen (10^7 bacteria per well) used in these experiments.

In some cases the proliferative response of separated surface-immunoglobulin-positive (sIg^+) and surface-immunoglobulin-negative (sIg^-) PBL was also examined. PBL were isolated and resuspended in L_{15} medium containing 5% fetal calf serum to approximately 3×10^6 cells per ml. A portion of these purified PBL were retained to give the unfractionated PBL response, and the rest were separated by a panning technique described by Graham and Secombes (6), using a mouse monoclonal anti-trout Ig antibody (I-14). The leukocytes were panned for 1 h at 18°C after which nonadherent sIg^- cells were collected and the adherent sIg^+ cells were scraped off. All three populations (unfractionated, sIg^+, and sIg^- PBL) were concentrated by centrifugation at $400 \times g$ for 5 min at 4°C, and the resultant cell pellets were resuspended in RPMI 1640 medium for use in the proliferation assay. In each assay the relative responses to T-cell (phytohemagglutinin) and B-cell (lipopolysaccharide) mitogens were also monitored by the method of Marsden et al. (12) to confirm that separation into sIg^+ and sIg^- cells was successful. Data of fractionated sIg^+ and sIg^- cells were presented as a stimulation index, obtained by dividing the counts per minute of these populations from fish vaccinated with live Brivax II by the counts per minute of the respective fractionated cells from fish vaccinated with dead Brivax II. Thus, an index greater than 1 indicated that there was a greater response of cells from fish given the live vaccine.

Antibody assays. Blood samples were collected from 13 to 15 fish per group at each time studied. The blood was allowed to clot and was stored at 4°C overnight prior to centrifugation to obtain serum. Serum samples were stored at −20°C prior to use. Antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA) as described by Thompson et al. (17) with the exception that the ELISA plates were coated with sonicated Brivax II at 0.5 mg/ml. Log_2 titers were expressed as means ± SEs.

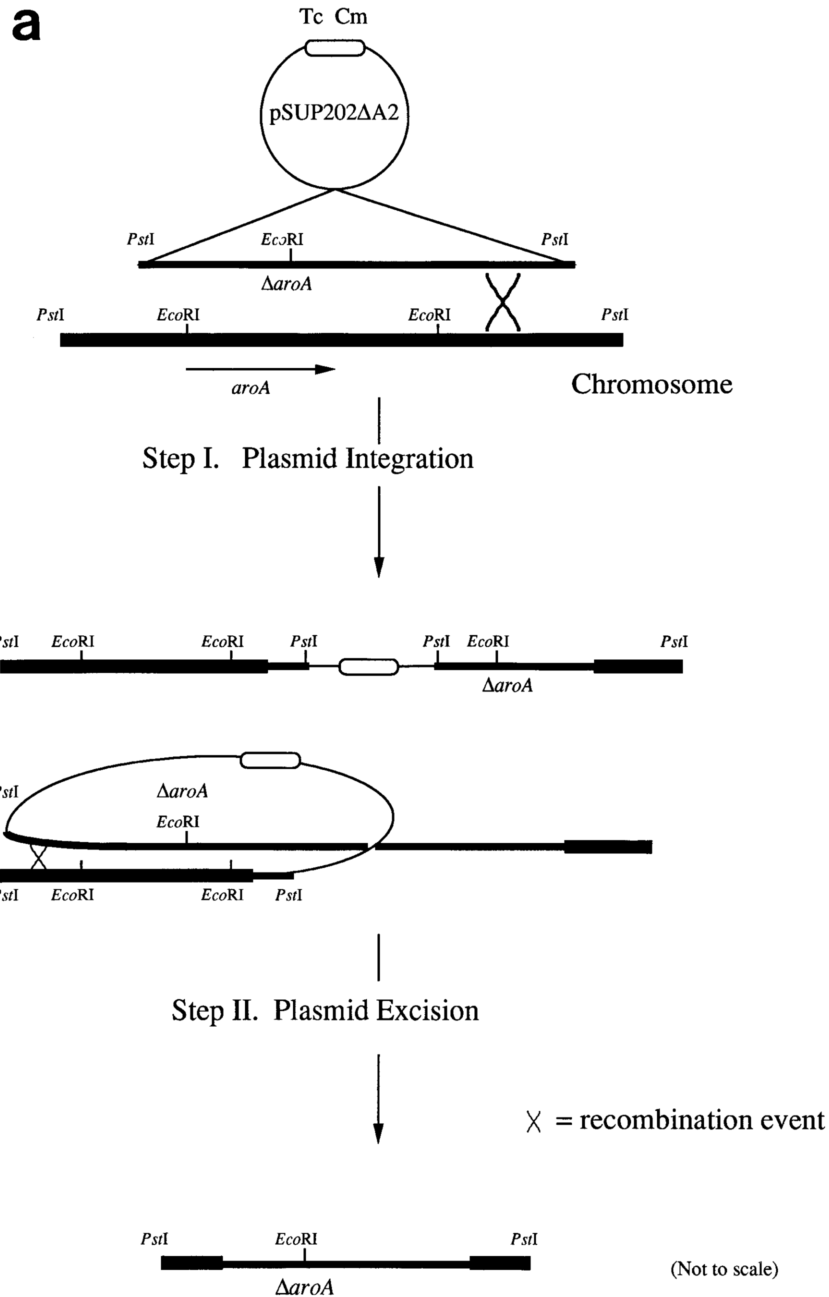
Statistical analysis. Obtained data were analyzed statistically by analysis of variance (ANOVA) and Student's *t* test, as appropriate.

RESULTS

Construction of *A. salmonicida* 644Rb Δ*aroA*. The suicide delivery plasmid pSUP202ΔA2 (19), harboring the 2-kb *Eco*RI *aroA* deletion flanked by 0.7 and 1.6 kb of DNA to provide homology for recombination, was introduced by conjugation from *E. coli* S17-1 into *A. salmonicida* 644Rb. Plasmid integrants were selected by plating dilutions of the mating mixture on TSA plates containing nalidixic acid and either chloramphenicol or tetracycline. Colonies were grown in broth lacking antibiotics and then plated for single colonies on drug-free agar (Fig. 1a). Aromatic-dependent mutants were identified from among these colonies by streaking on minimal agar lacking aromatic supplements. One such strain which failed to grow on minimal agar and had lost both chloramphenicol and tetracycline resistance was identified. The ability to grow on minimal agar was restored by the addition of aromatic supplements (19). The structure of the deleted *aroA* locus was verified by Southern hybridization, using the wild-type *Pst*I *aroA* fragment as a probe. This probe detected a 4.3-kb fragment in *Pst*I-digested wild-type DNA. In the mutant strain, the probe reacted with a band of 2.3 kb, which corresponds to the *Pst*I fragment containing the 2.0-kb *Eco*RI *aroA* deletion (Fig. 1b). No revertants to Aro^+ were detected among 10^{11} cells (18). The mutant strain grew normally on complete agar and retained a normal A layer, as shown by its ability to aggregate in broth and the Congo red binding assay (18).

Proliferation responses. As seen in Fig. 2, immunization of fish with 2×10^8 Brivax II gave good priming of lymphocytes, as assessed by proliferation in vitro in response to the wild-type strain. Control cultures without antigen typically gave counts of less than 1,500 cpm, and cultures without cells but containing 10^7 bacteria per well gave an average cpm of 527 ($n = 10$). Maximal proliferation was seen at week 2 with both antigen concentrations used to stimulate proliferation, and there was a significant ($P < 0.05$) time effect by one-way ANOVA. However, there was an inherent degree of variability in the proliferation assay from week to week, making such comparisons difficult to interpret. The effect of in vitro antigen concentration was also significant overall by two-way ANOVA ($P < 0.001$), with higher concentrations eliciting higher proliferation. However, at week 8 the effect was not significant as a result of a large SE in the higher concentration group. Controls to determine the specificity of the assay using cells from live vaccinated fish showed that while a degree of responsiveness to an unrelated antigen (*V. anguillarum*) was seen, the response to the specific antigen was significantly ($P < 0.05$) greater (Fig. 3). Indeed, the responses to *V. anguillarum* were no greater than those seen using leukocytes from unprimed fish in previous studies (unpublished data).

The effect of vaccine dose was investigated 4 weeks postvaccination by using both the wild-type strain and MT004 to stimulate proliferation in vitro (Fig. 4). In both cases a clear effect of vaccine dose was apparent, with a dose of 2×10^9 Brivax II per fish inducing the highest levels of proliferation and a dose of 2×10^6 Brivax II per fish inducing the lowest levels of proliferation in response to the various types and concentrations of *A. salmonicida* antigens used in vitro. While the effect of vaccination dose was apparent with all antigens used to stimulate proliferation, it was only at the lower antigen concentration (10^5 bacteria per well) that this effect was statistically significant ($P < 0.05$). The effect of in vitro dose was also



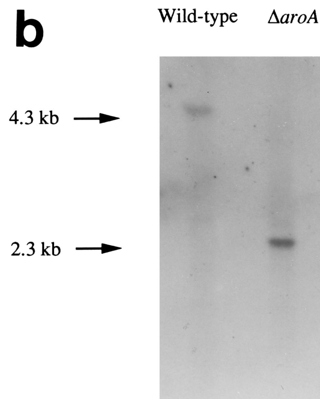


FIG. 1. (a) Schematic representation of the events involved in the construction of the *aroA* deletion. Step 1 involved the integration of the suicide vector harboring the Δ*aroA* allele. This was achieved via a single crossover, resulting in a Campbell-type integration event. The plasmid was subsequently excised (step II) following a second recombination which, if it occurred on the other side of the deletion, resulted in allele replacement as shown. The circle represents the suicide plasmid with genes for resistance to chloramphenicol and tetracycline (⬭) harboring the *Pst*I fragment containing the deleted 2.0-kb *Eco*RI *aroA* fragment (thin bold line). The wild-type chromosomal *Pst*I locus is represented by the thick bold line. The horizontal arrow indicates the *aroA* coding sequence. (b) Southern hybridization analysis of *A. salmonicida* 644Rb and the *aroA* mutant. Chromosomal DNA (3 μg) was digested with *Pst*I and separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose and probed with the wild-type *Pst*I *aroA* fragment labelled with [α-^{32}P]dATP.

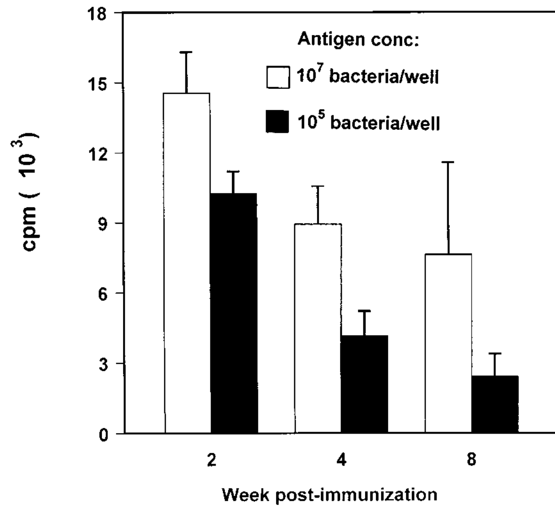


FIG. 2. Proliferation of PBL at various times postvaccination with Brivax II at 2×10^8 bacteria per fish in response to 10^5 and 10^7 killed wild-type (644Rb) *A. salmonicida* cells per well. Data are presented as mean (± SE [shown by error bar]) counts per minute for five fish. Antigen conc, Antigen concentration.

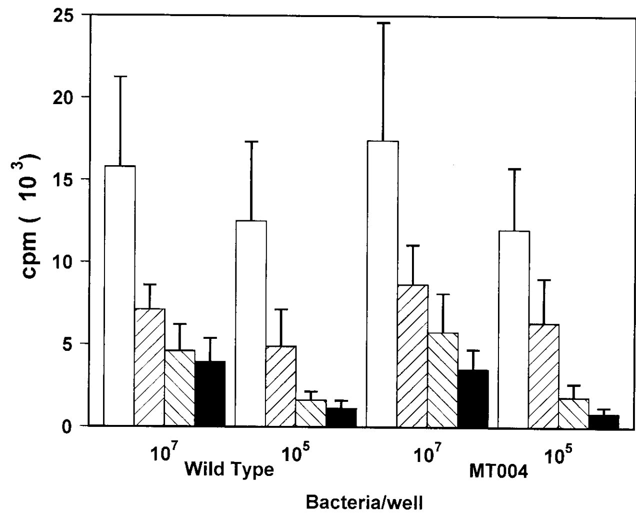


FIG. 4. Proliferation of PBL 4 weeks postvaccination with various doses of Brivax II in response to 10^5 and 10^7 killed wild-type (644Rb) and MT004 *A. salmonicida* cells per well. The fish were vaccinated with the following doses of Brivax II per fish: 2×10^9 (□), 2×10^8 (▨), 2×10^7 (▧), and 2×10^6 (■). Data are presented as means (± SE [shown by error bar]) counts per minute for seven fish.

more marked at the lower vaccine doses in this run compared with the above data in Fig. 2. Proliferation in response to the wild-type strain (A layer positive) and MT004 (A layer negative) was similar and not significantly different at all vaccine doses and in vitro antigen concentrations examined. Similarly, there were no significant differences between in vitro responses to the wild-type strain and Brivax II at this time, using a vaccine dose of 2×10^8 bacteria per fish (Fig. 5). Comparison of the responses of leukocytes from vaccinated fish with responses of leukocytes from control fish showed that, as expected, the former were significantly greater ($P < 0.05$) than the latter (Fig. 5).

The proliferative responses of unfractionated PBL from trout vaccinated with live or dead Brivax II at 2×10^8 bacteria per fish 4 weeks postvaccination are shown in Fig. 6. In all cases fish given the live vaccine had a significantly higher ($P < 0.001$) degree of proliferation in response to both the whole-cell and lysate antigen preparations used. Similar results were found at weeks 2 and 8 postinjection (data not shown). Fractionation of blood leukocytes into sIg^+ and sIg^- cells was confirmed by their mitogen responsiveness (Fig. 7). These populations contain 90 and 30% lymphocytes, respectively (12), and proliferate because of their B- and T-cell populations. Thus, while unfractionated cells could respond to both mitogens, sIg^+ (B) cells responded only to lipopolysaccharide, whereas sIg^- (T) cells responded only to phytohemagglutinin. Typically, B-cell responses to specific antigen were greater than T-cell responses across all antigen types and doses tested (Fig. 7). Since in-

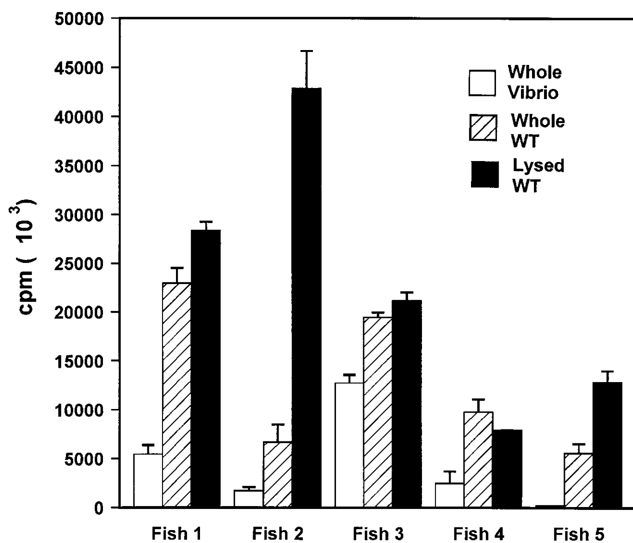


FIG. 3. Proliferation of PBL at 2 to 4 weeks postvaccination with Brivax II at 2×10^8 bacteria per fish in response to 10^7 killed wild-type (Whole WT) *A. salmonicida* cells per well, 5 μg of wild-type cell lysate per ml (Lysed WT), or 10^7 killed *V. anguillarum* cells per well (Whole Vibrio). Data are presented as mean (± SE [shown by error bar] counts per minute for three wells per treatment.

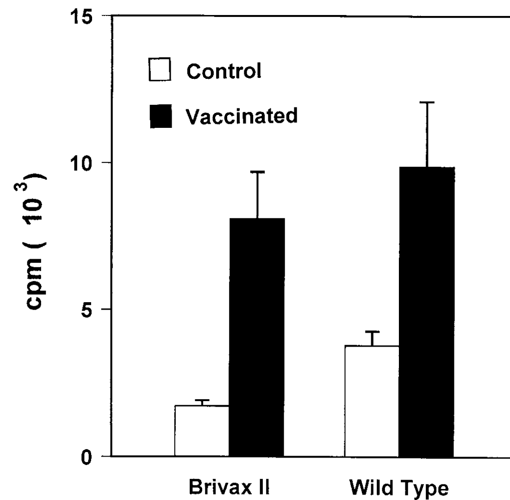


FIG. 5. Proliferation of PBL 4 weeks postvaccination with Brivax II at 2×10^8 bacteria per fish or postinjection with PBS (Control) in response to 10^6 killed Brivax II and wild-type (644Rb) *A. salmonicida* cells per well. Data are presented as mean (± SE [shown by error bar]) counts per minute for five fish.

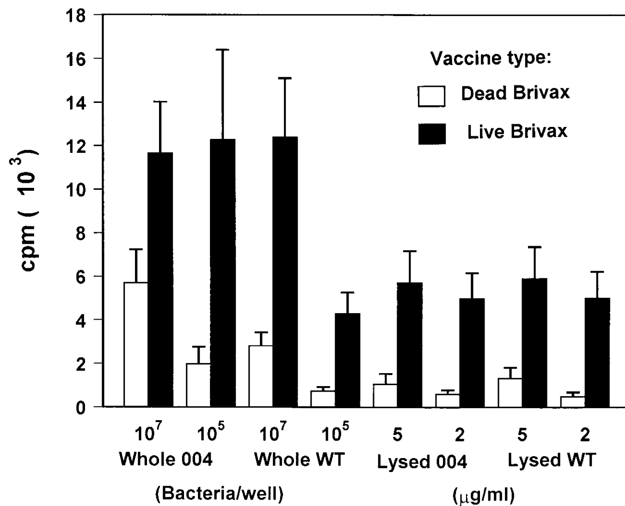


FIG. 6. Proliferation of PBL 4 weeks postvaccination with 2×10^8 live or killed Brivax II cells per fish in response to 10^5 and 10^7 killed wild-type (Whole WT) and MT004 (Whole 004) *A. salmonicida* cells per well or 2 and 5 μg of cell lysates (Lysed WT, Lysed 004) of the above strains per ml. Data are presented as mean (± SE [shown by error bar]) counts per minute for five fish.

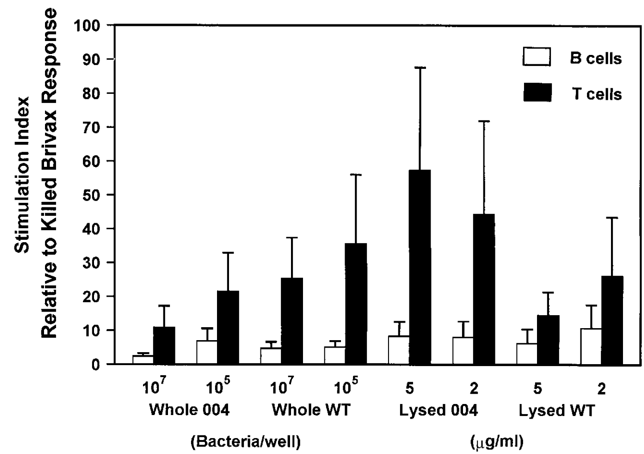


FIG. 8. Proliferation of sIg^+ (B cells) and sIg^- (T cells) PBL 4 weeks post-vaccination with 2×10^8 live or killed Brivax II cells per fish in response to 10^5 and 10^7 killed wild-type (Whole WT) and MT004 (Whole 004) *A. salmonicida* cells per well or 2 and 5 μg of cell lysates (Lysed WT, Lysed 004) of the above strains per ml. Data are presented as the mean (± SE [shown by error bar]) stimulation indices of the live Brivax response (expressed relative to the killed Brivax response [i.e., the killed Brivax response equals 1 in all cases]) for five fish.

creased T-cell responses would be expected if cell-mediated immune responses were preferentially stimulated by the live vaccine, the responses were analyzed relative to responses elicited by the dead vaccine. Responses of B and T cells to these antigens reflected the unfractionated cell responses, in that both populations from fish given the live vaccine were greater than the responses of respective cells from fish given the dead vaccine (i.e., the stimulation indices were above 1) (Fig. 8). However, from these experiments it was apparent that overall the magnitude of increase in proliferation seen following live vaccination was significantly higher ($P < 0.01$) in the T-cell population than in the B-cell population (i.e., T cells had higher stimulation indices than B cells).

Antibody responses. Antibody titers of anti-Brivax sera from trout vaccinated with different doses of live Brivax II and sera from control fish injected with PBS are shown in Fig. 9. Antibody titers increased steadily over the first 4 to 6 weeks and then began to decline slightly, stabilizing again at weeks 8 and 11. There was a clear vaccine dose effect, which was significant overall ($P < 0.001$) by two-way ANOVA. Indeed, each group was significantly different from the others in a pairwise comparison by two-way ANOVA ($P < 0.001$). In addition, 2×10^8 dead Brivax II cells were used to vaccinate a further group of

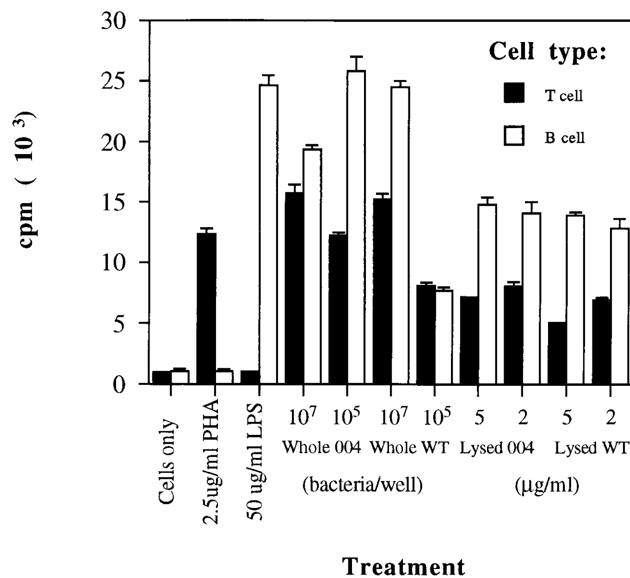


FIG. 7. Proliferation of sIg^+ (B cells) and sIg^- (T cells) PBL 4 weeks post-vaccination with 2×10^8 live Brivax II cells per fish in response to 10^5 and 10^7 killed wild-type (Whole WT) and MT004 (Whole 004) *A. salmonicida* cells per well or 2 and 5 μg of cell lysates (Lysed WT, Lysed 004) of the above strains per ml. Data are presented as mean (± SE [shown by error bar]) counts per minute from a representative experiment from five fish examined at this time point and three wells per treatment. PHA, phytohemagglutinin; LPS, lipopolysaccharide.

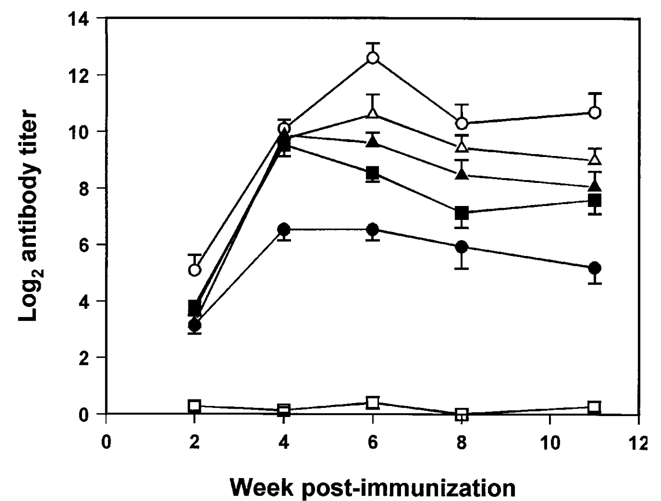


FIG. 9. Serum anti-Brivax antibody titers determined by ELISA at various times postvaccination with four doses of Brivax II, plus a killed vaccine preparation at 2×10^8 bacteria per fish and a negative control (PBS). The fish were vaccinated with the following doses of Brivax II per fish: Controls (□), 2×10^9 live bacteria (○), 2×10^8 live bacteria (△), 2×10^7 live bacteria (■), 2×10^6 live bacteria (●), and 2×10^8 dead bacteria (▲). Data are presented as means ± SEs of $\log_2$ titers for 13 to 15 fish.

trout, and as seen in Fig. 9, the titers from this group were significantly lower ($P < 0.05$) than the titers from fish vaccinated with the same dose of live Brivax. This effect was apparent from week 6 on, when titers were approximately 1 $\log_2$ unit lower than those of the corresponding live vaccinated group.

DISCUSSION

Good proliferative and antibody responses were elicited by vaccination of rainbow trout with a live (*aroA*) strain of *A. salmonicida*. These results are in agreement with the results of the earlier study of Vaughan et al. (19), using an *aroA* mutant generated by inserting a kanamycin resistance determinant within the *aroA* gene, where titers of up to 1:128 ($\log_2$ titer = 7) were present in brown trout 6 weeks postvaccination with 5.4×10^7 bacteria per fish. While antibody titers steadily increased over the first 4 to 6 weeks postinjection, proliferative activity was maximal much earlier in the response (week 2). Maximal proliferation 2 weeks postinjection has also been observed with a killed *A. salmonicida* preparation (10), where it was speculated that perhaps an ongoing primary immune response contributed to this early increase in addition to any generated memory cells. Proliferation was still highly significant at the last sampling at week 8, suggesting that good immunological memory had been established. Indeed, long-lived immunological memory is considered to be a characteristic of responses to live vaccines (1).

The dose-response experiment clearly demonstrated that the larger the vaccine dose, the larger the priming effect on proliferation and antibody production, although all doses elicited good responses. The highest dose used was far greater than in previous protection studies using live *A. salmonicida* vaccines (18, 19), where doses of 1×10^7 to 5×10^7 bacteria per fish were injected intraperitoneally and shown to give good protection. Thus, the lower vaccine doses may give rise to biologically relevant, albeit lower, responses. Indeed, even in the present study the responses of cells from fish given the lower vaccine dose were not significantly lower than the responses of cells from fish given the high vaccine dose when a high antigen concentration (10^7 bacteria per well) was used to stimulate proliferation.

One of the most interesting findings in the present study was the significant positive effect of using a live vaccine to prime for lymphocyte proliferation. This effect may be brought about by a difference in the real bacterial dose received by fish given the live vaccine compared with the killed vaccine, since live $AroA^-$ bacteria are likely to replicate in vivo for a few days before using up their folate reserves (7). Thus, the response to the dead vaccine may represent a response to a lower vaccine dose. However, it is also possible that the bacteria penetrated lymphoid organs in a more stimulatory manner than can be achieved with a dead vaccine.

Further experiments looking at differential effects upon trout T and B cells support the notion that a qualitatively different immune response was elicited with the live vaccine. Isolated sIg^+ and sIg^- PBL have been used in many previous functional studies to investigate fish B- and T-cell responses and have confirmed that discrete populations akin to mammalian B and T cells exist in fish (2, 6, 12). While the panning technique used in the present study gives quite pure sIg^+ cells (90% lymphocytes), the sIg^- cells contain a mixture of cell types (10), although only the lymphocytes present (30%) should proliferate in response to mitogens or antigens. While both B- and T-cell populations from fish given the live vaccine showed higher proliferative responses than these populations from fish given the killed vaccine, as with unfractionated PBL, the relative increase in proliferation was significantly higher in the T-cell population than in the B-cell population. This qualitative difference in the responses elicited by the live and killed vaccines may be due to the induction of a strong cell-mediated immune response with the former, as seen with live vaccines in mammals (16). This interpretation was also suggested by the antibody data, where although vaccination with live Brivax gave a significantly higher titer than vaccination with killed Brivax, the difference was relatively small, reflecting the smaller increase in B-cell activity compared with that of T-cell activity seen in the proliferation experiments. Whether cell-mediated or humoral immune responses are elicited is determined in mammals by the type of helper T cells stimulated and the cytokines they release. Thus, interleukin 2 (IL-2) and gamma interferon released from Th1 cells drive cell-mediated responses whereas IL-4, IL-5, and IL-6 released from Th2 cells drive humoral immunity. The Th1 and Th2 cells themselves have cytokine requirements for IL-12 and IL-4, respectively (9). The major source of IL-12 is infected macrophages, presumably explaining the ability of live vaccines to efficiently stimulate cell-mediated responses. Interestingly, the magnitudes of the proliferative responses of the isolated trout T- and B-cell populations were similar whether stimulated with *A. salmonicida* antigens or with phytohemagglutinin or lipopolysaccharide, respectively, as seen in another study (12). Since in fish immunological memory is due strictly to an increase in the B-cell precursor pool rather than an increased capacity of the precursors to proliferate (8), this may reflect a greatly enlarged antigen-specific lymphocyte pool, although the possibility of some polyclonal activation by the *A. salmonicida* antigens cannot be entirely excluded as seen in Fig. 3.

In conclusion, Brivax II appears to be a good stimulator of immune responses in rainbow trout, as seen in the proliferation and antibody experiments. Evidence is presented that live vaccines elicit larger responses than killed vaccines, although it may be difficult to directly compare vaccine doses because of in vivo replication of live bacteria. Nevertheless, it is apparent that live vaccines preferentially enhance T-cell responsiveness, and live vaccines may be a requirement for successful vaccination against some fish pathogens.

ACKNOWLEDGMENTS

This work was supported by a contract from the EC (contract AIR1-CT92-0441).

Thanks go to Mike van der Laak, Bjorn Weitzel, and Emma Collins for assistance with the proliferation assays.

REFERENCES

1. **Brown, F., G. Dougan, E. M. Hoey, S. J. Martin, B. K. Rima, and A. Trudgett.** 1993. Vaccine design, p. 130. Wiley, Chichester, England.
2. **Clem, L. W., N. W. Miller, and J. E. Bly.** 1991. Evolution of lymphocyte subpopulations, their interactions and temperature sensitivities, p. 191–213. *In* G. W. Warr and N. Cohen (ed.), Phylogenesis of immune functions. CRC Press, Boca Raton, Fla.
3. **Ellis, A. E., A. S. Burrows, and K. J. Stapleton.** 1988. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases and extracellular haemolysins. J. Fish Dis. **11:**309–323.
4. **Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn.** 1993. *Renibacterium salmoninarum* and bacterial kidney disease—the unfinished jigsaw. Annu. Rev. Fish Dis. **3:**87–104.
5. **Garduno, R. A., J. C. Thornton, and W. W. Kay.** 1993. Fate of the fish pathogen *Aeromonas salmonicida* in the peritoneal cavity of rainbow trout. Can. J. Microbiol. **39:**1051–1058.
6. **Graham, S., and C. J. Secombes.** 1990. Cellular requirements for lymphokine secretion by rainbow trout *Salmo gairdneri* leucocytes. Dev. Comp. Immunol. **14:**59–68.
7. **Izhar, M., L. DeSilva, H. S. Joysey, and C. E. Hormaeche.** 1990. Moderate immunodeficiency does not increase susceptibility to *Salmonella typhimurium aroA* live vaccines in mice. Infect. Immun. **58:**2258–2261.

8. **Kaattari, S. L.** 1992. Fish B lymphocytes: defining their form and function. Annu. Rev. Fish Dis. **2:**161–180.
9. **Kaufmann, S. H. E.** 1995. Immunity to intracellular microbial pathogens. Immunol. Today **16:**338–342.
10. **Marsden, M. J., D. Cox, and C. J. Secombes.** 1994. Antigen-induced release of macrophage activating factor from rainbow trout *Oncorhynchus mykiss* leucocytes. Vet. Immunol. Immunopathol. **42:**199–208.
11. **Marsden, M. J., A. DeVoy, L. Vaughan, T. J. Foster, and C. J. Secombes.** 1996. Use of a genetically attenuated strain of *Aeromonas salmonicida* to vaccinate salmonid fish. Aquacult. Int. **4:**55–66.
12. **Marsden, M. J., S. H. Hamdani, and C. J. Secombes.** 1995. Proliferative responses of rainbow trout, *Oncorhynchus mykiss*, T and B cells to antigens of *Aeromonas salmonicida*. Fish Shellfish Immunol. **5:**199–210.
13. **Norqvist, A., A. Bergman, G. Skogman, and H. Wolf-Watz.** 1994. A field trial with the live attenuated fish vaccine strain *Vibrio anguillarum* VAN1000. Bull. Eur. Assoc. Fish Pathol. **14:**156–158.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
15. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
16. **Thatte, J., S. Rath, and V. Bal.** 1993. Immunization with live versus killed *Salmonella typhimurium* leads to the generation of an IFN-γ-dominant versus an IL-4-dominant immune response. Int. Immunol. **5:**1431–1436.
17. **Thompson, I., T. C. Fletcher, D. F. Houlihan, and C. J. Secombes.** 1994. The effect of dietary vitamin A on the immunocompetence of Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. **12:**513–523.
18. **Thornton, J. C., R. A. Garduno, S. G. Newman, and W. W. Kay.** 1991. Surface disorganised, attenuated mutants of *Aeromonas salmonicida* as furunculosis vaccines. Microb. Pathog. **11:**85–89.
19. **Vaughan, L. M., P. R. Smith, and T. J. Foster.** 1993. An aromatic-dependent mutant of the fish pathogen *Aeromonas salmonicida* is attenuated in fish and is effective as a live vaccine against the salmonid disease furunculosis. Infect. Immun. **61:**2172–2181.

Editor: P. J. Sansonetti