

Molecular Chimerization of *Pasteurella haemolytica* Leukotoxin to Interleukin-2: Effects on Cytokine and Antigen Function†

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A chimeric recombinant protein composed of the *lktA* gene product from *Pasteurella haemolytica* fused to bovine interleukin-2 (IL-2) was made. The LKT-IL-2 chimera was compared with recombinant bovine IL-2 with regard to the ability to induce proliferative responses and LAK cell activity in bovine peripheral blood mononuclear cells in vitro. In both instances, chimerization had no effect on IL-2 activity. Similarly, the LKT component was unaffected in its ability to induce an effective immune response after immunization. The adjuvant properties of IL-2 have been established in a number of models, and this effect was tested by using the chimera. A multiple-injection protocol of LKT-IL-2 was compared with single-dose administration of LKT. The results obtained indicate that while there was no increase in specific antibody production, the IL-2 component of the chimera may be able to affect antigen-specific proliferation, as assessed by limiting-dilution analysis. Use of cytokine-antigen chimeras may provide a valuable antigen-adjuvant formulation that is simple to produce and purify and thus have economic advantages over conventional preparations. Furthermore, chimerization will also ensure that the adjuvant acts at the same site as the antigen, thus optimizing immunostimulatory activity.

Recombinant DNA technology and peptide synthesis are giving rise to many novel vaccines. Unfortunately, such vaccines often lack the immunogenicity of live preparations and there is a need to enhance their immunogenicity by using safe adjuvants. Gamma interferon, interleukin-1 (IL-1), and IL-2 have all been used effectively to enhance specific responses to subunit vaccines (9, 24), as well as whole virus particles (20, 21). We have described previously the use of recombinant bovine IL-2 (rBoIL-2) to enhance immunity to glycoprotein IV from bovine herpesvirus 1. By using a multiple-injection protocol, we established that as little as 0.5 µg/kg/day could enhance specific responses to this antigen (10). This dose of IL-2 represented a half-log dilution of the minimal biologically active dose (2). Further studies have also indicated that multiple-low-dose IL-2 treatment following immunization could induce immune responsiveness to extremely low doses of antigens in the absence of any nonspecific effects (11).

Although the precise mechanisms of IL-2 adjuvancy have not been elucidated, it is known that it does not bypass T-cell involvement and act directly on B cells, nor does it simply act as a foreign protein inducing noncognate help (7, 15). Our own studies have substantiated these claims, as IL-2 was able to enhance cytotoxic responses to bovine herpesvirus 1 glycoprotein IV through a T-cell-dependent pathway (10). Furthermore, IL-2 was able to enhance the response to minute doses of antigen, thus increasing the effectiveness of a subunit vaccine (11). This indicates that IL-2 would have an effect at an early stage of immune recognition-activation (i.e., T-cell help) rather than B-cell differentiation or proliferation. However, in all but a very few applications, formulation of the IL-2 and/or antigen in an emulsion appears to be

a prerequisite for optimal effect, as does multiple-dose cytokine administration (7, 10, 24).

The adjuvant effects of a cytokine may be further enhanced if the cytokine is linked to a (poly)peptide antigen. IFN-γ was chemically linked to avidin, and the conjugated antigen was much more effective than was the unconjugated mixture. The reason proposed for this increase was that gamma interferon was able to act on the cell that presented the antigen (9). While similar experiments have not been reported for IL-2, it is a logical assumption that antigen conjugated to IL-2 would be more effective than a mixture, as such a molecule would ensure that both the antigen and IL-2 would be present in the microenvironment of the lymph node draining the site of immunization.

Leukotoxin (LKT) from *Pasteurella haemolytica* is known to be an effective vaccine against bovine pasteurellosis (6), and serum antibody responses to LKT correlate with protection (16). Our own studies have also indicated that LKT can protect against a *P. haemolytica* challenge. We have produced a molecular chimera consisting of bovine IL-2 and LKT (LKT-IL-2). Our objective was to investigate the effects of chimerization on in vitro and in vivo responses to LKT and whether chimerization may be an effective way of administering antigen-cytokine adjuvant formulations.

MATERIALS AND METHODS

Animals. Hereford beef calves 7 to 8 months old that were seronegative for antibodies against *P. haemolytica* biotype A serotype 1 were used throughout the study. All immunization protocols were carried out by using Emulsigen Plus as an adjuvant as recommended by the manufacturer (MVP Laboratories), except where indicated. Animals were immunized with LKT, LKT-IL-2 molecular chimeras, or a mixture of LKT with an amount of IL-2 equimolar to that given to chimera-immunized animals (LKT plus IL-2). Immunization protocols varied between experiments and are as described in the text. Some animals with natural *P. haemolyt-*

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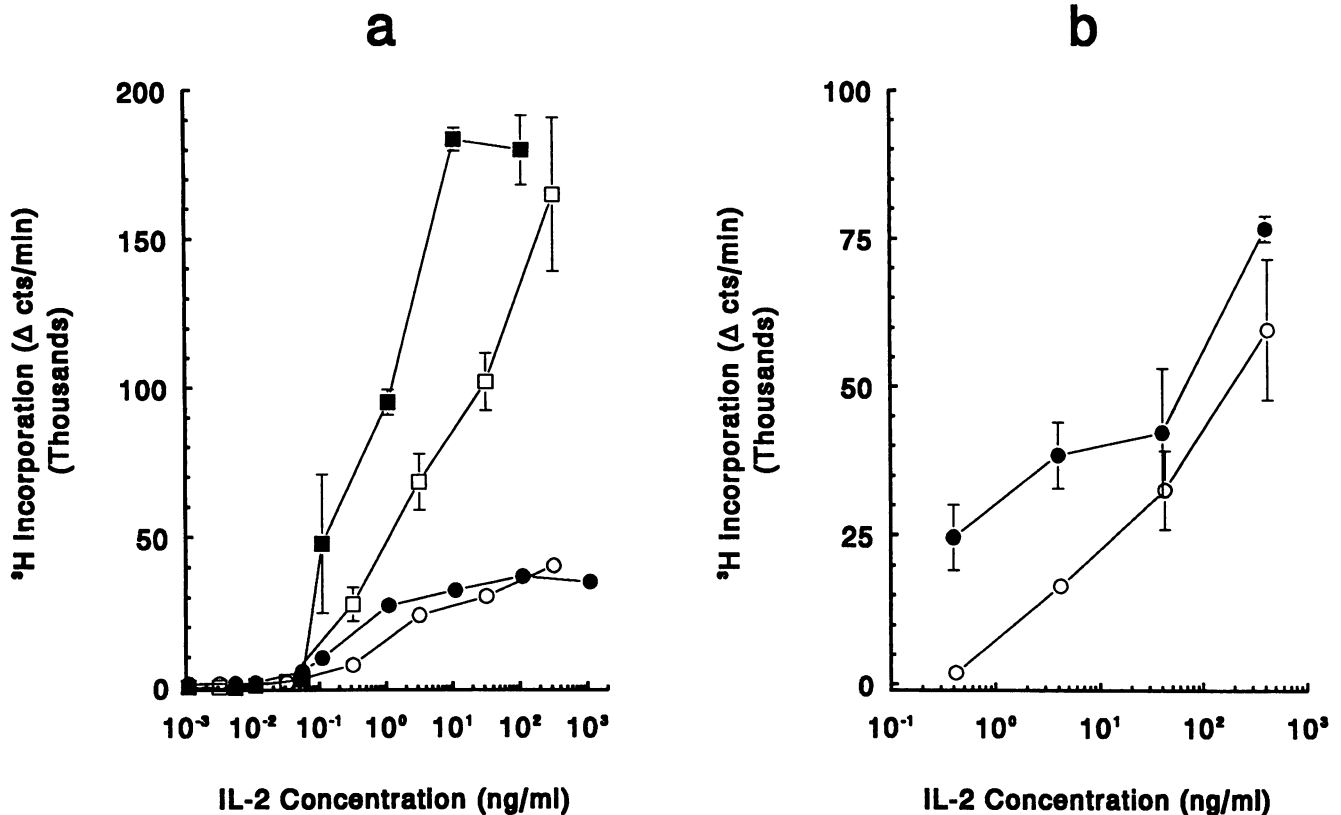


FIG. 1. Ability of rHuIL-2, rBoIL-2, and the LKT-IL-2 chimera to induce proliferation of bovine lymphocytes. Freshly isolated PBMC were plated at 2×10^6 /ml in the presence or absence of different concentrations of rHuIL-2, rBoIL-2, or a molar equivalent concentration of LKT-IL-2. Following 3 days of incubation, [3 H]thymidine incorporation was assessed and plotted as increased counts per minute over those of medium controls. The results are representative of five animals tested. (a) Comparison of the abilities of rHuIL-2 (□ and ○) and rBoIL-2 (■ and ●) to induce proliferation in PBMC (■ and □) or an IL-2-dependent T-cell line (○ and ●). (b) Comparison of the abilities of LKT-IL-2 (○) and rBoIL-2 (●) to induce proliferation in PBMC.

ica infections were used to assess secondary responses in vitro. These animals all had titers against *P. haemolytica* or LKT as described below of $>1/10,000$.

Antigens and cytokines. Recombinant LKT was prepared as previously described by Harland et al. (8). The LKT-IL-2 chimera was prepared as follows. The bovine IL-2 gene in plasmid pBovIL-2 (CIBA-GEIGY, Basel, Switzerland) was digested to completion with restriction endonuclease *Bcl*I, and the single-stranded ends were removed by mung bean endonuclease treatment. The DNA was then digested with *Eco*RI to excise the IL-2 gene fragment. This fragment was ligated into the vector pTZ19R (Pharmacia Canada), digested with *Eco*RI and *Sma*I. The resulting plasmid, pAA285, did not contain the IL-2 translational terminator and contained a *Bam*HI site at the 3' end of the gene. Plasmid pAA345, containing the *P. haemolytica* leukotoxin gene *lktA*, was digested with *Bam*HI and *Bgl*II, and the 2.75-kb fragment was ligated into *Bam*HI-digested pAA285. The resulting plasmid, pAA354, was digested with *Apa*LI, filled in with the Klenow fragment of DNA polymerase, and digested with *Bam*HI to excise the LKT-IL-2 fragment. This fragment was gel purified and ligated into *tac*-based expression vector pGH433, which had been cut with *Bgl*II, filled in with Klenow polymerase, and digested with *Bam*HI. The resulting clone, pAA356, contained the desired gene fusion under control of the *Escherichia coli tac* promoter. LKT-IL-2 was prepared by adding isopropyl- β -D-thiogalactopy-

ranoside (final concentration, 1 mM) to exponentially growing *E. coli* W1485/pAA356. After 3 h at 37°C, cells were harvested by centrifugation and lysed by sonication in sodium deoxycholate and Triton X-100. Aggregated LKT-IL-2 was harvested by low-speed centrifugation and washed with several changes of double-distilled water. This material was solubilized with guanidine hydrochloride and diluted to a concentration of approximately 200 μ g/ml, and the guanidine hydrochloride was removed by dialysis against several volumes of Tris-buffered saline, pH 8.0. Solubilized material was stored at -20°C . Sodium chloride extraction of *P. haemolytica* antigens was done by harvesting exponentially growing cells of *P. haemolytica* serotype 1 strain B122 (19), suspending them in 0.1 volume of 0.85% NaCl, and vigorously shaking them for 60 min at 37°C with glass beads. Cell debris was removed by centrifugation, and the supernatant was filter sterilized and stored at -20°C . Protein concentration was measured by using a Bradford assay (Bio-Rad Laboratories). rBoIL-2 (CIBA-GEIGY) had a specific activity of 10^7 U/mg. Recombinant human IL-2 (rHuIL-2; Boehringer Mannheim, Laval, Quebec, Canada) had a specific activity of 3.1×10^6 U/mg.

Lymphocyte blastogenesis. Lymphocyte blastogenesis was done as previously described (12). Peripheral blood mononuclear cells (PBMC) were isolated from venous blood and incubated in wells of 96-well cluster trays with serial dilutions of antigen, mitogen, or IL-2 in RPMI 1640 medium

(GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum (Hyclone, Logan, Utah). After 3 (mitogens) or 5 (IL-2 and/or antigens) days, cultures were pulsed with 0.4 μ Ci of [*methyl-3*H]thymidine (Amersham, Oakville, Ontario, Canada), harvested onto glass fiber filter strips, and prepared for β -spectroscopy. Control cultures comprised cells that were cultured in medium alone. Results are expressed as increased counts per minute compared with control (RPMI) cultures.

Precursor frequency determination. The frequency of cells responding to antigen, chimera, or IL-2 was assessed by using different immunization protocols or in vitro stimuli, as previously described (12, 23). For the assay, responder cells comprised T cells enriched by passage through nylon wool columns (14). Briefly, responder cells (2×10^5 per well to 1×10^3 per well) were plated into 96-well U-form plates in 100 μ l of RPMI 1640 with 10% fetal bovine serum and 2.5×10^4 autologous irradiated (1,500 rads) feeder cells. For assessment of cells responding to IL-2 or the IL-2 component of an LKT-IL-2 chimera, responder and feeder cells were incubated with 30 ng of IL-2 or 203 ng of LKT-IL-2 per ml. This dose of the chimera contained 30 ng of IL-2 per ml. Antigen-responsive cells were assessed similarly, by incubating cells in the presence of 20 μ g of LKT per ml. Following 4 (IL-2 plus the chimera) or 6 (LKT) days, cultures were pulsed with [*3*H]thymidine for 18 h and harvested onto glass fiber filters, and radioactivity was measured by β -spectroscopy. Control cultures comprised feeder or responder cells incubated alone in the presence or absence of the antigen. Test wells were considered positive when values exceeded the mean control value by 3 standard deviations. Linear regression analysis of the log percentage of wells negative against the number of responders plated yielded the line from which the minimal estimate of frequencies could be calculated as the number of cells corresponding to 37% negative wells (23).

Serology. Enzyme-linked immunosorbent assays using LKT or NaCl extraction of *P. haemolytica* were done as previously described (8).

Cytotoxicity. LAK cell activity was assessed in an 18-h 51 Cr release assay in which K562 cells cultured in the presence or absence of 10 U of rHuIL-2 (Boehringer Mannheim) per ml were used as targets (3). The percentage of specific cytotoxicity was calculated from the mean of quadruplicate cultures by using the following formula: % cytotoxicity = [(cpm with effector cells - cpm spontaneous release) / (cpm total release - cpm spontaneous release)] \times 100, where cpm is counts per minute.

RESULTS

Effects of chimerization on IL-2 activity. Preliminary in vitro studies were done to assess the ability of rBoIL-2 or rHuIL-2 and the LKT-IL-2 chimera to induce a proliferative response in PBMC or an IL-2-dependent T-cell line. There was no significant difference between the abilities of rBoIL-2 and rHuIL-2 to induce a proliferative response in IL-2-dependent T-cell (concanavalin A) blasts when they were used on a weight-per-volume basis. However, PBMC proliferative responses were the same only at optimal concentrations (Fig. 1a). Both cytokines were able to induce similar LAK cell cytotoxicities (data not shown). rHuIL-2 and rBoIL-2 were therefore used interchangeably for comparison in the initial in vitro studies, with 30 (rBoIL-2) or 300 (rHuIL-2) ng/ml inducing optimal proliferation (Fig. 1a). When IL-2 was incorporated as a molecular chimera, there was a similar trend in responsiveness, with 3 or 30 ng of

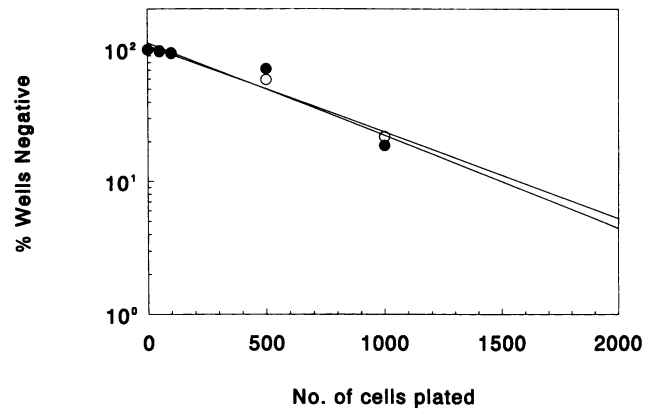


FIG. 2. Responsiveness of PBMC to rBoIL-2 and the LKT-IL-2 chimera, assessed by precursor frequency analysis. Cells from a seronegative calf were stimulated with 30 ng of rBoIL-2 per ml (○) or an equimolar amount of IL-2 in the LKT-IL-2 chimera (203 ng/ml; ●). Control cultures comprised feeders cultured in the presence of IL-2 or the chimera and Sephadex G-10-eluted cells cultured in medium alone. The frequencies of responding cells were 1 in 705 ($r = 0.988$) for IL-2 and 1 in 687 ($r = 0.955$) for the LKT-IL-2 chimera.

chimerized IL-2 per ml inducing the same response as the equivalent concentration of rBoIL-2 (Fig. 1b). Further comparisons were made by assessing the number of cells responding to either form of IL-2 by precursor frequency analysis. The frequency of cells responding to rBoIL-2 was 1 in 705, and the frequency of cells responding to chimerized IL-2 was 1 in 687 (Fig. 2).

Effects of chimerization on LKT responsiveness in vitro. PBMC from LKT-immune or *P. haemolytica*-infected animals generally gave a poor lymphoproliferative response, despite their high serum antibody responses ($>1:10,000$) to both *P. haemolytica* and LKT (Fig. 3). Responses to IL-2 were predictably high (cf. Fig. 1), but in most of the animals studied, response to the LKT-IL-2 chimera was higher than

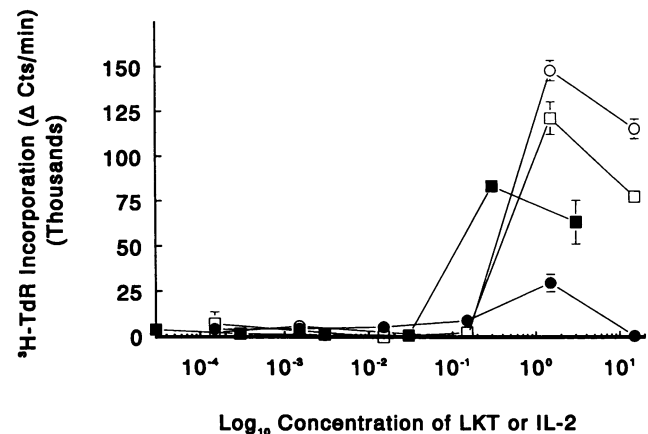


FIG. 3. In vitro proliferative responses to IL-2 and LKT. PBMC from a *P. haemolytica*-immune animal were incubated with various concentrations of LKT (●) or LKT-IL-2 (○), equimolar amounts of IL-2 (■), or LKT mixed with equimolar concentrations of IL-2 (□). Following 5 days of incubation, cells were harvested and [*3*H]thymidine incorporation was assessed. These results are representative of three animals tested.

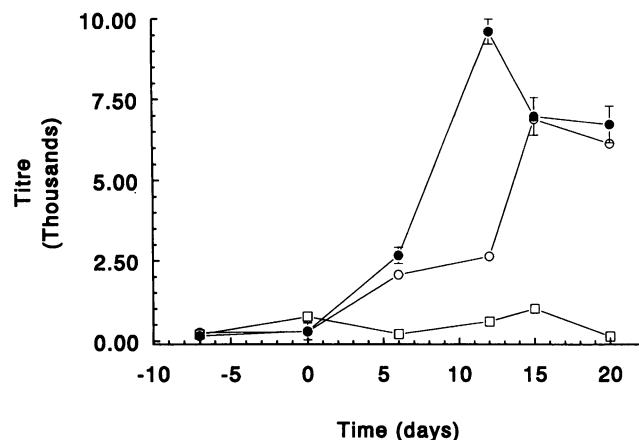


FIG. 4. Antibody responses following primary immunization with five doses (at 12-h intervals) of 100 μ g of LKT (●), 117 μ g of LKT-IL-2 (○), or a placebo (□). Animals were bled at approximately weekly intervals, and titers were assessed by regression analysis from an appropriate standard curve. Positive control serum consistently gave a titer of 10,000.

either the response to IL-2 or the responses to LKT and IL-2 mixtures (Fig. 3).

Effects of immunization with LKT or the LKT-IL-2 chimera. When animals were immunized with single- or multiple-dose regimens of LKT or the LKT-IL-2 chimera, there was no significant difference between serological responsiveness following primary immunization and that following secondary immunization ($P > 0.1$; data not shown). Despite the promising *in vitro* results described above, there was no significant difference among the abilities of LKT, the LKT-

IL-2 chimera, and LKT combined with an equivalent amount of IL-2 to elicit an antigen-specific response ($P > 0.1$; Fig. 4) (data not shown). Since there were also no changes in the phenotype of circulating PBMC, we decided to carry out a more sensitive assessment of immune reactivity by estimating the number of antigen-reactive cells. Limiting-dilution analysis was used to assess T-cell responsiveness following immunization with LKT or the LKT-IL-2 chimera. The frequency of cells responding to LKT following multiple-dose immunization with LKT was 1 in 10,900, and that following LKT-IL-2 chimera immunization was 1 in 9,100. Immunization using a single dose of the LKT-IL-2 chimera resulted in significantly fewer responding cells (1 in 93,000; Fig. 5a). The frequency of precursor cells following primary immunization was compared with that following infection or immunization and a boost with LKT. In all cases, the frequencies of cells responding were between 1 in 16,000 and 1 in 20,000 (Fig. 5b).

DISCUSSION

Pneumonic pasteurellosis, also referred to as shipping fever, is a severe form of disease that often develops following stress and infection with a number of bovine respiratory viruses. To control this disease complex, we have relied on killed or live vaccines (4), although more recently vaccination with LKT has become a viable alternative (8). We have cloned and expressed the *lktA* gene, and the LKT produced has the immunogenic advantages of conventionally isolated LKT but not the adverse cytotoxic activity for polymorphonuclear leukocytes (5). This gene product has therefore become a viable choice for a candidate subunit vaccine against pneumonic pasteurellosis, although as with other subunit vaccines, a suitable adjuvant that is

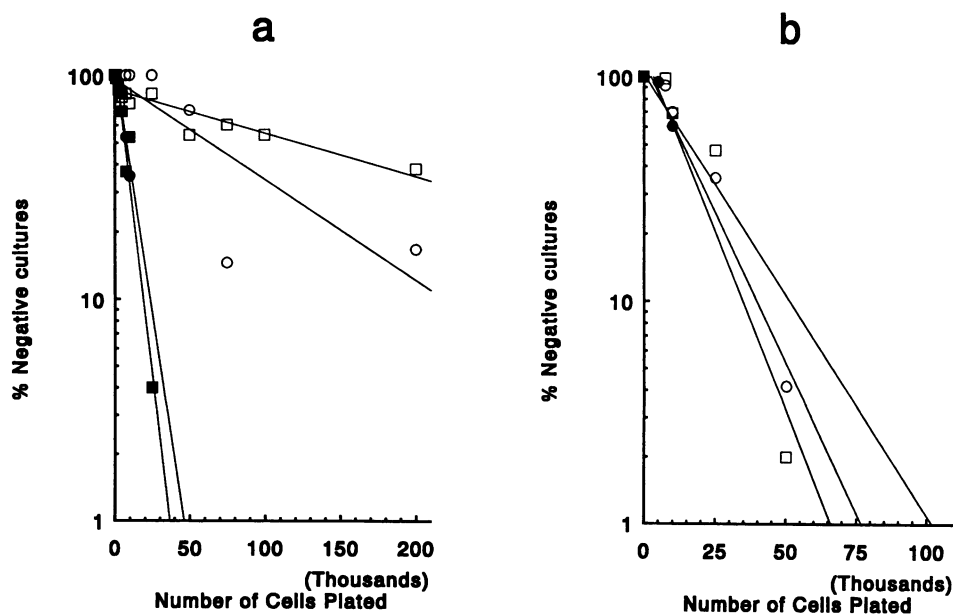


FIG. 5. (a) Precursor frequency (f_p) analysis of LKT-responding cells following immunization with a placebo (□; $r = 0.92$; $f_p = 1:189,700$), a single dose of 117 μ g of LKT-IL-2 (○; $r = 0.82$; $f_p = 1:93,000$), five doses of 100 μ g of LKT (●; $r = 0.95$; $f_p = 1:10,900$), or five doses of 117 μ g of LKT-IL-2 (■; $r = 0.98$; $f_p = 1:9,100$). Correlation coefficients and frequency determinations were assessed by linear regression. (b) f_p analysis of LKT-responding cells following infection with *P. haemolytica* (●; $r = 0.92$; $f_p = 1:22,500$), immunization and boosting with five doses of 100 μ g of LKT every 12 h (□; $r = 0.94$; $f_p = 1:17,125$), or primary immunization with five doses of 117 μ g of LKT-IL-2 every 12 h (○; $r = 0.98$; $f_p = 1:18,400$).

inexpensive yet highly efficient and safe to use needs to be an integral part of the formulation.

IL-2 is known to be an extremely effective adjuvant when used with a number of antigens, particularly when administered in a multiple-injection protocol (7, 10, 15), and linkage of the immunological adjuvant to an antigen has distinct advantages over a mixture. Preparation and delivery costs are lower, and the adjuvant is delivered to the same site as the antigen, thus activating those cells that may be involved in initiation of the response at the injection site or local draining lymph nodes. Use of a molecular chimera to this end has a further advantage in that there is no chemical process involved in ligating the two components which may later compromise the functional or immunogenic activity of IL-2 or LKT. This study has demonstrated the functional integrity of both components of the chimeric molecule consisting of LKT and IL-2.

First, we established that rHuIL-2, rBoIL-2, and the LKT-IL-2 chimera all induce the same proliferative responses in freshly isolated bovine PBMC and/or T-cell blasts. rHuIL-2 and rBoIL-2 share over 70% homology (1), and those regions essential for receptor binding and molecular configuration (13) are particularly well conserved, as are the p55 receptor sequences (25). It is likely that receptor-binding sites on both rHuIL-2 and rBoIL-2 are the same, i.e., through a region of highly conserved amino acids near the carboxy terminus. We have demonstrated that the functional integrity of IL-2 is kept, despite chimerization to LKT. Further, even when IL-2 chimerization is achieved at the carboxy terminus, there is no detectable effect on IL-2 function (12a). Thus, the structure and exposure of relevant binding sites is kept intact and there appears to be no steric hindrance by the larger LKT molecule.

IL-2 is well established as an immunological adjuvant. It is known to enhance responses to a number of antigens, including modified live bovine herpesvirus 1 (21), inactivated rabies virus (18), and subunit vaccines of medical and veterinary importance (7, 10). Recently, we have established the minimal biologically active dose (2) and used it in a multiple-injection protocol to demonstrate adjuvant activity with a bovine herpesvirus 1 glycoprotein IV vaccine (10). Cytokine adjuvants are known to be more effective when given in a multiple-injection protocol (10, 11, 21), and their activity may be further enhanced in a slow-release formulation (17). Therefore, we assessed the effects that the LKT-IL-2 chimera had on immune responses to LKT when administered in a multiple-injection protocol. The results indicate that T-cell reactivity may be enhanced following multiple administration. Furthermore, the number of cells responding to antigen following a primary immunization was similar to that following a secondary response. This indicates that in its present form, the chimera is able to enhance primary antigen responses to levels similar to those seen after boosting. However, in the *in vivo* trials, there was never any significant difference in antibody response among animals that were given the LKT-IL-2 chimera, LKT, or LKT with equimolar amounts of IL-2. These results are surprising, as previous studies have indicated that one of the most noticeable effects of IL-2 is to enhance serum antibody production (10, 20). This discrepancy may be explained by the fact that less IL-2 was administered in the chimera (17 µg per animal per dose) than has been used in other studies (approximately 100 µg per animal per dose). Should this dose be increased, then more overt adjuvant activity may be detected by means other than the highly sensitive limiting-dilution analysis.

Immunomodulation by IL-2 has been exploited in a chimera of IL-2 and *Pseudomonas* endotoxin with which autoimmune uveoretinitis was prevented by selectivity suppressing activated T cells (22). In this study, the LKT component of the LKT-IL-2 chimera was not modified and therefore still contained the binding site for polymorphonuclear cells. It is possible that under these conditions the LKT-IL-2 chimera is targeted to the incorrect cell population (i.e., polymorphonuclear leukocytes) for the desired effect of enhanced specific responses mediated by T and/or B lymphocytes. We are investigating ways both to enhance adjuvant activity and to overcome potential polymorphonuclear leukocyte targeting by LKT. Thus, we propose to enhance adjuvant activity by increasing the molar ratio of IL-2 by (i) adding multiple copies of IL-2 to LKT and/or (ii) epitope mapping the LKT molecule so that more IL-2 may be chimerized to a truncated form of the *lktA* gene. This would provide the advantages of the present system while improving immunogenicity, overcoming potential targeting problems, and raising adjuvant properties.

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