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Progress Report
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I. Work Summary

We are rushing along with the project, essentially following our proposed outlined experiments. We are somewhat ahead of our estimated timetable and have generated some unexpectedly positive findings regarding the biological activity of synthetic peptides based on the binding site of LBP. I have appended some of these results at the end of this report.

A. Progress on specific aim #1

This specific aim consists of identifying the LPS binding site of LPS binding protein (LBP). We have completed this goal aim as outlined in our proposal by generating overlapping synthetic peptides and testing for the ability to bind LPS in a slot blot capture system. Fine mapping using this technique suggests that amino acids 86 to 102 are important in the binding. A longer peptide (hLBP76-102) has slightly better binding affinity on a molar basis, perhaps due to stability or secondary and tertiary structure.

We initially selected the LBP peptides as perhaps the best candidate for peptide-IgG conjugates because we predicted that the binding affinities would be high and also especially because this is the only LPS binding protein naturally found in the bloodstream. In our hands, most LPS binding peptides that have been described bind with much lower affinity in blood. Since the native holoprotein of LBP mediates and increases LPS-induced cytokine production by greatly facilitating the interaction of LPS with the CD14 receptor on monocytes\macrophages, we were unsure of what the biological activity of the peptides would be. Accordingly, we studied the biological activities of the peptides noted above in our neutralizing assays. We were pleasantly surprised to find that the peptides inhibit and in some cases completely block IL-1, IL-6, and TNF production from adherent human monocytes and also in whole blood. These experiments were performed in ongoing collaboration with Jean-Marc Cavaillon at the Pasteur Institute in Paris, France. In contrast, the peptides did not neutralize the effect of LPS in the Limulus lysate assay. Representative experiments are appended.

This finding suggested that one major mechanism of action of the peptides may be to bind to LPS in blood and block its binding to native LBP in the plasma, thus inhibiting LPS-LBP binding to CD14. To study the mechanism of activity, we obtained Chinese hamster ovary cells that had been transfected with CD14, and studied the binding of LPS to CD14 on the cell surface using laser immunofluorescent confocal microscopy. As expected, we found that the binding of LPS to the cells was dependent upon the presence of CD14 and serum as a source of native LBP. Notably, hLBP76-102 and hLBP86-102 blocked this binding. Thus, we believe that a much of the inhibition of the cytokines is due to competition for LPS-native LBP binding. Since there is some inhibition of cytokine activity by the peptides even in the absence of serum, however, this cannot be the entire mechanism. Presumably the second mechanism is via binding to LPS and

block the active lipid A moiety by steric hindrance in a way that alters minimal cellular activation without blocking activation of Limulus lysate. Our findings are consistent with very recently published data that a 197 residue recombinant fragment of LBP blocks cytokine activation via blocking LPS binding to CD14 (J. Biol Chem. 269:8172-8175, March 1994). Although it is unclear if our work narrowing of the binding site to a 17AA residue is patentable, we have submitted our findings to the Office of Technology Affairs at the MGH. We hope to submit a manuscript within the next several weeks describing these data.

B. Progress on specific aim #2

Our work on peptides mimicking LBP has overshadowed for the moment our fine mapping of the LPS binding site of bactericidal permeability increasing protein (BPI). As noted above, LBP based peptides have the distinct advantage of mimicking a protein that is known to bind LPS in the bloodstream. BPI is a protein in granulocytes. We are however in the process of synthesizing overlapping proteins surrounding the region that is expected to best bind LPS based on its homology to LBP. Indeed, there is published information on synthetic peptides that retain the bactericidal activity of BPI (J. Biol Chem. 269:1865-1872, 1994). However, these investigators also found multiple peptides spanning the entire molecule that reportedly neutralized the effect of LPS. In our opinion this information casts some doubt on the experimental technique used for the LAL experiments and we will repeat them once we have peptides in hand. We predict that the region of BPI that will bind LPS with the highest affinity will be the section that is highly homologous to LBP in sequence and chemical properties (alternating hydrophobic and amphipathic amino acids) that extends from residues 85-102.

C. Progress on specific aim #3

We have prepared several lots of hLBP76-102-human IgG and hLBP86-102-human IgG using SPDP as a coupling linker as described in the grant protocol. This linker contains a disufide bond between the peptide and the IgG. We have also generated a single test lot of conjugate of these peptides with SMPT, a similar linker that contains large molecular groups surrounding this bond which block reduction, making the link more stable *in vivo*.

In similar parallel work, we generated conjugates of SPDP and SMPT linked IgG made with the LPS binding sites of Tachypleus anti-LPS factor (TALF) and cationic antimicrobial protein (CAP 18) for comparison. Some of the analysis of these conjugates preceded the work on the LBP conjugates because we had the sequence of the binding site earlier.

D. Progress on specific aim #4

We characterized the above conjugates for LPS binding activity by SDS-PAGE, radioimmunoassay, and Western blotting using the methods outlined in our grant. These data indicate that both the LBP and CAP18 conjugates with both linkers bind LPS with high affinity. The Western blots indicate that the peptide is cleaved from the conjugates upon reduction with 2-mecaptoethanol for the SPDP linked conjugates, but not for the SMPT conjugates.

An important issue for the further development of the conjugates will be to assess their ability to bind LPS in whole blood and to circulate in a stable form for relatively long periods in the bloodstream. As written in the original

grant, we have developed a magnetic bead assay to study these issues. The basis of the assay is to capture the conjugates from whole blood with anti-human IgG covalently coupled to magnetic immunobeads. Radiolabeled LPS is then added to whole blood and then the beads are added and magnetically removed and counted for activity. Thus, functional stability of the conjugate is assessed in the blood. We have established in preliminary *in vitro* experiments that the CAP18 (SPDP) conjugates are functionally stable for four hours in whole blood, but that activity is much less after incubation in whole blood for 24 hours. In contrast, in the one preliminary experiment that we have done, the CAP18 (SMPT) conjugate is stable for at least 24 hours, the longest time point studied. We thus believe that this linker will be preferable. We plan to use this same assay to evaluate the stability of the conjugates *in vivo*.

We assessed the CAP18 (SPDP) conjugates for *in vitro* neutralizing activity with respect to cytokine inhibition and Limulus lysate inhibition. The conjugates are as active as the peptides they contain. We are presently evaluating the LBP conjugates in an analogous manner.

E. Progress on specific aim #5

We assessed the pharmacokinetics of the CAP18 (SPDP) conjugate in several rabbits. After a 10 mg injection, there is an early peak, and then a plateau of conjugate in serum at approximately 100 ug/ml over 6 hour (based on human IgG as assessed by ELISA). This level is several fold higher than what we would expect is needed to bind even nanogram concentrations of LPS in the blood-stream. As noted above, we now need to assess the functional stability of the peptide over longer time periods. Ideally, this should be done with radiolabeled peptide so that we can assess if the peptide/IgG concentrations are stable *in vivo*.

II. **New knowledge since start of the grant**

1. We have defined the LPS binding site of LBP. Synthetic peptides mimicking this site bind LPS, block binding of LPS to LBP, block binding of LPS to the CD14 receptor on transfected Chinese hamster ovary cells, and block LPS-induced cytokine activity in whole blood and adherent human monocytes. Notably, these same peptides do not neutralize LPS as assessed by the Limulus lysate assay. These findings suggest to us that the LBP peptides are functioning mainly by blocking the binding of LPS to LBP (rather than globally sterically blocking all of the activities of lipid A). The peptides therefore appear to provide a means of specifically blocking the binding of LPS to CD14 on cells, thus inhibiting cellular activation. We have submitted this information to the MGH office of technology assessment for an opinion as to whether this is a discovery that should be patented.

2. We have made LBP-human IgG conjugates using these peptides. The LBP-IgG conjugates bind LPS with high affinity in all assays tested. We have not yet tested the behavior of these conjugates in the assays described above.

3. We have studied synthetic peptides based on the binding site of CAP18. These peptides neutralize LPS. CAP18-IgG conjugates bind LPS with high affinity in all of our assay systems. These conjugates neutralize LPS as assessed by inhibition of cytokine production and activation of Limulus lysate.

4. We have started to compare the production of conjugates with SPDP with the more stable conjugate SMPT. CAP18-IgG (SMPT) conjugates incubated in whole

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blood are more stable than CAP18-IgG (SPDP) conjugates with respect to binding LPS in preliminary experiments.

5. We have started to evaluate the pharmacokinetics of the conjugates in rabbits. The human IgG concentration in the rabbit serum is stable (about 100 ug/ml) over at least 6 hours after IV injection of 10 mg of CAP18 (SPDP) conjugate.

III. Technical problems

We have had no substantive technical problems.

IV. Future directions

We plan to continue on our outlined workplan without much deviation.

Our binding assays suggest that the LBP peptides and the CAP18 peptides bind LPS with the highest affinity. Since we had the sequence to the CAP18 binding site prior to the LBP binding site, much of our *in vitro* analysis has been with the CAP18 conjugates. We are now catching up by repeating these experiments with the LBP peptides. We now find that the LBP peptides not only bind LPS but block its binding to LBP (and thus block cytokine activity) . Thus we believe that it is especially important to rush ahead with these experiments. In the meantime, we are generating the peptides to the binding site of BPI. These conjugates will follow on the heels of the other two.

Specific issues that we think that will be important to address in the next trimester will be:

A. How well does each conjugate bind LPS in whole blood?
B. How stable is each conjugate in whole blood?
C. Are the biological blocking activities of the LBP peptides still present when the peptide is part of a conjugate?
D. What are the pharmacokinetics of the different conjugates *in vivo*?

Two reagents that we hope to get started on in the next trimester will be the generation of ^{14}C-peptides and their respective conjugates for *in vivo* studies and the generation of murine monoclonal antibodies to the LBP peptide.

V. Publications

The following manuscripts have appeared or are in the process of submission since the annual review of our last grant:

1. Wilz SW, Kurnick JT, Pandolfi F, Rubin RH, Warren HS, Goldstein R, Kersten CM, McCluskey RT. T lymphocyte responses to antigens of gram-negative bacteria in pyelonephritis. Clin Immunol Immunopathol 1993; 69:36-42.

2. Ge Y, Ezzell RM, Tomplins RG, Warren HS. Cellular distribution of endotoxin after injection of chemically purified lipopolysaccharide differs from that after injection of live bacteria. J Infect Dis 1994; 169:95-104.

3. Larrick JW, Hirata M, Zheng H, Zhong J, Bolin D, Cavaillon J-M, Warren HS, Wright SC. A novel granulocyte-derived peptide with LPS neutralizing activity. J Immunol 1994; 152:231-240.

4. Kersten CM, McCluskey RT, Warren HS, Kurnick JT. Responses of human T Cell to dominant discrete protein antigens of Escherichia coli and Pseudomonas aeruginosa. Scand J Immunol. In Press 1994.

5. Kloczewiak M, Black KM, Loiselle P, Cavaillon JM, Wainwright N, Warren HS. Synthetic peptides that mimic the binding site of horseshoe crab anti-lipopolysaccharide factor. Provisionally accepted to J. Infect Disease, final revision sent.

6. Warren HS, Cavaillon JM, Loiselle P, Ge Y, Black K, Zanzot E, Fitting C, Golenboch D, Vermeulen MW, Ezzell R, Kloczewiak M. Identification of a major LPS binding site of lipopolysaccharide binding protein. Manuscript being prepared for submission.

VI. Legends to Figures and Tables

Table 1. Binding of LPS to peptides based on LPS binding protein assessed by the slot blot capture assay. Data are expressed as minimum nanomoles that bind an excess of LPS. Thus, lower numbers reflect higher binding affinity. Methods of the assay are unchanged from our original grant proposal. The two peptides with highest binding affinity are hLBP76-102 and hLBP86-102.

Table 2. Whole blood cytokine assay of LBP peptides. Peptides (10 ug/ml) were preincubated with LPS before adding to whole blood. Cytokines were assayed as described in our grant. The findings are reflective of 4 separate experiments. Thus, hLBP76-102 and hLBP86-102 block TNF and IL-6 secretion in whole blood. IL-1 secretion is also inhibited. Cytokine induction is similarly blocked in adherent human monocytes, with and without preincubation. In these experiments, the inhibition is augmented in the presence of human serum containing LBP.

Figure 1. Sequence and coding of peptides based on LPS binding protein.

Figure 2. Binding of LBP-human IgG conjugates to E. coli O75 LPS (open symbols) and E. coli O4 LPS (closed symbols) in radioimmunoassay with 5 ug/ml tritiated LPS. The methods of assay are as in our original grant proposal. P349A (triangles) is a human IgG control. P349B (circles) is a hLBP86-102-human IgG conjugate coupled with SPDP. P348B (squares) is a hLBP76-102-human IgG conjugate coupled with SPDP. The IgG controls do not bind to either LPS, whereas both conjugates bind in roughly equalmolar concentrations to LPS.

Figure 3. Limulus lysate assay of neutralization of E. coli O111:B4 LPS by hLBP86-102 (closed triangles, RA517) and hLBP86-102-human IgG conjugate coupled with SPDP (closed squares, P349B) compared to no peptide control (open circles, saline). The IgG conjugate (50 ug/ml) contains 1.8 ug/ml peptide. Thus the concentration of peptide is equal in both samples. Neither the peptide nor the peptide-IgG conjugate neutralize in this assay, even at greater than 100,000-fold molar excess to LPS concentration.

Table 1 - Binding of Lipopolysaccharide (LPS) to Peptide Analogues of Human LPS Binding Protein (hLBP) : Minimal Peptide that Binds LPS.

	Mean ± S. E.M. (nanograms)	Mean ± S.E.M. (picomoles)
hLBP1-30	918 ±116	291 ±37
hLBP26-55	2148 ±293	612 ±83
hLBP51-80	2604 ±1053	785 ±317
hLBP76-102	118 ±36	38 ±11
hLBP86-102	156 ±44	74 ±21
hLBP86-99	2396 ±262	1315 ±144
hLBP86-106	1432 ±493	557 ±192
hLBP89-106	2081 ±300	978 ±141
hLBP94-106	9375 ±1804	6014 ±1157

Table 2

Whole blood cytokine assay with 20 ng/ml E. coli LPS

Peptide	TNF	IL-6
no peptide	3263	2463
hLBP 26-55	3370	3565
hLBP 76-102	<30	48
hLBP 86-102	<30	96

Whole blood cytokine assay with 10 ng/ml S. minnesota LPS

Peptide	TNF	IL-6
no peptide	2925	2643
hLBP 26-55	3087	2433
hLBP 76-102	501	165
hLBP 86-102	<30	78

Figure 1 - Synthetic peptides mimicking LPS binding protein

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                10        20        30        40        50        60
          ANPGLVARITDKGLQYAAQEGLLALQSELLRITLPDFTGDLRIPHVGRGRYEFHSLNIHS

hLBP1-30  ANPGLVARITDKGLQYAAQEGLLALQSELL
hLBP26-55                          QSELLRITLPDFTGDLRIPHVGRGRYEFHS

          50        60        70        80        90        100   106
          RYEFHSLNIHSCELLHSALRPVPGQGLSLSISDSSIRVQGRWKVRKSFFKLQGSFDV - COOH

hLBP51-80   YEFHSLNIHSCELLHSALRPVPGQGLSLSI
hLBP76-102                         LSLSISDSSIRVQGRWKVRKSFFKLQGC
hLBP86-102                                 RVQGRWKVRKSFFKLQGC
hLBP86-99                                  RVQGRWKVRKSFFK
hLBP86-106                                 RVQGRWKVRKSFFKLQGSFDV
hLBP89-106                                     RWKVRKSFFKLQGSFDV
hLBP94-106                                         RKSFFKLQGSFDV
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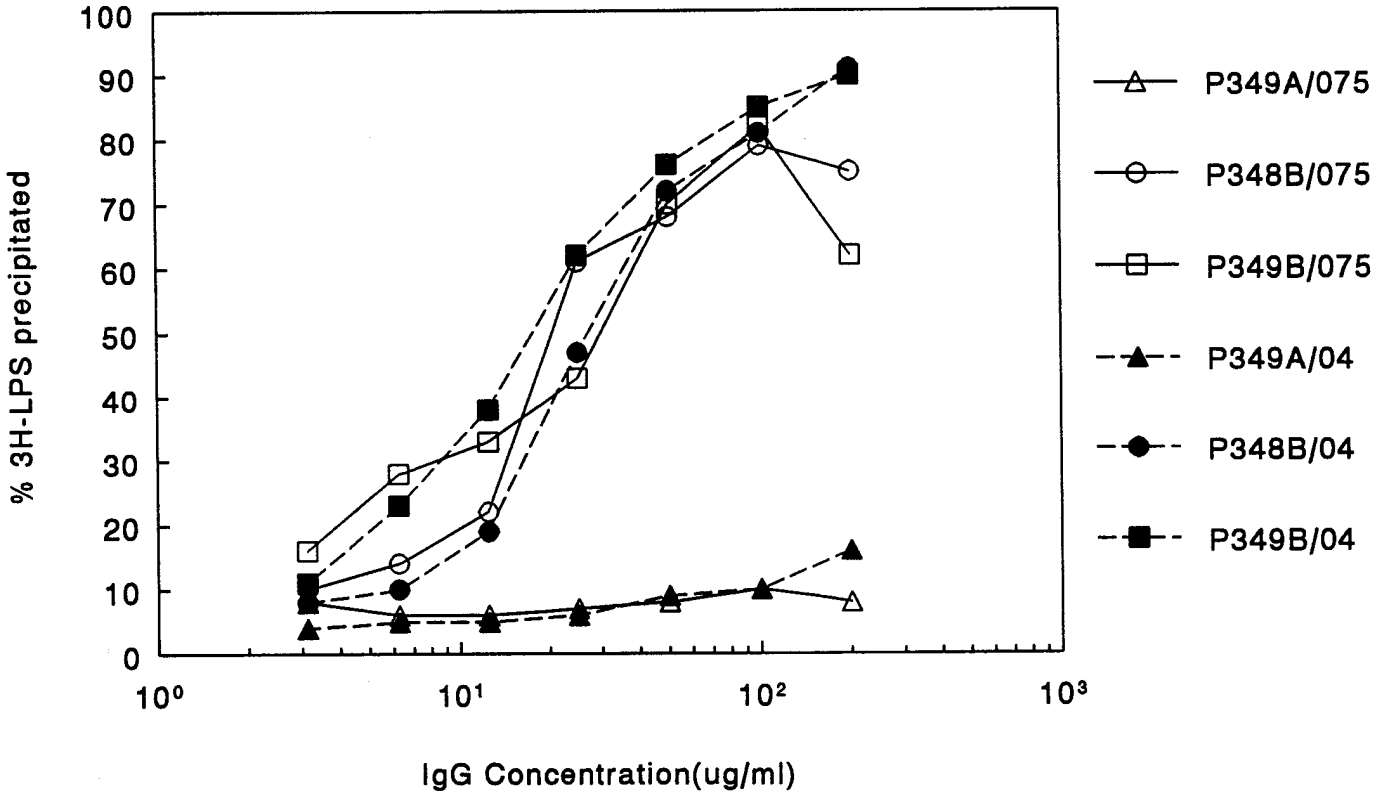
Binding of conjugates to E.coli 3H LPS



Neutralization of LPS by P349B/RA517

(50 ug/ml Conj, 1.8 ug/ml Peptide)