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I. Work Summary

In the third quarter of the grant we have continued to follow our original outlined plans. We are completely up and running in the new laboratory, and have prepared most of the needed reagents.

Our first approach to the purification of the LPS binding and neutralizing factor(s) is to affinity purify the substance(s) using anti-LPS immunoaffinity columns. Over the last several months we have incubated tritiated LPS prepared from S. typhimurium in normal and tolerant sera and passed the mixture over an anti-LPS column filled with affinity-purified rabbit anti-O-polysaccharide IgG. Approximately 50% of the radioactivity was recovered in the eluted fraction. SDS-PAGE analysis of this material showed no difference between normal and tolerant serum, with and without added LPS. There was, however, considerable non-specific binding. Furthermore, we were limited by the volume of the column to adding 20 ug LPS in 1.0 ml serum. To remedy these problems, we prepared a new column by coupling 17 mg of murine monoclonal IgG directed to the O-polysaccharide of E. coli 0111:B4 to 5 mls of CNBr-activated sepharose 4B. In the single experiment we have performed with this column, we affinity-purified 5 mg of E. Coli 0111:B4 LPS previously incubated in 8 mls of tolerant rabbit serum on the column. An SDS-PAGE of the eluted material contained bands staining for protein that were not present in a control gel of 8 mls tolerant serum passed over the column in the absence of LPS. We are hopeful that repetition and expansion of this experiment with additional controls will confirm our initial (and very preliminary) finding. We plan to use a similar protocol utilizing different combinations of normal and tolerant rabbit serum and homologous and heterologous LPS to the IgG on the column.

Our second approach involves classical protein purification techniques to progressively purify the LPS binding factors. We have completed the packing and calibration of a sepharose 6B-Cl molecular sizing column. We purified the lipoproteins from normal and tolerant serum by ultracentrifugation in KBr at a density of 1.21 g/ml. Greater amounts of $^{3}$H-LPS were precipitated by calcium and dextran following incubation in normal serum spiked with lipoproteins from tolerant rabbit sera. These data confirm our prior experiments and suggest that the lipoprotein fraction of tolerant serum binds LPS. We next passed these lipoproteins over the sepharose 6B-Cl molecular sizing column and obtained four distinct peaks, which we are in the process of testing.

We have refined our TNF inhibition assay and compared our pools of normal and tolerant rabbit sera for the ability to decrease the production

of TNF by the RAW 264.7 cell line. These experiments, now repeated 3 times, have shown that LPS incubated in tolerant sera is less able to induce TNF production. This phenomena has not been previously described. We plan to study several different LPS in the system, and also to study normal serum spiked with lipoproteins from tolerant serum in this system.

We have obtained the HPLC and are in the process of setting it up and ordering columns.

II. New Knowledge

As noted above:

1. In a quite preliminary experiment, we have found a band of approximately 27 KD (and several smaller bands of higher MW) in the SDS-PAGE of affinity purified LPS complexes formed in tolerant sera. Many controls need to be done to confirm and determine the reality and importance of these protein bands.

2. LPS incubated in tolerant serum is less active in inducing TNF from the RAW 264.7 macrophage cell line compared to controls.

III. Technical Problems

None at this time. Our technical problems of the last quarterly report have been resolved. We have eliminated (hopefully permanantly) the fungal contamination of our cell cultures. Our low pressure chromatography system is now working in a satisfactory manner. Finally, we have located an appropriate centrifuge head for our ultracentrifuge which appears to work well for the separation of lipoproteins from other serum proteins.

IV. Goals for the next quarter

a. Repetition and expansion of affinity purification of LPS-protein complexes using the anti-E.coli 0111:B4 LPS column, including multiple controls.

b. If (a.) confirms hopeful bands, modification of buffers to decrease non-specific binding, and further purification of eluted material by HLPC.

c. If (a.) confirms hopeful bands, and perhaps anyway, purification of anti-E.coli 018 LPS monoclonal IgG from acites fluid so as to make a similar anti-LPS column for further confirmation using a different LPS-antibody combination.

d. Testing of molecular sizing column fractions for $^{3}H$-LPS binding activity. SDS-PAGE of fractions. Further purification by HPLC.

e. Radiolabeling of E. Coli 0111:B4 with $^{3}H$-galactose and extraction of radiolabeled LPS.