RECEIVED

SEP 29 1997

OSTI

DOE/OR/21492--T2

Semi Annual Progress Report

Oak Ridge Research Institute

Contract No: DE-AC05-84OR21492

Title: Methods for Environmental Monitoring of DOE Waste
Disposal and Storage Sites

Period: 11-1-85 to 3-31-86

Authors: R. Blake
N. Revis

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

MASTER

DISCLAIMER

**Portions of this document may be illegible
in electronic image products. Images are
produced from the best available original
document.**

CONTENTS

	<u>PAGE</u>
1.0. INTRODUCTION.....	2
2.0. MATERIALS AND METHODS.....	2
2.1.1. Antigen preparation.....	2
2.1.2. Injection sequence.....	3
2.1.3. Animal bleeding.....	3
2.1.4. Titer tests.....	4
2.1.5. Assessment of antibody specificity.....	5
3.0. RESULTS.....	5
3.1.1. IgG produced.....	5
3.1.2. IgG specificity.....	5
4.0. DISCUSSION.....	7
5.0 OUTLINE FOR FUTURE WORK.....	8

(1. INTRODUCTION

This progress report contains an account of recent research efforts carried out at the Oak Ridge Research Institute, to raise antibodies in New Zealand White Rabbits which are specific for various strains of *Thiobacillus ferrooxidans* bacteria. It is then intended to use the antibodies, in a subsequent phase of the project, to develop one or more ELISAs, which would be used to analyze, both in the laboratory and in the field, the numbers and extent of dispersion of these microorganisms at acid mine drainage sites. This is important because it is the metabolic consequences of unrestricted dissemination of these bacteria which are responsible in large measure for the environmentally damaging acid run-off. Efforts to control the proliferation of these microorganisms have been relatively unfocused up to the present, because of the lack of suitably discriminating methods of assaying the effects of treatment. For example, current, non ELISA-based, methods for measuring these microorganisms depend on cell counting techniques, which are inadequate because of lack of specificity, and their inability to measure those organisms which adhere to the substratum.

In theory, these constraints will not apply to the ELISA system, where the key biochemical event is the recognition of the antibody for the antigen. This is inherently more specific than simple cell counting or turbidity measurements, and can occur whether or not the cells are adhering to soil particles. Thus it is envisioned that the ELISA may provide a powerful tool for the accurate, site specific measurement of these microorganisms, with the possibility of creating a system which can be used directly in the field, because of the compact size of 96 well microtiter plates and ELISA reading items.

In this progress report, we describe the work carried out in the first section of the project, which has been concerned with the raising of the antibodies to the *Thiobacillus ferrooxidans* cultures. We give an account of how the various cultures were grown, how they were treated before being injected into rabbits, the injection protocol, how the animals were bled, and how the formation of IgG and its degree of specificity was assessed. Finally we describe how large quantities of the various IgG fractions were prepared, and how and where they were stored.

2.0. MATERIALS AND METHODS

2.1.1. Antigen preparation

Cultures of 5 different strains of *Thiobacillus ferrooxidans* and related species were obtained from Dr. Robert Blake of Meharry Medical School. These cultures, designated 29047, M1, 23270, T.con, and 13598, had been grown in an acidic ferrous sulfate growth medium then frozen, for shipment to the Oak Ridge Research Institute.

The cultures were allowed to thaw then subdivided into smaller aliquots, for more convenient storage. One aliquot of each culture was retained at room temperature, and centrifuged at 2500 rpm for 25 minutes, to sediment the cells. The medium was removed and the cells washed with an equal volume of sterile physiological saline. This was the first of 3 cell washing steps, after which the cells were finally dispersed in the saline, and counted using a hemocytometer.

Based on the cell count obtained, the culture was serially diluted to give a cell concentration of 20000 cells/ml, in a volume of 10 ml. This culture was then injected into a vial containing 10 ml of Freund's Complete Adjuvant,

resulting in a final cell concentration in adjuvant of 10000 cells/ml. These suspensions of adjuvant and cells were then very vigorously mixed by shaking and vortexing, prior to injection into the rabbits.

2.1.2. Injection Sequence

A group of 3 New Zealand White rabbits was used for each antigen preparation. Four rabbits which received no injections served as controls, making a total of 19 animals in the study. The rabbits used were approximately 4 months old at the start of the study, and between 2.5-3.0 kg in weight.

Prior to injection, each animal in the treatment groups was clipped free of hair along its back. 1ml aliquots of freshly dispersed antigen preparation were injected subcutaneously, at 4-5 sites along the back. Following injection, each rabbit was fitted with a restraining collar to prevent the injection sites being disturbed. These collars were removed after approximately 24 hours.

Each animal received an identical series of booster injections after 21 days. After a further 21 days, each animal received similar injections, but with the cell number increased to 50000 cells/ml.

New batches of frozen cells were used for each series of booster injections.

The following table gives a list of the animals, and the antigens they received:

<u>Animal #</u>	<u>Antigen</u>	<u>Animal #</u>	<u>Antigen</u>
446	controls	461	T. con
464		462	
466		463	
468			
455	29047	459	13598
456		447	
457		448	
452	M1	449	23270
453		450	
454		451	

2.1.3. Animal bleeding.

5 ml aliquots of blood were obtained, via the medial ear vein, from all animals in the study. 350 units of Heparin, in a 20ul volume of water, was introduced into the tip of the syringe needle prior to bleeding, to prevent coagulation during bleeding.

The cells were sedimented by centrifugation, then treated with sodium azide to a final concentration of 0.02%. Each sample was then subdivided into two parts; one for storage at -20°C, and the other stored at 4°C.

Immunoglobulins were partially purified from each plasma sample by the addition of an equal volume of saturated ammonium sulfate. The precipitate was washed twice with 50% saturated ammonium sulfate, then dissolved in a small volume of 10mM ammonium bicarbonate, and exhaustively dialyzed against the same solution.

Samples of the plasma, kept at 4°C, were used to assay the extent of

Formation of IgG in response to the injection treatments. The assay used was based on the affinity of enzyme linked Protein A for IgGs. Aliquots of the IgG preparations were used to measure protein, and to assess antigen-binding specificity.

When the extent of IgG production and specificity had been assessed, a further bleeding of the animals took place. In this case, up to 35ml of blood was obtained from the medial ear vein, after which the animal was immediately euthanatized by lethal injection (T61). Preparation of partially purified IgG was essentially as described above. Dialyzed IgG preparations in 10mM ammonium bicarbonate were subdivided into serum bottles, frozen to -70°C , then lyophilized. The lyophilized preparations were further subdivided prior to shipping.

2.1.4. Titer Tests

The assay employed to assess the extent of production of IgG in the plasma samples was an ELISA, using the ability of soluble proteins to bind to the plastic of 96 well microtiter plates, and the ability of alkaline phosphatase-linked Protein A to both bind to the immobilized immunoglobulin, and to retain its enzymatic activity. The provision of excess enzyme-linked ligand, plus excess alkaline phosphatase substrate, allowed a semiquantitative assessment of immunoglobulin production to be made by the extent of colored product (p-nitrophenol) formation in the well.

Only plasma sample 452 was used initially, in a range-finding experiment, using serial dilutions up to 1:10000. 100ul volumes of these dilutions were pipetted into separate wells of a 96 well microtiter plate, and allowed to incubate overnight at room temperature.

The solutions were then sucked out at the vacuum pump, and the wells washed with a solution of 3% bovine serum albumin in 0.1M Tris buffer, pH 8.6. The washing process was repeated three times, and then the albumin solution was allowed to stand in the wells for 2 hours. This was to ensure that the rest of the protein binding sites on the plastic were saturated, so that subsequent immobilization of the Protein A conjugate would be due to IgG specific binding, and not to non-specific binding of the conjugate to the plastic.

Following removal of the bovine serum albumin solution from the wells, 100ul volumes of a solution of 100ug Protein A-alkaline phosphatase conjugate in 10ml of 0.1M Tris buffer, pH 8.6, were added to the wells, and allowed to incubate for 2 hours. After incubation, the solution was sucked out at the vacuum pump, and, using the bovine serum albumin solution, a further cycle of 3 washes of the wells was carried out. Finally, 100ul volumes of a solution of 300 mg p-nitrophenyl phosphate in Tris buffer were added to the wells, and the rate and extent of the subsequent development of the yellow p-nitrophenol was assessed. In the conditions of the assay, this rate was proportional to the amount of IgG contained in the original plasma dilution.

To quantify the extent of response, the reactions were stopped by transfer of the reaction mixture to a vial containing 1.0ml of 0.1M NaOH. The absorbance of these solutions was then read on a spectrophotometer against a water blank, at 405nm.

As is made clear in the RESULTS section, these range finding experiments indicated that the optimum range for a proportional response between color formation and protein concentration was between dilution ranges of 1:2000 and 1:10000. The rest of the plasma samples were therefore serially diluted to 1:4000 and 1:8000, and then assayed for IgG content as described above.

..5. Assessment of Antibody Specificity

The experiment to determine the specificity of the antibody preparations was designed in the light of the fact that the antigen consists of one or more chemical moieties on the surface of a living cell. This constraint might be expected to limit the use of immunodiffusion plates for visualizing the degree of antibody/antigen response. To examine this question, we set up a series of incubations containing unit protein concentrations of the different IgG preparations, together with a constant cell concentration of the particular culture under investigation. After an overnight incubation, the cells, together with any specifically bound IgG molecules which may have bound to them out of solution, were sedimented by centrifugation. The resulting, possibly IgG-depleted, supernatant was carefully separated from the cell pellet, then used as the source of antigen in the AP-Protein A ELISA already described. The difference in the ELISA color generated by this solution and its none cell-incubated control, expressed as δA , is a measure of the affinity an IgG preparation may have for a particular cell culture.

3.0. RESULTS

3.1.1. IgG Produced

Figure 1 summarizes the production of Protein A reactive IgG by the various animals in the study in response to the program of injections. The data consist of the meaned responses of the 1:4000 and 1:8000 plasma dilutions to the Protein A-alkaline phosphatase/p-nitrophenyl phosphate system, based on the extinction coefficient of p-nitrophenol at 405mu. On the average, the animals injected with antigen had at least 4 times the Immunoglobulins in their plasma compared to controls. By far the greatest response occurred in those rabbits receiving the 23270 strain, with up to 11 times the response of the control rabbits. By contrast, Animal #448 failed to display any increased immunoglobulin production compared to controls.

3.1.2. IgG Specificity

Table 1 summarizes the results of several ELISAs, in which comparisons were made between the amounts of Protein A reactive IgG contained in 1mg/ml protein concentrations of the various partially purified IgG preparations, with those amounts remaining after overnight incubation of the IgGs with aliquots of specific *Thiobacillus* strains. After incubation, the samples were centrifuged, and the cell-free supernatant removed from the sedimented cell pellets using pasteur pipettes. Duplicate 200ul aliquots of these solutions, together with identical volumes of untreated preparations, were then used as the target source for the sequence of binding events, in a 96 well microtiter plate, resulting in the proportional immobilization of Protein A/alkaline phosphatase conjugate and the proportional production of p-nitrophenol from the addition of excess p-nitrophenyl phosphate substrate. The difference in absorbance values, δA , between treated and control wells, is a measure of the binding and removal from solution of the immunoglobulin by a particular *Thiobacillus* strain, and thus an index of the degree of specificity of an antibody preparation for its own and related antigens.

Table 1. Removal of IgG from solution by incubation of partially purified IgG preparations with different strains of *Thiobacillus ferrooxidans*.

Difference of ELISA-generated absorbencies between treated and non-treated controls.

<u>IgG preparations/ treatment groups</u>		<u>Thiobacillus ferrooxidans strains</u>				
		<u>29047</u>	<u>M1</u>	<u>23270 (expt 1)</u>	<u>23270 (expt 2)</u>	<u>T. con</u>
controls	446	0.113	0.01	0.052	0.069	0
	464	0.05	0.087	0.051	0.073	0.053
	466	N/D	0.012	0.123	N/D	N/D
	468	0.073	0.002	0.05	0.088	0.047
29047	455	<u>0.295</u>	0.032	N/D	<u>0.171</u>	0.075
	456	<u>0.08</u>	<u>0.335</u>	N/D	<u>0.658</u>	<u>0.213</u>
	457	<u>0.166</u>	0.063	N/D	<u>0.285</u>	0.006
M1	452	0.025	0.014	N/D	0.016	0.008
	453	0.131	0.03	N/D	0.034	0.03
	454	0.113	0	N/D	0.22	0.003
23270	449	<u>0.334</u>	0.104	<u>0.213</u>	<u>0.269</u>	<u>0.167</u>
	450	0.017	0.014	<u>0.26</u>	<u>0.378</u>	<u>0.205</u>
	451	0.114	0	<u>0.165</u>	0.11	0
con.	461	0.019	0.055	0.072	0.09	0
	462	0.091	0.035	N/D	0.075	0.035
	463	0.018	0	N/D	0	0.033
13598	459	0.07	0.073	0.091	0.147	0.055
	447	0	0.044	N/D	0.009	0
	448	0.01	0	N/D	0.107	0.036

A statistical presentation of these data is provided in table 2, in which the δA values for each treatment group are meaned and compared to that of the mean of all the controls in the study.

Table 2. Comparison of the means of the absorbance changes for each treatment group, compared to those of the control group of animals.

<u>IgG preparations/ treatment groups</u>	<u>Thiobacillus ferrooxidans strains</u>				
	<u>29047</u>	<u>M1</u>	<u>23270 (expt 1)</u>	<u>23270 (expt 2)</u>	<u>T. con</u>
29047	0.18 ($\pm$ 0.11)	0.143 ($\pm$ 0.167)	-	0.371 ($\pm$ 0.255)	0.098 ($\pm$ 0.105)
	***	*		***	NS
M1	0.009 ($\pm$ 0.057)	0.015 ($\pm$ 0.015)	-	0.09 ($\pm$ 0.113)	0.014 ($\pm$ 0.014)
	NS	NS		NS	NS
23270	0.155 ($\pm$ 0.162)	0.039 ($\pm$ 0.056)	0.213 ($\pm$ 0.048)	0.252 ($\pm$ 0.135)	0.124 ($\pm$ 0.109)
	*	NS	***	***	*
T. con.	0.042 ($\pm$ 0.042)	0.03 ($\pm$ 0.028)	-	0.055 ($\pm$ 0.048)	0.023 ($\pm$ 0.02)
	NS	NS		NS	NS
13598	0.029 ($\pm$ 0.038)	0.049 ($\pm$ 0.042)	-	0.087 ($\pm$ 0.071)	0.03 ($\pm$ 0.028)
	NS	NS		NS	NS

* P<0.05, ** P<0.01, *** P<0.001.

4.0 DISCUSSION.

Reference to figure 1 makes the point that, in general, more Protein A reactive IgG molecules were produced in those animals receiving injections of 29047 and 23270, than in those animals receiving the other Thiobacillus ferrooxidans strains. Furthermore, It is clear from table 2 that the ability of antibodies from animals in these two groups to co-sediment with Thiobacillus cells is far greater than for those animals receiving other antigens.

The series of experiments designed to determine the degree of antigenic specificity has provided a complex picture (table 1), with, in general, an overall pattern of coidentity between the antigenic determinants on the different strains of Thiobacilli.

Detailed consideration of this table is worthwhile, however. If, first of all, we assume that, in those animals which received 23270, the antibody preparations are mixtures of components with a range of antigenic determinations, we can define the antibodies in preparation 449 as a1, a2, a3, a4, a5, etc, in 450 as b1, b2, etc., and 451 as c1, c2, etc. In theory, any or all of the a's, b's, and c's can be identical. From table 1, we now see that the a's and the b's react with T. con, but the c's do not. Thus, none of the c's share any coidentity with any of the a's and b's. We can also see that the a's produced by 449 interact with 29047, whereas the b's and c's produced by 450 and 451 do not. Thus none of the a's have coidentity with any of the b's and c's. Most interestingly, the c's of 451 appear to only have appreciable coprecipitability with 23270, and not with any other of the Thiobacillus strains tested. Therefore, this mixture of antibodies (in 451) might be a good candidate for use in the further development of an ELISA for 23270.

Similar considerations can be applied in the case of those animals which were injected with T. ferrooxidans culture 29047. In this case we can assume that animal #455 produces a mixture of antibodies described as d's, #456 produces e's, and #457 produces f's.

The inability of the e antibodies to coprecipitate with the target antigen, 29047, to any great extent, strongly suggests that there is no coidentity between these antibodies and those in the d and f series. However, there may be a considerable amount of coidentity between the d and f series. This does not include the a's however, because they do not react with T. con. Furthermore, the e's are dissimilar to the b's, because of their differing reactivity with M1.

Unfortunately, none of these antibody mixtures have a unique antigenic target. However, the possibility exists that the antibody preparations raised in animals 455 and 457 might have application in an ELISA where discrimination between 29047 and 23270 was not a major concern.

5.0. OUTLINE FOR FUTURE WORK

We have succeeded in raising antibodies in rabbits which have specificity for different strains of Thiobacillus ferrooxidans. These antibodies may have use in an ELISA which could be developed against the various strains of bacteria, as target antigen. In this scheme, an aliquot of the culture, or soil sample under investigation, would be introduced into the well of a 96 well microtiter plate, and allowed to incubate in conditions which would permit the adherence of the antigen to the plastic of the well. Excess solution, and other non-bound components would then be removed from the well by suction, and a solution of bovine serum albumin added to the well, and incubated for sufficient time for all the unoccupied protein binding sites on the plastic to be filled.

A solution of the antibody preparation would then be added to the well, which would bind to any target antigen adhering to the plastic. After incubation and washing out, the antibody preparation would be replaced by a solution of anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate, raised in goat. The cycle of incubation and washing out would then be repeated until a final addition of alkaline phosphatase substrate was made. The color generated in the well, if all intermediate additions were made in excess, would be proportional to the number of Thiobacillus cells in the culture or soil sample originally added to the well.

For reasons already referred to in the Discussion, the limiting factor in the application of these antibodies to an ELISA may be the lack of unique specificity of the antibodies for only its original strain of Thiobacilli.

Clearly, with the possible exception of 451, most of the antibody preparations described here would not be able to discriminate between one strain of Thiobacilli and another.

A presently unresolved question is the degree of interaction which might be expected between these antibodies and other non-Thiobacillus microorganisms. If the specificity were to be too broad, and included their ability to bind to a range of other unrelated microbial genera, then there would be cause for doubting whether these antibody preparations would have any use in the measurement of the numbers and distribution of certain Thiobacillus strains, exclusively. Alternatively, the possibility exists that although the antibodies recognize more than one strain of Thiobacilli, yet they may be useful in methods development because their specificity is sufficiently narrow to include only a certain number of closely related Thiobacilli. Antibody preparations 455 and 457 may come into this category.

The most discriminating antibodies which can be made are those developed using monoclonal methodologies. In this case, the antibodies are raised in mice whose spleen cells are then fused with a culture of mouse myeloma cells. The resulting hybrid cells are then counted, and plated out to give as near as possible to one cell per well. Thus, any clone which develops in a well has the possibility of being derived from just one cell, and perhaps of producing antibodies specific for only one antigenic determinant. This degree of specificity is then determined by carrying out antibody specific ELISAs on the medium in which the clone is bathed.

The disadvantage to the monoclonal techniques are that they are very expensive, time consuming, and labor intensive, with the possibility of a successful outcome of the studies within a year being very remote. It was for these reasons primarily that the decision was made to produce polyclonal antibodies to the Thiobacilli, in the hope of being able to use the prepared antibodies in an ELISA very quickly. We now seek to establish the degree of microbial specificity of these preparations, and, using them, to develop ELISAs which will be of use in the measurement of these microorganisms.