IMMUNOTOXICITY OF JET FUEL

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During our preliminary year of experimentation we have made an initial examination of the immunotoxicological effects of JP-8 jet fuel exposure. Inbred C57BL6 mice were exposed to varying concentrations (either 500, 1000 or 2500 mg/m3) of aerosolized JP-8 jet fuel for a period of 7 days with an exposure period of 1 hour per day. Animal exposure was performed via nose-only presentation while the animals were held in individual subject loading tubes. The tubes were nose cone fitted to receiving adapters that originated from a common anodized aluminum exposure chamber. Nose only exposure was utilized to minimize ingestion of jet fuel during self grooming. Animals were rotate on a daily basis through the 12 adapter positions on the exposure chamber. This rotation was done to minimize proximity to jet fuel source as a variable in exposure concentration or composition. Exposure concentration was determined by a seven stage cascade impactor, and were measured after each exposure (1,2). 24 hours after the last exposure the animals were sacrificed and examined for changes in immune system composition and function. The major immune system organ systems (i.e., spleen, thymus, lymph nodes, blood and bone marrow) were recovered and examined for changes in organ weight, total cell numbers, immune cell components (by differential histochemical staining),
and lymphocyte subpopulations by flow cytometric analyses. Assays were also performed to assess any changes in immune function in these organs. In some experiments the animals were administered an aerosolized concentration of the neuropeptide substance P (SP, 1 uM) for 15 minutes immediately following the JP-8 exposure in an effort to protect from or reverse the effects of JP-8 exposure (see Background section for rationale and further information). As controls, other animals were exposed only to air plus/minus SP (i.e., no JP-8 exposure).
FINAL TECHNICAL REPORT

"Immunotoxicology of Jet Fuel"
Grant F49620-95-1-0007

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IMMUNOTOXICOLOGY OF JP-8 JET FUEL EXPOSURE

OBJECTIVES

The original Specific Aims of the proposal were as follows.

1. Investigate the immunotoxicological effects of jet fuel exposure on the following immune system organs.
   A. blood-source of circulating immune cells
   B. spleen-source of primary (naive) T and B cells, macrophages and NK cells
   C. thymus-site of generation of the T cell lineage
   D. lymph nodes-source of secondary (memory) T and B cells
   E. bone marrow-site of generation of all blood and immune systems lineage cells

   (hematopoiesis)

2. Utilize the following assays to assess the immunotoxicological effects (the reasons for each assay are given). These assays will allow for the determination of any effects on any component of the immune system and its location (whether phenotypic or functional in nature).
   A. determination of organ weights, cell numbers and viabilities-to determine gross effects of exposure and required for statistical analysis of other data
   B. cell differentials-determination of morphological changes in blood and/or immune systems cellular components (e.g., lymphocytes, monocytes, granulocytes, etc)
   C. flow cytometric analyses (FACS)-a detailed and complex analysis of changes in blood and immune systems components
   D. mixed lymphocyte responses (MLR)-analysis of the immune responsiveness of T and B cells (e.g., to stimuli such as antigens, mitogens and growth factors)
   E. limit dilution analyses (LDA)-statistical analysis of the frequency of responding lymphocyte populations to any particular stimuli after exposure; allows for an accurate and quantitative analysis of exposure effects
   F. NK and LAK cytotoxicity-analysis of the nonspecific immune system components implicated to be involved in both immunoregulation and tumor surveillance
   G. cytokine production-analysis of the ability of the immune system components to produce the soluble factors required for the function of the immune system
   H. colony forming unit assays (CFU)-assessment of the hematopoietic potential of the bone marrow to regenerate the blood and immune systems
   I. thymus organ culture (TOC)-assessment of the effects of exposure on the ability to generate the T lineage immune components
All of Specific Aim #1 and Specific Aim #2A-D have been accomplished or will be accomplished by the end of the granting period (end date 10/31/95). Due to the magnitude of the effects observed in #2A-D, and after consultation with AFOSR, Specific Aims #2E-I were postponed while additional work was performed to understand the cause and mechanism of the effects observed in Specific Aims #2A-D. This additional work includes the effects and mechanisms of the protective effects of Substance P, potential reversibility of the immunotoxicological effects of jet fuel exposure, additional kinetics and further dose-response for jet fuel exposure. These additional experiments should be completed by 10/31/95. Specific Aims #2E-I have been incorporated into the proposal to AFOSR to continue this work for a further 2 years.

STATUS OF EFFORT

During our preliminary year of experimentation we have made an initial examination of the immunotoxicological effects of JP-8 jet fuel exposure. Inbred C57BL6 mice were exposed to varying concentrations (either 500, 1000 or 2500 mg/m³) of aerosolized JP-8 jet fuel for a period of 7 days with an exposure period of 1 hour per day. Animal exposure was performed via nose-only presentation while the animals were held in individual subject loading tubes. The tubes were nose cone fitted to receiving adapters that originated from a common anodized aluminum exposure chamber. Nose only exposure was utilized to minimize ingestion of jet fuel during self grooming. Animals were rotated on a daily basis through the 12 adapter positions on the exposure chamber. This rotation was done to minimize proximity to the jet fuel source as a variable in exposure concentration or composition. Exposure concentration was determined by a seven stage cascade impactor, and were measured after each exposure (1,2). 24 hours after the last exposure the animals were sacrificed and examined for changes in immune system composition and function. The major immune system organ systems (i.e., spleen, thymus, lymph nodes, blood and bone marrow) were recovered and examined for changes in organ weight, total cell numbers, immune cell components (by differential histochemical staining), and lymphocyte subpopulations by flow cytometric analyses. Assays were also performed to assess any changes in immune function in these organs. In some experiments the animals were administered an aerosolized concentration of the neuropeptide substance P (SP, 1 uM) for 15 minutes immediately following the JP-8 exposure in an effort to protect from or reverse the effects of JP-8 exposure (see Background section for rationale and further information). As controls, other animals were exposed only to air plus/minus SP (i.e., no JP-8 exposure).

As shown in Figures 1A and 1B, exposure of the mice to JP-8 jet fuel had significant effects on the total numbers of cells recovered from each of the major immune system organs. Even at an exposure concentration as low as 500 mg/m³, a significant reduction in the total number of immune cells recovered from the spleen and the thymus was observed. At higher JP-8 exposure concentrations the reductions in cell numbers was even greater (reaching a high of approximately 50% for the spleen and 70% for the thymus). No apparent concentration-response to differing levels of JP-8 exposure were observed indicating that the effects were well over the threshold for JP-8 (i.e., we probably were not yet on the linear portion of the concentration-response curve). Decreases in cellularity were correlated with concomitant decreases in organ
weight for the spleen and thymus (data not shown). Administration of SP after JP-8 exposure was observed to be able to reverse some of the effects found (i.e., 100% reversal of effects on the spleen and greater than a 50% reversal of the effects on the thymus). The effects of JP-8 exposure on the lymph nodes, bone marrow and blood were quite different, however. In terms of the lymph nodes (a secondary immune organ) there appeared to be a linear concentration-response of JP-8 exposure up to the concentration of 1000 mg/m³ that caused an increase in total cell numbers. At the 2500 mg/m³ levels however, there was a significant reduction in total cell numbers recovered which may indicate that at this concentration the JP-8 was toxic. Administration of SP was not able to significantly overcome the effects of JP-8 at this concentration. Exposure of the animals to any of the concentrations of JP-8 did not affect the total numbers of cells recovered from the bone marrow. Interestingly, administration of SP resulted in a significant increase in total cells recovered. The effects of JP-8 exposure on the blood was similar to that seen with the lymph nodes. SP administration did not significantly reverse the effects of 2500 mg/m³ concentration of JP-8. It should be noted that animals exposed to SP alone showed no differences from the results seen with sham exposed animals.

Due to the drastic changes observed in immune cells, experiments were performed to determine if any particular subset of immune cells was preferentially affected (or depleted) due to JP-8 exposure. Flow cytometric analyses (i.e., FACS analyses) were performed on the cells recovered from each of the immune system organs. The cell populations were stained with monoclonal antibodies directed against the following cell surface antigens to distinguish various immune cell populations: anti-CD4 (helper T cells), anti-CD8 (cytotoxic T cells), anti-CD3 (total T cells), Mac-1 (monocytes and macrophages), and anti-CD45R (B220, B cells). As shown in Figures 2A and 2B, FACS analysis of spleen cell populations revealed that at any concentration of JP-8 used that there was a reduction in the percentage of CD8+ T cells, a small change in CD4+ T cells, and a reduction in total T cells that reflected the loss of cytotoxic T cells. An approximate 40% reduction in CD8+ T cells was observed that was found to be significant. Further, JP-8 exposure resulted in an increase in inflammatory cells (i.e., Mac-1-positive, approximately 50% increase) as well as a large increase in B cells (approximately 2-fold). Administration of SP had minimal effects on the T cell or B cell populations, but was able to normalize the inflammatory cell levels. Administration of SP alone had an effect no different than that seen in unexposed animals.

Analysis of the thymi of exposed animals (the site of T cell generation and T cell education) revealed that JP-8 exposure caused a small increase in mature T cell subpopulations in this organ but resulted in a more significant reduction in double-positive T cells (Figures 3A and 3B). The double-positive T cells are responsible for the generation of the mature single-positive T cells. These observations in combination with the severe reduction in thymus cell numbers would indicate that T cell production had been compromised and that T cell education (that occurs at the double-positive stage) might have been negatively affected. Administration of SP was able to reverse or at least partially reverse the effects of JP-8 exposure. Administration of SP alone had no effect as compared to sham controls.
The effects of JP-8 exposure on immune cells obtained from the lymph nodes of mice are shown in Figures 4A and 4B. It was again observed that JP-8 exposure resulted in a significant decrease in CD8+ cytotoxic T cells with minimal changes in CD4+ helper T cells. Not surprisingly, there were no changes in inflammatory cells observed (as this organ contains few cell of this type) but interestingly there was a significant increase (of approximately 3-fold) in B cells. These results again indicate that T cell function might be compromised and that B cells may have been activated and could pose a problem through abnormal antibody secretion. Preliminary results with SP administration after JP-8 exposure (data not shown) have indicated that SP may be capable of normalizing these cell populations after exposure.

Finally, as shown in Figures 5A and 5B, exposure of animals to JP-8 resulted in increased percentages of mature T cells (probably a result of other cell type depletion), a decrease in Mac-1+ cells, and an increase in B cells (also probably a result of the loss of other cell types) in the bone marrow. These results indicate that the microenvironment (i.e., stroma that is important for hematopoiesis and B cell development) may have been affected by JP-8 exposure. This result would indicate that hematopoiesis might not be able to compensate for immune cell depletion in peripheral immune organs (e.g., spleen and thymus), and that B cell education that occurs in the bone marrow may be compromised. The end result of these effects might be an individual unable to replace its depleted immune system leaving it susceptible to infectious disease and cancer, as well as the improper education of B cells that may then be able to induce autoimmune disease. Administration of SP was found to be able to normalize these effects in the bone marrow to a great extent. Administration of SP alone had no effect.

Due to the significant effects of JP-8 exposure seen in the experiments described above, assays were performed to analyze the effects on immune function. To assess immunocompetence blastogenesis assays utilized. This assay allows one to examine T cell function, in terms of the ability to secrete cytokines and to respond to mitogen. In the blastogenesis assay the cells obtained from the spleens of exposed animals (only spleen cells were examined as it contains the greatest concentration of mature functional immune cells and should be the organ in which functional changes are most easily seen) were stimulated with either IL 2 (to analyze for the presence of T cells activated by the exposure either directly or via activation of an inflammatory reaction), the mitogen Concanavalin A (Con A, which activates all T cells, stimulation with Con A alone also assesses the ability of the T cells not only to proliferate in response to the stimuli but also to secrete cytokines needed for proliferation), and Con A plus IL 2 (which should provide the strongest stimulus for immune response as the need for cytokine secretion is bypassed).

Unexposed or sham-exposed mice did not respond to IL 2 stimulation by itself as mice normally do not possess T cells that display IL 2 receptors without prior activation. At the highest concentration of JP-8 exposure (2500 mg/m³) a 60-fold increase in T cell stimulation by IL 2 alone was observed. This observation is significant in that it implies that a large percentage of T cells have in some fashion been activated due to JP-8 exposure and express IL 2 receptors on their cell surface. This effect could be direct due to JP-8 itself or could be the result of an inflammatory response triggered by JP-8 exposure. In either case, such T cell activation could result in significant harm to the individual via an exhaustion of the immune system that leaves the individual susceptible to infections, and could eventually lead to autoimmune disease through dysfunctional immune regulation or activation of self-reactive T cells. Administration of SP after
JP-8 exposure was capable of completely reversing these effects and bringing the immune response back to normal levels. It should be noted that SP administration alone had no effect. JP-8 exposure at any concentration resulted in a significant reduction in immune responses to both Con A and Con A + IL 2 (up to a 90% suppression in immune stimulation). The results found with the Con A + IL 2 stimulation indicated that the results of JP-8 exposure were not due solely to the inability of immune cells to secrete cytokines but also must be due to direct effects on the immune cells themselves. As all assays were conducted using the same number of viable cells, the results also were not due to a difference in viable cells plated for the assays. Substance P administration was capable of partially reversing some of the effects due to JP-8 exposure (to a level of approximately 30-60% that of normal). These results indicated that exposure of individuals to JP-8 caused a significant impairment in immune function which could leave the individual susceptible to infectious disease as well as to the development and/or progression of malignancy.

It should be noted that in experiments not shown here (due to their preliminary nature) similar exposures to JP-8 were performed using mice deficient in two important enzyme systems thought to help protect from hydrocarbon exposure, the aryl hydrocarbon hydroxylase and N-acetyltransferase enzymes. Identical results were observed in these animals as those seen in the normal mice. Thus, the effects of JP-8 exposure were significant in normal mice.

The completion of the preliminary project will encompass several additional experiments that are currently in progress. Additional kinetics experiments are underway to determine the host response to JP-8 exposure. That is, animals are currently exposed for a period of 7 days. In the current ongoing experiments the animals are being exposed for periods of 1 day, 3 days, 5 days and 2 weeks in order to determine the minimal amount of exposure time for significant effects on the immune system, and to analyze whether different levels of toxicological damage result from such exposure regimens (i.e., is the immune response damage linearly related to days of exposure?). Also, additional concentrations of JP-8 will be examined for effects on the immune system. Currently, it has been determined that 500 mg/m³ is sufficient to induce profound effects on the immune system. We also are analyzing whether lower concentrations of JP-8 exposure (i.e., 100 mg/m³ and 250 mg/m³) are able to induce the same or similar effects on the immune system, and if the damage seen in the immune system is linearly correlated with JP-8 concentration. Finally, in our collaboration with Dr. Mark Witten we are correlating the immune system changes seen at each concentration and exposure time with changes in lung pathology of the exposed mice. To do so we are examining serial sections of exposed lungs and analyzing cellular populations found in bronchoalveolar lavage fluid (BALF). That is, do immune system changes correlate with lung changes? Or, are changes in the immune system a more sensitive indicator of toxicological damage due to JP-8 exposure? These experiments are in progress and will be completed and analyzed by the end of October 1995, allowing us to definitively determine the JP-8 concentration and exposure time for the experiments to be performed in the current application.
ACCOMPLISHMENTS/NEW FINDINGS

In conclusion, the current results from the preliminary experiments can be summarized as follows:

1. Short-term exposure of normal C57BL6 mice to low concentrations of JP-8 jet fuel (500 mg/m³, 1 h/day, 7 days) resulted in significant decreases in immune organ weights and in lymphoid cells recovered from the major immune system organs.

2. Exposure to JP-8 jet fuel appeared to diminish all T lymphoid subpopulations equally, with concomitant increases in inflammatory and B cells being observed.

3. Significant and profound depression of immune function resulted from short-term (7 day), low concentration (500 mg/m³) exposure to JP-8 jet fuel.

4. The majority of the effects of JP-8 jet fuel exposure on the immune system could be significantly reversed (or prevented) by substance P administration to the exposed animals. The mechanism of substance P action is unknown at present. It is also unknown if substance P acts via the prevention or the reversal of the effects of JP-8 exposure.

5. No significant differences due to gender in the effects of JP-8 jet fuel exposure on the immune parameters measured were observed.

6. Changes in immunological competence such as those observed after exposure to JP-8 jet fuel may have significant effects on an individual’s susceptibility to infectious disease, as well as future development and/or progression of cancer and autoimmune disease.

From the preliminary experiments that have been performed to date it appears that the immune system may be a sensitive indicator of toxicological damage (both to the immune system as well as to other physiological systems) incurred by the individual due to JP-8 jet fuel exposure. Not only do significant and profound changes occur in the immune system at low concentrations after short-term exposures (in contrast to the type of pathological changes seen or not seen in the lung at similar exposure levels), but such changes may have significant implications for the health and medical treatment of exposed individuals. If changes such as those observed were to persist for any period of time it might lead to increased susceptibility to infectious disease, cancer and autoimmune disease.
PERSONNEL SUPPORTED

The current grant has provided salary support for the following personnel.

1. David T Harris, Ph.D. : Principal Investigator of the grant : Summer salary support
2. Debbie Sakiestewa : Research Technician : 100% effort on the grant
3. Pattie Parker : Research Technician : 50% effort on the grant
4. Christy Lyons : Undergraduate Honors Student : 100% effort on the grant

PUBLICATIONS

The work performed to date on this grant has resulted in (1) abstract and (1) presentation that was presented at the AFOSR conference held in Dayton, Ohio in May 1995. Also, we currently have in preparation (and expect to submit in the next month) a manuscript detailing our observations on the immunotoxicological effects of JP-8 jet fuel.

TRANSITIONS

Conferences
Results from the work performed on the grant were presented at the annual AFOSR meeting held in Dayton, OH in May 1995. We expect to submit an additional abstract for presentation in 1996.

Transitions
The significant effects of JP-8 exposure on the immune system, even at the lowest doses tested to date (500 mg/m³) should have significant clinical implications (at this dose in below the recommended personnel exposure level). It is expected that prolonged exposure to JP-8, even at low doses, would result in an increased risk of infectious disease, increased incidence of cancer, and increased development of autoimmune disease. However, the powerful and significant protective effects of Substance P may provide a clinical avenue for reversal of and protection from these immunotoxicological effects. It is expected that the results obtained in these studies will be instrumental in establishing safe exposure guidelines for USAF personnel working with JP-8 jet fuel, and medical measures that can be taken in cases of overexposure.

NEW DISCOVERIES

No patents or inventions to report.
HONORS/AWARDS

The following honors were received by David T. Harris, Ph.D. during the course of the granting period.

1994  Elected to American Men and Women in Science
1995  Elected to "Who's Who in the West"
1995  Elected to "Who's Who in America"
1995  Elected to "Who's Who Worldwide"
Figure 1A: Effects of JP-8 Jet Fuel Exposure on Cell Recovery. Animals were exposed to the indicated doses of JP-8 and total cells enumerated. The SEM were less than 10% of the mean.

Cell Recovery

![Cell Recovery Bar Chart](image-url)

- Spleen
  - N: 12, 9, 13, 7, 5
- Thymus
  - N: 12, 9, 13, 7, 5

- 0
- 500
- 1000
- 2500
- 2500+SP

Cell Numbers (x10^6)
Figure 1B. Effects of JP-8 Jet Fuel Exposure on Cell Recovery. Animals were exposed to the indicated doses of JP-8 +/− SP and cells enumerated. The SEM's were less than 10% of the means.

Cell Recovery

<table>
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<tr>
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<th>Bone Marrow</th>
<th>Blood</th>
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<td>11 9 12 7 5</td>
<td>12 9 12 4 5</td>
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<tr>
<td>Cell Numbers (x10^6)</td>
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<td>18</td>
<td>16</td>
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</table>

- 0
- 500
- 1000
- 2500
- 2500+SP
Figure 2A. Effects of JP-8 Jet Fuel Exposure on Spleen Immune Cells. Animals were exposed to the indicated doses of JP-8 +/- SP and spleen cells analyzed by flow cytometry. The SEM's were less than 10% of the means.
Figure 2B. Effects of JP-8 Jet Fuel Exposure on Spleen Immune Cells. Animals were exposed to the indicated doses of JP-8 +/- SP and spleen cells analyzed by flow cytometry. The SEM's were less than 10% of the means.
Figure 3A. Effects of JP-8 Jet Fuel Exposure on Thymus Immune Cells. Animals were exposed to the indicated doses of JP-8 and thymus cells analyzed by flow cytometry. The SEM's were less than 10% of the means.

Thymus FACS Summary

- 0
- 500
- 1000
- 2500
- 2500+SP

Percentage Positive

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</tr>
<tr>
<td>2500+SP</td>
<td>5</td>
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</table>

N

15 - 9417686.PPT - 5/25/95
Figure 3B. Effects of JP-8 Jet Fuel Exposure on Thymus Immune Cells. Animals were exposed to the indicated doses of JP-8 +/- SP and thymus cells analyzed by flow cytometry. The SEM's were less than 10% of the means.

Thymus FACS Summary

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Figure 4A. Effects of JP-8 Jet Fuel Exposure on Lymph Node Immune Cells. Animals were exposed to JP-8 +/- SP (at the indicated doses) and lymph node cells analyzed by flow cytometry. The SEM's were less than 10% of the means.

Lymph Node FACS Summary

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<tr>
<td>CD3</td>
<td></td>
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</tbody>
</table>

Percentage Positive

N  6  3  8  3

ND

ND

ND
Figure 4B. Effects of JP-8 Jet Fuel Exposure on Lymph Node Immune Cells. Animals were exposed to the indicated doses of JP-8 and lymph node cells were analyzed by flow cytometry. The SEM's were less than 10% of the means.

Lymph Node FACS Summary

- 0
- 500
- 1000
- 2500
- 2500+SP

Percentage Positive

MAC-1

- N
- 6
- 3
- 8
- 3

CD45R

- 6
- 3
- 8
- 3

ND
Figure 5A. Effects of JP-8 Jet Fuel Exposure on Bone Marrow Immune Cells. Animals were exposed to the indicated doses of JP-8 +/- SP and bone marrow cells analyzed by flow cytometry. The SEM's were less than 10% of the means.

**Bone Marrow FACS Summary**

<table>
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<tr>
<th></th>
<th>LY2</th>
<th>L3T4</th>
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<tbody>
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<td>2500+SP</td>
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</table>
Figure 5B. Effects of JP-8 Jet Fuel Exposure on Bone Marrow Immune Cells. Animals were exposed to the indicated doses of JP-8 +/- SP and bone marrow cells analyzed by flow cytometry. The SEM's were less than 10% of the means.

Bone Marrow FACS Summary

![Bone Marrow FACS Summary diagram]

- Percentage Positive

MAC-1: N 11 9 11 4 5
CD45R: N 11 9 11 4 5

- 0
- 500
- 1000
- 2500
- 2500+SP
Figure 6. Effects of JP-8 Jet Fuel Exposure on Immune Cell Function. Animals were exposed to the indicated doses of JP-8 +/- SP and the spleen cells tested for functional activity in a MLR assay. All SEM's were less than 10% of the means.

MLR Summary

![MLR Summary Diagram]

- 0
- 500
- 1000
- 2500
- 2500+SP

Stimulation Index

N  11 9 12 7 5 11 9 12 7 5 11 9 12 7 5

IL2 Con A Con A+IL2
Dear Walt,

Please find the final report for year 01 of my AFOSR grant. Hopefully, they will now release my money. Talk to you soon.

Yours,
David