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Bioprocessing Development: Immune/Cellular Applications - AntiIg Autoantibody and Complement-Mediated Destruction of Neoplastic Cells

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[Signature]
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1. Introduction.

This space bioprocessing contract effort was comprised of four general objectives. These were; 1) the evaluation of current separation processes; 2) the identification of problems relevant to the separation of important biologicals; 3) the identification of ground-based assay methods needed for pre- and postflight analysis of space bioprocessing separation technology; and 4) the establishment of methods to determine the efficiency of space bioprocessing separation procedures.

Several biological disciplines may have been appropriate as an avenue for pursuit of these objectives. However immunology was deemed advantageous for this purpose because of the diversity of cells and cell products involved and the extensive interest being given to their separation. Upon recognition of a cellular or molecular agent as foreign to the body, the immune system becomes activated to produce cells whose function is to destroy that agent and cell products whose function is to inactivate the agent and assist in its destruction. Long after the agent is removed from the body, some cells remain in a state of readiness to continue these destructive actions specifically against that agent should further exposure to it occur. This is the basis of acquired immunity to disease.

The ability of selected non-human mammals to produce immune agents suitable for human diagnostic and therapeutic use can be utilized if the specific entities involved are obtainable in sufficient purity and quantity. This commercial productive capability is limited by the fact that...
sought. For this reason, advances in separation technology have enjoyed a strong interest from the immunology community. The potential contributions of space bioprocessing to separation technology would seem at least equally appropriate in this respect.

Several separation methods were utilized in the contract objectives pertaining to the evaluation of current separation procedures. Density gradient centrifugation, filtration, adsorption chromatography, electrophoresis, and laser activated cell sorting were studied by various approaches to achieve a perspective of current separation technology in relation to the potential advantages of space bioprocessing. With some methods, actual trials were performed using immune-related biologicals and with other methods, a theoretical analysis of the separation capabilities was performed using literature sources as an important information base.

The objective to identify problems relevant to the separation of important biologicals was very compatible with the need for improved separation capabilities for immune-related cells and cell products. Therefore the separation of several cell types and products was studied using current techniques as a means of directly observing these problems. Lymphocytes, tumor cells, and rheumatoid factors were among these biologicals.

A third objective, that of identifying ground-based assay methods needed for pre- and postflight analytical support of space bioprocessing technology, was approached through the study of specific immunological applications of generalized assay procedures. Included in these procedures were immunodiffusion, cytotoxicity, and staining methods. Assays
for a variety of cell types and products were encompassed by the range of applications of these procedures.

A fourth objective to establish methods for determining the efficiency of space bioprocessing separations was approached by consideration of the requirements for earth-based applications of isolated biologicals. The potential quantity and quality of product were considered with respect to that required for a justified benefit to earth-based operations.

The approaches used to pursue these objectives will be presented in further detail in subsequent sections. The results of this study will then be presented after which recommendations for further efforts compatible with the goals of space bioprocessing will be given. An expenditure summary will then follow. The last two sections will deal respectively with a general summation of the contract effort and the conclusions resulting therefrom.

2. Evaluation of Current Separation Processes.

In order to assess the potential advantages of space bioprocessing technology for separation applications, a preview and preliminary evaluation of several ground-based separation methods were conducted. The requirements for obtaining adequate ground-based control data and the possible advantages of complimentary separation procedures in the Shuttle Spacelab environment were considered in this aspect of the contract effort. Included in the separation methods considered were density gradient centrifugation, electrophoresis, adsorption chromatography, filtration, and laser activated cell sorting systems.
a. Density Gradient Centrifugation.

Because of the versatility of density gradient centrifugation as evidenced by the wide range of research areas in which it is currently used, this method was examined in detail as a cell separation technique. The increased gravitational force applied to the cells can be controlled by the centrifuge speed and the counteractive buoyancy force on the cells can be varied by the specific gravity of the gradient medium. Discontinuous and continuous gradient columns can be used to yield a finite or theoretically infinite number of fractions respectively. Further refinements of separation capability may be achieved by optimizing the centrifugation period. Temperature must be controlled so that heat damage to living cells is negligible. In spite of the extensive range of controllable factors which can influence the quality of separation, cells are still separated on the basis of density. The rather limited range of cell densities, particularly between cell types of a common parentage, is responsible for a restricted applicability of this separation method. Also there must be a sufficient correlation between cell function and density for the technique to be practical in isolating specific cells with functional homogeneity. Such correlation is not present in many cell lines.

Density gradient centrifugation may be enhanced by the induction of alterations in cell density on a pre-selected basis. Density modification techniques such as antibody complex formation, rosette formation, and heavy particle ingestion, are subject to disadvantages of instability, irreversible changes in function, and impaired viability. The large gravitational forces exerted on these cells may also be contributory to
cell damage and affect subsequent assay procedures such as cytotoxicity measurement and viability determination. Therefore density gradient centrifugation methods were found to have a rather limited utility.

b. Electrophoresis.

Electrophoresis is applicable to the separation of cells and cell products. Separation is based upon several determinants. Size, mass, and net surface charge are the primary determinants. The combined effect of these determinants and their correlation to function are such that functional homogeneity of the resultant fractions was more promising than with many other separation methods.

Electrophoresis technology has been developed into a variety of systems. Modifications of buffer, field strength, flow rate, sample introduction, supporting medium, geometry, and spatial orientation have been used to improve the separation capabilities of this basic method. In the early electrophoretic systems, gravity was not considered much of a limiting factor for obtaining purity or quantity of product. As electrophoresis technology progressed, the effects of gravity in limiting the separation capabilities became more significant.

Two major influences of gravity upon electrophoretic systems are sedimentation and convection. Sedimentation of particles impedes their mobility and may also promote a clumping effect. Convection currents arise from heat produced by the electric field current in a one-gravity environment. A horizontally rotating electrophoresis system has been developed as one means of reducing these gravitational effects.

The system can only minimize the effects of gravity and dis-
tribute them uniformly throughout the column but this can not be con-
sidered the equivalent of a near-zero gravity environment. In addition
these rotational forms of electrophoresis systems have been burdened
with their own unique distorting influences.

Other variations of the electrophoretic basis of separation have
resulted in a diversity of systems. Some designs are directed toward a
high quality of purity in separation and others toward a high volume of
sample throughput. One approach, continuous flow electrophoresis, seems
to incorporate both of these advantages. It is this approach which
seems most promising as a space bioprocessing separation method because
of this dual utility.

c. Adsorption Chromatography.

Adsorption chromatography is similar to electrophoresis in that
separation occurs as a suspending medium is passed through a matrix.
However with adsorption chromatography, the matrix particles are coated
with a molecular species having a selective affinity for a particular
solute. This affinity is based primarily on chemical structure and is
only as specific as the selectivity of the binding agent. The use of
immune-reactive compounds as binding agents renders this system of some
potential value in isolating antibodies and antigens. It is also pos-
sible that if sufficiently selective antibodies to functionally homog-
 enous cell populations could be obtained, affinity chromatography would
have a potential capability for separating cell types as well as cell
products.
A relatively large volume of sample can be handled by this method and the binding agent can be recovered for reuse. Gravity is not considered a major interfering factor so that there is no apparent advantage of using this system for inflight space bioprocessing. However it may be of benefit in pre- and postflight procedures.

d. Filtration.

The filtration methods examined consisted of ultrafiltration and gel filtration. Ultrafiltration is a separation technique applicable to macromolecular separations and is based upon size alone. For this reason, it has a very limited value in isolating specifically reactive immunoglobulins in a suitably pure state. It does seem useful for preliminary preparative procedures however.

Gel filtration utilizes a combination of determinants, size and diffusability, to separate molecular entities. The basis of gel filtration is that the flow of smaller molecules through a column of gel beads under proper conditions, can be retarded by diffusion of these smaller molecules through the gel matrix. Because larger molecules are not able to enter the matrix pores, they flow through the column at a faster rate. Although size and diffusability in the gel medium are the primary determinants, adjustments of pH, gel density, homogeneity of column packing, and temperature can be optimized for particular separations. The relative insensitivity of gel filtration for biochemically similar but functionally distinct compounds is sufficient to severely restrict its usefulness. Thus, it can be considered a candidate for preparative separation of molecules prior to more sensitive and sophisticated techniques.
Laser Activated Cell Sorting.

The laser activated cell sorting method was investigated because it is considered by many as the most recent major advancement in separation technology. It can be used for separation of cells or particles of macromolecular size. The cells or particles are coated with a fluorescent material and individually passed through a narrow channel. Fluorescence occurs when the cell or particle is struck by a laser beam directed across the channel. The fluorescence is detected and measured. The cell or particle type is categorized by the amount of fluorescence measured. This information is then used by a computerized control system to determine the deflection of the flow path to preselected receptacles.

Several advantages are present in this method of separation. A separation of cells or particles on an individual basis is possible and a very large throughput can be handled automatically. A large amount of flexibility is possible by reprogramming the computerized controller processor. The selectivity of this system seems limited by the number of fluorescent coatings available. However, there is promise of an increased number of coatings as the potential applications of this system are developed.

The major disadvantage of this system is economics. The current price is approximately $116,000. The sophistication of technical personnel required for its operation is also an economic drawback.

However, the quality of separation appears very promising. Although its use as an inflight procedure does not seem justified, it would be very useful in preparative separation of cell or molecular mixtures prior...
to space bioprocessing separation methods within the Shuttle Spacelab environment.

3. Identification of Problems Relevant to the Separation of Important Biologicals.

Laboratory exercises in representative immunologic applications of current separation technology were performed to provide first-hand experience with research problems that may benefit from space bioprocessing separation procedures. These applications will be discussed with emphasis of their relevance to space bioprocessing. Three specific separation applications were studied: isolation of tumor cells, separation of lymphocyte subgroups, and isolation of rheumatoid factors.

a. Isolation of Tumor Cells.

Tumor cells present a unique challenge to the surveillance function of the immune system. In one respect, they may not be easily recognizable as foreign to the body because they arise from endogenous precursors. In another aspect, their usually degenerative nature renders them physiologically foreign to the body. This endogenous parentage of tumor cells is thought responsible for a weakened antigenicity of these cells. This decreased antigenicity and the interspersion of normal cells within tumorous tissue were considered justification to attempt the isolation of tumor cells from tumorous tissue for possible clarification of the immune mechanisms in operation.

The method of cell separation considered initially was centrifugation within a density gradient of Ficoll-Hypaque (Ficat, 1969; Terasawa et al., 1972). Centrifugation at a speed of 10,000 rpm (400 G) for
twenty minutes in a solution of 10 ml. of 9.0% Ficoll and 2.75 ml. of 50% sodium hypaque removed erythrocytes from the suspension but had little additional merit.

Therefore a second approach of density gradient centrifugation was attempted using discontinuous albumin solutions as the gradient column (Dickie, 1975). The colloidal nature of albumin in solution is a retardant to the mixing of different layers in the gradient column, thus enabling a multiphase density separation column. Albumin, like Ficoll-Hypaque, is non-toxic to cells.

Several combinations of albumin solution were tried for the purpose of determining the combination most suitable for tumor cell separation. The range of concentrations of these solutions was from 7% to 27% albumin. Cell viability, measured by a dye exclusion (Hanks and Wallace, 1958), was used to indicate fractions with an enrichment of living cells. The purity of fractions was determined by staining methods. Although initial results were indicative of some potential for this method of tumor cell separation, the variation in purity and viability between tumor cell sources and between fractions seemed too great to achieve a predictable separation pattern by centrifugation.

An earth-based method more appropriate for separation of tumor cells may be laser activated cell sorting. However no opportunity to test this possibility was available. The applicability of laser activated cell sorting for tumor cell separation is also dependent upon the availability of an adequate fluorescent marker for these cells.
A very tenuous viability of tumor cells upon removal from a host environment was observed in these studies. This may render them a poor candidate for space bioprocessing separation procedures. However they should not be ruled out on this basis alone since it may be possible to establish conditions that would overcome this disadvantage as the Shuttle program develops.

b. Separation of Lymphocytes.

Lymphocyte subgroups seem more appropriate candidates for space bioprocessing separation. Techniques for extensive cell culture, requirements for prolonged viability, several assay methods, and a broad spectrum of clinically relevant research interest have been established for these cell types.

Lymphocytes are critical to a normal immune response. Their role in the various steps required for immune function is accomplished by several distinct subgroups. At least five categories of lymphocytes are now thought to exist. These are B-lymphocytes, T-lymphocytes, effector cells, suppressor cells, and killer cells. The availability of homogenous preparations of these cell types would be of great importance in the study of their mechanism of action. However, an even more important advantage of their isolation may exist because of the immune-mediator substances they may produce. The culture of pure subgroups may enable the earth-based recovery of these products on a commercial scale. Therefore a preliminary study of current methods of lymphocyte separation was considered very compatible with the potential development of space bioprocessing applications.
Two density gradient centrifugation methods were evaluated for the separation of lymphocytes. A continuous phase of Ficoll-Hypaque solution was the gradient in one approach and a discontinuous phase of albumin solutions was the gradient in the other. The method with Ficoll-Hypaque is a commonly used procedure for separating lymphocytes from erythrocytes in solutions of whole blood. However no apparent advantage of this method for separating lymphocytes into subgroups was evident in these studies.

The use of albumin solutions as a gradient seemed more appropriate for preparative separation of lymphocytes into subgroups. A sharp delineation of fractions and an extensive versatility were possible by utilizing different combinations of albumin solutions differing in concentration. Because a very limited number of cells can be handled per gradient column, this method is not very suitable for large scale preparations of lymphocyte subgroups. Also the poor homogeneity observed in the fractions was not very supportive of this method for isolation of these subgroups.

c. Isolation of Rheumatoid Factors.

A macromolecular separation method based upon adsorption was used in the isolation of rheumatoid factors from plasma. Elevated titers of rheumatoid factors are found in the plasma of patients afflicted with rheumatoid arthritis. These rheumatoid factors are immunoglobulins, presumably of the IgM class, and have an affinity for IgG immunoglobulins. Such affinity is utilized in removing the rheumatoid factors from plasma. Subsequent manipulation of Ig will remove this affinity.
releasing the rheumatoid factors. This separation procedure served as an excellent example of molecular separation techniques involving affinity by weak bond formation. Rheumatoid factor was considered an important candidate for separation because of the observation in this laboratory that it may be responsible for complement-dependent injury to tumor-derived cells but not cells from normal tissues. This was suggestive of a functionally specific subgroup of this immunoglobulin class.

Therefore plasma with high rheumatoid factor titers was adsorbed using aggregated human gammaglobulin and then eluted with glycine-phosphate buffer (pH 2.5) (DeCarvaino et al., 1964; Torrigiani et al., 1970). The eluted rheumatoid factor was resuspended in phosphate-buffered saline (pH 7.2) and reduced by vacuum dialysis to the volume of the original plasma (Heimer and Schwartz, 1961).

Semi-quantitative assays for rheumatoid factor activity were performed on the dialysate and approximately 50% of the original activity was isolated in the dialysate. However immuno-assays of the dialysate were indicative of substantial impurities. A continuous flow electrophoresis separation method was used in an attempt to remove these impurities from the dialysate. Four fractions resulted but rheumatoid factor activity was detected in only three of these. The fourth fraction, although void of rheumatoid factor activity, contained 43% of the protein present in the original dialysate sample tested (Griffin, 1976). This was evidence of the need for improved purity of immune-related compounds.

The specificity of adsorbing agent appeared to be the limiting factor in the adsorption separation method. A superiority of electrophoresis over adsorption methods was also demonstrated by this exercise. The advan-
tages of electrophoresis may be even greater in electrophoretic systems developed for space bioprocessing inflight use.

4. Identification of Ground-Based Assay Methods Needed for Pre- and Post-flight Analysis of Space Bioprocessing Technology.

It would be impossible to precisely and completely identify the ground-based assay methods needed for analytical support of space bioprocessing at this early stage of its development. Therefore some basic assay methods with a broad spectrum of existing and potential applications were studied and evaluated for their general applicability to space bioprocessing. These methods were immunodiffusion, cytotoxicity, and cell specific staining procedures.

a. Immunodiffusion.

Immunodiffusion is a method which was developed into extensive analytical capabilities by Ouchterlony (1958). It is useful primarily for detecting and measuring immune-reactive compounds. The rheumatoid factor dialysate was used as the test material in this effort. Diffusion was carried out in an agarose medium against several different immune-reactive groups. It appeared that the major protein impurity in this dialysate was of the IgG class of immunoglobulins. Immunodiffusion was found to be extremely sensitive and could detect even trace amounts of immunoglobulins.

b. Rossette Formation.

Another bioassay method studied was that of rosette formation.
ulins on cell membranes. A common variation of this method is used in assaying for B- and T-lymphocytes. Certain lymphocytes are able to form rosettes by binding with untreated sheep red blood cells or with immune-activated sheep red blood cells. The technique is not as reproducible as desired and individual technical skill seems a significant factor in its successful performance and reproducibility.

Another significant variable in this assay method is the source of sheep red blood cells. An adequate standardization source for these assays is not yet available but perhaps a purified cell fraction obtained via space bioprocessing could be used to initiate this standardization. In spite of the variation in this assay method, acceptably consistent results are possible if careful technique is exercised and the sheep erythrocyte source is kept constant.

c. Cytotoxicity.

Under appropriate immune stimulation, cells can be made susceptible to damage in the presence of fresh complement. In this manner, cytotoxicity assays may be used to measure the effector substance or the target cells. This dual capacity renders this assay method of great potential value.

Because rheumatoid factor appeared to be cytotoxic to tumor-derived cells, it was used as an avenue to study cytotoxicity assay techniques. Incubation of target cells with rheumatoid factor and fresh plasma was conducted by previously established procedures (Klein, 1971; Stewart and Goldstein, 1974). Controls were similarly treated except the plasma was heated to inactivate the complement. At this point, cell viability could be measured by the Trypan Blue exclusion method (Hanks and Wallace,
1959). However this method seemed sensitive only to cells with considerable damage. A more sensitive method was found to be protease digestion of damaged cells (Stewart and Goldstein, 1974).

This cytotoxicity assay method was used to evaluate differences in the fractions obtained from density gradient centrifugation. The results were not suggestive of any suitable degree of purity in these fractions but they did suggest a trend towards two distinct cell populations. The development of antibodies against specific cell types might be a valuable adjunct to the application of cytotoxicity methods for cellular assay capabilities.

d. Staining Methods.

Another approach to analysing the cell separations performed in this contract effort was that of histochemical staining procedures. In addition to commonly used Wright's Hematoxin stain for the identification of leukocytes, a recently developed immuno-peroxidase method specific for certain sub-groups of lymphocytes was also examined. The applicability of this immuno-peroxidase method is presently limited to lymphocytes containing IgG or IgM on their surface but can be expanded as more immunoglobulin classes and subclasses are isolated in sufficient quantity and quality for the production of additional staining agents (Taylor and Skinner, 1976; Taylor and Burns, 1974).

The specificity of immunoglobulins and their receptors on cell membrane surfaces are considered by many to be a principle functional determinant in effecting cell-mediated immune reactions (Marchalonis, 1975; Rowe-Young and Donald, 1974; Vitetta and Uhr, 1975; Hunt and Williams,
Therefore further development of immunoperoxidase staining techniques may enable assays for lymphocyte subgroups that are highly relevant to their functional status. A very desirable feature observed in the use of these immunoperoxidase stains was that the cells containing the appropriate immunoglobulin on their surface were extremely evident while other cells were completely void of any evidence of reaction with the stain. This apparent all-or-none response would be very useful in eliminating error rising from subjective interpretation of cell types.

5. Efficiency of Space Bioprocessing Separation Procedures.

In considering the efficiency of space bioprocessing separation procedures, attention must be given to the expected minimum requirements to effectively achieve earth-based applications of these procedures. This will include functional integrity, purity, and quantity. The product that is recovered must satisfy the functional requirements to be usable. Therefore it is essential that decreased viability or altered function does not occur in the product as a consequence of the process. The required purity of product may vary depending on the specific compounds involved. For example, some compounds are very antigenic and as impurities may induce a disproportionate amount of immune reactive substances if antibodies were produced from the separated product. Requirements pertaining to quantity also will vary from one separation to another. This is particularly true for immune-related substances. The purification of antigens by space processing for frequent production of antibodies is representative of this variation in requirement. Some antigens
are capable of eliciting antibody production of as much as 100,000 times the weight of antigen. Others are not as stimulating to the immune system.

6. Results.
As a consequence of these efforts, several features related to current separation technology, to problems associated with the separation of biologicals, to assay methods, and to determining the efficiency of space bioprocessing technology became apparent.

Separation methods based upon size and mass do not seem very promising for advancements in the separation of functionally specific biologicals. Centrifugation and filtration methods are among these. There did not seem to be sufficient differences in size and mass of the biological entities whose separation was desired to enable their separation on that basis. Since these separation methods are not significantly hindered by gravity, there was no apparent advantage to their use as primary inflight space bioprocessing procedures.

Separation systems whose principle of action is selective affinity binding also do not appear directly advantageous for inflight space bioprocessing procedures. The significant limiting factor observed in these systems was the specificity of the binding agent. Gravity is not considered directly responsible for limiting the utility of these systems but indirectly it may be a factor in restricting the availability of more specific binding agents.

Electrophoresis utilizes size and mass and also electrochemical
limited by gravity and therefore may be enhanced by operation in the near weightlessness of the Shuttle Spacelab. Of the separation systems examined in this study, those based upon electrophoretic methods seemed most appropriate for inflight space bioprocessing procedures. Also the quality of separation even under ground-based restrictions, was found advantageous over other methods in purifying the test material used in this study.

The separation of biologicals presented some unique challenges to separation technology. In addition to being able to isolate the biological entity, a biological separation procedure must often be protective of the functional ability of the product. This requires very delicate handling prior, during, and after the separation process. In cases of cell separation, the membrane surface often contains the determinants that render a cell functionally distinct from other cells of similar structure. Electrophoresis and affinity binding systems utilized this surface chemistry for separation and appeared most sensitive to functional differences in separating biologicals. However as stated previously, electrophoresis was the only method that appeared of potential advantage for inflight space bioprocessing procedures.

A major drawback observed for cell electrophoresis was the absence of suitable standards. A purified cell line of stable and extensive availability would provide a means of predicting mobility for space bioprocessing separations and enabling an improved correlation of cell assays between laboratories. One result of the rosette formation assay procedures was a strong indication of the need for a standardized cell preparation for interlaboratory correlation of these assays.
An indirect relationship of other separation systems to space bioprocessing was apparent in this study. The binding agents used in constructing the affinity separation columns could be purified by space bioprocessing procedures and used for earth-based separations. Since many of these agents are recoverable from the columns and their production may be cultured by animal immunization procedures, the usage derived from one flight may be quite extensive.

Another separation problem that may be alleviated by space bioprocessing is that of uneven packing of gel filtration columns. Column preparation on earth does not result in an absolutely homogenous density distribution of matrix material. This can be a limiting factor the quality and efficiency of some separations.

Of the assay procedures studied, none seemed completely adequate to meet the potential requirements of space bioprocessing. However these assay procedures were dependent in varying degrees upon existing earth-based separation technology. As separation technology is advanced by space bioprocessing, the opportunity for improved assay procedures should follow. Therefore these assay procedures in their present state should be considered only as temporary supporting measures for space bioprocessing technology.

Cytotoxicity-based assays seemed to most sensitive but these are a destructive form of assay. The amount of material required for cytotoxic analysis may be a drawback in some cases. An apparent alternative was that of cell-specific staining methods as represented by the immunoperoxidase stains. Very little material was required since this is a
seemed currently limited by a lack of the specifically reactive agents. This limitation may be lessened by space bioprocessing separation procedures as well as further earth-based developments of these agents.

Purity, quantity, and viability were often unsatisfactory and were the limiting factors in the successful application of many earth-based separation techniques. Although purity and quantity may be improved by space bioprocessing, viability may not. A dependence of viability on proper handling was observed in this study and may become a major factor in the limitations of space bioprocessing. In this study the increased sensitivity of the protease digestion method in comparison to dye exclusion measurement for detecting minimal cell damage was very evident. There was no evidence that this minimal damage did not influence cell function. Therefore the protease digestion method would seem the preferred one for the assessment of viability under space bioprocessing conditions.

Tumor cells exhibited an extremely poor viability within a short time after their removal from the host environment. In spite of the potential research value of their separation, this poor viability might render unsuitable their separation by space bioprocessing.

Lymphocytes seemed more compatible with space bioprocessing in this respect. Their viability was far greater and enduring. The amenability of lymphocytes to maintenance through established culture techniques, the broad range of clinical research interest given them, and the inadequacy of current separation methods in isolating functional subgroups would also support them as space bioprocessing candidates.
Immunoglobulins and antigens were found to contain an extremely vast number of worthwhile components whose isolation would be of great significance and value to the biomedical community. The potential benefits of this capability to therapeutics, diagnostics, and research were found to be equally impressive.


Throughout the contract period, a portion of the effort was directed toward support of the activities of the NASA/JSC bioprocessing office. These activities were varied and dependent upon the requests of the bioprocessing office at that time. Supporting documentation for Shuttle experiment proposals, responses to administrative correspondence, assistance in planning and arranging a Colloquium on Bioprocessing held in March, surveys of technical literature, and technical input to bioprocessing related developments were among these activities. Proposals relating to the separation of cytotoxic cell factors, cell culture biosynthesis, electrophoretic behavior of plant cells, and multiphase partition separations were involved in these efforts. Extensive support was also given to the preparation of a program plan for space bioprocessing including technical evaluation of objectives, mechanisms for efficient development and implementation of experiments, and identification of projected hardware development requirements.
6. **Experiment Proposals**

Technical support of the Bio-processing program at JSC resulted in an example experiment submitted to the Life Sciences Spacelab Mission Development test series and a formal proposal to the Announcement of Opportunity for the first Spacelab Mission (AO-055-76-1).

Proposals:
1) "Separation of Cytotoxic Cell Factors", proposal no. 92, on June 19, 1976.

9. **Recommendations.**

In pursuing these contract activities several aspects related to space bioprocessing became evident. Some aspects were strongly plausible and others were not. Therefore it was deemed appropriate that some recommendations should be expressed based upon what was learned during the conduct of this contract.

a. **Cell Separations.**

Several areas of cell separation were identified as potential benefactors of space bioprocessing. The separation of lymphocytes subgroups would be of importance to the clarification of immune response and regulatory mechanisms. Pure preparations of B- and T-lymphocytes might serve as primary standards for associated assays. Alternatively, a pure preparation of sheep erythrocytes specifically reactive with B- or T-lymphocytes could be obtained by space bioprocessing for this purpose. With appropriate ground support from animal geneticists and the Bureau of Standards, secondary standards could be established and maintained.
Such a standardized cell preparation could also be used for comparing electrophoretic mobility measurements from one system to another. The use of a standard living cell line would be of significant benefit in establishing predictive indices of mobility of unrelated cell types under consideration as candidates for space bioprocessing separation technology. Such a predictive capability would facilitate the optimization of the separation system prior to flight and also support advances in ground-based electrophoretic separation of cells.

Other potential candidates for cell separation in space bioprocessing are cells derived from endocrine tissues. Hormone-producing cells could be cultured following their isolation and used for commercial sources of their respective products. This concept was tested in the Apollo-Soyuz mission when urokinase-producing renal cells were separated and analyzed for productive potential. The results of this experiment are very encouraging to further space bioprocessing applications.

b. Cell Products.

The separation and purification of cell products by electrophoresis within the Spacelab environment could offer several potential applications of space bioprocessing. Antibodies, antigens, and cell mediators are included in this category. Antibodies produced in response to specific disease agents could enable the development of diagnostic tests for these agents. Purified antibodies of specific affinity for other biologicals could also be used in conjunction with absorption methods of separation to achieve improved purity with those methods in one gravity conditions.
It may be even more productive in some cases to isolate antigens by space bioprocessing and use them for earth-based production of antibodies in laboratory animals. Antigen purification may be preferred because some antibodies can have cross reactivity.

An example of the potential impact of this application is exemplified by tumor-related antigens. In many cases tumorous tissue is thought to release molecular entities characteristic of the embryonic form of the parent tissue when damaged by physiologic responses. These molecular fragments can be made antigenic by proper animal immunologic techniques. The resultant antibodies could then form the basis of immunoassay screening tests for the tissue-specific neoplasms. A combination of such assays for the more prevalent cancers could enable a relatively inexpensive and practical means of mass screening capability for the early detection of cancer. The potential economic value of such a diagnostic benefit is indicated by considering the current expenditure for cancer detection methods practiced at present. An assay system of this type has been developed for carcinoembryonic antigen which has some correlation to stomach and upper intestinal neoplasms. However this concept could be greatly enhanced by improved separation capabilities.

Regulatory factors, such as those associated with immune-responsive cells and synaptic junctions would be useful in purified form for their structural characterization and subsequent synthesis of pharmaceutical analogs. The medical implications of this achievement would be significant to both research and clinical advances.
c. Technology for Bioprocessing Laboratory at NASA/JSC.

As potential applications of space bioprocessing became apparent, the need for a bioprocessing support laboratory located at the Johnson Space Center became more evident. Preliminary hardware and analytical requirements for such a basic support laboratory can be developed from experience gained with relevant laboratory methods.

The minimum hardware requirements should consist of a refrigerated centrifuge, light microscope, fluorescent microscope, carbon dioxide incubator, refrigerator, freezer, weighing balance, ultra-filtration cell, dialysis system, sterilizer, and gel electrophoresis system. Additional requirements should be expected in response to further development of space bioprocessing applications. A tremendous advantage would result if sufficient support were also available to provide a laser activated cell sorting system.

Support for a broad spectrum of interests in space bioprocessing experiments would be possible if techniques associated with cell culture, identification of B- and T-lymphocytes, fluorescein conjugation assays, dialysis, molecular filtration, and immune-cell specific staining were available. The forementioned hardware requirements are compatible with these analytical capabilities.

Perhaps a suitable material for use in developing cell separation methods within this bioprocessing laboratory would be lymphocytes derived from mouse spleens. This is a readily available source and would be representative of many cell separation characteristics. A secondary benefit could be forthcoming early in the space bioprocessing program if commercial preparations of B-lymphocytes can be obtained.
the development of separation technology. A very sensitive thymic hormone assay valuable to immunologic diagnosis has been developed recently. However this method requires B-lymphocytes in relatively pure fractions. The purity of these cells is obtainable currently only from athymic mouse spleens but this requires the tremendous expense of maintaining isolated colonies of these unique mice. A means of obtaining B-lymphocyte fractions from normal mice would obviate this expense and place this capability for thymic hormone assay within the budgetary constraints of most laboratories.

d. Separation Criteria.

Periodic reviews of separation criteria should occur as separation candidates for space bioprocessing are developed. Viability, required purity and quantity, and consideration of current advances in earth-based technology should be included in these reviews. This will ensure that separation candidates are not flown unnecessarily and that the latest relevant separation technology can be incorporated into space bioprocessing procedures.

e. Hardware Development and Testing Requirements.

The availability of the Shuttle flight simulation facility should be taken advantage of at least twice prior to final approval of any hardware systems. The fidelity of space bioprocessing experiment simulations should be as realistic as possible and encompass such areas as logistics, storage, waste disposal, power and timeline requirements, compatibility with other experiment requirements, exercises in correction of probable malfunctions, and anticipated pre-launch support.
10. Summary.

During the twelve months of this contract effort, experience was gained in the areas of immunology research, separation procedures, assay methods, and space bioprocessing requirements and was utilized in developing potential applications of space bioprocessing. Separation methods of cells and cell products were examined, including density gradient centrifugation, affinity chromatography, filtration, electrophoresis, and laser activated cell sorting. Cytotoxicity analysis, rosette formation, immunodiffusion, viability, and staining procedures were among the assay methods examined. Several potential applications of space bioprocessing were identified and recommendations were made pertaining to the further development of space bioprocessing. Some requirements for in-house support of space bioprocessing were also identified. Based upon this experience, several conclusions were reached that would suggest that current technology is not entirely satisfactorily for support of space bioprocessing but will have to be developed further in selected areas concomitantly with space bioprocessing technology.

11. Conclusions.

This contract effort was a very appropriate means to study space bioprocessing applications to biomedical research areas associated with immunology. Based upon this study, several conclusions were suggested:

a) Current separation methods are inadequate to meet the future needs for separation of biologicals important to biomedical research and industry.

b) Electrophoresis appeared to be the only separation method suitable as a primary space bioprocessing element system.
c) Separation systems based on chemical affinity such as adsorption chromatography may be improved indirectly through space bioprocessing by a greater availability and purity of binding agents.

d) Gel filtration methods may benefit indirectly from space bioprocessing through improved homogeneity in column packing.

e) Satisfactory assay systems were not apparent for functionally specific cells and cell products to the degree of refinement potentially required for analytical support of space bioprocessing.

f) A lack of acceptable standards in many cellular assay systems was suggested and will have to be developed before or concomittantly with space bioprocessing separation techniques for a meaningful analysis of the results.

g) Cell preparations with standardized electrophoretic mobility are needed for both space bioprocessing and earth-based systems.

h) Lymphocyte subgroups, tumor-related antigens, and immune function mediators seem the more promising candidates initially for space bioprocessing separations.
REFERENCES


FINAL REPORT OF
CONTRACT EFFORT #NAS 9-14820

Appendix:

1. Publication
2. Research Accomplishments
Rheumatoid factor and tumor-host interaction
(antibody response with tumor therapy)


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Communicated by R. A. Good, March 23, 1976

ABSTRACT In this survey for rheumatoid factor (RF) seropositivity on patients with neoplasms, an 85% rate of positive screening tests was recorded under certain circumstances. This high rate of RF seropositivity occurred after irradiation and/or chemotherapy of breast and lung cancers. Treated patients with breast cancers who had no evidence of residual tumor had an 89% rate of positive RF tests. Conversely, the incidence of RF seropositivity was low among untreated patients with similar tumors and treated patients with glioblastomas or multiple myeloma. The administration of cytotoxic drugs (e.g., azathioprine) was not itself associated with RF production even in renal allograft recipients. The data indicate that RF production occurs frequently after therapy of certain tumors and suggest that in these circumstances RF may be an expression of tumor-host interaction.

Rheumatoid factor (RF) is detected by routine laboratory techniques in serum from ≤4% of healthy adults under 65 years of age (1–5). About 13–20% of selected patients with malignancies are RF seropositive (1, 4, 5), and RF has been elicited from neoplastic but not from adjacent normal tissues (5). The relevance of this antibody response to tumor-host interactions is not known, nor are circumstances that cause it to occur.

Most RFs are IgM antibodies to antigenic determinants in IgG. In general, IgM is a more effective activator of complement than IgG (6, 7). However, the effect of IgM-RF on complement-mediated reactions is variable (8–11). For example, IgM-RF induces complement-mediated neutralization of herpes simplex virus complexed to IgG antibody (9). However, RF may inhibit complement-mediated phagocytosis (10) and lysis (11) of erythrocytes sensitized with IgG antibody. The basis for this difference in the interactions of complement, RF, and antibodies of the IgG class is not known.

This report presents evidence that RF is readily detected in serum from many patients with solid tumors and that conventional therapy of these tumors contributes to the occurrence of this antibody response.

PATIENTS AND METHODS

All subjects included in this study were under 66 years of age. The diagnosis was confirmed microscopically in all cases of malignant disease. The following subjects were included in the serologic survey for RF: (a) 56 patients with breast cancer, (b) 42 patients with squamous cell carcinoma of the lung, (c) 21 patients with multiple myeloma, (d) 12 patients with glioblastomas, (e) 21 patients without known malignancy receiving cytotoxic drugs for the purpose of immunosuppression, and (f) 21 healthy volunteers. Thirty-seven of the patients with breast cancer and all 42 patients with lung cancer had underwent and widely metastasized tumors. Treatment included irradiation (3500–4500 R or 35–45 J/kg) from a cobalt source and administration of various chemotherapeutic agents intravenously in dose schedules dictated by patient weight, clinical status, and drug tolerance. These drugs included cyclophosphamide, vinblastine, methotrexate, 5-fluorouracil, thio-TEPA, and steroids. Multiple myeloma was treated with irradiation, melphalan, and, in selected cases, steroids. Glioblastomas were treated with irradiation to the head and with steroids. Most patients had received prolonged courses of therapy before their serum was tested. Nineteen patients with breast cancer had no apparent residual tumor after radical mastectomy and had received "prophylactic" irradiation and chemotherapy before they were tested. Of the patients receiving cytotoxic drugs, which were administered for immunosuppression, 11 were renal allograft recipients and 10 had multiple sclerosis. The renal allograft recipients were receiving 100–175 mg of azathioprine and up to 20 mg of prednisone daily; patients with multiple sclerosis were receiving azathioprine at 50 mg/day. Sera were tested for RF at 1:20 dilutions with glycine-saline buffer using latex particles coated with Coons Fraction II (Hyland Laboratories).

RESULTS

The results of the serologic survey for RF are listed in Table 1. Twenty of the 21 patients with breast cancer and 15 of the 21 patients with lung cancer who had extensive tumor involvement and had prior irradiation and/or chemotherapy were RF seropositive. The findings did not appear to be influenced by the type or duration of treatment. Despite the absence of detectable residual tumor, 17 of the 19 treated patients with resected breast cancer were also seropositive. These rates were significantly higher than those recorded on the 21 healthy subjects, none of whom was seropositive (P < 0.001 by chi square tests). In contrast to the treated patients, only one out of 15 patients with metastatic breast cancer and two out of 21 patients with metastatic lung cancer, who were tested prior to therapy, were seropositive; these values were not significantly different from our control experience. Only one out of 21 patients with metastatic breast cancer and two out of 21 patients with metastatic lung cancer, who were tested prior to therapy, were seropositive; these values were not significantly different from our control experience. Only one out of 21 patients with metastatic breast cancer and two out of 21 patients with metastatic lung cancer, who were tested prior to therapy, were seropositive; these values were not significantly different from our control experience. Only one out of 21 patients with metastatic breast cancer and two out of 21 patients with metastatic lung cancer, who were tested prior to therapy, were seropositive; these values were not significantly different from our control experience.

DISCUSSION

A routine screening test demonstrated the presence of RF in serum from 85% of patients studied with breast or lung cancer. The presence of RF in patients with breast cancer and lung cancer is suggestive of a tumor-host interaction. The high rate of RF seropositivity in these circumstances suggests that RF may be an expression of tumor-host interaction.
who had received prior irradiation and/or chemotherapy. This remarkably high rate of RF seropositivity with cancer reflected patient selection (1, 4, 5). Therapy contributed to the occurrence of this humoral immune response; the prevalence of seropositivity was not increased among other patients with similar neoplasms who had not been treated. Yet the production of RF cannot be ascribed directly to the use of cytotoxic drugs since patients without known neoplasms treated with these therapeutic agents were seronegative. The presence of RF in serum from 89% of treated patients without evidence of residual breast cancer is of particular interest and could be related to residual foci of tumor or retained tumor antigen(s). However, the combination of allantogens and cytotoxic drug therapy in renal transplant recipients was not associated with RF production in this study. Furthermore, a high coincidence of RF and therapy was not observed with all types of neoplasms, as exemplified herein by glioblastoma and multiple myeloma.

The basis for the high frequency of circulating RF with certain neoplasms after irradiation and/or chemotherapy is not understood. Such therapy is likely to increase the amount of antigenic material released from tumor tissue. An immune response, which may include IgG, is likely to take place if tumor-related antigens reached competent lymphoid tissues. It has been postulated that antigen-antibody complexes, which may be related to immune responses to tumors, circulate in patients and animals with malignancies (12–15). When complexed with antigen, IgG is probably conformationally altered, which would increase the immunogenicity of IgG and favor RF production (16, 17). Thus, sequestration of tumor antigens by an intact blood-drain barrier (18) could explain the negative tests for RF on treated patients with glioblastoma. Humoral immunity is often impaired with multiple myeloma (19). Perhaps patients with multiple myeloma do not have the capacity for an RF response.

The frequent occurrence of RF seropositivity with certain commonly occurring neoplasms in association with their therapy has potential importance to tumor-host interactions. We have preliminary evidence that RF from the plasma of patients with rheumatoid arthritis may be toxic in the presence of complement to cells freed from some neoplasms.

The relevance of RF to tumor-host interaction is presently unknown. Tumor tissue from RF-seropositive patients showed a greater degree of necrosis than did tumor tissue from other patients who were seronegative in the present study. Since RF is a macromolecular substance, it is unlikely that RF released into the circulation could easily reach tumor tissue across the walls of blood vessels with a normal degree of permeability. Thus, if RF does have potential for damaging tumor cells, its effect in vivo is likely to be most apparent within the circulation. In that case, RF may reduce the risk of establishing blood-borne metastases. However, it should be pointed out that the incidence of malignant disease is not reduced among patients with rheumatoid metastases, who usually maintain high circulating titers of RF.

We thank Dr. K. M. Welch for serum from patients with multiple sclerosis, Dr. Lloyd Old for most helpful suggestions, and Ms. C. Keltner for assisting with the manuscript. This research was supported by NCI Grants 5 RO1 CA15533, AM17555, and HL17629.

![Table 1. Serologic survey for RF](image)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age in years (mean ± SD)</th>
<th>Irradiation and/or chemotherapy</th>
<th>Subjects tested</th>
<th>RF seropositive subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic breast carcinoma</td>
<td>46 ± 9</td>
<td>Yes</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Breast carcinoma, no evidence of residual tumor</td>
<td>45 ± 11</td>
<td>Yes</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Metastatic breast carcinoma</td>
<td>52 ± 10</td>
<td>No</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Squamous cell carcinoma of the lung</td>
<td>55 ± 7</td>
<td>Yes</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Squamous cell carcinoma of the lung</td>
<td>55 ± 8</td>
<td>No</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>56 ± 6</td>
<td>Yes</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>56 ± 7</td>
<td>Yes</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Renal allograft recipients</td>
<td>29 ± 9</td>
<td>Yes</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>29 ± 8</td>
<td>Yes</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>49 ± 12</td>
<td>No</td>
<td>21</td>
<td>0</td>
</tr>
</tbody>
</table>

Research Accomplishments

Tumor tissue is comprised of malignant cells, invading immune reactive cells and stromal cells. The tumor cells are the specific target of interest in immunologic studies on tumor-host interaction. In other words, interpretable data must relate specifically to injury caused to malignant cells as opposed to the other cell types.

The thrust of this work pertains to studies into the potential of rheumatoid factor (RF) positive blood being involved in a humoral cytotoxicity system to cancer cells. The impetus for this interest relates to our observation that about 85% of patients with cancer who have received conventional chemotherapy or irradiation therapy convert to RF seropositivity (Twomey, J.J., et al., Proc. Nat. Acad. Sci. USA 73:2106-2108, 1976). It is necessary that such studies be initiated in vitro. It was also our feeling that any cytotoxic effect that pertained to RF involved complement fixation.

The first nine months of the funding period was devoted to efforts in cell separation in an attempt to separate viable tumor cells from stromal and immune reactive cells freed from fresh human tumor tissue. A number of maneuvers that involved density gradient sedimentation over graded concentrations of albumin were attempted as tumor material became available for study from various operating rooms. These efforts proved unrewarding. This pinpoints the need for the development of new approaches to upgrade cell processing. This, from an immunobiologic frame of reference, should be well-suited for the attention of the aerospace bioprocessing research program.

During the latter part of funding, our efforts shifted to a different approach. Malignant melanoma tumors were the subject of these studies. Malignant melanoma
tumors were the subject of these studies. Malignant melanoma was selected because the neoplastic cells contain brown pigment, which readily distinguishes them from other cells freed from tumor tissue. Two sources of human malignant melanoma were used: (a) Implants of human tumors grown in athymic nude mice and (b) when fewer cells were required for experiments, melanoma cells were derived from long-term tissue cultures.

When human melanoma cells were incubated with RF positive plasma from patients with rheumatoid arthritis or from patients with treated cancer, a complement-dependent, cytotoxic effect was not observed. Similarly, a cytotoxic effect was not observed when incubations included normal plasma. However, when melanoma cells were incubated for 90 minutes with both rheumatoid and normal plasmas, a complement-dependent, cytotoxic effect was observed (Table 1). It can be seen from the data that a significant percentage of the tumor cells were injured in these incubations. Cytotoxicity was observed when RF plasma was derived either from patients with rheumatoid arthritis or from patients with treated cancer. The cytotoxic effect was lost when RF was adsorbed with glutaraldehyde cross-linked human gammaglobulin from the test plasmas.

<table>
<thead>
<tr>
<th>Tumor Number</th>
<th>Cell Source</th>
<th>Normal (%)</th>
<th>RA (%)</th>
<th>RF Positive Cancer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MI*</td>
<td>8</td>
<td>48</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>MI</td>
<td>1</td>
<td>36</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>MI</td>
<td>0</td>
<td>32</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>MI</td>
<td>2</td>
<td>43</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>TC**</td>
<td>0</td>
<td>45</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>MI</td>
<td>3</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>TC</td>
<td>0</td>
<td>53</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>TC</td>
<td>6</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>TC</td>
<td>2</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>TC</td>
<td>0</td>
<td>21</td>
<td>16</td>
</tr>
</tbody>
</table>

*Mouse implant

**Tissue culture
APPENDIX 2 (Cont.)

Experiments were done using a single source of melanoma cells and the same normal human plasma collection. Cytotoxicity with six different rheumatoid arthritis plasmas with high RF titer was tested. It can be seen from Table 2 that not all RF positive plasmas supported a comparable level of tumor cell cytotoxicity. One plasma lost its cytotoxic activity after six months of storage at -70°C (#3). It should be noted that this loss of cytotoxicity was not accompanied by a loss of RF.

TABLE 2

<table>
<thead>
<tr>
<th>Test Plasma</th>
<th>RF Titer</th>
<th>Per cent Cells Injured</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:2560</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>1:2560</td>
<td>32</td>
</tr>
<tr>
<td>3a</td>
<td>1:2560</td>
<td>32</td>
</tr>
<tr>
<td>b</td>
<td>1:2560</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1:1280</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>1:1280</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>1:1280</td>
<td>13</td>
</tr>
</tbody>
</table>

Studies were performed using serial dilutions of rheumatoid arthritis plasma with undiluted normal plasma and a single preparation of melanoma cells. Conversely, similar studies were performed using serial dilutions of normal plasma with undiluted rheumatoid plasma and the same preparation of tumor cells. It can be seen from the Figure that cytotoxicity was gradually lost with increasing dilutions of rheumatoid plasma. However, the cytotoxic contribution of the normal plasma was mostly dissipated by as little as a 1:2 dilution.

Our studies indicate that RF positive plasma contributes to tumor cell injury in a complex, complement-dependent humoral immune system. This involves a substance present in relatively high titer in RF positive plasma from either rheumatoid or cancer patients, plus a second substance present at much lower titer in normal plasma. A similar humoral cytotoxicity system may be operative in vivo and may be a significant factor in clinical improvement observed with...
chemotherapy or irradiation therapy which induces an RF response. If indeed this proves to be the case, this immune system is deserving of much attention since, through its direct application, it may prove ultimately possible for patients to derive benefit from same without prior cytotoxic or immunosuppressive exposure to cytotoxic therapy.

A considerable volume of additional research has substantiated and extended the studies listed above. These studies have been conducted in our laboratory since this contract was terminated. We intend to pursue these studies further. It is our belief that the aerospace program could make considerable contribution to this area of research. May I suggest two areas for thought: (1) The separation of tumor from other types of cells freed from fresh tumor tissue and, thereby, extend the types of cancer that are amenable for similar studies. 2) The separation
of substances present in rheumatoid and normal plasma that contribute to
the observed phenomenon so that they can be characterized biochemically, studied
functionally, and perhaps, later applied to clinical medicine in our quest
for an answer to the cancer problem.