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Title: ANTI-Ig AUTOANTIBODY AND COMPLEMENT-MEDIATED DESTRUCTION OF NEOPLASTIC CELLS

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Title: Anti-Ig Autoantibody and Complement-Mediated Destruction of Neoplastic Cells

Phase One: Summary 10/1/75 - 3/31/76

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PHASE I

Contract # NAS 9-14820

SIX MONTH SUMMARY REPORT
(10/1/75 - 3/31/76)

Contents:

LEGENDS FOR TABLES

1. Introduction.

Recognition and destruction of foreign cells and other matter is a normal function of the immune system. Under pathologic circumstances, the regulation of immunologic homeostasis is altered so that normal autologous cells are destroyed, resulting in autoimmune disease (Boyd, 1970, pp. 157-176). One form of autoimmune disease is rheumatoid arthritis (Boyd, 1970, p. 173).

Some immune response are effected through immunoglobulins (Ig), of which five classes have been recognized, namely, IgA, IgD, IgE, IgG, and IgM (Franklin, 1976). These Ig's represent a heterogenous collection of antibodies that can be distinguished from one another by certain physical characteristics such as sedimentation coefficient, electrophoretic mobility, and antigenic specificities. Variable regions within each Ig class are believed responsible for functional and other heterogeneity within each class (Richards et al., 1975; Marx, 1975; and Kolata, 1974).

Auto-antibodies associated with rheumatoid arthritis, termed rheumatoid factors (RF) react with antigenic determinants on IgG heavy chains. RF has predominantly but not completely IgM specificity. A number of laboratory tests have been developed such as the IgG-coated, latex particle agglutination test (Kabat and Mayer, 1967, p. 90; Bartfield and Epstein, 1969). We have observed that RF is detected in the majority of patients with certain solid tumors who have received prior chemotherapy or radiotherapy (Twomey et al., 1976). This auto-anitbody response was not detected in treated patients with primary brain tumors (where tissue is sequestered from the immune system by an intact blood-brain barrier) or with multiple myeloma where humoral immunity is usually impaired. In addition, the prevalence of RF is not increased with solid tumors prior to initiation of chemotherapy or radiotherapy. It is proposed that RF is related to prior chemotherapy or radiotherapy of tumors anatomically accessible to immunologic tissues capable of antibody responses. A primary IgG response occurs, antigen-antibody complexes form, complexed IgG becomes immunologic, and an RF response results.

It was further observed in this laboratory that RF caused complement-dependent injury to cells released from bladder tumors. This cytotoxicity was target specific in that cells from a variety of normal tissues were not damaged by RF. The specificity of the cell that is the target for RF-related cytotoxicity and amboceptor requirements for the phenomenon become issues of immediate importance in the field of tumor-host interactions.

Three areas for future efforts were identified; 1) to study the specificity of RF-related target cells, 2) to identify requirements for RF-related tumor cytotoxicity and to distinguish this host response from other toxic substances in blood of cancer patients, and 3) to determine factors and kinetics related to this RF response in vivo, including the antigen specificity of this RF relative to RF from patients with rheumatoid arthritis. The isolation and purification of RF from plasma is critical to this projected work. These procedures complement objectives of the Bioprocessing Office at the Johnson Space Center of the National Aeronautics and Space Administration. Therefore a NASA-funded contract to support these efforts was approved on October 1, 1975.

2. Isolation of Rheumatoid Factor from Plasma.

It was first necessary to extract RF from plasma to permit its individual study and, conversely, to test for cytotoxicity in plasma depleted of RF. Plasma from volunteer patients with rheumatoid arthritis was selected for pilot studies. Heparinized blood was used in these studies.

a. RF Separation Procedure.

Plasma with high RF titers (latex-binding test[1]) was adsorbed using aggregated human gammaglobulin and then eluted with glycine-phosphate buffer (pH 2.3) (DeCarvalho et al., 1964; Torrigiani et al., 1970); eluted RF was resuspended in phosphate-buffered saline (pH 7.2) (Heimer and Schwartz, 1961). Volume was reduced by vacuum dialysis to the original number of ml. plasma. The concentrations of RF in the original plasma, the dialysate, and in the adsorbed plasma were determined. The

[1]RA Test, Hyland, Division Travenol Laboratories, Inc., Costa Mesa, California, 92626.

original plasma had an RF titer of 1:1280, the dialysate a titer of 1:640, whereas the adsorbed plasma was negative in a dilution of 1:20.

b. Purity of Separated RF.

Once separated in this manner, the purity of the dialysate for RF was considered. Of foremost concern was the presence of Ig without RF activity or Ig of a class other than IgM (IgG contamination could lead to confusion through amboceptor activity). Immunodiffusion in 0.8 % agarose medium was therefore performed on the dialysate (Ouchterlony, 1958).

In addition to IgM, the dialysate contained some molecules with IgG specificity. The most likely source of this IgG was the adsorbant with test plasma but an alternative possibility. This IgG also could possess RF activity (Franklin et al., 1957; Edelman et al., 1958). Efforts are currently underway to remove this IgG. On immune adsorbant columns, some but not all IgG contaminant was removed. Failure of complete removal may be due to denaturation. Further efforts to overcome this obstacle are underway; preliminary experience is promising. In support of this purification effort, through collaboration with the Space Bioprocessing Office of the Johnson Space Center, a small amount of dialysate was submitted to the Space Sciences Division of General Electric for further separation on a very sophisticated continuous flow electrophoresis system designed for use in space flight conditions. In the preliminary experiments, four fractions were identified, three of which have varying amounts of RF activity, the total of which contains 100 % of the RF activity and only 57 % of the protein content present in the original sample (Griffin, 1976). The IgG content of these four fractions is under test.

3. RF Mediated Cytotoxicity Studies.

Once sufficient preparations of RF was obtained (even though not completely pure), its effects on tumor cell suspensions were studied. Tumor tissue was obtained from surgical specimens. This was done with approval of the attending physicians and of the appropriate institutional

human studies committees. Specimens proved variable in the viability of freshly freed cells. This is a technical area presently the subject of technological study.

Tumor specimens were immediately placed in cold, sterile, minimal essential medium[2]. Cells were gently teased with an eighteen gauge needle while concomitantly perfusing the tissue with minimal essential medium. The cells were then washed three times at 4° C. and resuspended in minimal essential medium. The number of live cells was determined using the Trypan Blue exclusion (Hanks and Wallace, 1958) after which the cell density was standardized at 5×10^6 live cells per ml.

The complement-mediated cytotoxic influence of rheumatoid factor on this cell suspension was then evaluated (Klein, 1971; Stewart and Goldstein, 1974). Cell death was determined in incubations that included 0.05 ml. of fresh normal plasma (complement), 0.05 ml. of dialysate, 0.05 ml. of saline, and 5×10^5 cells in 0.1 ml. of medium paired. Incubations included heat-inactivated complement. Then 0.25 ml. of pronase (5 mg. per 100 ml. saline) was added and incubation continued for a further thirty minutes at 37° C. Pronase solubilizes injured cells (Stewart and Goldstein, 1974). Thus a decline in hemocytometer cell count relative to the count in the paired decomplemented incubation (100 % viability) was reflective of cell damage.

The cytotoxic effect of RF varied from one tumor to another. The observed percentages of cells damaged by RF are listed in Table I. Although these percentages are variable (21.4 to 91.0 %), significant cytotoxicity is evident. The range of this data is in agreement with that observed in other investigations of immune-related tumor cell-specific cytotoxicity (O,Toole et al., 1974; Hellström and Hellström, 1974).

4. Separation of Tumor-Associated Cell Populations.

A problem related to the question of whether RF was toxic to tumor cells or to other cells present in tumor tissue. Other cells include

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[2]MEM, Grand Island Biological Company, Grand Island, New York, 14072.

Table I

Percent Cell Death Associated with Rheumatoid Factor in Cell Suspensions from Various Tumors.

Tumor Tissue Source	Percent Cell Death
Bladder tumor	87.0
Bladder tumor	69.5
Bladder tumor	37.3
Bladder tumor	21.4
Myeloma cells from an effusion	38.2
Malignant pleural effusion (type unknown)	91.0

lymphocytes, macrophages, granulocytes, and stromal cells. An additional problem was the removal of cells that were injured before being added to the test incubation. Such damaged cells could interfere with test reactions by adsorption of essential reagents such as RF and thereby sequestering them from the reaction under test. Consequently it was felt desirable to develop a method of separating these cell types so that the specificity of RF cytotoxicity could be identified with particular target cells.

The method of cell separation considered initially was centrifugation within a density gradient of Ficoll-Hypaque (Böyum, 1968; Terasaki, 1972, p. 50). Centrifugation at a speed of 1250 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 by sedimentation, but had little additional merit.

Therefore a density gradient method using human albumin was evaluated (Dickey, 1975). The colloidal nature of albumin in sclution is a retardant to the mixing of different layers in the gradient column, thus enabling a multiphase density separation column. Albumin, similar to Ficoll-Hypaque, is non-toxic to cells.

Initially several combinations of albumin solutions were used in constructing the column to determine the combination most suitable for cell separation. The range of concentrations of these solutions was from 7 % to 27 % albumin. Cell suspensions from twelve tumor specimens were separated in this manner. Cell viability, determined by dye exclusion (Hanks and Wallace, 1958), was measured in the fractions and compared to the viability of the suspension prior to separation. The difference between the percentage of live cells found in a particular albumin fraction and that found in the original suspension was calculated as a measure of the viable cell enrichment achieved in that fraction. There was little difference apparent in the fractions of albumin concentration less than 17 % or greater than 25 %. Therefore these measurements were grouped together with those of the 17 % and 25 % albumin fractions respectively in the data summarized in Table II. Some success is evident from the data. However considerable variability with different tumors is apparent.

Table II

Increase in Percent of Viable Tumor Cells by Separation on an Albumin Density Gradient.

Tumor Cell Source	Fraction (Albumin Concentration) 17 %	21 %	23 %	25 %	Bottom
Bone marrow	-38.4	-14.9	0.7	0.2	-8.3
Bone marrow	-62.3	4.1	8.5	3.3	0.6
Breast	2.0	-1.9	-1.9	3.2	-6.2
Bladder	34.1	15.1	18.1	4.1	-9.9
Larynx	57.6	41.6	2[illegible].6	50.6	-30.5
Neck mass	27.6	23.6	7.6	16.4	-2.4
Breast	3.2	2.8	0.6	-1.7	-2.2
Breast	22.6	2.2	3.8	-9.4	-4.0
Bladder	35.3	5.5	-4.0	-12.0	-15.4
Bone marrow	-8.7	31.0	25.0	12.0	-1.5
Bone marrow	-11.3	-7.2	1.1	2.1	-1.8
Pleural effusion	0.4	-0.8	4.1	2.7	-6.6

In cells from malignant effusions (Table III), enrichment in viable cells was increased in the 21 %, 23 %, and 25 % albumin fractions, with the greatest enrichment in the 23 % fraction. A decreased percentage of vaible cells in comparison to that of the original cell suspension, which can be interpreted as an enrichment of dead cells, was observed in the top (17 % albumin) and bottom (solid pellet in bottom of centrifuge tube) fractions.

The results of separating cell suspensions derived from the seven solid tumors (Table IV) are also suggestive of a separation of viable cells from dead cells but the pattern of separation is quite different from that observed with fluid type tumor sources. With solid tumors, the greatest percentage of live cells was found in the 17 % albumin fraction and the enrichment of live cells became less as the albumin concentration was increased. An actual enrichment of dead cells was evident in the bottom (solid pellet) fraction of the gradient column.

The separation of viable cells suggested by these results was encouraging, particularly with respect to solid tumors. An expansion of the range of concentration gradients was then considered as a possible means of improving the delineation of these cell populations. To evaluate this possibility, albumin concentrations of 14 %, 18 %, 22 %, and 26 % were used. As before, the tumor cell sources are classified as either fluid or solid. The results are presented in Tables V and VI respectively.

The measurements of enriched viable cell content in suspensions from fluid tumors was not suggestive of an improvement in separation using this new combination of albumin solutions. However these results are based upon only two specimens.

In contrast, use of this expanded range of albumin concentrations seemed to result in a much sharper separation of cells derived from solid tumors. Not only were the albumin fractions more enriched with viable cells but the increased percentage of dead cells in the bottom pellet was even greater with this gradient combination. Since it is the live cells that are of interest to the present studies of cytotoxic effects, these results are quite relevant.

Table III

Increase in Percent of Viable Tumor Cells from Malignant Effusions Separated by Albumin Density Gradient Centrifugation.

Tumor Source	Fraction (Albumin Concentration) 17 %	21 %	23 %	25 %	Bottom
Bone marrow	-38.4	-14.9	0.7	0.2	-8.3
Bone marrow	-62.3	4.1	8.5	3.3	0.6
Bone marrow	-8.7	31.0	25.0	12.0	-1.5
Bone marrow	-11.3	-7.2	1.1	2.1	-1.8
Pleural effusion	0.4	-0.8	4.1	2.7	-6.6
Mean	-12.1	2.4	6.3	4.1	-8.3

Table IV

Increase in Percent of Viable Cells from Solid Tumors and Separated by Albumin Density Gradient.

Tumor Source	Fraction (Albumin Concentration) 17 %	21 %	23 %	25 %	Bottom
Breast	2.0	-1.9	-1.9	3.2	-6.2
Bladder	34.1	15.1	18.1	4.1	-9.9
Larynx	57.6	41.6	23.6	50.6	-30.5
Neck mass	27.6	23.6	7.6	16.4	-2.4
Breast	3.2	2.8	0.6	-1.7	-2.2
Breast	22.6	2.2	3.8	-9.4	-4.0
Bladder	35.3	5.5	-4.0	-12.0	-15.4
Mean	26.1	12.7	8.8	9.3	-10.1

Table V

Increase in Percent of Viable Cells Separated from Malignant Effusions by Albumin Density Gradient.

Tumor Source	Fraction (Albumin Concentration) 14 %	18 %	22 %	26 %	Bottom
Bone marrow	-33.0	-24.1	-4.6	1.1	-8.3
Pleural effusion	-48.7	8.2	12.6	-1.2	6.8
Mean	-40.9	-8.0	4.0	-0.1	-0.8

Table VI

Increase in Percent of Viable Cells from
Solid Tumors and Separated by Albumin Density Gradient.

Tumor Source	Fraction (Albumin Concentration) 14 %	18 %	22 %	26 %	Bottom
Prostate	-11.7	8.6	-0.9	5.6	-5.8
Bladder	26.5	16.1	-3.2	-23.0	-38.7
Penis	38.5	46.6	45.7	32.0	-29.7
Ovary	53.2	63.5	54.3	46.7	-21.7
Bladder	45.8	48.1	42.9	36.3	-34.2
Mean	30.5	36.6	27.8	19.5	-26.0

Not only was it considered desirable to remove the dead cells from the cell suspension but it was also considered important to identify and isolate the various populations of cells involved in the cytotoxic mechanism. In this particular study, it would be valuable to isolate the neoplastic target cells from the effector cell group or groups. In order to assess the efficiency of the albumin gradient method in separating these cell types, slides were made of the cell population obtained from each fraction, subsequently stained with Wright's Stain, and analyzed microscopically for differential cell composition. These differential cell counts were compared to that of the original cell suspension to determine if enrichment of any cell type had occurred as a result of the separation procedure.

There was no evident pattern of separation based upon cell types in the results of this part of the study. Cytological analysis of additional specimens and improved staining methods may be of help in clarifying a possible pattern of separation.

One such staining method being considered is specific for IgG or IgM on the cell membrane surface (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 factor in effecting cell-mediated immune reactions such as cytotoxicity of tumor cells (Marchalonis, 1975; Rowlands and Daniele, 1975; Vitetta and Uhr, 1975; Hunt and Williams, 1974; Scorm, 1976; Metzgar et al., 1975; Kolata, 1975; Kulczycki et al., 1974; Irie and Norton, 1974). These membrane surface immunoglobulins, because of their specificity, may enable an improved system of cell identification by means of immunoglobulin-specific staining reactions. This possibility is further substantiated by the investigative efforts of Choi et al. (1974) who have outlined a general method for isolation and recovery of B lymphocytes bearing specific receptors. Apreliminary test of the peroxidase-conjugated immuno-specific staining method described by Taylor (1976) was performed on a small number of slides of the cells separated by the albumin gradient method in the present study. These preliminary results are indicative of an excellent staining specificity. Should

further tests of this staining method substantiate this potential value, this method may be very useful in identifying immune responsive cells of refined functional specificity, which would be very applicable to further clarification of the mechanisms of tumor immunology.

5. Problems.

Tissue availability has to some extent slowed progress. Successful negotiations to have a reservoir of human tumors implanted into a large colony of nude mice should now overcome this difficulty. This also eliminates the presence of human lymphocytes and renders amboceptor ammenable to experimental control. In addition, the use of tumors such as malignant melanoma will overcome much of the difficulties experienced in this part related to cell identity. We propose this approach as being likely to provide us with an adequate supply of human material suitable for our cytotoxicity.

6. Expenditure Summary.

The expenditures as of 3/31/76 are listed in Table VII. The funds remaining seem adequate for the anticipated expenditures in the completion of this contract.

7. Conclusions.

During the initial six months of this contract, advances were made in several areas relevant to the complement-mediated destruction of neoplastic cells. Methods of separating mixed cell suspensions into fractions of viable and non-viable cells were examined and one, an albumin gradient centrifugation method appears to be applicable in this respect.

Adsorbed RF was prepared and submitted for electrophoretic analysis, which resulted in the identification of several fractions varying in activity.

A roxidase-conjugated immuno-specific staining method for identifying classes of Ig present on cell membrane surfaces was tested and appears promising as a research tool in further studies of cytotoxic action.

Table VII

Summary of Expenditures to 3/31/76

Category	Dollars Spent	Dollars Remaining
Salaries	8,049.97	10,208.24
Supplies	1,844.89	3,652.11
Travel	352.00	148.00
Fringe Benefits	750.51	985.88
Indirect Costs	1,988.00	2,521.00

Lastly, through these efforts, a general understanding of the requirements for studying this cell-mediated cytotoxicity was achieved and will be a sound basis for approaches to further selected studies in this area.

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