To my parents,

Agnes and Robert Tong

CYTOFLUOROMETRIC EVALUATION

OF

ANTIGEN SPECIFIC ROSETTE FORMING CELLS IN HUMANS

by

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A THESIS

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STATEMENT OF THE PROBLEM

Immune responses involve the differentiation of resting lymphocytes into effector lymphocytes that mediate an immunospecific event. The clonal selection theory as proposed by Burnet (15) states that each animal carries a wide array of lymphocyte subpopulations, each expressing a unique antigen receptor specificity that is genetically predetermined.

In 1967, Naor and Sulitzineau (105) demonstrated the presence of lymphocytes in mice which could recognize a foreign antigen specifically without prior exposure. By using autoradiographic techniques, the antigen reactive cells binding previously radiolabelled antigens were visualized as lymphocytes with grains (photodeveloped radiolabelled antigen) on their cell surfaces. Since then, antigen binding cells (ABCs) have been demonstrated both in normal and immune animals, invariably with an increase in specific ABC levels following immunization (for review see 40, 136). ABCs detected against a given antigen represent specific immunocompetant cells, since their selective killing or inactivation resulted in abrogated immune reponse to that antigen (2,7,21,131).

The detection of ABCs is direct evidence for the clonal selection theory. In addition, the study of ABC populations has added to the understanding of various immunological phenomena. The differences in immunological responsiveness between genetically determined high and low responder strains (78,80), type I and II T independent (TI) humoral response[a](38), or tumor rejection by normal

[a]see footnotes, P.29

or compromised tumor bearing hosts (96,113,126) is manifested by variations in the level or composition of antigen binding cells. However, to date few procedures have utilized antigen binding cell evaluation for routine assessment of immunity, although it is well established that immunity correlates with increase in specific antigen binding cells. Techniques currently available, such as microsopic analysis of rosette formation or radioautography for detecting radiolabelled antigens are tedious and impractical for routine analysis.

The purpose of this thesis is to develop a rosette forming assay using laser cytofluorometry for the routine evaluation of antigen binding cell responses in humans. Antigen binding cell response analysis may be particularly useful in immunologically compromised situations, such as in tumor bearing patients where evaluation of tumor specific antigen binding response could help distinguish defects in initial recognition, or in amplification/efferent functions. In the first part of this study, the effectiveness of this assay in detecting antigen specific rosette formation among PPD and KLH immune donors is examined. Secondly, tumor specific rosette formation among patients with squamous cell carcinoma of the head and neck is evaluated, using 3M KCl extracts from various tumors. The specific aims of the research are:

1 a. To define the parameters of the rosette forming cell assay by using a model system of donors immunized to conventional antigens KLH and PPD.

b. To demonstrate a correlation between donor immunity and significant levels of specific antigen binding cells.

c. To describe the mechanism and cell types associated with specific antigen binding.

2 a. To apply this assay in evaluating tumor antigen rosetting response of tumor patients (primarily patients with squamous cell carcinoma of the head and neck) using tumor antigen extracts with previously defined specificity.

b. To describe the mechanism and cell types associated with specific tumor antigen binding.

LITERATURE REVIEW

A. Detection of antigen binding cell

1.Heterogeneity of ABCs

A variety of techniques have been developed for the detection and isolation of specific antigen binding cells. A brief description of each method and its respective merits are presented in Table I.

Antigen binding cells to a given antigen exhibit a wide range of binding avidity as well as receptor density (1,6,32,48,136) ,and the level and proportion of different antigen binding cell subpopulations detected is dependent on the condition and methods used. Early studies using radioautography had limited sensitivity and primarily detected B-ABCs, whereas rosette formation demonstrated both T and B ABCs (6,23,30,31,40,135). In a study comparing the rosette forming assay with radioautography, Bankhurst and Wilson

FOR THE DETECTION OF ANTIGEN BINDING CELLS

Disadvantage	References
1. Requires tedious photodeveloping techniques 2. Absolute number of ABCs detected varies with contact time between antigens and cells, and exposure time during photo-developing 3. Preferentially detects antigen binding cells with high receptor density, has limited sensitivity in detecting ABCs of low binding avidity. 4. Does not allow for ABC isolation	Naor & Sulitzeanu (1969) Ada & Byrt (1969) Davie & Paul (1970) Roelants (1972) Hammerling & McDevitt (1974) Swain & Coons (1976)
1. Technique is relatively time consuming 2. Detection requires optimizing enzyme-substrate interaction conditions as well as antigen-cell interaction conditions	Warner (1974) Miller et al (1971) Rotman & Cox (1971) Haas & vonBoehmer (1978)
1. Offers little information on correlation between recovered ABCs and enumerated ABCs 2. Isolation is time consuming and may lead to loss of ABCs during process 3. Extensive overlap in intensity between specific and nonspecific fluorescein-stained cells, limiting specificity of purified cells	Julius et al (1976) Haas & von Boehmer (1978) Greenstein et al (1980)
1. Maximum enrichment of specific ABCs inevitaly leads to nonspecific retention 2. Does not allow quantitation of ABCs 3. Requires large cell volume for extraction 4. Recovery efficiency of ABCs is not well documented; recovered population may represent only part of total ABCs retained	Maoz et al (1976) Wekerle et al (1972) Ly & Mishell (1974) Taniguchi & Miller (1977)
1. Enumeration by microscope requires time consuming visual efforts 2. % ABC determined is usually based on small sampling size (<500 cells) thus limiting accuracy of the technique	Bankhurst & Wilson (1971) Sjoberg & Moller (1972) Elliot & Haskill (1973) Kontianen & Andersson (1975) Higgins & Choi (1978)

(6) reported that more ABCs to the antigen chicken globulin were detected by autoradiography than rosette formation. More T-ABCs, however, were detected using the rosette forming cell (RFC) technique. They suggested that rosette forming cells exhibited the most avid receptors, and cells with low surface receptor density form rosettes but bind too few molecules of radiolabelled antigen in the selected experimental condition to appear as a labelled cell (i.e. less than 4,000 molecules). Cells with high receptor density could form rosettes and also binds sufficient radiolabelled antigens for detection. The observation that most rosette forming cells are θ-positive but were not radiolabelled also suggests that T cells display a much lower density of antigen binding receptor than B cells.

Using I^{125} labelled synthetic polypeptide {(Tyr-Glu)-Ala-Leu}, (TGAL), Hammerling and McDevitt (49) demonstrated antigen binding by T and B lymphocytes. Detection of T lymphocytes was favored by a longer exposure time as well as higher incubation temperature, although this had no influence on B cell detection.The presence of thymus and bone marrow derived ABCs have since been demonstrated in different laboratories (23,30,40,48,61,120,121). It is now clear that the ABC population is heterogenous and participates in a variety of humoral and cell mediated effector and regulatory functions.

2.Methods of detection

Recent studies on ABCs have concentrated on functional characterization of ABC subpopulations, (20,27,30,31,38,40,61,62,76,

77,88,106,107,138) and thus require techniques that allow enrichment and/or isolation of ABCs. These include the fluorescent activated cell sorting (FACS), immunoadsorption, or rosette formation techniques.

The Fluorescent Activated Cell Sorter (FACS) utilizes electronic capability in enumerating and selecting for ABCs binding fluorescein conjugated antigens. Cell sorting provides a rapid in vitro technique for isolation and characterization of viable ABC subpopulations. However, in all of the studies reported so far (38,40,75), only B-ABCs are recovered, suggesting that the method may have limited sensitivity in detecting other ABC subpopulations.

Immunoadsorption utilizes antigens fixed onto insoluble matrix, such as gelatin (40,92,93), Sephadex beads (89,138) or plastic surface (40). Cells binding antigens are retained when passed over the matrix, which can in turn be eluted and characterized. Immunoadsorption has proven to be an extremely useful technique in enrichment for ABC subpopulations, since binding conditions can be adjusted to optimize yield (40). Recent reports have shown that low avidity binding T lymphocytes can be routinely isolated using this technique (40,92,93,138). Maximum recovery, however, invariably resulted in extraction of nonspecific antigen adherent cells. Together with the high variability in recovery during ABC elution, immunoadsorption represents a relatively insensitive method in monitoring changes in specific antigen binding cell levels.

Formerly used for enumeration of antibody forming cells through the adherence of large particulate antigens, rosette formation has

also been found effective in detecting antigen binding immunocompetent cells. Chemical coating of antigens including protein (6,125), polysaccharides (98,116) and hapten determinants (97,98) onto autologous or syngeneic erythrocytes have permitted the quantitation of RFCs to a wide range of antigen systems. The rosette forming assay is relatively simple to perform, highly reproducible, and does not require elaborate experimental or technical conditions. In addition, antigen binding rosetted cells can be isolated by density gradient type techniques due to their considerable size and density difference relative to unrosetted cells (11,29-31,62,76, 135). Under standard conditions, rosette formation is capable of detecting ABCs of low avidity, such as T antigen binding cells. Studies that have utilized the RFC assay invariably require microscopic scanning of large numbers of cells per sample (200-500 cells) to determine the level of ABCs, and is not practical for day to day screening of large volume experiments.

Recently we have developed the cytofluorometric technique to assess antigen specific RFCs. Electronic analysis allows the evaluation of % RFCs per 10^4 cells per sample. The mean ($\pm$ S.D.) RFC response to each antigen can be determined using 3 to 5 samples, so that the level of RFC response to a panel of antigens can be evaluated and analysed statistically within a short period of time.

B. Correlation with immunity

1.ABCs in normal animals

Antigen binding cells are detectible in animals without apparant prior exposure to the antigen. Specific antigen binding cells to bovine serum albumin (BSA), Keyhole limpet hemocyanin (KLH) and the hapten dinitrophenol (DNP) can be detected in normal mouse spleens (75,105,114), and DNP- ABCs were detectible in lymph nodes, peripheral blood, and bone marrow in unimmunized guinea pigs (23). In humans, naive donors carry ABCs to radiolabelled flagellin, and horseshoe crab hemocyanin (HCH) in thymus, spleen, and peripheral blood leukocytes (26). ABCs isolated from normal unimmunized Balb/CN mice were found to be precursors of antibody forming cells and are monospecific (75). Both T and B ABCs can be detected against TGAL in lymph nodes and thymuses (48,49) . To consider the possibility of prior exposure before examination, the level of antigen binding cells to β -galactosidase was examined in germ free colostrum deprived piglets (25) and mice (116). Germ free animals possessed levels of ABCs that are not significantly different from normal controls. It is not known if these animals could have been exposed to E. coli β-galactosidase through normal feeding. However, together with other studies, it appears that ABCs with predetermined specificity can be found in normal animals capable of interaction with incoming antigenic challenge.

Antigen recognition appears to be an immune process acquired at an early age (24). Animals at half term including mouse at the yolk

sac embryo stage, or chick embryo can demonstrate a wide range of receptor specificities against β -galactosidase, fluorescein isothiocyanate (FITC), keyhole limpet hemocyanin (KLH), horse spleen ferritin (HSF), and horseradish peroxidase (HRP). Antigen binding function appears shortly after the emergence of stem cells and lymphoid cell precursors, and precedes the appearance of most organized lymphoid tissues. Antigen binding cells were also found in early mouse or chicken thymus, although the level decreases with age. It is not known, however, if this reflects a decrease in T-ABCs, since the T cell nature of these ABCs has not been demonstrated.

2. ABCs in immune animals

Immunization invariably results in clonal expansion of ABCs. Significant elevation of antigen binding cells can be demonstrated in a variety of systems after immunization. A 40- to 100- fold increase in the level of detectible specific ABCs can be found in immunized mouse spleens and lymph nodes (92,132). Increase in both T and B ABC subpopulations can be observed upon immunization with bovine serum albumin (BSA, 114) or TGAL (48,80). Hapten immunization resulted in increases in T-ABCs among ABC populations (97,98). Induction of cell mediated immunity after allogeneic red cell immunization is paralleled by increased T and B rosette forming cells (29-31,50,77). Antigen specific T-ABCs can be observed in bursectomized chickens after immunization (61,62). In addition, antigen specific antibodies generated after immunization can facilitate antigen binding through adsorption to Fc receptor-bearing

cells. This mechanism has been demonstrated to represent a significant level of ABCs detected in mice (78,113), guinea pigs (19), and chickens (22,72,137), although some studies have precluded the involvement of cytophilic antibody recognition in the ABC population detected (6,19,25,61).

Antigen binding cells in immunized animals have increased binding avidity after immunization. By using hapten that is cross reactive to the immunizing antigen, Davie and Paul (23) were able to show that immune ABCs have a higher binding avidity than ABCs in normal animals; the cross reactive hapten could no longer effectively compete with specific antigen binding. This was confirmed by Moller (97), who showed that hapten specific RFCs in immunized mice have a higher binding avidity than normals. Prange et al (112) also showed that immunization resulted in increased avidity in antigen binding, which is accompanied by increased IgG clonal expansion and antibody production.

It is apparant that immunization results in the expression of higher avidity antigen specific receptors in T, B and monocyte populations than normals resulting in an increased pool capable of enhanced specific interaction with that antigen. Presently, due to the selectivity of each study in detecting ABC of only a predetermined range of avidity (40,136), it is difficult to correlate the distribution of various ABC subpopulations as demonstrated in various reports. Thus the question of whether a certain regimen of immunization directly results in the induction of a particular antigen binding subpopulation remains unverified by ABC studies.

C. Antigen Binding Cells: Subset analysis

The only common denominator among antigen binding cells to a particular antigen may well be the presence of specific receptors that allows recognition and binding to a unique antigen. These cells exhibit a wide range of binding avidity and stability (6,23,48,61,97,112,120,136) and are comprised of T and B lymphocytes as well as cytophilic antibody armed monocytes and macrophages. More recent studies have indicated that cells bearing antigen specific receptors participate in a variety of effector and regulatory functions in both cell mediated and humoral responses. The involvement of various ABC subpopulations in immune function is discussed below.

1. B antigen binding cells

There is no doubt that B cells can bind antigens and can be detected with all methods available (1,6,23,40,135). The expression of antigen binding capacity parallels ontogenic expression of constant region genes (136). ABCs appeared in the bursa of 14 day embryos simultaneously with the first recognizable lymphoid cells, and this corresponds with the first appearance of detectible intracellular immunoglobulins. Antigen binding cells purified by FACS transfer humoral immunity (38,75), and ABCs extracted by affinity chromatography bear membrane immunoglobulin and have been found to be antibody forming cell precursors (38,40,88,75). Plasma cells

bearing membrane immunoglobulins bind antigens, and can be extracted by rosette forming techniques (40,62,98,112).

Clonal expansion of B ABCs following immunization is paralleled by changes in isotype expression (77). Murine lymphocytes immunized with sheep erythrocytes (SRBCs) showed a 10- to 20- fold increase in antigen specific rosette forming cells. Virtually all binding is inhibitable by anti-Ig, suggesting a predominance of membrane-immunoglobulin (M-Ig) bearing B ABCs. While there was no change in the percent of M-IgM ABCs between nonimmune and immune animals, the % of M-IgG bearing cells increases by nearly 50% after immunization. In contrast, M-IgD bearing RFCs declined after immunization. Most IgG and IgD bearing ABCs also expressed IgM. Data analysis have shown that a maturation process of $M^+D^+G^-$- bearing into $M^+D^+G^+$- bearing lymphocytes has occured.

Heterogeneity of B- ABCs can also be defined by receptor avidity which correlates with humoral responsiveness. Greenstein et al (38) used unprimed anti- Thy I - complement treated mouse splenocytes and extracted TNP- B ABCs with fluorescein conjugated TNP-BSA. When the antigen binding B cells were separated into cells binding a moderate amount of TNP-BSA and cells binding a large amount of the antigen it was found that B cells responsive to TNP- or DNP-Ficoll (Type II, TI[b] response) bound moderate amounts of antigen, whereas cells binding TNP-LPS or TNP-SRBC (Type I, TI response and TD[c] response respectively) bound either moderate and/or large quantities of antigen. This study indicates that B cell function may be correlated with the density of antigen receptors, since only B cells with a low density

b,c see footnotes, P.29

of antigen receptors are capable of responding in a Type II TI humoral response.

2. T antigen binding cells

T antigen binding cells have generally been shown to have low avidity as well as low receptor density for antigen specific binding (2,6,50,62). In addition, their association with antigen is often unstable and requires stringent condition for detection, such as higher incubation temperature, i.e. 37 C instead of 0 C (30,31), longer periods of antigen exposure (48,50) or fixative techniques after interaction with antigens (40). More recent reports using better defined experimental conditions have established the involvement of T-ABCs in various animal models. T lymphocytes capable of antigen recognition and binding can be extracted using rosette formation (6,30,31,62,97,98), radioautography (6,48,49,61, 120,121) and various immunoadsorption techniques (20,92,93,106, 107,123). Some authors have suggested that induction of T-ABCs is dependent upon the regimen of immunization (20,27) although this was not been confirmed by others (29,30,31,62,92). The involvement of T antigen binding cells in specific immune function can be demonstrated indirectly by studies which indicate that inactivation of T ABCs with radiolabelled antigens leads to the loss of antigen specific precursor and effector T cells (27) ; and T ABC depleted cell fractions after immunoabsorbant passage do not exhibit specific T cell functions (92,138). In addition, enriched T ABC fractions were associated with both regulatory and effector functions,

including transfer of delayed type hypersensitivity (DTH, 30), cytotoxic activity (30,92,93,138), and suppressor (106,107,123) and to a lesser extent, helper functions (31,76).

2a. T antigen binding effector cells

Early studies by Wekerle et al (138) and others have demonstrated that cytotoxic T cells adhere specifically to xenogeneic and allogeneic target cell monolayers. Elliot et al (30) showed that DTH can be transferred using immune mouse spleen cells binding to the immunizing antigen, sheep erythrocytes. The ability to transfer delayed type hypersensitivity was mediated by T cells, since anti-θ but not anti-Ig treatment abrogated the response. These T-RFCs also demonstrated in vitro cytotoxic response which was also sensitive to anti-θ treatment. The T-ABCs capable of DTH transfer and cytotoxic activity were small and medium size lymphocytes, indistinguishible by size, anti-θ sensitivity, antigen binding capacity or kinetics of induction.

2b. T antigen binding regulatory cells

A variety of reports have indicated that isolated T-ABCs carry suppressor function (92,93,107). T cells from an animal tolerized with TNP-KLH (enriched by passage over a KLH-collagen column) were found to suppress plaque forming cell (PFC) response to TNP. Enrichment of T antigen binding cells from in vitro sensitized lymphocytes also resulted in a 100- to 1000- fold enrichment of suppressor activity (27,92). Similarly, idiotype specific suppressor cells

isolated using Fab cross reacting idiotype (CRI) can also be generated by treatment with antiidiotype antisera before immunization (107). Antigen specific immune T cells isolated from immunoadsorbant columns can suppress secondary antibody response to DNP-KLH in vivo by transfer, and carried the phenotype of IJ^+ $Ly2,3^+$ [d] (27) . Other reports using other immunoadsorbant techniques were also able to demonstrate specific suppression by T-ABCs (92,123).

Presently, it is not entirely clear if helper T cells carry receptors that can recognize antigen in its native form. Helper T cells could not be isolated either by rosette formation (30,76) or immunoadsorbant techniques (116). However, in one study by Maoz and Feldman (92) antigen specific T-ABCs enriched 100- to 1,000 fold by using antigen- conjugated collagen columns exhibited helper function against TNP-KLH when mixed with normal spleen cells. From antigen binding cell enrichment studies , it is difficult to ascertain if the difficulty of isolating T helper cells is due to their low avidity of antigen binding (29,30) or simply because they do not recognize antigen in its native form. Other studies (132), however, have indicated that helper T cells require corecognition of processed antigen together with Ia, and thus would not be extracted through native antigen binding.

Antigen specific T-ABCs have been shown to participate in a variety of effector functions. Whether this functional heterogeneity is due to distinct subpopulations or T cells at different stages of differentiation is not known. By examining the binding properties of

[d] see footnotes, P.29

T-ABCs before and after *in vitro* immunization with SRBCs, Eardley et al (27) demonstrated a shift in the distribution of T-ABC subsets. T-RFCs are Thy^+, Ig^- and nonadherent to nylon wool. Upon *in vitro* sensitization, these antigen binding cells developed the capacity to adhere to nylon wool. Additionally, T-RFCs bear phenotypes of Ly $1,2,3^+$ or Ly $2,3^+$ depending on the immunization dose of antigen. The Ly $2,3^+$ T-RFCs appeared to be derived from Ly$1,2,3^+$ cells since depletion of Ly $1,2,3^+$ cells abrogates generation of Ly $2,3^+$ cells. T-RFCs generated in this assay only participated in amplification of suppressor events, but not effector functions. Since this study does not detect T-ABCs with other activities, such as transferrable helper, suppressor or effector functions, correlation with other studies is difficult. In fact, each report on T-ABC enrichment utilizes techniques that favor both the generation and recovery of the subpopulation of interest, and cannot be correlated to other studies on the distribution of T-ABC subpopulations. Although one can conclude that T-ABCs participate in a variety of immunological specific activities, the relative importance of various antigen receptor bearing T subpopulations *in vivo* is still poorly understood.

3. Antigen binding mediated by cytophilic antibodies

Early reports that demonstrated antigen binding by T and B lymphocytes bearing specific receptors have rarely considered the involvement of cytophilic antibody mediated specific antigen recognition (1,6,19,26,78). Recently, several reports have examined

the role of cytophilic antibody and suggest that this mechanism may play an important role in antigen recognition (19,22,72,78,114,137).

Kapp and Benacerraf (78) showed that spleen cells from normal mice acquired antigen specific rosette forming ability when treated with antisera from immune animals. In addition, double rosettes were observed when normal cells were incubated either with serum from a doubly immunized animal, or sera from two animals immunized to different antigens. Rosette forming cells were macrophages as well as lymphocytes, since removal of adherent cells led to substantial decrease but not total loss in the total number of single and double rosette forming cells. In the primary immunized serum, cytophilic antibody was found only in the IgM fraction and its appearance was transient. Both IgM and IgG fractions contained cytophilic antibodies in the secondary serum, but the IgG fraction was many times more active than the IgM fraction.

Serum dependent antigen recognition was also demonstrated among guinea pigs immunized with myelin basic protein in complete Freund's adjuvant (CFA-MBP). Immunized guinea pigs exhibited significantly higher I^{125} BP binding than normals or those just immunized with CFA (19). The enhanced antigen recognition can be transferred by sera, since normal guinea pig lymphocytes demonstrated the high level of antigen binding response after preincubation with immune serum. The transferrable binding capacity of immune serum resides in IgG_2. While the same phenomenon could not be demonstrated among rats, these data suggest cytophilic antibody-mediated BP recognition may occur in the guinea pig and may be an important factor in in

vivo recognition of the antigen.

Lymphocytes from agammaglobulinemic chickens can bind antigens via antigen specific cytophilic antibodies (Webb & Cooper,1973). Peripheral blood lymphocytes from normal chickens immunized to sheep red blood cells have an enhanced rosette forming response, but this was not observed among agammaglobulinemic chickens. Upon incubation with serum from immune birds, cells from agammaglobulinemic chickens could form significant numbers of rosettes to SRBCs. The arming activity can be adsorbed with sheep erythrocytes, and appeared to be IgM antibody with specific reactivity. The rosette forming response observed was not affected by macrophage depletion by filtration through a cotton wool column, suggesting that antigen specific rosette formation in agammaglobinemic chickens represents primarily T cells bearing cytophilic antibodies to SRBCs.

Jensensius et al (72) demonstrated that binding of radiolabelled antigen in bursectomized chickens was also mediated by cytophilic antibodies. Binding of TIGAL (radiolabelled iodine derivatized synthetic polypeptide TGAL) by non-bursa derived lymphocytes was abolished by anti-chicken light chain or antigen-antibody complexes. The antigen binding cells were found in both adherent and non-adherent populations, and may involve both monocytes and T lymphocytes.

These observations indicate that antigen binding cells in a variety of animal systems may include a subpopulation that recognizes antigen through cytophilic antibody adsorption. It is not known if cytophilic antibodies were involved in early studies that

reported only T and B lymphocyte antigen binding, since the possibility was rarely considered (19,22,72,78,137). A variety of mononuclear cells, including B, and helper and suppressor T lymphocytes (83,101), as well as monocytes (99,134,138,140) carry Fc receptors and can acquire cytophilic antibodies. Though the exact role of cytophilic antibody armed antigen specific recognition in immune responses is not established, reports have indicated that circulating immunoglobulins may act as a regulatory element via cytophilic adsorption. Stout and Johnson (119) reported that poly-AU treatment of immune lymphocytes resulted in the release of antibody receptors and a loss of secondary response upon antigenic challenge. This response could be restored if depleted cells were incubated with supernatants containing 7S immunoglobulins. Thus cytophilic antibody may be involved in the induction of secondary response , and its presence on cell surface appeared to be a requisite for memory expression.

In a study to compare the responsiveness in high and low responder mouse strains, Kapp and Benacerraf (78) reported that sera obtained from high or low responder mouse strains are equally proficient in arming high responders for antigen recognition response. However, serum from neither high nor low responders is capable of potentiating response in low responder mice strains. It appears that antibody mediated antigen recogniton may reflect specific response of a Fc bearing subpopulation, whose function is correlated with the immunoresponsiveness of the animal.

In bursectomized chickens, where B cell functions are absent, no

ABCs to conventional antigens were found (22,72,137). This is of interest since T cell function was intact. It appears that generation of ABCs requires the interaction of T and B lymphocytes. The ability to arm T lymphocytes with cytophilic antibodies may suggest an acquired antigen recognition process , although the study did not examine if the chickens acquired any subsequent immunological response capability other than antigen recognition, such as cell mediated immunity (CMI) that was absent before.

The presence of antibody dependent antigen recognition may modulate effector mechanisms after exposure to the antigen. More recently, immunity to particulate antigens or tumor cells can be demonstrated by antibody dependent cellular cytotoxic response. (51,82,102,109,130,133). It is possible that antibody dependent antigen recognition represents afferent process in ADCC reaction, although correlation of ADCC with antibody mediated specific recognition has not been documented. In any case, studies presented so far indicate that antibody dependent antigen recognition is largely an immune specific process and can only be acquired after immunization. Detection of cytophilic antibody-mediated antigen recognition is thus an appropriate indicator of donor immunity.

D. Immune recognition in tumor bearing hosts

1. Demonstration of tumor immunity

Immune recognition of tumor antigens *in vivo* is well documented.

Spontaneous remission is common among tumor bearing hosts that have failed to respond to treatment (4,5,17,56). Animals immunized with syngeneic tumor cells reject a subsequent challenge (5,82), and primary tumor bearers can demonstrate concommitant rejection responses when challenged with small numbers of their own tumor cells (36,82).

Proficient cell mediated as well as humoral immunity can be demonstrated in vitro in primary tumor bearers. Tumor antigen stimulated lymphocyte transformation (71,95,128), delayed cutaneous hypersensitivity or DCHR (17,33,55-57,64,65), lymphokine production (9,43,95), one way mixed lymphocyte culture (13,52,56) as well as syngeneic tumor cell killing by microcytotoxicity (17,53,85,86, 133), tumor challenge (36,56,128) and antibody mediated cellular cytotoxicity or ADCC (51,74,82,87,104,108) are tests frequently used to monitor cell mediated immunity. Tumor immunity assessed in vitro however, does not necessarily correlate with tumor control in vivo. Patients that exhibited strong in vitro responses frequently fail to control autochthonous tumors (4,5,52).

The inability of host to control tumor growth can be partially explained by factors that may act in vivo to interfere with the effectiveness of the host's immune response. Serum factors (5,12,14,17,54,70,110) as well as suppressor lymphocytes (34,42,69) have been found to cause generalized as well as tumor specific immune suppression. In rats (4,5), mice (5,13,51,128) and rabbits (5,17), cytotoxic activities of immune lymphocytes were abrogated after preincubation with the tumor bearer's serum. Similar serum

'blocking' effects have been demonstrated in patients with high tumor burden melanoma (10,53), and colon (41), breast and ovary carcinoma (54,118) and were believed to be mediated by specific antibody complexed with shed tumor antigen. In mouse tumor models cultured T cells from tumor bearing animals also produced soluble factors that could inhibit in vitro killing of tumor cells by immune splenocytes (5). Similarly, Fujimoto et al (34) demonstrated the presence of specific suppressor T cells in tumor bearing animals, which on transfer into syngeneic immune recipients diminished their ability to reject tumor cells . The presence of suppressor T cells has been poorly documented in human tumor systems (8), although they were active in tolerance (68), genetically associated high low response to antigen (79) and diseases with immunological aberrations (70).

2. Application to human tumor immunity evaluation

In order to be useful in patient tumor immunity evaluation, it is necessay for an in vitro assay to a/ measure tumor specific immunity; and b/ consider any in vivo regulatory elements.

Various cytotoxic assays have been utilized extensively to demonstrate cell mediated immunity in tumor patients. Although they were apparantly useful in establishing the immunoproficiency of patients tested, the ability of these assays to detect tumor specific immunity has been under severe criticism (56,86,87). Patient testing in vitro frequently requires using cultured tumor cell lines as targets, and patients were found to have lymphocytes

cytotoxic to tumor cells unrelated to their own neoplasm. A high level of reactivity also exists among normal individuals to normal and unrelated neoplastic cells. The existence of natural killing (NK) cytotoxic responses (56) have thus limited the applicability of the cytotoxic assays in tumor specific evaluation. More recent efforts (85,86) have been able to demonstrate tumor specific cytotoxic response under more stringent conditions to control for NK activity, but these studies have been limited in application and did not speak to the clinical or in vivo relevance of the reactions.

When the results of DCHR, cell mediated cytotoxicity and lymphocyte proliferation response in acute leukemia patients were compared (84), only DCHR reactivity consistently correlated with the patient's immune status. Similarly, when the in vivo tumor neutralization assay (Winn test)[e] was compared to microcytotoxicity and chromium release lymphocytotoxicity assays, results of the Winn test reflected tumor immunity of the intact animal better than either of the cytotoxicity assays (69). Both cytotoxic assays, aside from being from unable to predict concomitant immunity, also differ between themselves in their kinetics as well as intensity of tumor cell killing, and thus are likely to represent responses of different effector populations.

These considerations point to the necessity of developing new assays that can more accurately monitor a patient's concomitant immunity. Both the DCHR and Winn test carries the advantage of allowing the participation of in vivo regulatory elements that are probably absent in in vitro assays that require cell culture

[e]see footnotes, P.29

conditions. Presently, both assays have limited application in patient evaluation. The Winn test is not applicable for human subjects for obvious reasons. Although DCHR has been reliable in monitoring tumor patient's clinical status in the past (18,64,65, 84,94), ethical and human rights considerations have made routine skin testing with tumor antigens difficult.

Recently, an in vitro assay has been developed by Halliday and Miller (46) to measure tumor specific response in humans. Known as the Leukocyte Adherence Inhibition (LAI) test, the assay measures the change in adherence property of immune leukocytes upon exposure to the appropriate tumor antigens. Although studies indicate that there is more than one mechanism mediating the reaction (dependent on the experimental protocol) (37,39,45,63,66,111,124) it is widely accepted that the LAI can measure tumor specific response among patients bearing melanoma, squamous carcinoma, neuroblastoma and other kinds of malignancies (14,39,43,66,111,124,127). The assay can be performed in a few hours and does not require culturing of patients leukocytes that may alter the patient's in vivo responsiveness. In addition, the blocking effects of tumor bearer's serum can also be evaluated simultaneously with this assay (14,39,46,66, 111,127). The LAI thus offers considerable promise in the evaluation of tumor specific response in vitro. However, among patient groups with a high frequency of anergy, such as among patients with squamous cell carcinoma of the head and neck, only a low frequency of LAI responsiveness was observed (14). In these situations, the LAI cannot differentiate between a negative response that represents

generalized immunosuppression and a lack of tumor specific response. It is of advantage for prognostic purposes to develop an assay of higher sensitivity that can detect tumor specific immune response in a larger proportion of patients, such as one that directly assesses tumor specific recognition.

3. Demonstration of tumor antigen binding cells

In conventional antigen systems, the detection and evaluation of antigen binding cells have offered valuable information to the understanding of immunological phenomena such as immunity, high/low responders (78,80), or tolerance induction (1,23,98,136). Evaluation of tumor antigen binding cells in patients with various states of malignancy is likely to provide information on the ability of tumor bearing hosts to recognize tumor antigens at various stages of the disease, as well as factors that may affect the process. Studies on tumor antigen binding cell evaluation and identification, however, has been poorly documented.

Using tumor antigens conjugated to a solid phase matrix, specific cytotoxic cells can be isolated from tumor immunized mice (93). Spleen cells from tumor immunized rats that can recognize tumor associated antigens are retained when passed over tumor cell membrane extracts bound to collagen. The adherent population exhibited a 10- to 15- fold enrichment in specific T cytotoxic activity, whereas the nonadherent population was depleted of tumor specific cytotoxic response. This assay, however is not suitable for assessing tumor antigen binding cell levels, since the experimental

conditions have been adapted for optimal extraction of tumor antigen binding cells and invariably also resulted in nonspecific binding. Any subtle change in the level of tumor antigen binding cells in different tumor bearing situations would not be detected with this assay.

Tumor antigen binding cells can be demonstrated in mice following IP injection of viable syngeneic leukemia cells (96,113). After immunization, peritoneal exudates can demonstrate tumor specific recognition by tumor cell rosette formation. The response is tumor specific and parallels macrophage dependent cytotoxic response. This tumor specific rosetting response is largely mediated by macrophages, and is cytophilic antibody dependent. Pretreatment of mice with hyperimmune tumor antisera before immunization abrogated this response, and may reflect a biological suppressive mechanism that interfered with immune recognition of tumor cells, as well as macrophage effector functions. In this situation, the demonstration of tumor antigen binding cells bears a direct correlation to tumor immunity in vivo. The same approach however, has not been extended to tumor patient evaluation.

In another study, tumor related antigen recognition was reported in man. Wolberg (139) was able to demonstrate significantly increased binding of 3M KCl extracts of tumor membrane fragments among patients with colon carcinoma patients relative to normals or patients with high tumor burden using radioautography and enzyme labelling techniques. The process was largely mediated by T and B lymphocytes, and was not affected by preincubation with the

patient's serum . However, no difference was observed in binding of normal colon versus cancer tissue membranes, and it is not known if the determinants recognized were tumor specific.

E. Summary

Antigen binding cells are detectible in both normal and immune animals. Antigen binding cells in immune animals represent an expanded cell pool bearing receptors capable of antigen recognition. They participate in a variety of antigen specific regulatory and effector functions, including antibody production, DTH, cell mediated cytotoxic response as well as suppressor and perhaps helper activity. Antigen recognition is predominantly mediated by T and B lymphocytes bearing endogenously generated antigen specific receptors. Fc receptor bearing cells in immune animals can also recognize antigens by adsorption of antigen specific cytophilic antibodies. Although the exact role of these cells in antigen specific response is not known, they have also been known to participate in both regulatory and effector functions.

Relatively little has been done to document the presence of tumor specific antigen binding cells in tumor bearing animals, although specific responsiveness can be demonstrated routinely in vitro. This is probably due to the poor definition of tumor specific antigens, especially in man. The study of tumor specific antigen binding cells, however, would provide considerable information on

specific tumor-host interaction, as similar studies have added to the understanding of immune response to conventional antigens.

[a,b] Antigens such as DNP-Ficoll or TNP-LPS can activate B cells (antibody production) without the requirement of helper function from T lymphocytes, and are known as T independent (TI) antigens. In addition, TI antigens are divided into TI Type I antigens (which trigger an immature population of B cells) and TI Type II antigens (which activate a more mature population of B cells). These two classes of antigens also differ, among other things, in their ability to induce antibody responses in neonatal mice and susceptibility to anti-δ immunosuppression. Moreover, Type I TI, but not Type II TI antigens, can give an additive response to TD antigens. (Mosier, D.E., J.J. Mond & E.A. Goldings. J. Immunol. 119:1874-1878 1977.)

[c] T dependent (TD) antigens such as TNP-SRBC require the interaction of helper T cells with B cells for antigen specific humoral response

[d] Co-expression of surface antigen coded by Ia-J locus of the major histocompatibility complex and T antigens Ly 2,3 is believed to be characteristic of suppressor T cells (132).

Lymphocytes from tumor bearers are intradermally injected together with syngeneic tumor cells into normal syngeneic recipients. Immunity is measured by the extent of tumor growth after a period of time, as compared to growth of tumor following tumor cell injection without immune lymphocytes.

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Manuscript 1

CYTOFLUOROMETRIC EVALUATION OF ANTIGEN SPECIFIC ROSETTE FORMING CELLS IN HUMANS

I. Correlation of immunity to conventional antigen with antibody mediated monocyte recognition.

ABSTRACT

A rapid in vitro assay was developed to assess circulating antigen binding cells in man. Mononuclear cells from KLH or PPD responsive donors, when tested for rosette formation by laser cytofluorometry and fluorescent microscopy with HSA-, KLH- & PPD-conjugated autologous erythrocytes, were found to demonstrate elevated levels of rosette forming cells to the appropriate recall antigens. Specificity of rosette formation was suggested by a) significant elevation in antigen specific rosette forming cells among immunized donors; and b) the dose dependent inhibition of rosette formation by soluble antigen preincubation. This antigen binding appears to be mediated by cytophilic immunoglobulins. Preincubation with polyvalent anti-human-Ig goat $F(ab')_2$ abrogates antigen specific rosette formation. Preincubation with antigen specific antibody can arm naive donor cells to form specific rosettes,but was ineffective if the naive cells were pretreated with excess human IgG. Microscopic analysis of enriched RFC fractions after acridine orange or esterase staining reveals the involvement of monocytes predominantly and to a lesser extent lymphocytes in antigen specific rosette formation. Further evidence implicating the monocyte includes reduction of the rosette forming response after monocyte depletion with either G-10 passage or carbonyl iron treatment. Based on these observations this rosette forming technique appears to measure specific interaction between antigens and cytophilic antibody armed monocytes & lymphocytes.

INTRODUCTION

Immune function is dependent upon recognition and processing of antigens. Cells capable of antigen recognition can be detected in humans and a variety of animal systems (for review see 7,17,29,37). ABCs are present in both normal (8,9,18,19,24,31,34) and immunized (11-13,20,26,28,38) animals, and have been found to participate in effector (16,17,24) and regulatory (10,28,30,32,33) functions. Although it has been well established that immunity correlates with elevated levels of specific antigen binding cells, this approach has been utilized rarely for immune evaluation in humans.

In this study, we attempt to evaluate donor immunity by assessing the donor's specific ABC level to that antigen. A variation of the rosette forming cell (RFC) assay is adapted, using antigens conjugated to autologous erythrocytes. The technique requires minimal manipulation of donor mononuclear cells and offers the option of enriching for RFCs by using density gradients (4, 11-13, 22,37). By using the laser cytofluorometric technique, a donor's rosette forming cell response to a panel of control and test antigens can be evaluated using large sampling size (i.e. up to 10^4 nucleated cells per sample), with triplicate sample determination to give the mean RFC response. The reactivity to a given antigen can be statisically analysed by comparison with the individual's response to control antigens.

This study indicates that RFC detection is correlated with donor immunity. Antigen specific RFCs to the appropriate antigen can be consistently demonstrated cytofluorometrically and microscopically among immunized donors, but not unimmunized donors. Under the given experimental condition, it appears that the RFCs detected represent antigen recognition by Fc receptor bearing mononuclear cells armed with antigen specific cytophilic antibodies.

METHODS AND MATERIALS

Preparation of Donor Mononuclear Cells

Heparinized blood (10 ml with 10 U heparin/ml) from donors (with donor's informed consent) previously tested for dermal reactivity or lymphocyte transformation responses to KLH and PPD was combined with 2 ml of 6% dextran (> 250,000 MW) and incubated in an inverted plastic syringe for 20 minutes at 37 C. The white cell rich supernatant was resuspended in 10 ml RPMI 1640 (GIBCO, Grand Island, N.Y.) and washed twice at 160 g for 10 minutes. The pellet was resuspended in 10 ml of RPMI 1640, gently layered over 5 ml Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, N.J.) in a 15 ml conical plastic tube, and centrifuged at 400 g for 30 minutes. The mononuclear cell interface was collected, washed twice in RPMI 1640 at 150 g for seven minutes, and resuspended in RPMI 1640 to a final concentration of 2-4 x 10^6 cells/ml.

Preparation of Antigen Conjugated Autologous Erythrocytes

The erythrocyte fraction remaining after dextran sedimentation was washed twice at 180 g for 10 minutes. The packed RBCs were then resuspended to 2.5% (v/v) with phosphate buffered saline (PBS) at pH 7.4. Antigen conjugation using chromium chloride as described by Goding et al (15) was adapted. One milliliter of 2.5% (v/v) erythrocytes in Tris buffered saline (TBS) was added to equal volume of TBS containing 0.15 mg of PPD (tuberculin, purified protein derivative; Connaught Laboratories, Toronto,Canada), KLH (keyhole limpet hemocyanin, courtesy of Dr. M. Rittenberg) or HSA (human

serum albumin; Sigma Chemicals, St. Louis, Mo). One milliter of chromium chloride (0.1%) in TBS was then added in a dropwise fashion, and the mixture gently vortexed and left undisturbed for 4 min at 23 C. The reacted mixture was then washed twice in RPMI-1640 and reconstituted to 0.25% (v/v).

Rosette Formation:

Rosetting was carried out at 4 C for 60 minutes with prepared mononuclear cells (2-3 x 10^6 per ml) and an equal volume of 0.25% antigen-conjugated autologous erythrocytes (Fig. 1). After incubation the reaction mixture was diluted with 2.0 ml of RPMI 1640 at 4 C.. One-half milliliter of acridine orange (Allied Chemicals, N.Y.) at 0.1 mg% was added to the diluted mixture for nuclear staining approximately 1 minute before reading, and aliquots of 0.25 ml were introduced into the cytofluorograf for rosette enumeration. In each experiment, the % specific RFC is determined by the formula:

$$\% \text{ specific RFC} = \% \text{ RFC}_{\text{test Ag-RBC}} - \% \text{ RFC}_{\text{HSA-RBC}}$$

for background correction.

Visual Microscopic Enumeration of Rosette Forming Cells

After appropriate incubation, the rosetted mononuclear cell preparation was stained with 50 ul of acridine orange @ 1 mg% for nuclear staining. An aliquot of the reacted mixture was extracted by a pasteur pipette and examined under a fluorescent microscope (Leitz SM-Lux). Either 200 or 300 nucleated (green fluorescent) cells were counted per sample. Nucleated cells with 2

or more erythrocytes attached were considered as rosettes. The % RFCs to a given Ag-RBC conjugate represents the calculated mean % rosettes per 2- or 300 nucleated cells out of duplicate preparations.

Identification of background in cytofluorometric determination

In order to determine the nature of "backgrond rosettes" in cytofluorometric determinations, rosetted samples with control antigen (HSA-RBC) were examined both microscopically and cytofluorometrically. Since cytofluorometric determination cannot discriminate between WBC clumped with RBC from rosettes, the % RFC (WBCs with 3 or more RBCs attached) as well as cell clumps (cell aggregates with 2 or more WBCs , or with irregular RBC cluster) was determined microscopically and compared to the cytofluorometric background value. In five separate determinations (appendix A), the background implicated in cytofluorometric determination correlates with the % cell clumps (A) and the low level of RFCs (B) to the control antigen (Fig.2). Antigen specificity of the background cannot be demonstrated, since neither cell clumping nor RFCs to control antigen was affected by relevant antigen preincubation. Thus they were considered to be background inherent in the assay and subtracted from each determination of antigen specific rosette formation.

Antigen Inhibition of Rosette Formation:

Mononuclear cells at 3-4 x 10^6 were incubated with equal volume of RPMI 1640 containing the appropriate antigen at 37 C for

30 minutes. The incubation mixture was washed with RPMI 1640 and centrifuged twice at 160 g for 7 minutes and resuspended to original concentration. Rosette incubation was carried out as previously described. The percent inhibition of antigen specific RFC is calculated as follows:

$$\text{Percent Inhibition} = 1 - \frac{\%\text{ Ag specific RFC following recall antigen preincubation}}{\%\text{ Ag specific RFC after control antigen preincubation}}$$

where

$$\text{antigen specific RFC} = \%RFC_{\text{test Ag-RBC}} - \%RFC_{\text{HSA-RBC}}$$

Rosette Quantitation by Laser Cytofluorometry:

The Cytofluorograf contains a laser which emits a light beam (4880 A) incident to a flow channel through which the cell suspension passes. Photosensors can simultaneously register both fluorescence and scattered light from each particle passing through the flow channel. Nucleated cells stained with acridine orange fluoresce in the green region (5000 A) in direct relation to their nuclear contents. The nucleated cells, (rosetted or otherwise) passing through the flow channel represent the total cells evaluated. Red cells are excluded in the total count enumeration due to their lack of fluorescence. The cytofluorograf also distinguishes particles of different sizes by their respective degree of forward angle scatter of the incident laser beam.

Mononuclear cells are observable as a compact population with a limited size distribution. The rosetted mononuclear fractions, can be differentiated from the unrosetted fractions by virtue of their increased size. The "selected count" electronic window is set with pure mononuclear cell sample used in the experiment to exclude greater than 99% of the unrosetted cells. RFCs in rosetted samples introduced for analysis are registered in the selected count window and expressed as percent antigen binding cells per 10^4 nucleated cells. Single or clumped red cells were not included in the total or rosetted cell windows since they are not fluorescent.

Monocyte Depletion

Aliquots of hypaque ficoll extracted mononuclear cells at 1 x 10^7 per ml in RPMI 1640 were incubated at 37 C for 30 min with 0.01 gm of carbonyl iron in a nonwettable Petric dish. Petri dishes were rinsed three times with RPMI 1640 to collect residual cells. Treated cells were then layered over Ficoll-Paque and spun at 400 g for 20 minutes. The monocyte depleted lymphocytes were collected at the interface and washed twice before being reconstituted to 2 x 10^6 /ml.

Monocyte depletion was also carried out using Sephadex G-10 passage. Briefly 5 ml. of G-10 beads (Pharmacia, Piscataway, N.J.) that have been equilibrated overnight in RPMI 1640 with 5% newborn calf serum (NCS) is poured into a 10 ml syringe attached with a stop valve in an upright position. Donor mononuclear cells suspended in

RPMI 1640 5% NCS at $1X10^7$ is layered on top of the beads and incubated at room temperature for 15 minutes. The eluate was collected and chased with 5X vol. of RPMI-1640/5% NCS. Monocyte depleted fractions are 99% monocyte free by Wright staining.

Lymphocyte Transformation

Lymphocyte transformation was carried out as previously described (Burger, et al., 1976). Briefly, leukocytes were isolated by dextran sedimentation and washed three times (160 g for 10 minutes) and cultured at 2 x 10^6 cell/2 ml RPMI 1640 HEPES buffered at pH 7.2 with 10% normal AB serum added. Antigens (5 ug PPD, or 10 ug KLH) were added to the cultures. Lymphocyte transformation was assessed at 5 days for antigen stimulation, the last 24 hours with 1 uCi tritiated thymidine. Stimulation index (S.I.) was calculated by the following formula:

$$S.I. = \frac{\text{CPM in cultures with antigen}}{\text{CPM in cultures without antigen}}$$

Stimulation ratios greater than 2 were considered positive.

Preparation of Antigen Specific Antibodies

Antigen specific antibodies were isolated from immune serum by column immunoabsorption. For PPD specific antibodies, serum from PPD immunized donors with positive RFC response was used. Briefly, the immune serum is spun at 15,000 rpm (Sorvall RC2B) for 10 minutes. The supernatant is then passed over a PPD conjugated Sepharose CN-4B column (10mg/ml) and chased with 4x volume of TBS.

PPD antibodies adsorbed to the column are eluted with 3M sodium isothiocyanate (approx. 50 ml) and dialysed and concentrated with TBS using a ProDiCon apparatus (Bio-Molecular Dynamics, Beaverton Or). Specific reactivity and antibody nature of the protein eluate was examined using PPD, KLH, HSA, and antihuman Ig antibodies in Ouchterlony reactions. A similar procedure is also used to elute KLH specific antibodies from KLH positive donor serum.

Specific antibody arming of normal donors

Antibody preincubation was carried out at 4 C for 30 min. with the indicated concentration of specific antibody per 4 x 10^6 cells/ml. Cells are then washed twice with RPMI and centrifuged twice at 160 g for 7 minutes and resuspended to the original concentration.

Enrichment of RFCs

RFCs are enriched by gently layering rosetted samples (using 1 ml WBC + 1 ml Ag-RBC) over 2 ml of Ficoll-Paque and spun at 400 g for 30 minutes. Supernatant is poured off and the pellet gently resuspended in 0.25 ml of RPMI.

Heterorosetting Incubation

Heterorosette formation is carried out using 0.25 ml of WBC (at 4 x 10^6 /ml.) with equal vols. of 1% SRBC (sheep red blood cell) and antigen conjugated chicken red blood cell (Ag-CRBC) at 4^oC for one hour. Rosette forming cells are identified as WBCs with 3 or more Ag-CRBCs attached. Cells which bind Ag-CRBCs as well as at

least three SRBC attached are T-RFCs. Monocyte RFCs are identified by preincubation of test samples with 0.01 gm of carbonyl iron per $1X10^7$ cells at 37C for 30 min. followed by washing. Monocyte-RFCs are identified as WBCs with monocyte morphology with Ag-CRBCs attached and identifiable carbonyl iron pairs in the cytoplasm or cytoplasmic membrane.

RESULTS

The first part of this study examines the effectiveness of the assay in detecting donor RFCs to recall and irrelevant Ag. For initial experiments we have chosen previously immunized donors who demonstrated significant KLH or PPD lymphocyte proliferation responses. Control group donors include those with no previous exposure to either of the antigens. The RFC response of each donor was evaluated after incubation with 0.25% KLH- or PPD-RBCs. These values are compared to the background level of RFCs determined with erythrocytes conjugated to the human serum albumin, HSA (Table I). The mean ($\pm$ S.D.) RFC level of each antigen is determined using triplicate samples. In each experiment, a donor is considered to carry specific rosette forming cells to KLH or PPD if the level of rosette formation is significantly higher than background by student's t test ($p < 0.05$)

The KLH and PPD RFC responses of 22 donors tested in this fashion are summarized in Fig.3. In each case donor cells were coded and tested with all three Ag-RBCs. Their RFC responses are then categorized according to their immunity as previously determined by lymphocyte transformation and/or skin testing. Among PPD sensitized donors tested in this manner, greater than 80% (10/12) carried a significantly elevated level of PPD-RFCs($p < .05$). None however, exhibited a significant response to KLH. Alternatively, all KLH immunized donors (9/9) have a significant RFC response to KLH, but not to PPD (0/9). KLH (+) PPD (+) donors have

significant RFC responses to both KLH and PPD (5/6 PPD'+', 6/6 KLH'+'). Control groups do not demonstrate significant RFC response to either antigen (0/6).

RFC Detection by Cytofluorometry & Microscopy

Our findings agree with previous studies (8,17,29,38) suggesting that donor immunity correlates with elevation of specific RFCs. The cytofluorometric technique carries an added advantage of bypassing the time consuming microscopic evaluation technique used in other studies. It is important, however, to determine if the % specific RFC detected by the two techniques correlate with one another. A comparison study with 19 parallel determinations of specific rosette forming cells to either KLH or PPD is presented in figure 4. Rosetted samples were acridine orange stained and the specific RFC level to either KLH or PPD was determined in a blind parallel fashion by fluorescent microscope or cytofluorometry. Linear regression analysis indicates a strong correlation between the level of RFCs determined by the two methods over a range of values commonly observed ($p < .01$, $df = 18$).

Antigen Inhibition of Rosette Formation

The above observations that a) 90% of immunized donors formed significant levels of RFCs to recall antigen over background, and b) elevated levels of rosette formation are invariably restricted to the recall antigen, are suggestive evidence that these are antigen specific rosette forming cells. In order to examine further the

question of specificity, rosette formation of immune donors were examined before and after preincubation with the soluble form of either relevant or irrelevant antigens. Specific RFC response to PPD from a PPD(+) KLH (-) donor was completely reduced to background level after preincubation with 0.2 mg. PPD, but was not affected by preincubation with 0.2 mg KLH (Fig 5a). Similar results were observed with a PPD(+) KLH(+) donor (Fig. 5b); elevated levels of KLH rosette forming cells could be abolished by KLH preincubation, but was not affected by preincubation with either HSA or PPD; and PPD rosettes were only inhibited by PPD preincubation. Thus, the formation of elevated levels of RFCs to recall Ag-RBCs in immunized donors is inhibitable by specific antigen preincubation, but not by cross inhibition with an irrelevant antigen. However, in non-immunized donors, the level of RFCS to test antigens KLH and PPD is not significantly different from background (HSA-RFC). In addition, preincubation of cells from nonimmune donors with 0.2 mg of HSA, PPD or KLH does not signficantly alter the respective RFC responses to any of the antigens (Fig. 6)

Further experiments indicate that the inhibitory effect of antigen preincubation is dose dependent. Results of two experiments with mononuclear cells from KLH (+) PPD(-) and a PPD(+)KLH(-) donor are represented in Figures 7a and 7b. In each case increasing the preincubation dose resulted in proportionate inhibition of rosette formation. Pre-exposure to high concentrations of antigen (2.0 mg) produced both significant variations in the background as well as a decrease in the level of RFCs to the sensitizing antigen. This

change in background level of rosettes has thus precluded establishing 100% inhibition of antigen specific rosette formation. In any case, over the ranges of 0-90% inhibition, direct correlations by semilogarithmic regression analysis (R= 0.91 and 0.96., $p < .01$) can be demonstrated between the concentration of the preincubating relevant antigen and inhibition of PPD or KLH specific rosette formation.

Mediation of rosette formation by serum factors

The comparatively high (> 0.5%) and variable antigen binding observed in this study suggests the involvement of cytophilic antibody arming of Fc bearing cells in specific rosette formation. In order to ascertain immunoglobulin involvement, RFCs were incubated with goat anti-human-Ig $F(ab')_2$ in an attempt to block rosette formation (Fig. 8). Whereas normal goat immunoglobulin does not affect specific rosette formation, preincubation with 250 ug of antihuman Ig antibody effectively abrogated the donor's ability to form specific PPD(A & B) or KLH (C) rosettes. Preincubation of cells from donor III with 25 ug. of $F(ab')_2$ anti-antibody also resulted in decreased specific rosette formation. Thus, it is apparant that most, if not all, of the RFCs detected in this assay bind antigen through surface immunoglobulins.

Normal donor cells can also acquire specific rosette forming response following preincubation with specific antibodies. Following preincubation with PPD specific antibodies (which were previously extracted from an immune donor serum by affinity chromatography),

normal donor cells (4/4) acquired the capacity to form PPD specific rosettes (Fig. 9). This arming phenomenon is observed at all of the concentration of specific antibody used, i.e., from 250 ug to 2.5 ug per 4 x 10^6 cells, and is antigen specific, since it does not significantly alter the donor's rosetting response to another antigen, KLH. KLH(+) (donor A) remained KLH responsive after acquiring PPD reactivity, whereas KLH(-) donor (B) acquires PPD but not KLH activity after PPD antibody preincubation.

A similar response can be observed when KLH specific antibody was used for arming. The results shown in figure 10 are representative of 4 experiments in which naive donor cells gained KLH rosette response after incubation with a wide range (2.4 - 240 ug) of KLH antibody concentrations. In one case, treatment of even 2.4 ug Ab/4 x 10^6 cells (donor B) resulted in a positive response. Similar to the experiments described with PPD arming, KLH antibody arming did not affect preexisting antigen binding reactivity to another antigen, PPD.

In subsequent experiments, we attempted to block antibody "arming" by saturating Fc receptors with pooled normal IgG (without reactivity to PPD or KLH). Two of the four experiments performed under this protocol are represented in Fig.11. The ability of KLH (-) donor to form KLH rosettes after KLH Ab incubation was reduced after princubation with 200 ug of normal human Ig (Fig.11a). The IgG preincubation did not appear to significantly alter PPD response that was already present. A similar effect can be observed on acquired PPD RFC response (Fig.11b). The donor's acquired PPD

response was significantly reduced by 100 ug human IgG, and pre-incubation with 200 ug completely abrogated the specific response. KLH-RFC response of the donor was again not significantly affected These data support the hypothesis that rosette forming cells detected in this assay are comprised to a large extent of Fc bearing cells, whose antigen specific rosette forming cell ability is mediated by cytophilic antibodies.

Monocytes as rosette forming cells

In preliminary experiments designed to evaluate the importance of monocytes and macrophages in specific rosetting response, phagocytic cells were depleted by G-10 passage or carbonyl iron treatment. In three donors (Fig.12), specific rosette forming response to KLH or PPD were either totally abolished (B) or significantly suppressed (A & C) after carbonyl iron depletion of monocytes.

An alternate approach utilizing Sephadex G-10 for moncyte depletion also yielded similar results. Specific rosette formation was abrogated after G-10 depletion (Table 2), and this observation held true whether cytofluorometric or microscopic evaluation was used. Thus it appears that specific rosette forming cells are largely of the monocytes/macrophages series.

Characterization of RFCs

Positive identification of RFCs can be enhanced by enriching rosette forming cells by hypaque ficoll centifugation of rosetted

samples at 4 C. The % RFC responses of several donors before and after enrichment is shown in Table 3. Up to 10- fold enrichment can be achieved with proportionate increase in both background as well as antigen specific RFCs. In addition, the % RFCs to recall antigens remains significantly higher than background after enrichment. When enriched samples are examined in the fluorescent microscope, the proportion of macrophage among RFCs can be identified morphologically as esterase positive mononuclear cells with monocyte morphology after acridine orange and/or esterase staining. Both methods have yielded very similar results in identifying the monocytes as the predominant rosette forming cell (75 $\pm$ 4.9 % of total RFCs from 4 experiments, Table 3).

In order to identify more clearly the relative role of lymphocytes and monocytes in rosette formation a heterorosetting technique has been adapted. Donor cells were incubated with carbonyl iron to identify iron ingesting monocytes and then subjected to rosette formation with 1% sheep red blood cells and 1.0% or 0.25% antigen conjugated chicken red cells. Rosette forming cells are identified as cells binding three or more antigen-conjugated chicken erythrocytes. In addition, rosette forming monocytes can be identified by their ability to ingest carbonyl iron in the cytoplasm, while T-RFCs are those that bind elliptical nucleated Ag-CRBCs as well as enucleated sheep red blood cells.

Under the standard experimental conditions, (4 C, 0.25% Ag-RBCs) heterorosetting observations supported earlier cell depletion experiments suggesting that the majority of RFCs are monocytes

(Table 4). Only a low % of T-RFCs was observed (Expts.I to IV), whereas carbonyl iron ingesting monocytes constitute a major proportions of heterorosettes, i.e. 60% (2.4/4.0) in Expt. III, and 79% (3.3/4.3) in Expt. IV. However, the level of rosette formation by both T lymphocytes and other cell types can be enhanced either by increasing the concentration of Ag-RBCs (Expt.I), or incubating temperature (Expt. II).

DISCUSSION

In this study we were able to demonstrate antigen specific rosette forming cells among donors immunized to the conventional antigens KLH and PPD. Significant levels of rosette forming cells to recall Ag-RBC over background were consistently demonstrated (19/21) and were primarily restricted to the antigens to which the donors were immunized. In addition, prior incubation of donor cells with the appropriate recall antigen abrogates this response, thus suggesting antigen specificity. The inhibitory effect by antigen preincubation also appears to be dose dependent, since linear correlations can be established between preincubating antigen concentration and % inhibition of specific PPD and KLH rosettes respectively. Thus this assay appears to be sensitive for detecting antigen specific immunity to conventional antigens PPD and KLH. Overall, the RFC assay utilizing cytofluorometry promises to be a relatively simple in vitro assay that can consistently predict donor immunity with a low incidence of false positives (0%).

Studies in the past have indicated that antigen binding cells are present in unprimed normal animals (8,9,18,19,24). These cells recognize antigens specifically since their depletion resulted in unresponsiveness to the antigen (2,3,6,37). While our study showed the persistence of certain level of 'background' binding to control Ag-RBCs both by cytofluorometry and microscopy, antigen specificity of these background rosettes could not be demonstrated by inhibition with relevant antigen preincubation. Microscopic evaluation reveals

that a large part of the background observed cytofluorometrically can be attributed to cell clumps in the preparation (Fig. 2) which cannot be differentiated during electronic analysis from rosettes. However, preincubation of donor cells with high concentration of antigen that effectively abrogates specific rosette formation did not alter the level of background rosettes or cell clumps. This observation holds true whether the samples were examined microscopically or cytoflurometrically, and suggests that the background as observed in this assay is an antigen nonspecific phenonomenon, probably representing random adherence to sticky red cell surfaces. In any case, donors tested with a panel of erythrocyte conjugated control antigens routinely demonstrated a uniform level of background to different control Ag-RBCs that do not differ statistically from one another. This value can thus be treated as 'noise' inherent to the assay and does not affect the identification and quantitation of antigen specific rosette formation.

Electronic analysis allows the assessment of % RFCs in samples containing 10^4 WBCs and triplicate samples can be evaluated to provide statistical distribution of RFCs to a given antigen. The cytofluorometric technique therefore offers a theoretical advantage over microscopic evaluation, in which the level of specific RFCs are commonly based on a sampling size of 2-500 WBCs. The strong correlation ($p < 0.01, r = 0.6, n = 19$) between visual and cytofluorometric enumeration indicates that cytofluorometric determination is a compatible but more effective technique than visual enumeration RFC determination. Since the response of one or more donors to

several Ag-RBCs can be evaluated within hours, donor immunity to KLH or PPD can be routinely assessed without visual determination.

Specific antigen binding has been demonstrated in the past in B and T lymphocytes (17), as well as Fc bearing cells that bind antigens via cytophilic antibodies (5,7,23,26,34,38). In each study, it appears that the nature and proportion of specific antigen binding cells detected depends on the method as well as the immunization regimen. In this assay, we have been able to demonstrate that rosette formation was largely mediated by cytophilic antibodies. The involvement of cytophilic antibody arming in specific rosette formation also serves to explain the comparatively high (> 0.5%) and variable levels of specific rosettes detected in this assay. The ability to abrogate immune donor specific rosette response by $F(ab')_2$ goat anti-human-Ig suggests that a majority of rosette forming cells carry immunoglobulins which either serve as specific antigen receptors or are closely related to them. In a retrospective study on the sera of immune donors previously tested for specific RFCs, all were found to carry specific IgG to the relevant antigen/s by Ouchterlony immunoprecipitation (data not shown). In addition, antigen specific antibodies can be routinely isolated from responsive donors and utilized for 'naive' donor arming to give specific rosetting response. The ability of normal IgG preincubation to block 'arming' without affecting the recipient's response to other recall antigens is further documentation that 'arming' acts via the acquisition of cytophilic antibodies onto mononuclear cells bearing Fc receptors.

Both direct and indirect evidence supports the prediction of macrophage involvement in specific rosette formation. Removal of phagocytic cells by carbonyl iron treatment resulted in significant decrease in the specific RFCs, and depletion of > 99% monocytes by G-10 also resulted in a loss of specific rosette forming ability. Morphological examination of both enriched and unenriched rosette samples indicate that antigen specific rosette forming cells are predominantly esterase positive. Enriched RFCs to control antigens were comprised of approximately equal proportions of lymphocytes and monocytes (data not shown), but they did not appear to be antigen specific since formation was not affected by relevant antigen preincubation. Together with the evidence of antibody dependence discussed above, our data strongly suggest that specific rosette formation as demonstrated in this assay is mediated by antigen specific cytophilic antibody arming of monocytes.

The utilization of a heterorosetting technique also indicates a predominance of carbonyl iron ingesting, non-T mononuclear cells in rosette formation. Both T and non T lymphocytes were observed, but under standard experimental condition they only made up a small proportion in the rosette forming cell population. This is not surprising since the RBC:WBC ratio (1 : 3) has been kept relatively low in our rosetting assay. Our data agrees with previous studies (17,18,19) that both T and non-T lymphocyte rosette formation can be enchanced by increasing reaction temperature (to 37 C) and Ag-RBC : WBC ratio; but this also causes coincidence in electronic circuitry that is unfavorable for cytofluorometric analysis.

This assay offers a rapid in vitro evaluation of antigen recognition by antibody mediated Fc receptor bearing cells in donors subcutaneously immunized with conventional antigen. Application of this assay in clinical evaluation has also yielded promising results. When applied to malignant situations (see following manuscript), this assay could detect tumor specific recognition and was capable of discriminating clinically distinct tumor patient populations.

Additionally, we (Vandenbark et al, manuscript 3) have demonstrated specific RFCs to myelin basic protein (MBP) in multiple sclerosis patients in exacerbation, but MBP-RFCs are not detectible in remission. The association of antigen specific cells and active disease is intriguing, but the functional prosperties and pathogenic importance of MBP-RFCs have not yet been established. Whether or not the mechanism of rosette formation is similar to that reported here is currently under investigation.

Table 1

Rosette Formation by PPD and KLH Sensitized Donors

Donor response by lymphocyte transformation[a]	Donor Response by Rosette Formation[b]			RFCs to test antigens[c]	
	HSA-RFC	PPD-RFC	KLH-RFC	PPD	KLH
I. PPD(+) KLH(-)	1.40 ± 0.10	2.40 ± 0.18	1.34 ± 0.15	1.9[d]	(-0.06)
	1.13 ± 0.03	2.16 ± 0.30	1.09 ± 0.24	0.82[d]	(-0.04)
II. PPD(-) KLH(+)	1.90 ± 0.11	1.86 ± 0.09	2.45 ± 0.25	(-0.04)	0.55[d]
	0.42 ± 0.22	0.32 ± 0.16	0.94 ± 0.14	(-0.10)	0.52[d]
III. PPD(+) KLH(+)	1.18 ± 0.15	1.87 ± 0.49	1.93 ± 0.16	0.69[d]	0.75[d]
	1.84 ± 0.61	2.23 ± 0.27	2.57 ± 0.07	0.39[d]	0.73[d]
IV. PPD(-) KLH(-)	1.58 ± 0.31	1.59 ± 0.16	1.52 ± 0.09	(-0.01)	(-0.06)
	2.74 ± 0.13	2.80 ± 0.24	2.94 ± 0.15	(0.16)	(0.20)

[a]Donors were previously PPD(+) or sensizized by subcutaneous injection with KLH. Immunity can be demonstrated by lymphocyte transformation with stimulation indices of greater than 2.

[b]Rosette forming response of donors is evaluated cytofluorometrically after 1 hour incubation at 4 C with equal volumes of 0.25 % appropriate Ag-RBCs.

[c] Antigen specific RFCs to test antigens = % $RFC_{\text{test Ag-RBC}}$ - % $RFC_{\text{HSA-RBC}}$ for background correction.

[d] Statistically significant values (Student's t test, $p < 0.01$)

Table 2

Effect of Monocyte Depletion on Antigen Specific Rosette Formation

Donor reactivity	Cell treatment	% Antigen specific rosette forming cells[a] KLH-RFC determined by Cytofluorometry	KLH-RFC determined by Fluorescent microscopy[b]	PPD-RFC determined by Cytofluorometry	PPD-RFC determined by Fluorescent microscopy
I. PPD(+)KLH(+)	none	0.37 ± 0.12[c]	0.36[c]	0.48 ± 0.15[c]	0.30[c]
	G-10 eluant	(-0.04 ± 0.15)	0	(-0.05 ± 0.16)	ND
II. PPD(+)KLH(-)	none			1.13 ± 0.15[c]	0.67[c]
	G-10 eluant			(0.09 ± 0.04)	0
III. PPD(+)KLH(-)	none			0.78 ± 0.20	0.38[c]
	G-10 eluant			(-0.04 ± 0.18)	0

[a] % Antigen specific RFC = % $RFC_{\text{test Ag-RBC}}$ - % $RFC_{\text{HSA-RBC}}$

[b] rosetted samples are acridine orange stained and 500 cells were counted to determine % rosette formation.

[c] values are statistically significant by Student's t test, ($p<0.05$)

Table 3

Morphological Identification of Antigen Specific RFCs

Experiment	RFC enrichment[b]	% Antigen specific RFC[a]		% Monocytes[c]
		PPD	KLH	
I.	unenriched		0.70	100 (2/2)
	RFC enriched		10.30	80 (17/21)
II.	unenriched	0.90		ND
	RFC enriched	1.30		75 (6/8)
III.	unenriched	1.0		100 (4/4)
	RFC enriched	4.0		75 (26/34)
IV.	unenriched	1.0		60 (3/5)
	RFC enriched	1.5		68 (8/14)

[a] % antigen specific RFC = $\%RFC_{\text{test Ag-RBC}} - \%RFC_{\text{HSA-RBC}}$: rosette forming cells are identified by fluorescent microscopy as WBCs with three or more RBCs attached. Three hundred nucleated cells were enumerated to determine % RFCs in each sample.

[b] Rosette forming cells are enriched by isopycnic centrifugation using Ficoll Paque (4 C, 400 g,30 min.)

[c] Monocytes are identified morphologically following esterase staining (Expts.I & II) or acridine orange staining (Expts.I,II,III & IV)

Table 4

Morphological Identification of Antigen Specific RFCs

Donor reactivity	Incubation[a] condition	Antigen Specific Rosette Forming Cells[b] T cells	Non-T cells	Monocytes	Total
I. PPD(-) KLH(+)	4 C; 0.25%	0 %	0.4 %		0.4 %
	4 C; 1.0 %	0.65	1.3		1.95
II.PPD(-) KLH(+)	4 C; 0.25%	0.25	5.0		5.25
	37 C; 0.25%	0.5	3.0		3.5
III.PPD(+) KLH(-)	4 C; 0.25%	0.6	1.0	2.4 %	4.0
IV.PPD(-) KLH(-)	4 C; 0.25%	0	1.0	3.3 %	4.3

[a]Donor cells were incubated with equal volumes of 1 % SRBC and indicated concentration of antigen conjugated chicken red blood cells.

[b]% antigen specific RFC = % $RFC_{\text{test Ag-RBC}}$ - % $RFC_{\text{HSA-RBC}}$: in each case 600 nucleated cells were enumerated to determine the % rosette formation. T-RFCs are lymphocytes with Ag-conjugated CRBC and 2 or more SRBCs attached to their surfaces(heterorosettes). In experiments III and IV, donor mononuclear cells were preincubated with carbonyl iron before rosette formation. Monocyte RFCs are identified as carbonyl iron ingesting mononuclear cells with 3 or more CRBCs attached.

Figure 1: Diagramatic representation of *in vitro* analysis of donor rosette formation response to PPD and KLH.

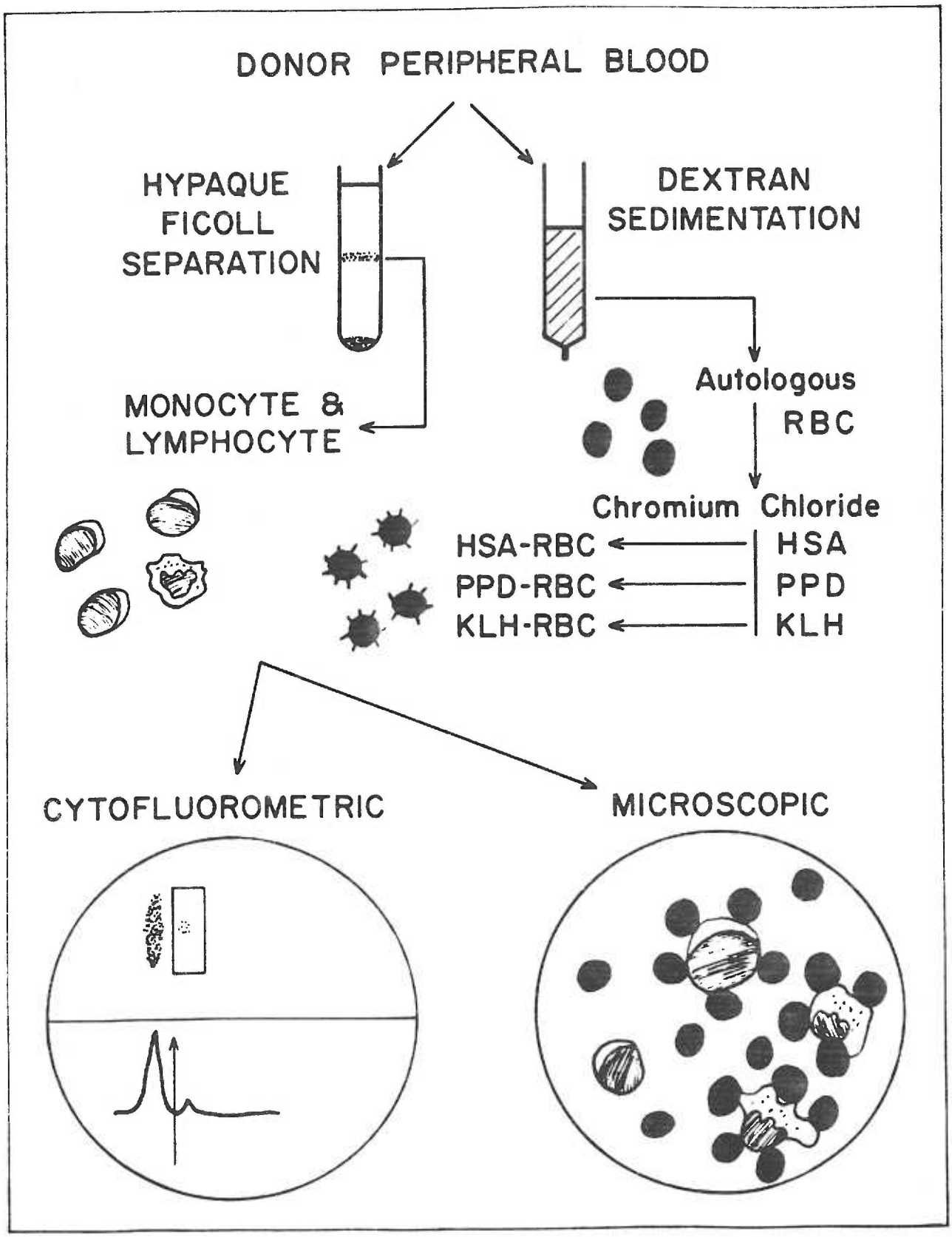

DONOR PERIPHERAL BLOOD
HYPAQUE
FICOLL
SEPARATION
DEXTRAN
SEDIMENTATION
MONOCYTE &
LYMPHOCYTE
Autologous
RBC
Chromium Chloride
HSA-RBC
PPD-RBC
KLH-RBC
HSA
PPD
KLH
CYTOFLUOROMETRIC
MICROSCOPIC

Figure 2 : Microscopic analysis of "background" implicated in cytofluorometric (CFM) evaluation. Rosetted samples that have been evaluated by cytofluorometry are analyzed in parallel by fluorescent microscopic analysis. Rosettes are identified as WBCs with 3 or more Ag-RBC surrounding their surface. The number of WBC aggregates as well as irregular red/WBC aggregates are also determined since electronic analysis fail to differentiate these from actual rosettes. The level of RFC to an irrelevant antigen, HSA (B) is subtracted from the level of RFC to test antigens to eliminate background inherent to the assay to give the level of specific RFCs (C).

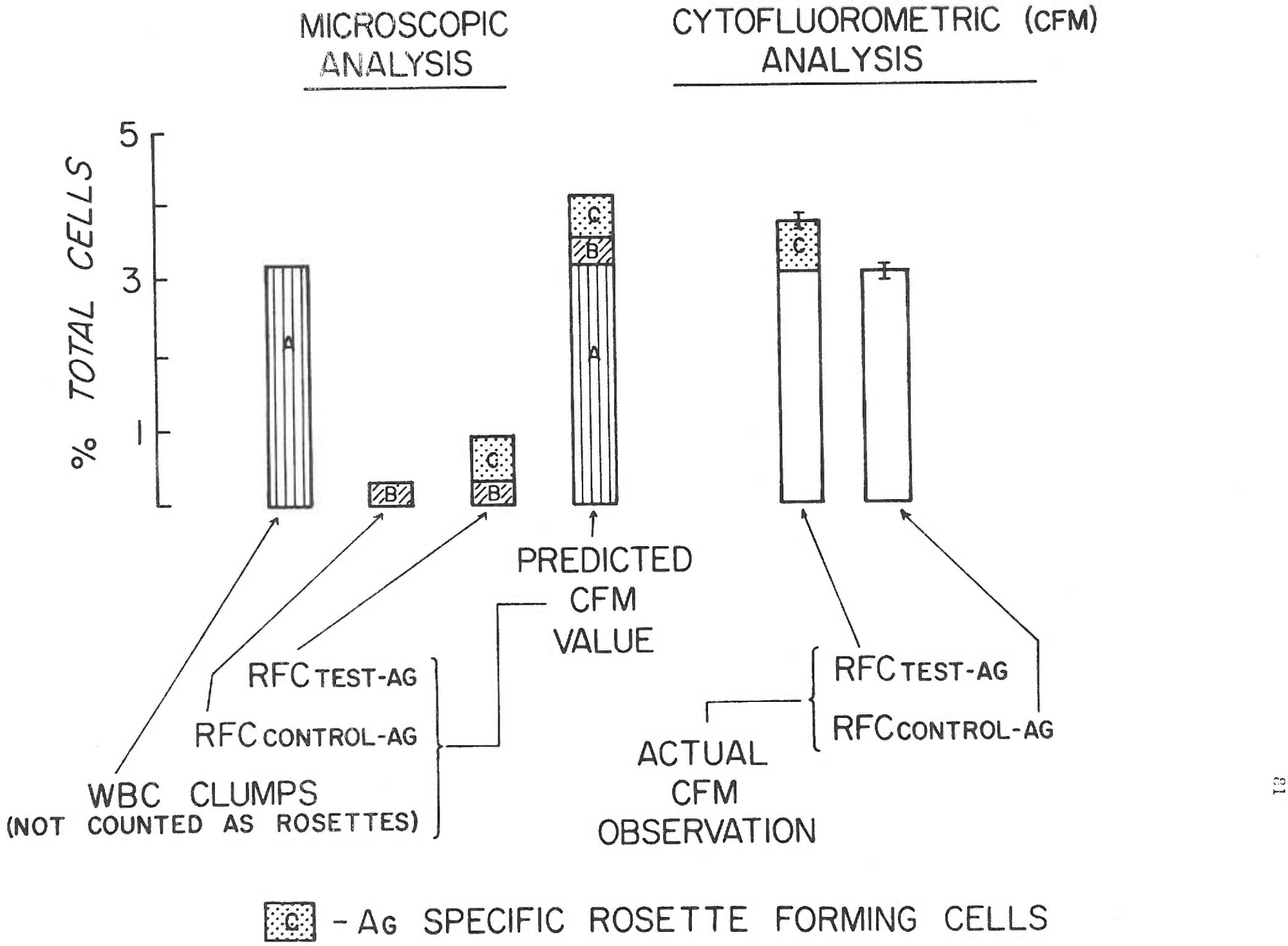

MICROSCOPIC ANALYSIS
CYTOFLUOROMETRIC (CFM) ANALYSIS
% TOTAL CELLS
5
3
1
A
B
C
PREDICTED CFM VALUE
RFC TEST-AG
RFC CONTROL-AG
WBC CLUMPS
(NOT COUNTED AS ROSETTES)
ACTUAL CFM OBSERVATION
RFC TEST-AG
RFC CONTROL-AG
C - AG SPECIFIC ROSETTE FORMING CELLS

Figure 3 : Antigen specific rosette forming response to PPD and KLH by controls and PPD and KLH responsive and nonresponsive donors. Each donor's PPD or KLH specific rosette forming cell response is determined and compared to level of RFC to HSA-RFCs. Each symbol represents either a statistically significant (o) or not significant (●) response above background to the respective antigen by Student's t test ($p < 0.05$).

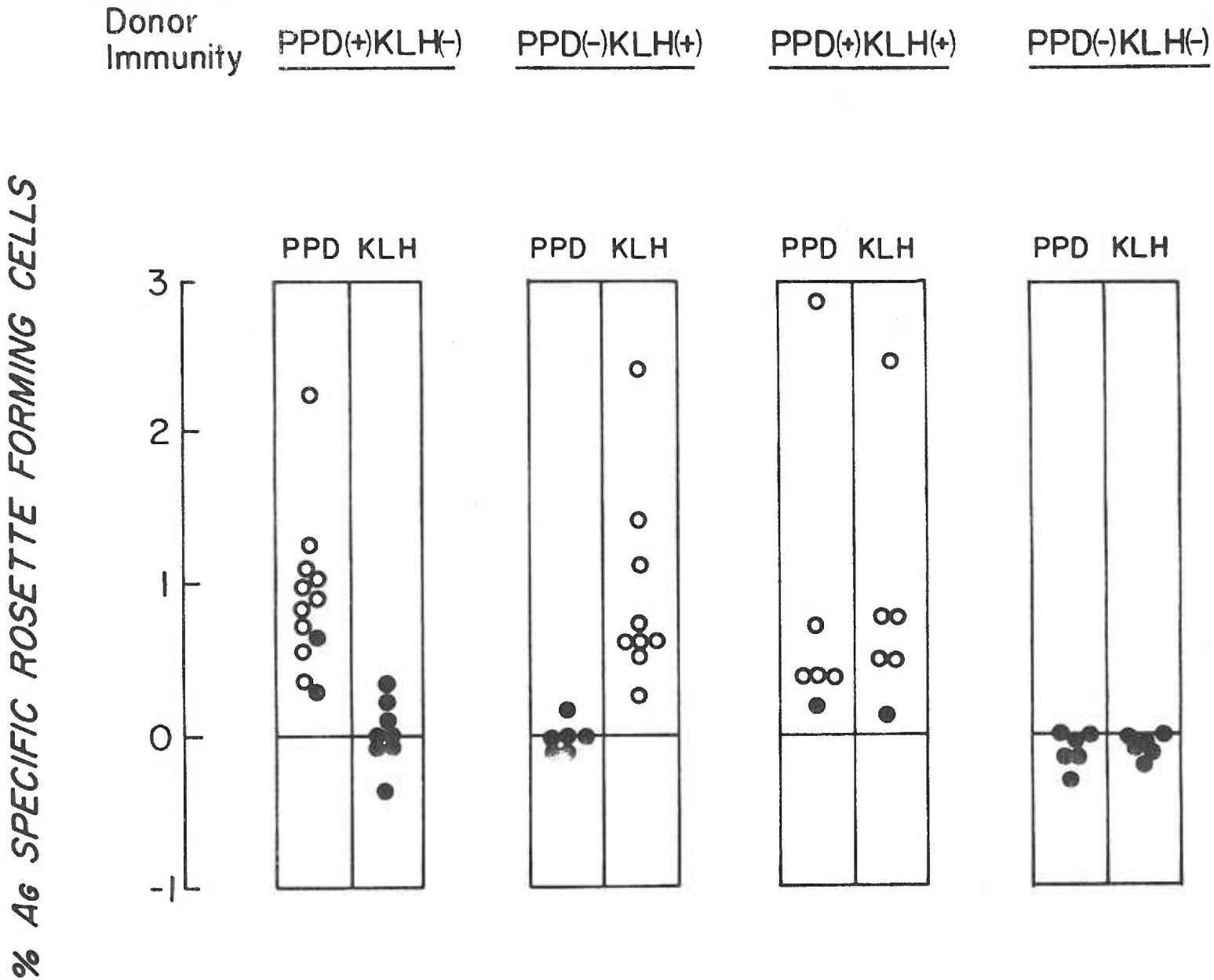

Donor Immunity
PPD(+)KLH(-)
PPD(-)KLH(+)
PPD(+)KLH(+)
PPD(-)KLH(-)
% Ag SPECIFIC ROSETTE FORMING CELLS
3
2
1
0
-1
PPD KLH
PPD KLH
PPD KLH
PPD KLH

Figure 4: Correlation of the level of antigen specific rosette forming cells as determined cytofluorometrically and microscopically. Each point represents the level of specific (see text) RFCs determined cytofluorometrically and microscopically in parallel. Data is analysed by linear regression and dotted line represents 95% confidence limits. ($R=.61$; $n=19$, $p < .01$)

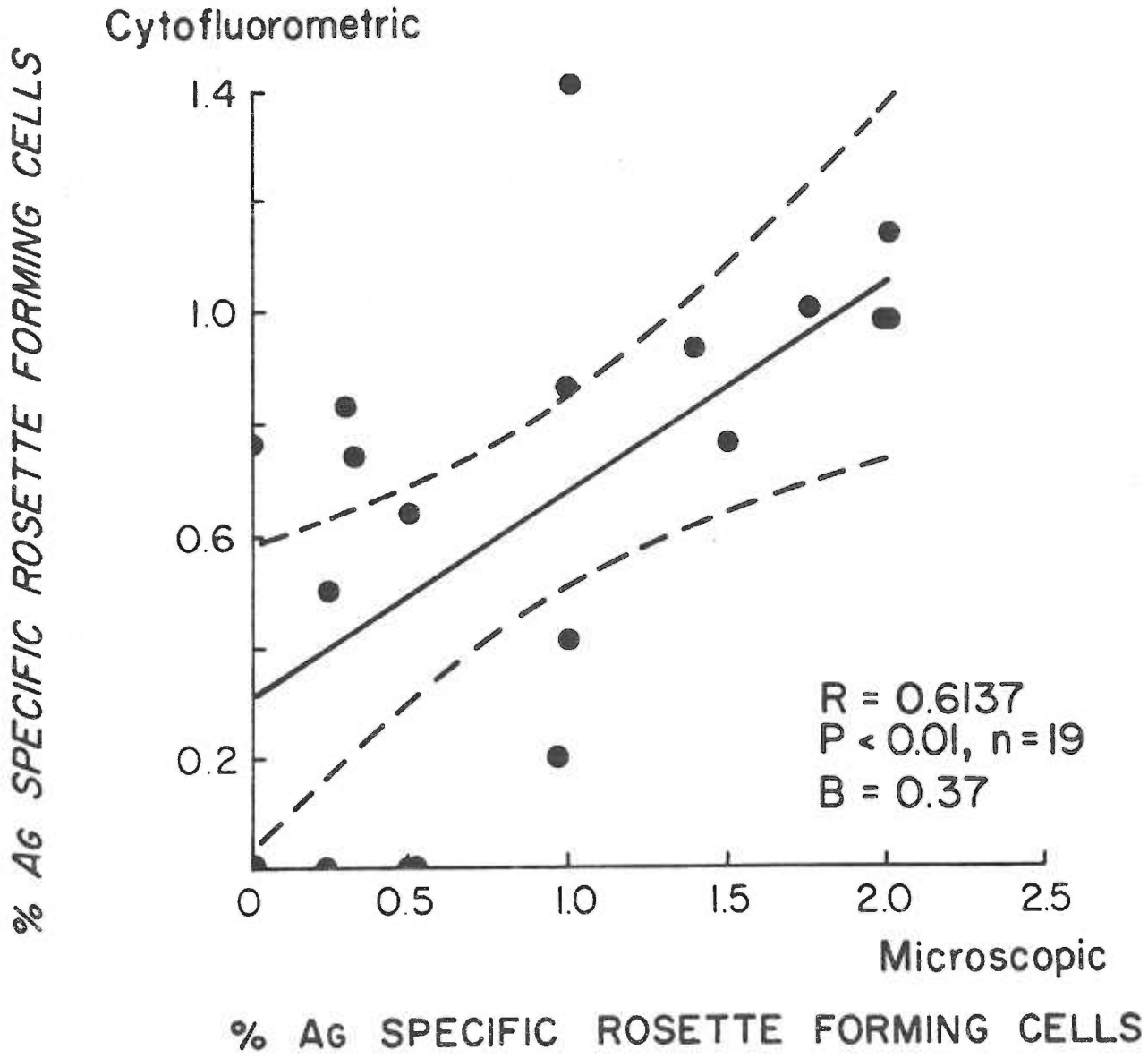
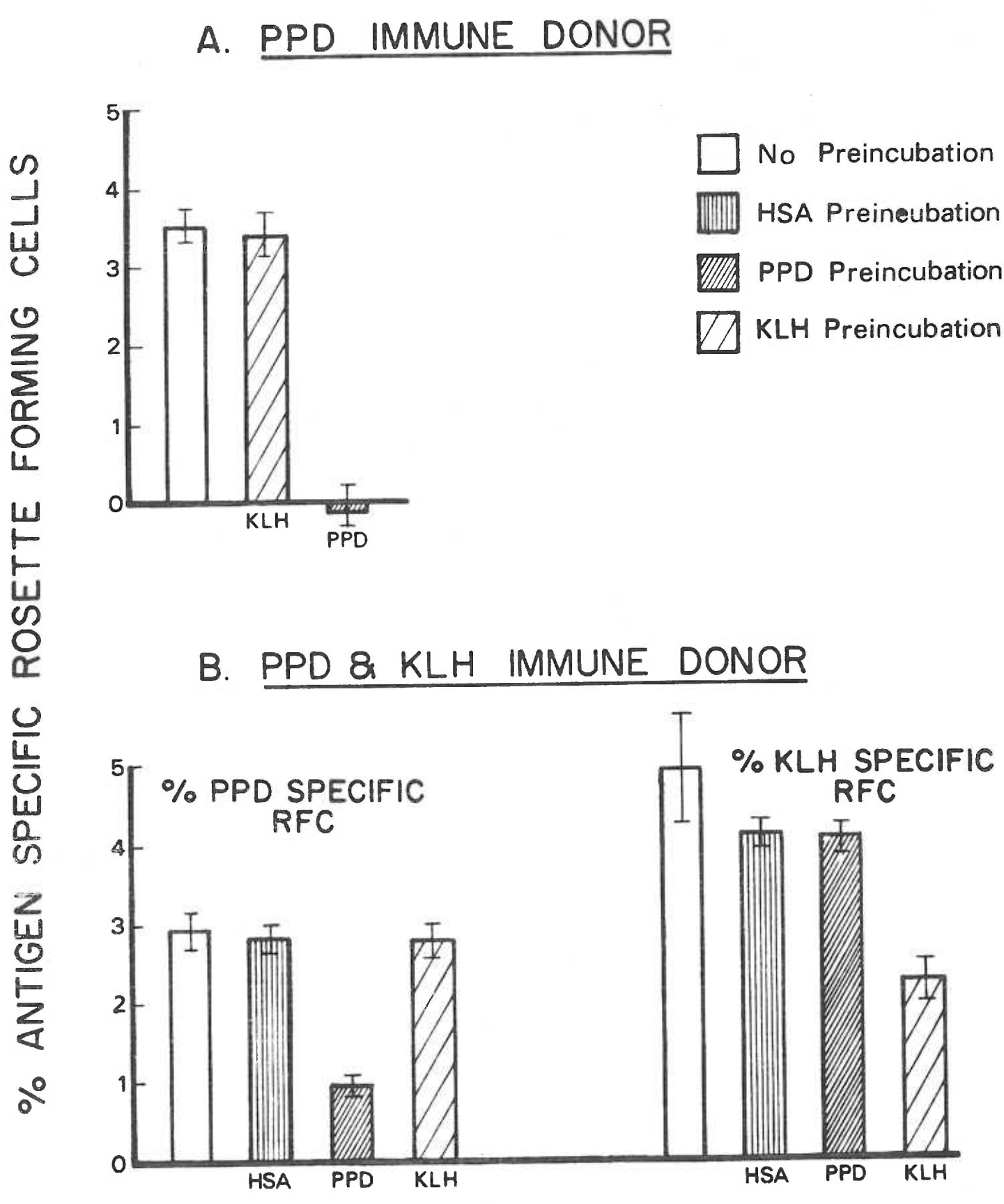

Cytofluorometric
% Ag SPECIFIC ROSETTE FORMING CELLS
1.4
1.0
0.6
0.2
0
0.5
1.0
1.5
2.0
2.5
R = 0.6137
P < 0.01, n = 19
B = 0.37
Microscopic
% Ag SPECIFIC ROSETTE FORMING CELLS

Figure 5: Effect of antigen preincubation on antigen specific rosette formation. The level of PPD specific RFC (A) in a PPD immune donor, and PPD specific RFC and KLH specific RFC and KLH specific RFC (B) are shown after preincubation of donor cells with 0.15 mg of HSA (▥), PPD (▨) or KLH (▨).


A. PPD IMMUNE DONOR
% ANTIGEN SPECIFIC ROSETTE FORMING CELLS
5
4
3
2
1
0
KLH
PPD
No Preincubation
HSA Preineubation
PPD Preincubation
KLH Preincubation
B. PPD & KLH IMMUNE DONOR
% PPD SPECIFIC RFC
% KLH SPECIFIC RFC
HSA
PPD
KLH
HSA
PPD
KLH

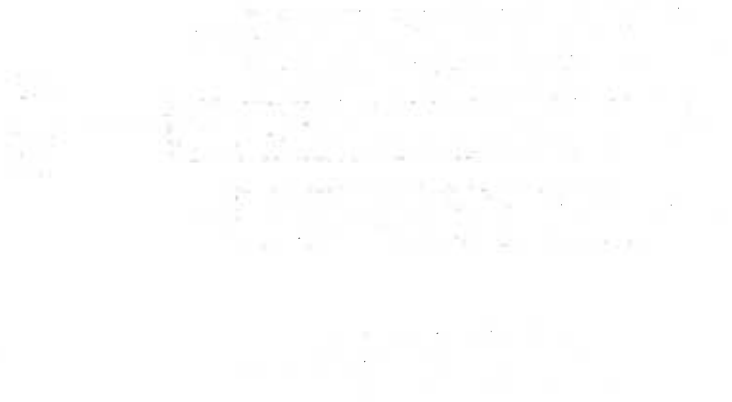


Figure 6: Level of RFCs determined cytofluorometrically of a KLH(-)PPD(-) donor following preincubation with no antigen (□), or .2 mg of HSA (▥), KLH (▨) or PPD (▧).

KLH(-) PPD (-) DONOR

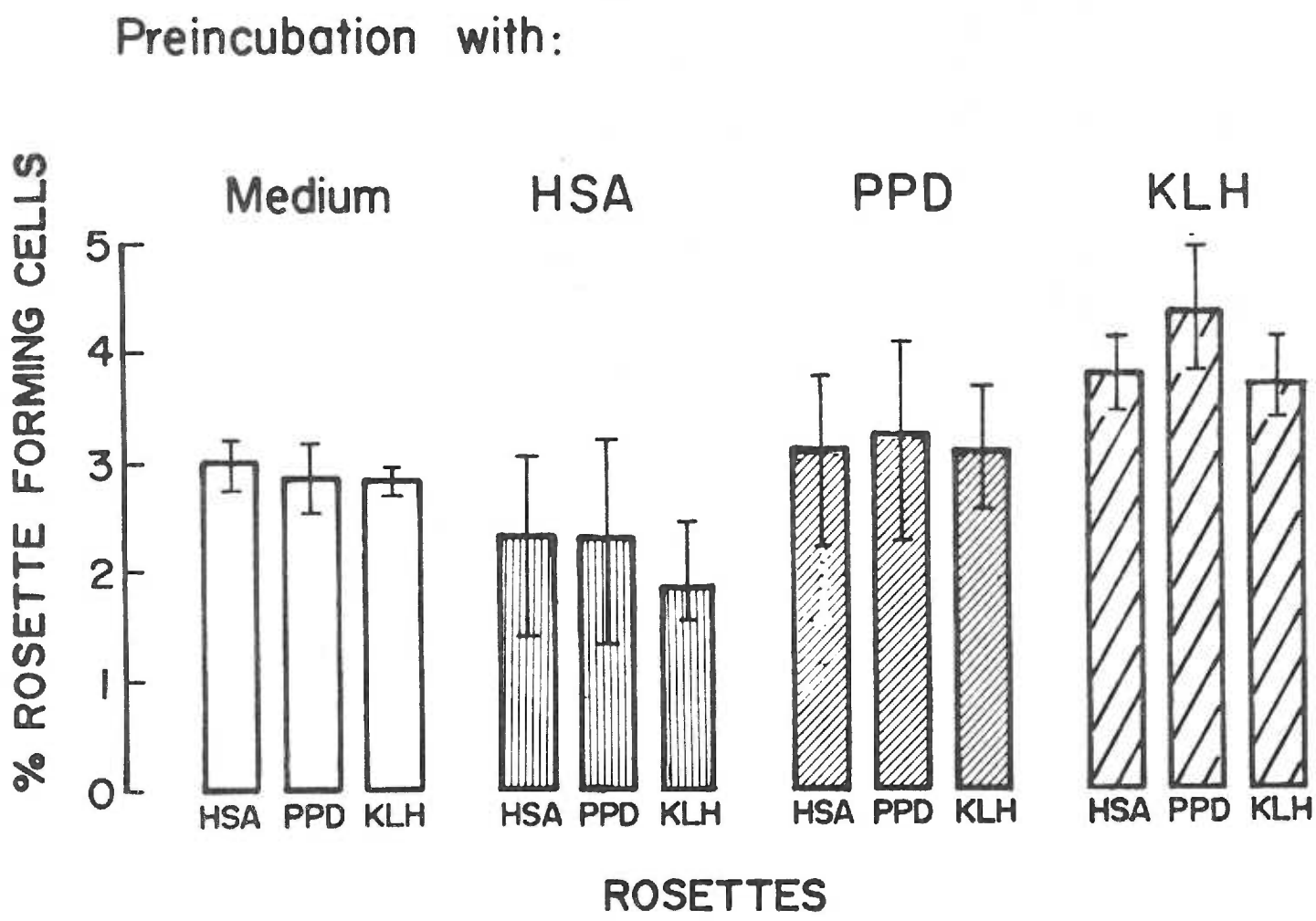


Figure 7: Inhibition of antigen specific rosette formation of a KLH (A) and PPD (B) sensitized donor after preincubation with different concentrations of appropriate recall antigens.

% Inhibition of antigen specific rosette forming cells (⊙)

$$\left\{ 1 - \frac{(\%\ RFC_{\text{recall Ag-RBC}} - \%\ RFC_{\text{HSA-RBC}})\ \text{after recall Ag preincubation}}{(\%\ RFC_{\text{recall Ag-RBC}} - \%\ RFC_{\text{HSA-RBC}})\ \text{after control Ag preincubation}} \right\} \times 100$$

% nonspecific inhibition is determined by evaluating the change in HSA-RFC level after recall antigen preincubation (●).

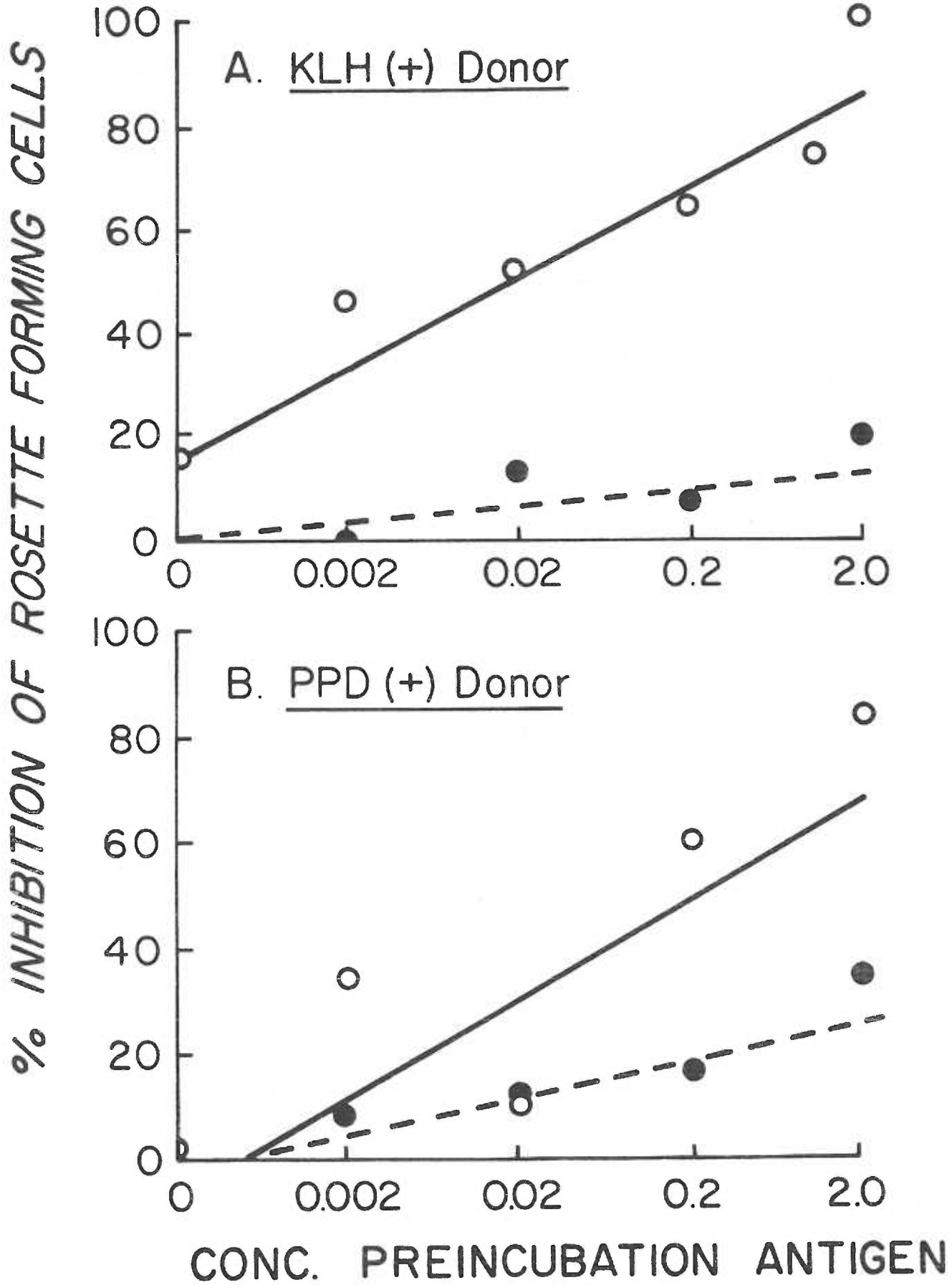

% INHIBITION OF ROSETTE FORMING CELLS
A. KLH (+) Donor
B. PPD (+) Donor
100
80
60
40
20
0
0
0.002
0.02
0.2
2.0
CONC. PREINCUBATION ANTIGEN

Figure 8 : Antigen specific rosette formation is inhibited by preincubation with anti- human Ig antibodies. Specific RFC response of (A) PPD (+) KLH (-); (B) PPD (+) KLH (-) and (C) PPD (-) KLH (+) donors is determined after preincubation with the indicated concentration of goat $F(ab')_2$ anti-human-Ig. Incubation with the same concentrations of normal goat IgGs did not significantly affect the level of specific RFCs (data not shown).

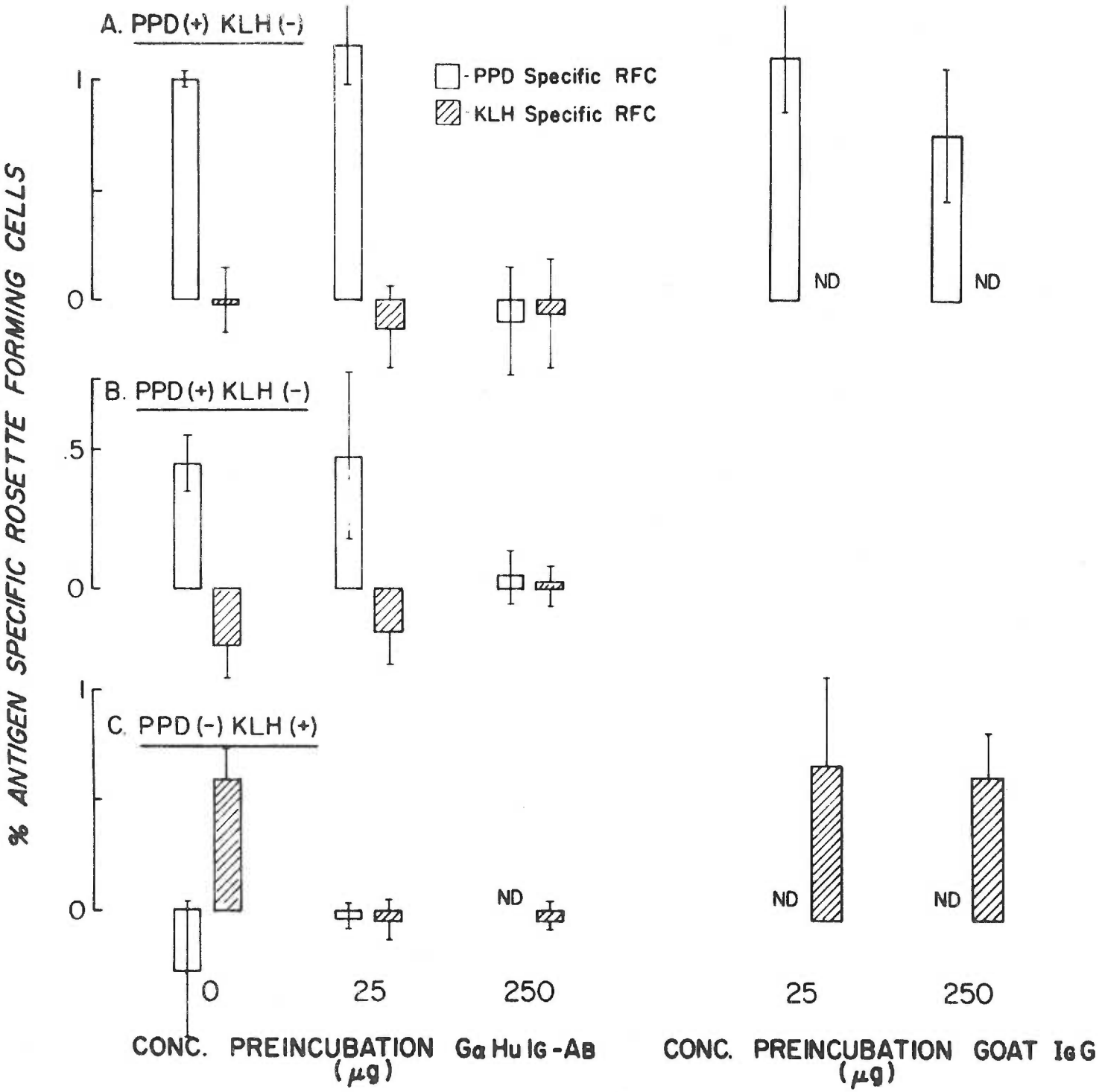

A. PPD (+) KLH (-)
B. PPD (+) KLH (-)
C. PPD (-) KLH (+)
PPD Specific RFC
KLH Specific RFC
% ANTIGEN SPECIFIC ROSETTE FORMING CELLS
1
.5
0
ND
0
25
250
CONC. PREINCUBATION (μg)
Gα Hu Ig-Ab
GOAT IgG

Figure 9 : Specific rosette forming response of A PPD(-)KLH(+) and B PPD(-)KLH(-) donors following preincubation with indicated concentration of PPD antibodies.

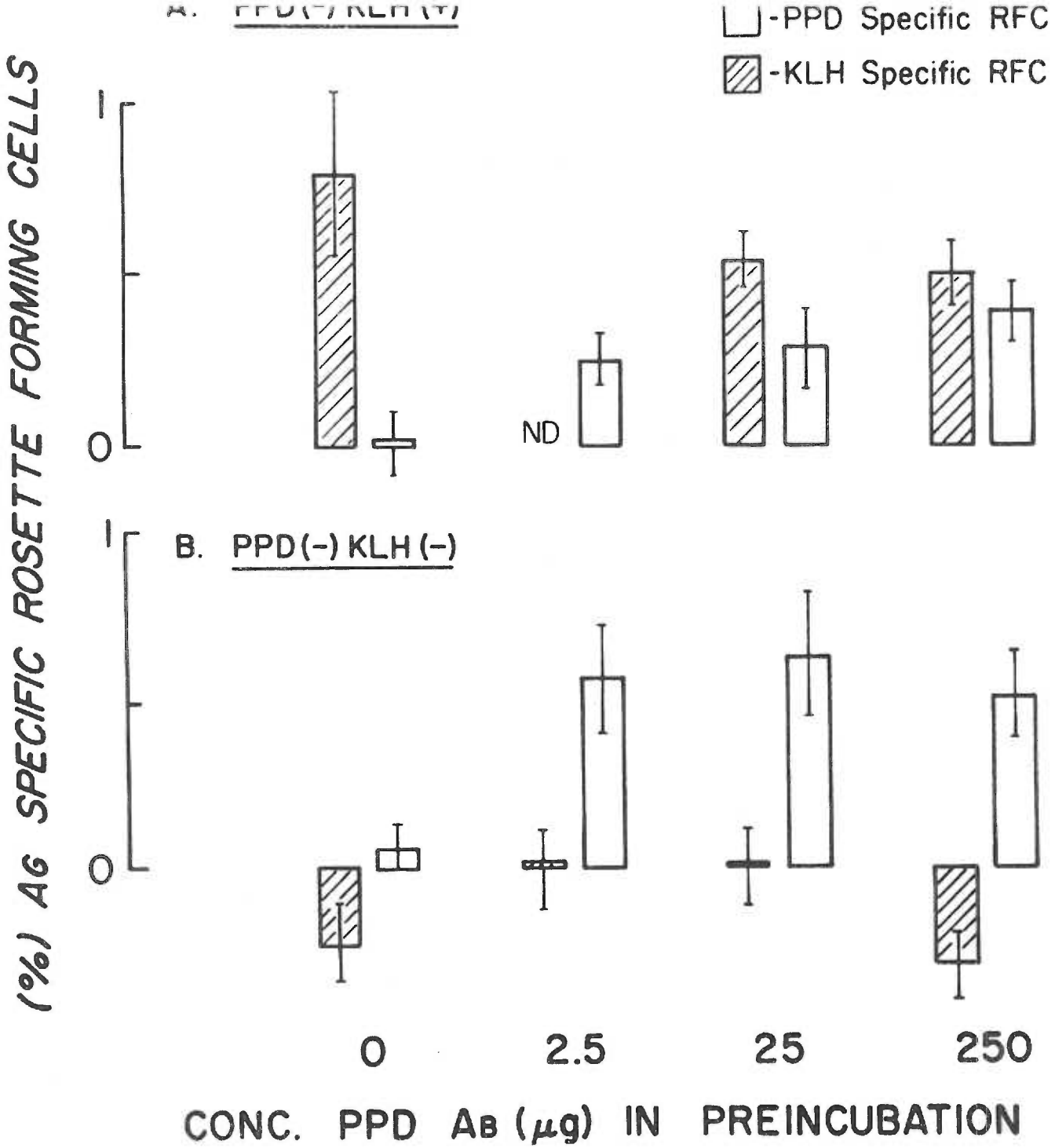

-PPD Specific RFC
-KLH Specific RFC
A. PPD(-) KLH(+)
B. PPD(-) KLH(-)
1
0
ND
(%) Ag SPECIFIC ROSETTE FORMING CELLS
0
2.5
25
250
CONC. PPD Ab (μg) IN PREINCUBATION

Figure 10: Specific rosette forming response of PPD(+)KLH(-) and PPD(-)KLH(-) donors after preincubation with indicated concentration of KLH antibodies.

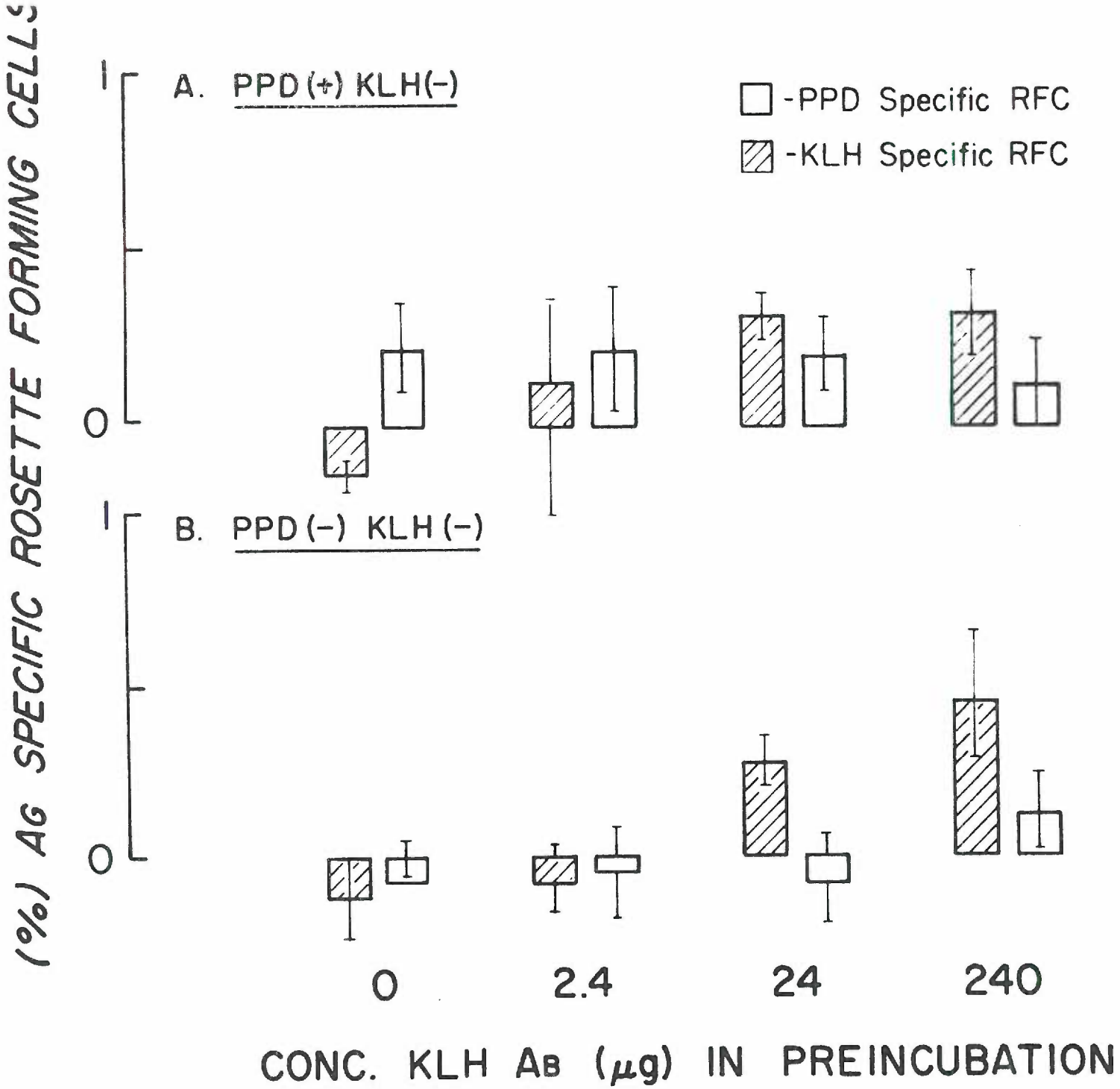

(%) Ag SPECIFIC ROSETTE FORMING CELLS
A. PPD (+) KLH (-)
-PPD Specific RFC
-KLH Specific RFC
1
0
B. PPD (-) KLH (-)
1
0
0
2.4
24
240
CONC. KLH Ab (μg) IN PREINCUBATION

Figure 11 : Pretreatment of donor cells with normal IgG blocks arming of rosette formation by specific antibodies. PPD(+)KLH(-) and PPD(-)KLH(+) donor cells were pre-incubated with the indicated concentration of human IgG at 37C for 30 minutes and then incubated with KLH or PPD antibodies at 4C.

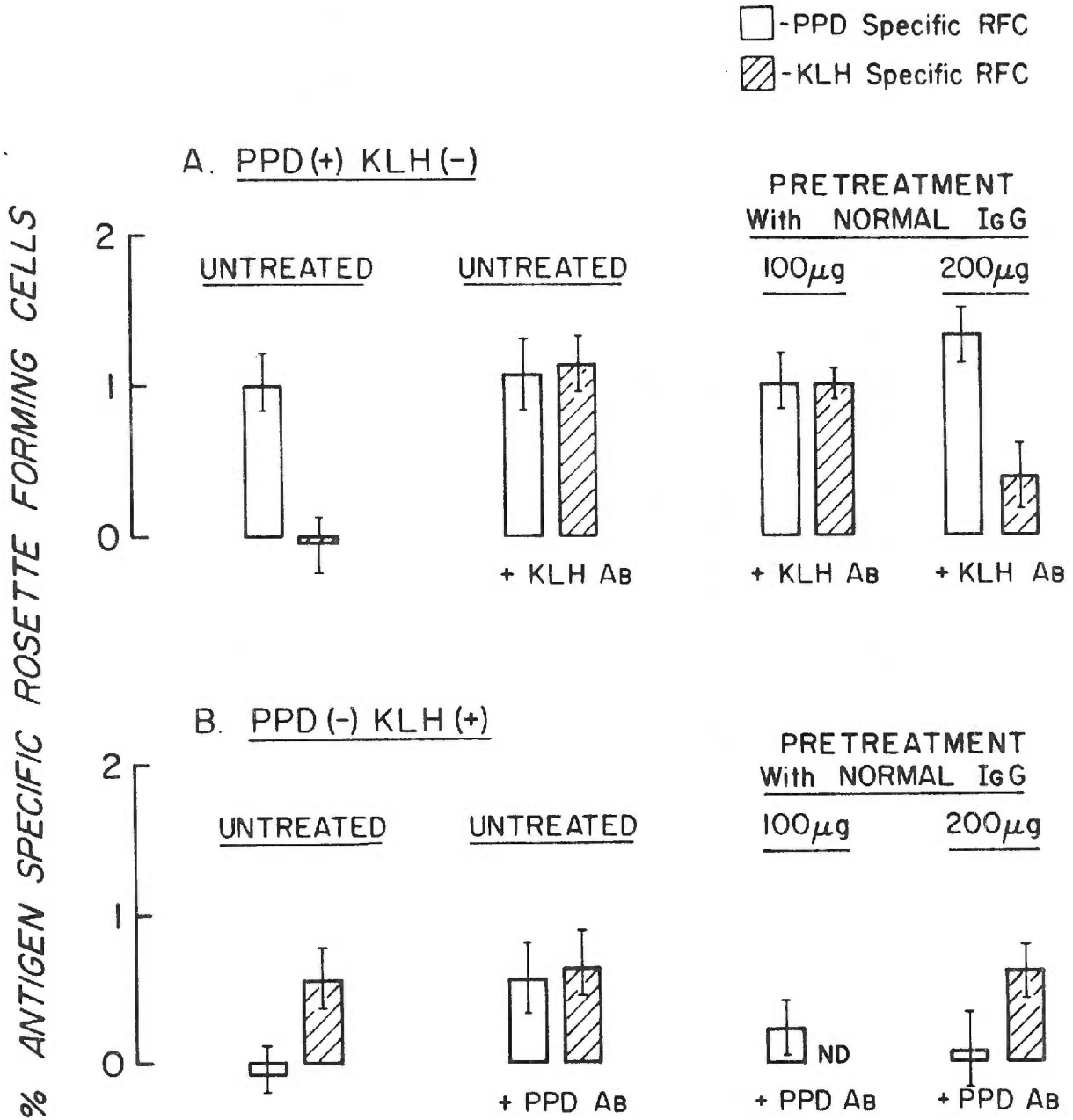
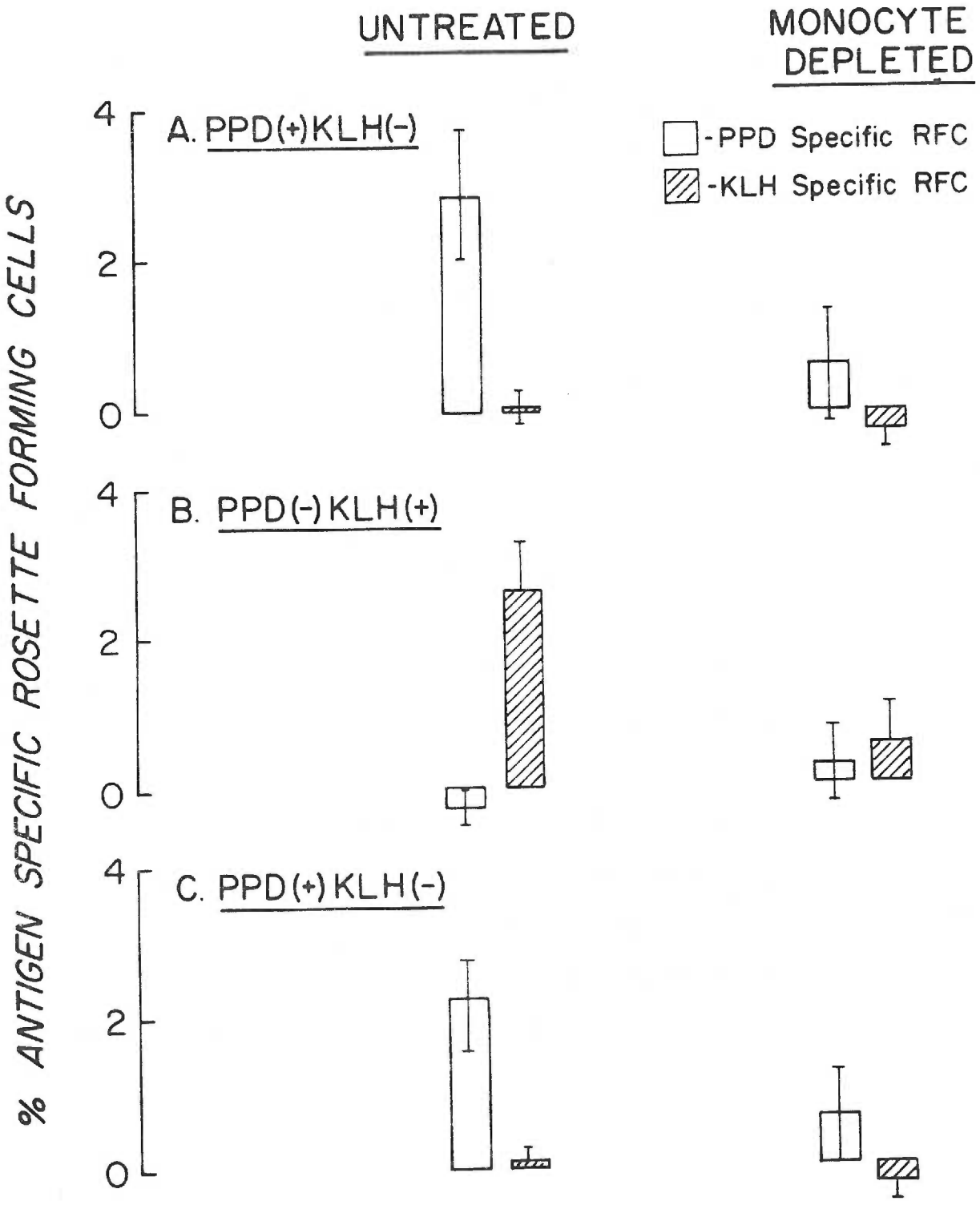

□-PPD Specific RFC
▨-KLH Specific RFC
A. PPD(+) KLH(-)
PRETREATMENT
With NORMAL IgG
UNTREATED
UNTREATED
100μg
200μg
2
1
0
+ KLH Ab
+ KLH Ab
+ KLH Ab
B. PPD(-) KLH(+)
PRETREATMENT
With NORMAL IgG
UNTREATED
UNTREATED
100μg
200μg
2
1
0
ND
+ PPD Ab
+ PPD Ab
+ PPD Ab
% ANTIGEN SPECIFIC ROSETTE FORMING CELLS

Figure 12 : PPD and KLH specific rosette forming response of three donors before and after monocyte depletion.
Donor cells were depleted of monocytes by incubation with carbonyl iron at 37C for 30' followed by hypaque ficoll depletion of iron ingesting cells


UNTREATED
MONOCYTE DEPLETED
-PPD Specific RFC
-KLH Specific RFC
A. PPD(+)KLH(-)
B. PPD(-)KLH(+)
C. PPD(+)KLH(-)
4
2
0
% ANTIGEN SPECIFIC ROSETTE FORMING CELLS

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Manuscript 2

CYTOFLUOROMETRIC EVALUATION OF ANTIGEN SPECIFIC ROSETTE FORMING CELLS IN HUMANS

II. Assessment of tumor specific recognition by patients with squamous cell carcinoma of the head and neck

ABSTRACT

We (Tong et al) have developed a rosette forming assay to examine tumor antigen recognition in patients with squamous cell carcinoma of the head and neck (SQCC-HN). Laser cytofluorometry is utilized to evaluate the rosette forming response of patient mononuclear cells using 3M KCl tumor antigen extracts conjugated to autologous erythrocytes. Patients with no evidence of malignancy or malignancies of other histological types do not form tumor specific rosette forming cells (RFCs) with SQCC-HN tumor extract. On the other hand, > 85% (5/6) of treated tumor free SQCC-HN patients, and over 70% (22/30) of patients with primary malignancy of SQCC-HN form tumor specific rosettes with SQCC-HN tumor extract, but not to extracts of histologically distinct tumors. The frequency of responsiveness among patients with primary malignancy is directly correlated with increased tumor burden.(TNM staging, $p < 0.05$). Patients with recurrent SQCC-HN, however, are not responsive to SQCC-HN or any other tumor extracts. Rosette formation appears to be mediated by tumor specific cytophilic antibodies. Reactivity is lost after 24 hour cell culture, but can be restored by incubation with autologous serum or serum from other responsive patients. Serum arming capacity resides solely in the IgG rich fraction (by DEAE chromatography). Microscopic analysis of enriched RFC fractions following acridine orange or esterase staining indicates that the monocyte is the predominant RFC. Unresponsiveness of recurrent patients is likely to be due to defects in both humoral and cellular

functions. Recurrent patient cells after culture could not acquire rosette forming capacity after incubation with responsive patient serum, and recurrent patient serum cannot arm autologous or normal cells for rosette formation.

INTRODUCTION

Many approaches have been used to evaluate immunological responses to tumor extracts in humans (4,10,11,15,16,18,20,22,29, 31,33,34,35,50) but few have shown clinical usefulness either in diagnosis or staging. Patients with squamous cell carcinoma of the head and neck have been difficult to evaluate partially because they often exhibit depression of certain immune functions. These patients have impaired T function including suppression of lymphocyte proliferative response to mitogens and antigens in vitro (3,21,45,46), as well as depressed delayed cutaneous hypersensitivity reaction (DCHR) to common recall antigens (4,44,46). DCHR and Leukocyte Adherence Inhibition (LAI) tests utilized to evaluate tumor specific reactivity could only detected a low frequency of tumor specific response in these patients (< 40%). Since a positive response in these assays requires both intact tumor recognition as well as secondary efferent functions, unresponsive patients may have underlying defects in either or both functions. In addition, the low frequency of responsiveness during initial evaluation may restrict application of these assays for prognostic and longitudinal followup evaluation on patients with squamous cell carcinoma of the head and neck.

Few reports have documented the presence of specific tumor antigen binding cells (32,36,43,50) in tumor bearing hosts, although concomitant immunity can be demonstrated with other in vitro assays (4,10,11,15,22,29,44). Detection of specific tumor antigen binding

cells would provide direct information of the ability of host cells to recognize tumor associated antigens, and its absence would indicate a lack of tumor specific response related to the ABC subpopulation detected in that assay.

In a previous study we have modified the rosette forming cell (RFC) technique for routine assessment of specific antigen binding cells. For donors that have been previously immunized to conventional antigens KLH and PPD, immunity can be demonstrated through the detection of antigen specific rosette forming cells (manuscript 1). The use of a cytofluorometric technique permits routine RFC evaluation to a panel of antigens as well as statistical analysis of the level of rosette forming cells to each antigen. This assay also carries the added advantage of requiring minimal manipulation of donor cells, thus precluding artifacts that may arise if in vitro culturing were required.

We have applied the rosette forming assay using 3M KCl tumor extracts conjugated to autologous RBCs to assess tumor specific recognition by patients with squamous cell carcinoma of the head and neck at various stages of malignancy. The tumor antigen extracts were previously utilized in LAI and DCHR assays, and have demonstrated functional specificity (4,44,46). A high frequency of rosette forming response to tumor antigen extracts of the same histological type can be demonstrated among patients with primary malignancy as well as treated, tumor free patients. This response appears to be tumor specific, since these patients do not form specific RFCs with other tumor antigen extracts. On the other hand,

patients with recurrent disease do not exhibit tumor specific RFC response. A major portion of rosette forming cells detected among responsive patients are found to be monocytes armed with cytophilic antibodies capable of tumor specific recognition. The lack of tumor specific rosette forming cell response in recurrent patients may be attributed to both monocyte and humoral dysfunctions.

MATERIALS AND METHODS

Patients

The study population included in-patients as well as returning patients at the Portland Veterans Administration Hospital and University of Oregon Health Sciences Center. Eighty patients (volunteers after informed consent) were evaluated for specific rosette forming response, including 45 patients with squamous cell carcinoma (30 with primary malignancy, 6 without tumor undergoing followup therapy, 9 with recurrent tumors), 21 patients without evidence of malignancy, and 14 patients with biopsy proven non-head and neck malignancies or head and neck nonsquamous tumors. The latter patients were newly diagnozed, were tumor free following therapy, or had persistent tumor. Of the nine patients studied with recurrent squamous cell carcinoma of the head and neck, three had received radiation, surgery and chemotherapy, four had received irradiation and surgery; and two patients had received only surgery. Two of the patients were known to be nutritionally depleted. Thirty patients that had newly diagnosed squamous cell carcinoma of the head and neck were evaluated before treatment. Returning patient group represents patients with treated primary malignancy of squamous cell carcinoma of the head and neck who had remained tumor free for at least 30 days. All patients are males ranging from 50 - 70 years of age.

Tumor antigen extraction

Tumor tissues were obtained within four hours of excision from

patients with histologically proven tumors. Tissue was collected only from primary and regionally metastatic lesions from patients for whom surgery was the primary mode of therapy. Tissues were finely minced, washed repeatedly with saline and freeze-dried. The dried tissue was stored at -20 C until extraction with 3M KCl according to the method of Meltzer et al (Fig. 1)

Rosette forming cell assay

Preparation of donor lymphocytes and antigen conjugation to autologous erythrocytes have been described in the preceding paper (manuscript 1). Briefly, autologous erythrocytes obtained from patient peripheral blood after dextran sedimentation were reconstituted to 2.5% (v/v) in Tris buffered saline (TBS) and added to equal volume of TBS containing 0.15 mg of the appropriate tumor antigen extract. Equal volume of chromium chloride (0.1%) in TBS was added dropwise and left at 23 C (room temperature) for 4 minutes. After appropriate washing, antigen conjugated erythrocytes were reconstituted to 0.25% (v/v) and incubated with Ficoll Paque (Pharmacia, Piscataway, N.J.) extracted mononuclear cells from the same donor for 1 hour at 4 C. The % RFCs in each sample was determined cytofluorometrically after nuclear staining with acridine orange (final concentration 0.01 mg%).

Monocyte depletion

Five milliliters of G-10 beads (Pharmacia, Piscataway,N.J.) that have been equilibrated overnight in RPMI-1640 with 5% newborn calf

serum (NCS) is poured into an upright 10 ml syringe with an attached stop valve. Donor mononuclear cells suspended in RPMI-1640 -5% NCS are layered on top and incubated at room temperature for 15 minutes. The eluate was collected and washed with 5x volume of RPMI-1640 -5% NCS. Monocyte depletion fractions are 99% monocyte free by Wright's staining.

In vitro mononuclear cell incubation

For evaluation of rosette forming response after in vitro culture, responsive patient mononuclear cells were incubated at 37 C for 24 hours with RPMI-1640 with 5% NCS. Cultured cells were washed twice with RPMI-1640 and resuspended in serum free media to the original concentration for cytofluorometric analysis.

Cultured mononuclear cells were incubated with either autologous or allogeneic serum from responsive patients to evaluate the effect of serum arming . Serum for each patient was obtained by centrifugation of whole blood sample at 250 g for 10 minutes. Plasma was freeze thawed once before use. Serum incubation was carried out at 4 C for 30 min. in the ratio of the relative yield of WBC: plasma during extraction. For example, if 10 ml of plasma and 2 X 10^7 mononuclear cells were obtained from the patient's whole blood sample, then 2 ml of serum is used per 4 x 10^6 cells. After incubation WBCs were washed twice with RPMI-1640 and then resuspended to original concentration for rosette forming cell evaluation.

DEAE Serum Fractionation

Sera from either responsive primary or recurrent patients with squamous cell carcinoma were pooled and immunoglobulin fraction obtained by 50% NH_4SO_4 precipitation. The precipitate was pelleted by centrifugation at 10,000 rpm or 800 g (Sorval Hi-Speed RC2B), resuspended in 0.01M phosphate buffer and dialysed for 72 hours. The preparation was filtered to remove aggregates and then passed over a DEAE column (Pharmacia, Piscataway, N.J.) previously equilibrated with 0.01 M phospate buffer. IgG enriched fraction (Fraction I) was collected as the first major peak eluted off the column. Protein retained by DEAE passage was eluted with 0.1 M phosphate buffer as the IgG depleted fraction (Fraction II). Protein concentration in each fraction is determined by spectrophotometric absorbance at 280 nm.

RESULTS

Evaluation of tumor specific rosette formation

In a previous study we have reported the detection of antigen specific rosette forming cells to PPD and KLH among appropriately immunized donors. In each determination the level of antigen specific RFCs is significantly higher than background (determined using HSA-RBCs) and was retricted to the antigen/s to which the donors were previously immunized. This approach as adapted for tumor specific rosette forming evaluation is shown in Table I. Mononuclear cells from normals and squamous cell carcinoma of the head and neck (SQCC-HN) patients were coded and tested in a blind fashion against autologous erythrocytes conjugated with HSA, or 3M KCl tumor extracts from squamous cell carcinoma of the head and neck (SQCC-HN), adenocarcinoma of the breast (AdCB), and melanoma (Mel). Normals invariably fail to exhibit antigen specific RFCs to all of the tumor antigens tested, whereas squamous patients only respond to SQCC-HN-RBCs.

Figure 2 shows the RFC response to SQCC-HN tumor extract by patients with the same malignancy as compared to control patient groups. Twenty-two out of 30 (22/30) patients with untreated biopsy proven squamous cell carcinoma of the head and neck demonstrated specific rosette forming cell response. Similarly, a high frequency of reactivity (5/6) can be observed among the small number of returning patients with treated primary malignancy who have remained tumor free at the time of evaluation. This pattern of responsive-

ness, however, is absent among patients with a recurrent malignancy. None of the 9 (0/9) recurrent patients tested in this manner showed any significant rosette forming cell response. When normal controls or patients with other types of tumors were tested for SQCC-RFCs, only a low level of responsiveness could be observed. One out of 14 tumor patients with non head and neck squamous tumors or head and neck non squamous malignancies responded to SQCC-HN extracts, and only 1 out of 21 normal control group donors with no evidence of malignancy exhibited this response. Similarly, when the primary and returning patients were tested with other tumor antigen extracts, only a low frequency of cross reactivity (3/36) was observed (Figure 3).

Correlation of response with tumor staging

The level as well as the frequency of responsiveness of patients with primary malignancy has been correlated to tumor burden : localized malignancy($T_{1-3}N_0M_0$), early lymph node ($T_{1-3}N_1M_0$) and extensive regional lymphoid ($T_{1-4}N_{2-3}M_0$) involvements(Figure 4). The frequency of response of patients with extensive lymphoid involvement ($T_{1-4}N_{2-3}M$) was significantly higher than the other two groups ($p < 0.05$, chi square and Kruskal-Wallis one way analysis of variance). In addition, the mean levels of response of $T_{1-4}N_1$ and $T_{1-4}N_{2-3}$ patients (including unresponsive patients in each group) are significantly higher than $T_{1-3}N_0$ group ($p < 0.05$).

TNM staging as utilized by the American Joint Committee for Cancer staging and End Results Reporting (AJCCS) is adopted. Tumor burden is defined according to size of primary tumor (T_{1-4}), nodal involvement (N_{0-3}) and presence (M_1) or absence (M_0) or distal metatasis.

Specificity of rosette forming cell response

The exclusive responsiveness to the same tumor antigen extract by patients with SQCC-HN together with the low level of reactivity among other tumor patients and normals suggest that rosette formation to SQCC-HN is tumor specific. To examine this question further, responsive donor cells were evaluated before and after preincubation with relevant and irrelavant tumor antigens. RFC response as shown in Figure 5 is representative of the data evaluated with five responsive patients tested after preincubation with either SQCC-HN or melanoma. In all cases, SQCC-specific rosette formation was inhibited following preincubation with the same tumor antigen extract, but not with control tumor antigen.

Mediation of tumor specific rosette formation by serum factors

In order to examine the nature of antigen receptors on RFCs, responsive patient cells were cultured in vitro with 10% newborn calf serum and then evaluated for specific RFC response. Incubation for 24 hours invariably resulted in a loss of donor reactivity (Figure 6). Attempts to regenerate tumor antigen specific receptors endogenously by culturing for extended period (up to 4 days) did not result in regeneration of tumor specific RFC response (data not shown). However, tumor specific RFC reactivity could be regained if cultured mononuclear cells were preincubated with autologous serum for 30 minutes. Overnight culture of responsive cells did not enhance specific rosette formation, since cells 'rearmed' after autologous serum incubation did not exhibit any higher level of

rosette formation compared to uncultured cells from the same patient. Normal AB (+) serum was ineffective in regenerating RFC response (Figure 6c).

Tumor specific RFCs carry surface immunoglobulin

The above observations indicate that tumor specific rosette formation is likely to be mediated by factor/s present in immune sera, such as cytophilic antibodies with tumor specific reactivity. To determine if rosette formation is dependent on surface immunoglobulins, the effect of goat $F(ab')_2$ anti-human-Ig treatment on tumor specific rosette forming response was evaluated. As shown in Table 2, donors who previously demonstrated significant SQCC-HN RFC response lost their reactivity following preincubation with anti-Ig, thus suggesting that a majority of specific RFCs carry surface immunoglobulins.

Next we evaluated the effect of $F(ab')_2$ anti-human-Ig treatment on cultured cells (Figure 7). Similar to the above described observation, reactivity lost after 24 hour culture of the responsive patient cells was regained after incubation with autologus serum. The reacquired rosette forming response to SQCC-HN was again abolished after treating 'rearmed' cells with anti- antibody.

Involvement of monocytes in tumor specific RFCs

The above observations agree with our previous study involving donors responsive to conventional antigens (manuscript 1) which indicated the involvement of cytophilic arming of monocytes in

rosette formation. To evaluate the participation of monocytes in tumor specific rosette formation, donor response was tested prior to and after passage through Sephadex G-10 for monocyte depletion. Tumor patients who exhibited a tumor specific rosette forming response to SQCC-HN invariably lost their reactivity following monocyte depletion (Figure 8). In five separate experiments, 95% loss of tumor specific rosette forming response was observed following monocyte depletion.

The identity of tumor specific rosette forming cells was examined further after isopycnic enrichment of rosetted samples using Ficoll Paque centrifugation. Enriched rosetted samples were either esterase or acridine orange stained and examined under the light microscope. Morphological identification confirmed monocyte depletion data and reveals a predominant involvement of monocytes in tumor specific RFC populations (Figure 9). In five separate determinations, a mean value of 90% (90 $\pm$ 10 %) of RFCs was found to be esterase positive mononuclear cells. The participation of lymphocytes is relatively minor, ranging from 0 - 10% (10 $\pm$ 10 %) in five experiments.

Serum dysfunction of recurrent patients

Since tumor antigen recognition as detected in this assay depends on the availability of cytophilic antibodies as well as Fc receptor-bearing monocytes, we wished to determine if unresponsiveness among recurrent patients was due to defects in either or

both of these functions. To define more clearly the effect of serum arming, sera from 3 responsive or 2 recurrent squamous patients were pooled and fractionated into IgG rich (Fraction I) and IgG depleted (Fraction II) fractions by DEAE chromatography. The ability of both whole sera , as well of the respective fractions to arm normal control donors to effect tumor specific rosette forming response is then compared. As was shown in Figure 9, unfractionated sera from responsive patients can "arm" normal control cells to generate a tumor specific rosette forming response; the arming ability resides solely in the IgG enriched fraction (Fraction I) as predicted, since depletion of serum IgG (Fraction II) abrogates serum arming ability. However, neither pooled serum nor IgG enriched serum fraction (Fraction I) from recurrent patients is capable of arming normal donors (Figure 9b).

Monocyte dysfunction of recurrent patients

While it is evident from the above observation that recurrent donors lack available cytophilic antibodies for monocyte arming, it is not known if a defect in monocyte function also exists. Recurrent patient mononuclear cells were cultured overnight and then tested for rosette forming response following incubation with serum from responsive patients. Two recurrent patient cells examined (Table 3) remained unresponsive after overnight culture. Reactivity was not acquired after incubation with either autologus serum or sera that have previous demonstrated arming ability. In addition,

cells from one recurrent tummor patient could not generate a KLH specific RFC response after incubation with KLH specific antibodies that have been used used previously to arm normal cell populations.

DISCUSSION

In this study we have used a modified RFC assay to examine the ability of tumor patients to recognize tumor associated antigens. Correlation of immunity with elevated levels of specific antigen binding cells is well documented in conventional antigen systems (for review see 48), but few reports could directly demonstrate specific tumor antigen binding in tumor bearing hosts. This assay thus represents an unique approach in assessing specific tumor immunity in humans.

By using the cytofluorometric evaluation, we were able to routinely assess each donor's RFC response to a panel of relevant and control tumor antigen extracts. In addition, since the response of each patient can be evaluated within a few hours , physiologic changes that may be induced by in vitro cell culturing .

The RFC response detected in SQCC-HN patients appears to be tumor specific . Responsive primary carcinoma patients demonstrated RFCs to conjugated 3M KCl tumor antigens of the same histological type, which have demonstrated functional specificity by DCHR and LAI response (4,44). These patients only exhibited a low level of reactivity when tested with other tumor antigens, including neuroblastoma, colon carcinoma, melanoma and adenocarcinoma of the breast. RFC responsiveness of SQCC-HN can be inhibited by preincubating donor cells with the same tumor antigen extract, but not other tumor antigens. Normal controls, as well as patients with head and neck nonsquamous or non-head and neck squamous tumors, do not

respond to SQCC-HN or any of the other tumor antigen extracts tested. Thus it is unlikely that the RFC response among primary SQCC-HN patients represents reactivity against contaminating antigens such as HLA or carcinoembryonic antigens commonly found among the various tumor extracts (24,28,34). Rather, this response suggests specific recognition of unique antigen determinants expressed in the SQCC-HN tumor extract by patients bearing the same histological tumor type.

A dichotomy of responsiveness can be observed within patients bearing squamous cell carcinoma of the head and neck. A high proportion of patients with detectible primary malignancy carry significant levels of RFCs to SQCC-HN antigen extracts, and the frequency of reactivity increases with increased nodal involvement. The highest frequency of response was observed in patients that have had their tumors resected who are tumor free at the time of evaluation. The in vivo relevance of the rosette forming cell response evaluation is underscored by the striking difference in response pattern between tumor free primary patients (5/6 RFC'+') and recurrent patients (0/9 RFC '+'). While only a small number of patients were tested, it is evident that none of the recurrent patients responded to the relevant tumor extract (or to any of the tumor extracts tested). It appears that tumor immune recognition as demonstrated among primary patients, which becomes more prevalent with increasing tumor burden and remains detectible during followup is absent among recurrent patients. Presently, it is not clear if unresponsiveness represents an inability of the immune system to

respond or recognize the tumor thus leading to recurrence, or is the result of tumor recurrence . Our preliminary study on recurrent patients, however, suggests defects in both the cellular and humoral components.

A variety of mechanisms have been reported on specific antigen binding and recognition. Antigen specific T and B lymphocytes bind antigens by specific receptors (7,8,9,12,13, 19), while nonspecific effector cells such as macrophages, NK cells and PMNs can acquire antigen specific responsiveness through Fc adsorption of antigen specific cytophilic antibodies (6,23,25,49). In this study, the comparatively high (> 0.5%) and often variable level of RFCs among responsive donors led us to consider the involvement of cytophilic antibodies. Pretreatment of responsive donor cells with goat F(ab') anti-human-Ig resulted in a loss of tumor specific RFC response, suggesting that RFCs detected in this assay bind through surface immunoglobulins or other receptors in close physiological approximation. Responsive patients lose their RFC reactivity after a 24 hour culture period, probably after receptor shedding. Extended culture did not result in regeneration of RFC response,suggesting that tumor specific receptors are not endogenously derived. Tumor specific reactivity, however, can be restored after a 30 minute preincubation with autologous serum, or serum from other responsive patients. Restored RFC response can again be blocked if the armed cells were treated with anti-human-Ig $F(ab')_2$. Preliminary evidence indicates that the arming element is cytophilic IgG antibody with tumor specific reactivity, since arming activity resides solely in

the IgG serum fraction.

Monocytes as well as B and T lymphocyte subpopulations carry Fc receptors and can acquire cytophilic antibodies for specific antigen recognition (5,6,23,25,39,47). In this study,the predominant involvement of monocytes can be established by both monocyte depletion as well as RFC enrichment techniques. Monocyte depletion by Sephadex G-10 passage invariably resulted in complete loss of tumor specific RFC reactivity among responsive donors. In addition, when enriched RFC fractions of various responsive donors were examined by light microscopy, RFCs can be found to be comprised predominantly ($\geq$ 90%) of esterase positive mononuclear cells with monocyte morphology. These observations confirmed our previous study (manuscript 1) implicating antibody armed monocytes as the predominant RFC detected by cytofluorometry. However, RFC response appears to involve only a subpopulation of monocytes. After 24 hour culture (which allows shedding of surface receptors) followed by autologous serum incubation, RFC response were restored to levels observed prior to culture. In no instances did this treatment improve RFC response, even though one might expect that after culturing more monocytes with free Fc receptors would be available for arming. It is also possible that the inabilility to enhance rosette formation may be due to the limitation of this assay in detecting monocyte-antibody - tumor antigen interaction of only a selected avidity.

The evaluation of tumor specific recognition response by cytophilic antibody armed monocytes is particularly relevant in

tumor bearing host situations. Macrophages and monocytes were believed to play an important role in antineoplastic immunosurveillance (for review see 59). Peripheral blood derived marophage infiltration correlates with good prognosis in both human (37,52) and animal (7,38,140) tumor systems. More recently, Wood et al demonstrated the parallel distribution of antibodies and macrophages in a variety of human (26,38,52) and animal (37,51) tumors in situ. Antibodies can be bound to Fc receptors of macrophages and can be appropriately eluted (52). Other studies have demonstrated tumor specific cytostatic (15,31,42) and cytotoxic (14,27,42,47) reactions by cytophilic antibody armed macrophages . In addition, mice injected with hyperimmune antiserum acquired tumor specific immunity which can be demonstrated both by peritoneal macrophage mediated tumor specific rosette formation and tumor rejection responses upon challenge.

Although squamous cell carcinoma patients as a group showed a low frequency of responsiveness in cell mediated immune reactions (LAI, DCHR, cell mediated cytotoxicity), this defect evidently is not extended to monocyte mediated tumor specific recognition. A high frequency of primary malignant bearing patients are responsive by the RFC assay, with an increase in frequency with increased tumor burden. Longitudinal followup studies on a single patient have demonstrated tumor specific RFC response before and after resection of a primary malignancy, but loss of specific RFCs upon recurrence after 6 months. It is not clear if this is representative of the RFC response of patients likely to recur. However, the pattern of unres-

ponsiveness within the recurrent group tends to indicate that tumor specific RFC evaluation is useful in prognostic evaluation.

Presently it is not entirely clear why recurrent patients failed to respond in RFC assay. Preliminary evidence based on two patients indicated defects in both monocyte and antibody functions. Preincubation with recurrent patient whole sera, IgG enriched or IgG depleted fractions cannot arm either autologous or normal cells for rosette formation. These patients either lack cytophilic antibodies or more likely, carry antibodies that have been complexed with tumor antigens (1,2,73) and are unavailable for tumor specific recognition. In addition, cells from recurrent patients remained unresponsive after 24 hour cell cultures in an attempt to arm with either autologous or responsive sera. In one recurrent patient, preincubation with specific antibody against KLH also did not result in acquired KLH responsiveness as observed among normal donors (manuscript 1). This defect cannot be explained by the absence of monocytes, since all of the recurrent patients examined have normal or elevated monocyte counts (15 $\pm$ 3 %). In a study comparing antigen binding response between high and low responder strains of mice, Benacereaf and Kapp (25) demonstrated that low responders lack a monocyte subpopulation that can acquire cytophilic antibodies for antigen specific recognition. A similar defect may be present in patients with recurrent malignancies. Present studies include experiments to elucidate further recognition defects in recurrent patients, as well as continued evaluation of incoming SQCC-HN patients at various stages of malignancy, and patients with other types of malignancies.

Table 1

Tumor Specific Rosette Formation by Tumor Patients

Tumor type	% Rosette Formation				Specific rosette forming cells[a]		
	HSA-RFC	AdcB-RFC	SOCC-RFC	Mel-RFC	AdcB	SQCC	Mel
Control	4.82±0.20	4.60±0.47	4.91±0.35	4.70±0.34	(-0.22)	(0.09)	(-0.12)
SOCC-HN	2.95±0.21	2.92±0.37	3.95±0.11	3.60±0.50	(-0.03)	1.0[b]	(-0.35)
Mel	2.45±0.27	ND	2.60±0.17	3.07±0.15	-	(0.15)	0.62[b]

HSA: human se rum albumin; AdCB: adenocarcinoma of the breast; SQCC: squamous cell carcinoma of the head and neck; Mel: melanoma: ND: not done.

[a] % antigen specific RFCs = % $RFC_{tumor\ antigen}$ - % RFC_{HSA} .

[b] values are statistically significant by Student's t test, ($p < 0.05$).

Table 2 : Effect of Anti-Human-Ig treatment on tumor specific Rosette-Formation of SQCC-HN patients

P. No / Patient	TREATMENT	SPECIFIC ROSETTE FORMATION[a] TO	
		SQCC-HN Ag	MEL Ag
F.N. ($T_1N_0M_0$)	none	.38 ± .13[c]	(.04 ± .05)
	GαHuIg[b]	(-.10± .22)	ND
. G.A. ($T_3N_0M_0$)	none	.61 ± .21[c]	(.09 ± .37)
	GαHuIg	(-.04± .14)	(.03 ± .11)
I.A.B. ($T_2N_0M_0$)	none	.50 ± .28[c]	(.05 ± .31)
	GαHuIg	(-.14±.15)	(0 ± .18)
. A.S. ($T_2N_0M_0$)	none	1.06 ± .13[c]	(.03 ± .21)
	GαHuIg	(- .13± .08)	ND

pecific Rosette Formation = % $RFC_{Tu-Ag-RBC}$ - $\%RFC_{HSA-RBC}$

oat $F(ab')_2$ with anti human immoglobulin (G+M+A+D+E) specificity

statistically significant by student's t test

Table 3

Antibody Mediated Tumor Antigen Recognition in Patients

Donor cell	% Antigen specific rosette formation to SQCC-HN [a]			
	Untreated	after 24 hour culture [b]		
		+ Autologous serum	+ Arming serum [c]	+ KLH specific antibody [d]
I. Control	(-0.20 ± 0.30)	(-0.13 ± 0.05)	0.49 ± 0.09 [e]	0.71 ± 0.38 [e]
II. Primary SQCC-HN ($T_4N_1M_0$)	0.73 ± 0.27 [e]	0.60 ± 0.37 [e]	0.74 ± 0.20 [e]	ND
III. Recurrent SQCC-HN	(-0.06 ± 0.32)	(0.09 ± 0.29)	(0.08 ± 0.37)	ND
IV. Recurrent SQCC-HN	(-0.64 ± 0.32)	(-0.21± 1.22)	(-0.66 ± 0.83)	(-0.02± 0.27)
V. Recurrent SQCC-HN	(-0.08 ± 0.60)	(0.28 ± 0.50)	(0.23 ± 0.25)	(-0.53± 0.63)

[a] % Antigen specific $RFC_{SQCC-HN}$ = % $RFC_{SQCC-HN}$ - % RFC_{HSA} for background correction.

[b] Donor mononuclear cells were cultured overnight at 37 C with 5% newborn calf serum.

[c] Arming serum was derived by pooling sera from SQCC-HN responsive patients.

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IgF specific antibody is extracted from RSV immune donors by affinity chromatography

[c]Values are statistically significant by Student's t test ($p<0.05$)

Figure 1 : Preparation of tumor extracts.

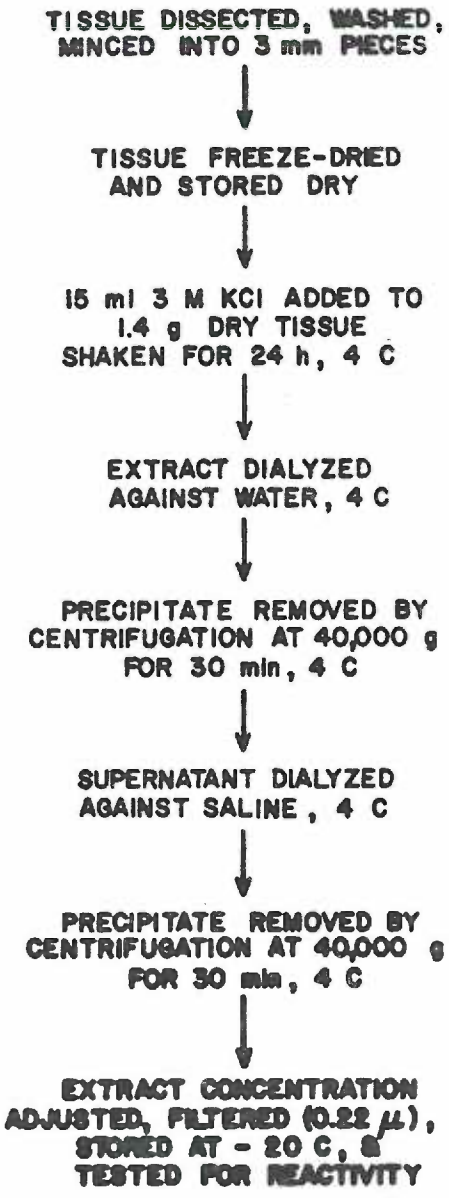

TISSUE DISSECTED, WASHED, MINCED INTO 3 mm PIECES
TISSUE FREEZE-DRIED AND STORED DRY
15 ml 3 M KCl ADDED TO 1.4 g DRY TISSUE SHAKEN FOR 24 h, 4 C
EXTRACT DIALYZED AGAINST WATER, 4 C
PRECIPITATE REMOVED BY CENTRIFUGATION AT 40,000 g FOR 30 min, 4 C
SUPERNATANT DIALYZED AGAINST SALINE, 4 C
PRECIPITATE REMOVED BY CENTRIFUGATION AT 40,000 g FOR 30 min, 4 C
EXTRACT CONCENTRATION ADJUSTED, FILTERED (0.22 μ), STORED AT - 20 C, & TESTED FOR REACTIVITY

Figure 2: Rosette forming response to SQCC-RBCs of various patient groups. RFC response of each patient is evaluated using control and SQCC-HN tumor extracts. Each value represents a patient's specific RFC response to SQCC-HN that is significantly higher ($p < 0.05$) or not different from response to control tumor antigens.

A. Patient with primary malignancy of SQCC-HN.

B. SQCC-HN patients who had remained tumor free after treatment.

C. Patients with recurrent malignancy of SQCC-HN.

D. Non-head and neck squamous and head and neck non-squamous tumor patients.

F. Control group with no evidence of malignant disease.

% SQCC-HN RFC response = % total RFC_{SQCC} - % RFC_{HSA} for background correction.

(o): Statistically significant response by Student's t test; $p < 0.05$.

(●): Statistically non-significant response.

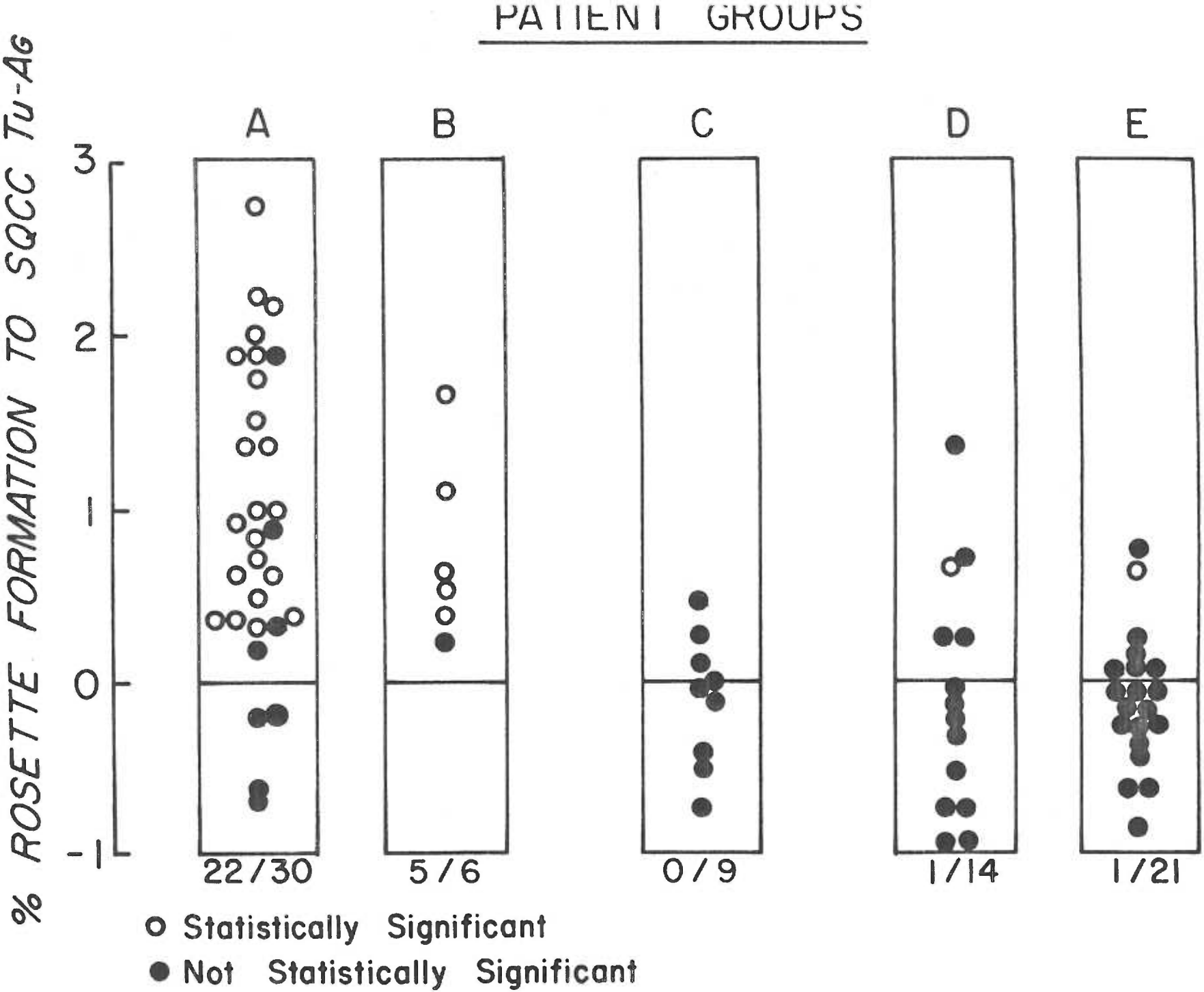

PATIENT GROUPS
% ROSETTE FORMATION TO SQCC Tu-A6
A
B
C
D
E
3
2
1
0
-1
22/30
5/6
0/9
1/14
1/21
Statistically Significant
Not Statistically Significant

Figure 3 : Tumor specific rosette forming response of patients with primary malignancy of squamous cell carcinoma of the head and neck. Patients were tested with 3M KCl extract of squamous cell carcinoma of the head and neck (SQCC-HN), melanoma (Mel) and colon carcinoma (CC) tumors.

(⊙): statistical significant response by Student's t test, ($p < 0.05$).

(●): statistically not significant response by Student's t test, ($p > 0.05$)

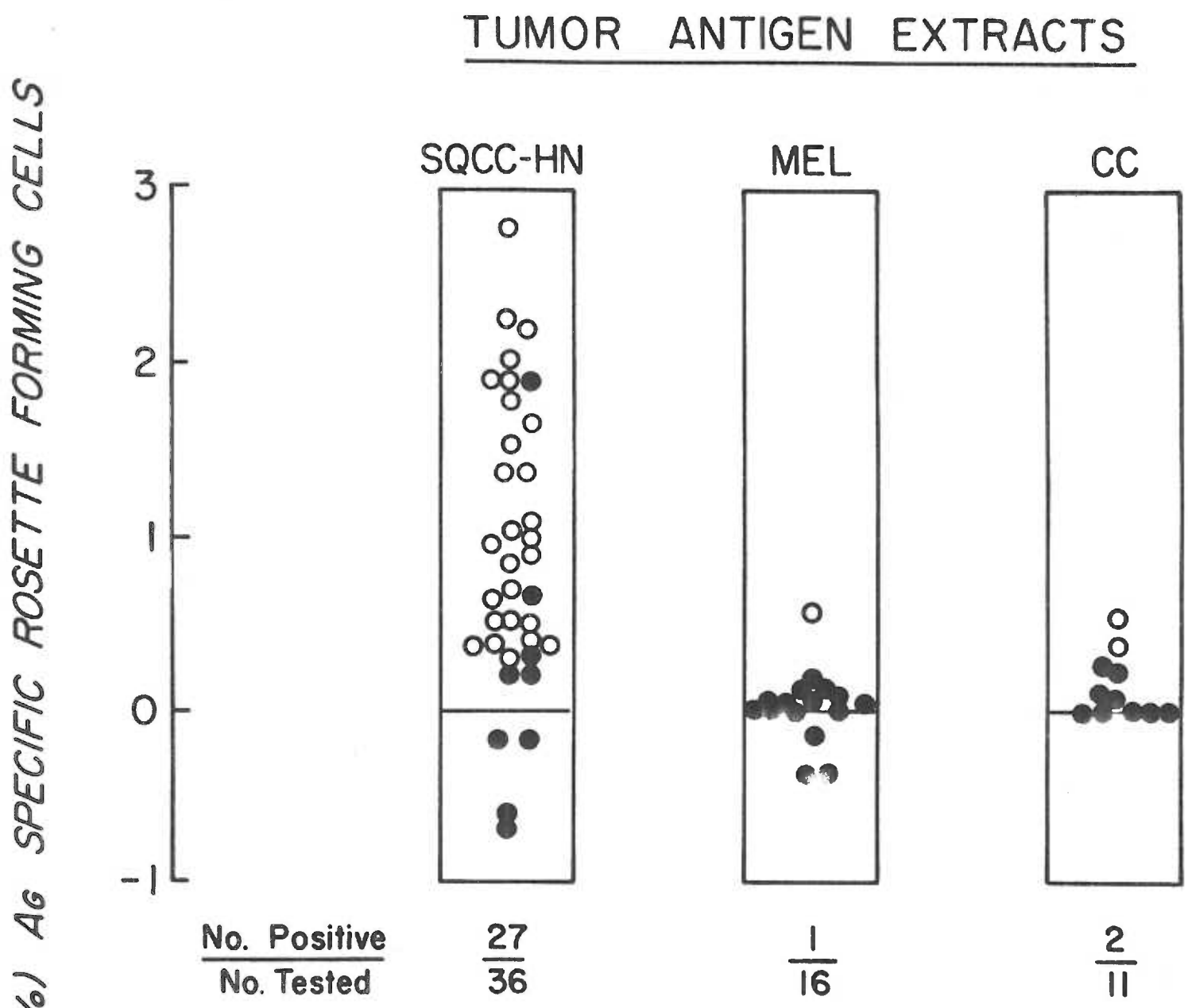

TUMOR ANTIGEN EXTRACTS
(%) Ag SPECIFIC ROSETTE FORMING CELLS
3
2
1
0
-1
SQCC-HN
MEL
CC
No. Positive / No. Tested
27/36
1/16
2/11

Figure 4 : Rosette formating cell response to 3M KCl SQCC-HN tumor extract by SQCC-HN at different stages of primary malignancy

% ROSETTE FORMATION TO SQCC Tu-Ag
3
2
1
0
-1
STAGING
T1-3N0M0
T1-3N1M0
T1-4N2-3M0
8/13
6/12
7/7

Figure 5 : SQCC-HN specific RFC response following preincubation with relevant and irrelevant tumor antigens. Responsive donors were tested following preincubation with with 0.2 mg of SQCC-HN or Mel tumor antigen extracts at 37 C, 30'. (Representative of 3 separate experiments).

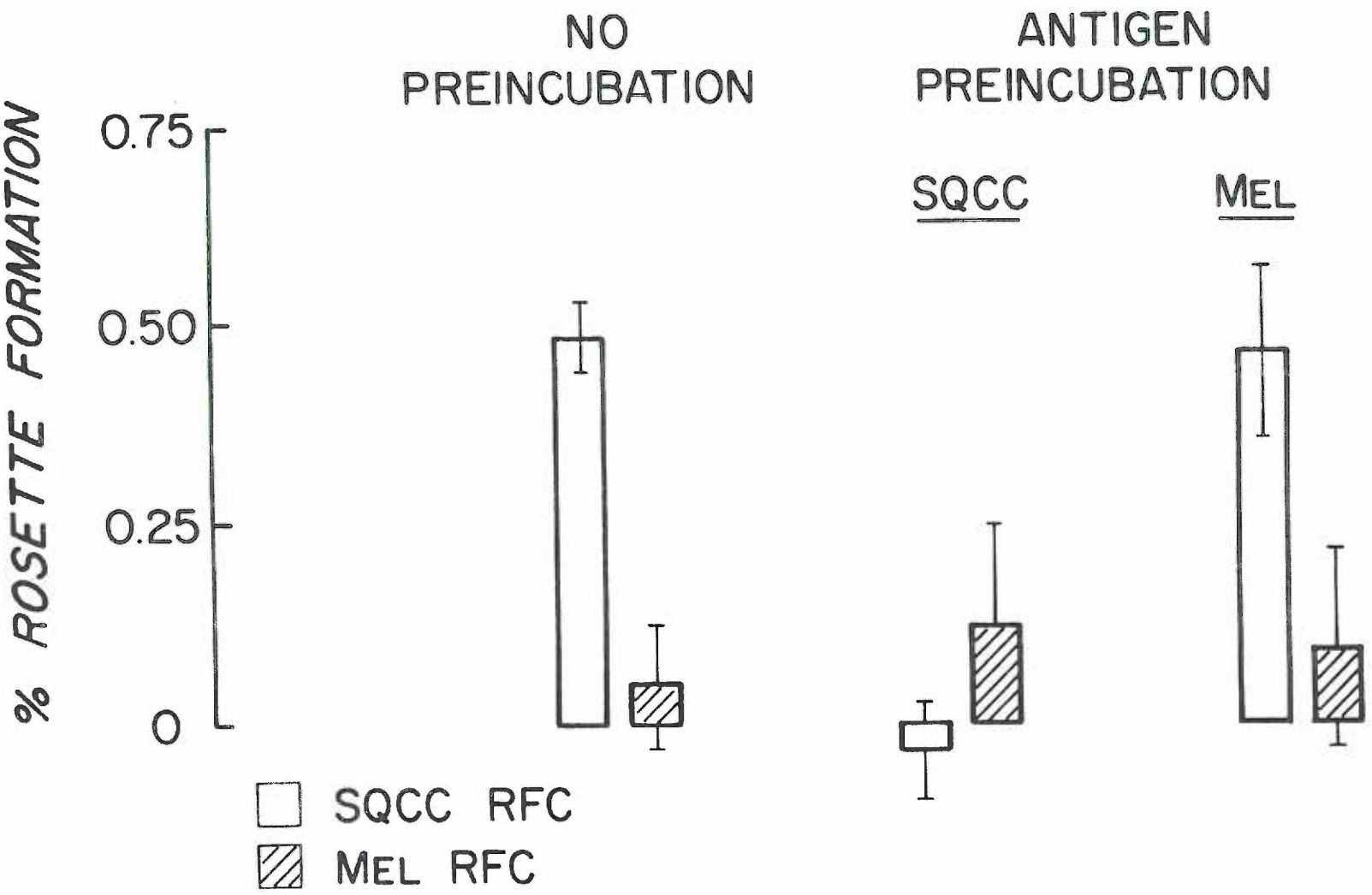

SQUAMOUS CELL CARCINOMA PATIENT
NO PREINCUBATION
ANTIGEN PREINCUBATION
SQCC
MEL
% ROSETTE FORMATION
0.75
0.50
0.25
0
SQCC RFC
MEL RFC

Figure 6 : Abrogation of SQCC-HN specific rosette forming response by overnight culture of responsive donor cells can be restored by incubation with autologous serum. The SQCC-HN RFC response of three responsive donors were tested before and after overnight culturing; and also following incubation with autologous serum for 30 minutes at 37 C.

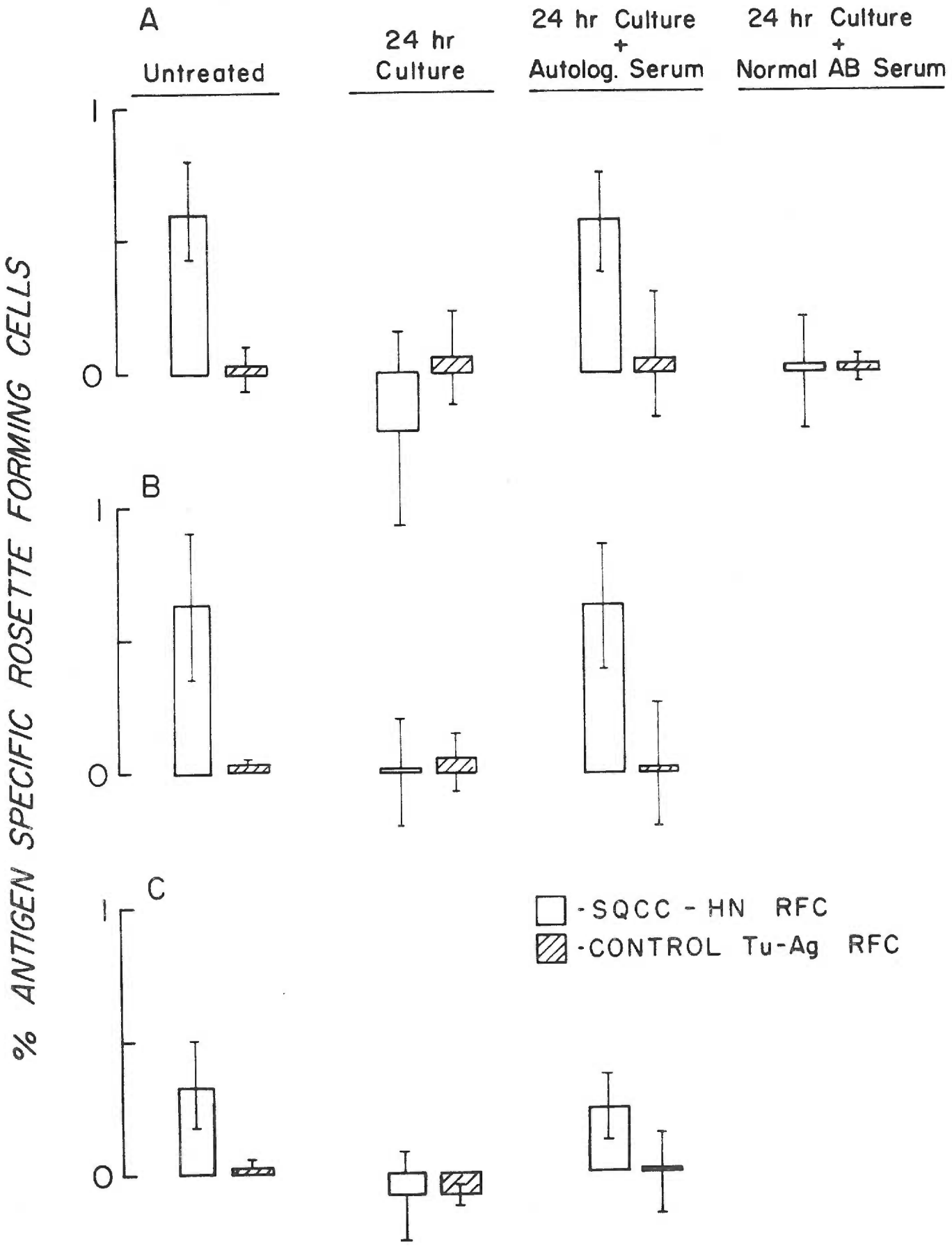

A
Untreated
24 hr Culture
24 hr Culture + Autolog. Serum
24 hr Culture + Normal AB Serum
B
C
% ANTIGEN SPECIFIC ROSETTE FORMING CELLS
1
0
-SQCC - HN RFC
-CONTROL Tu-Ag RFC

figure 7 : Restored SQCC-HN specific RFCs by autologous serum incubation can be inhibited by anti- human Ig antibodies. SQCC -HN RFC response restored after preincubation with auto logous serum was inhibited after preincubation with goat anti- human Ig $F(ab')_2$.

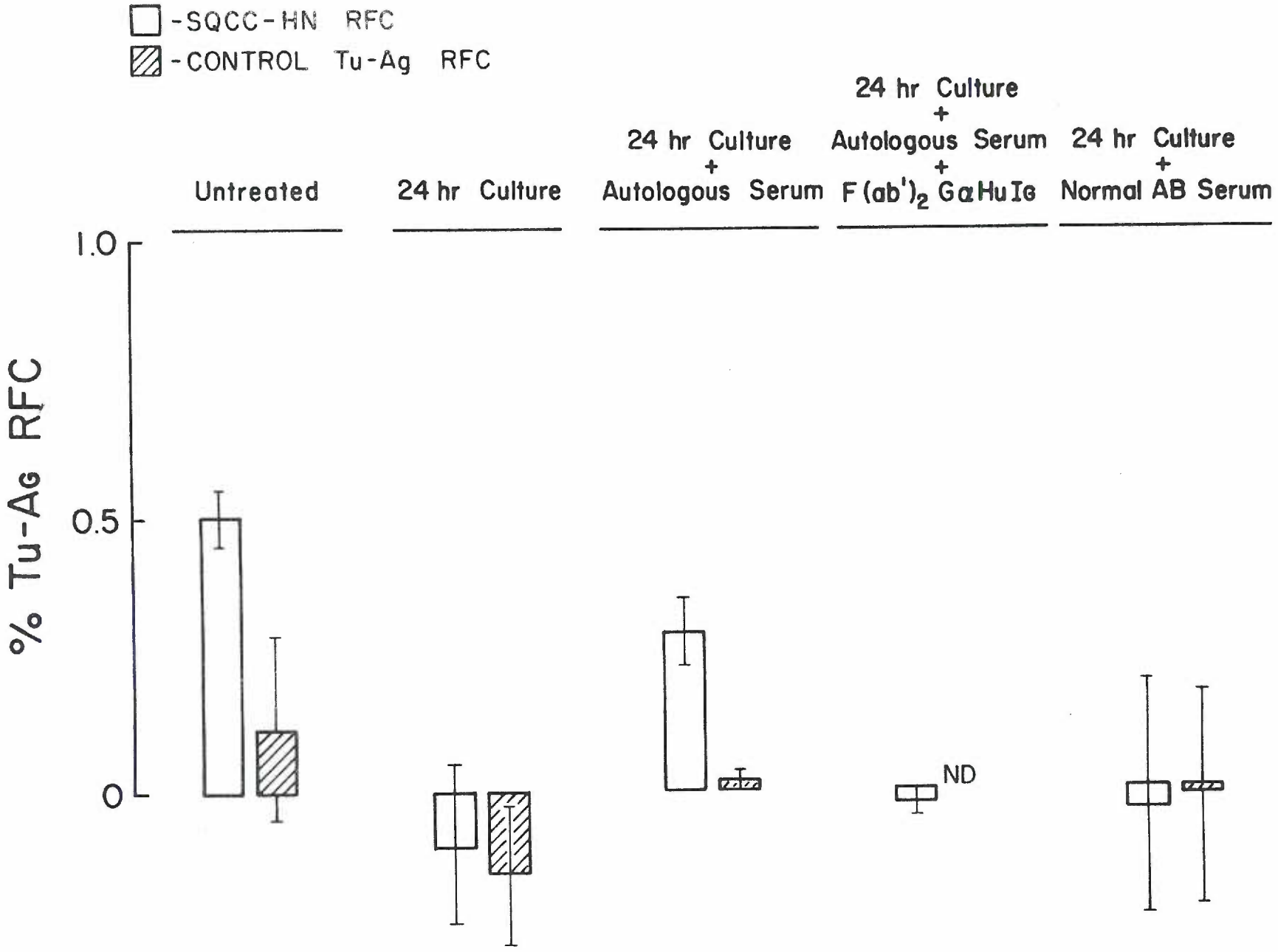

-SQCC-HN RFC
-CONTROL Tu-Ag RFC
Untreated
24 hr Culture
24 hr Culture + Autologous Serum
24 hr Culture + Autologous Serum + F(ab')2 GαHuIg
24 hr Culture + Normal AB Serum
% Tu-Ag RFC
1.0
0.5
0
ND

Figure 8: Effect of monocyte depletion on tumor antigen RFC response of SQCC reactive donor. Tumor antigen rosette forming cell response was tested before and after monocyte depletion by passing donor cells through Sephadex G-10. Data is representative of 5 separate experiments.

SQUAMOUS CELL CARCINOMA PATIENT

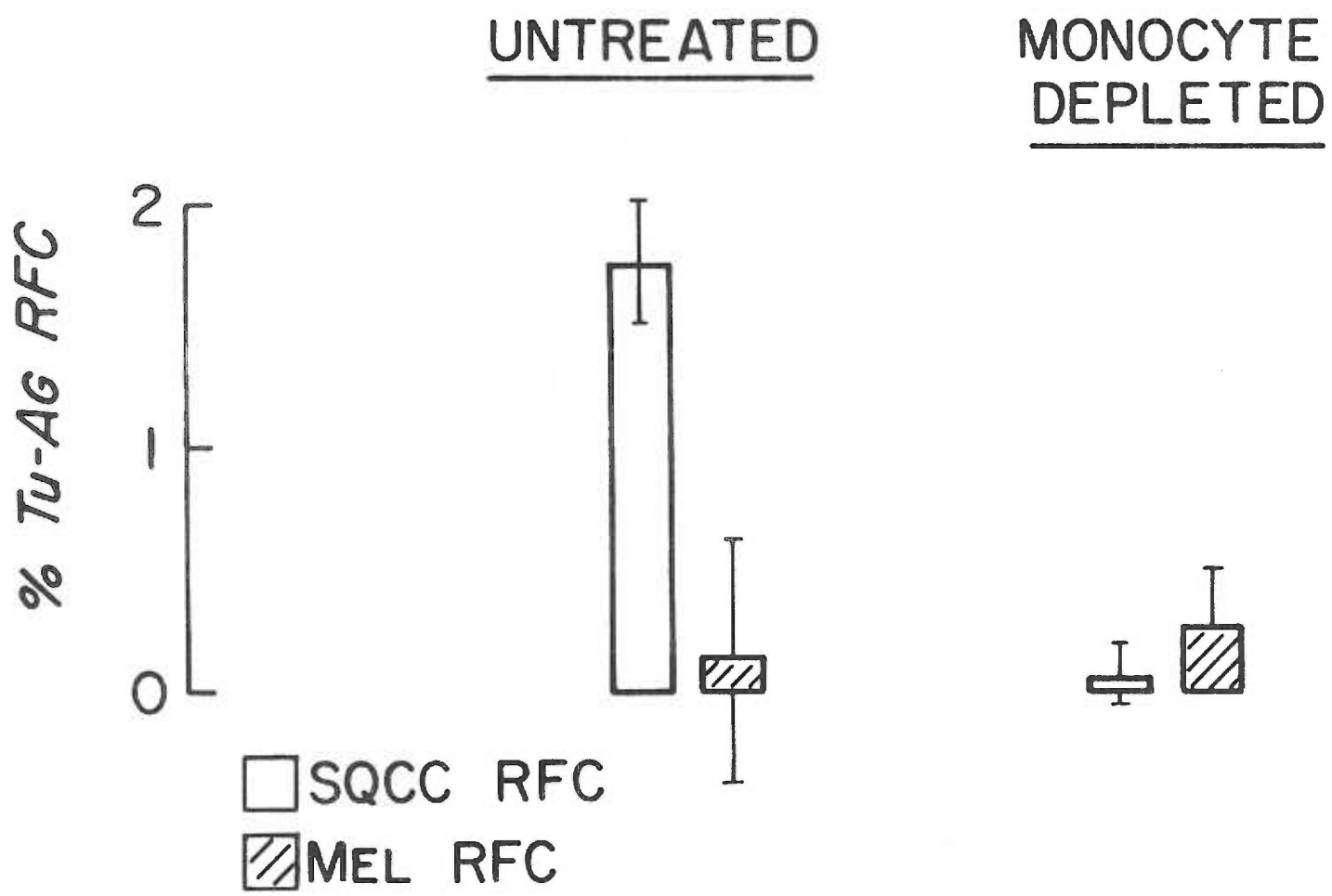


Figure 9: Identification of tumor Ag-RFC using RFC enriched fractions. RFCs are enriched using hypaque ficoll separation (4C, 400 g, 30 minutes) are then examined under fluorescent microscope. RFCs are identified as WBCs with three or more RBCs attached. Monocytes are mononuclear cells bearing monocyte morphology and are esterase positive. Data is representative of 6 total experiments.

(□): monocytes; (▨): lymphocytes

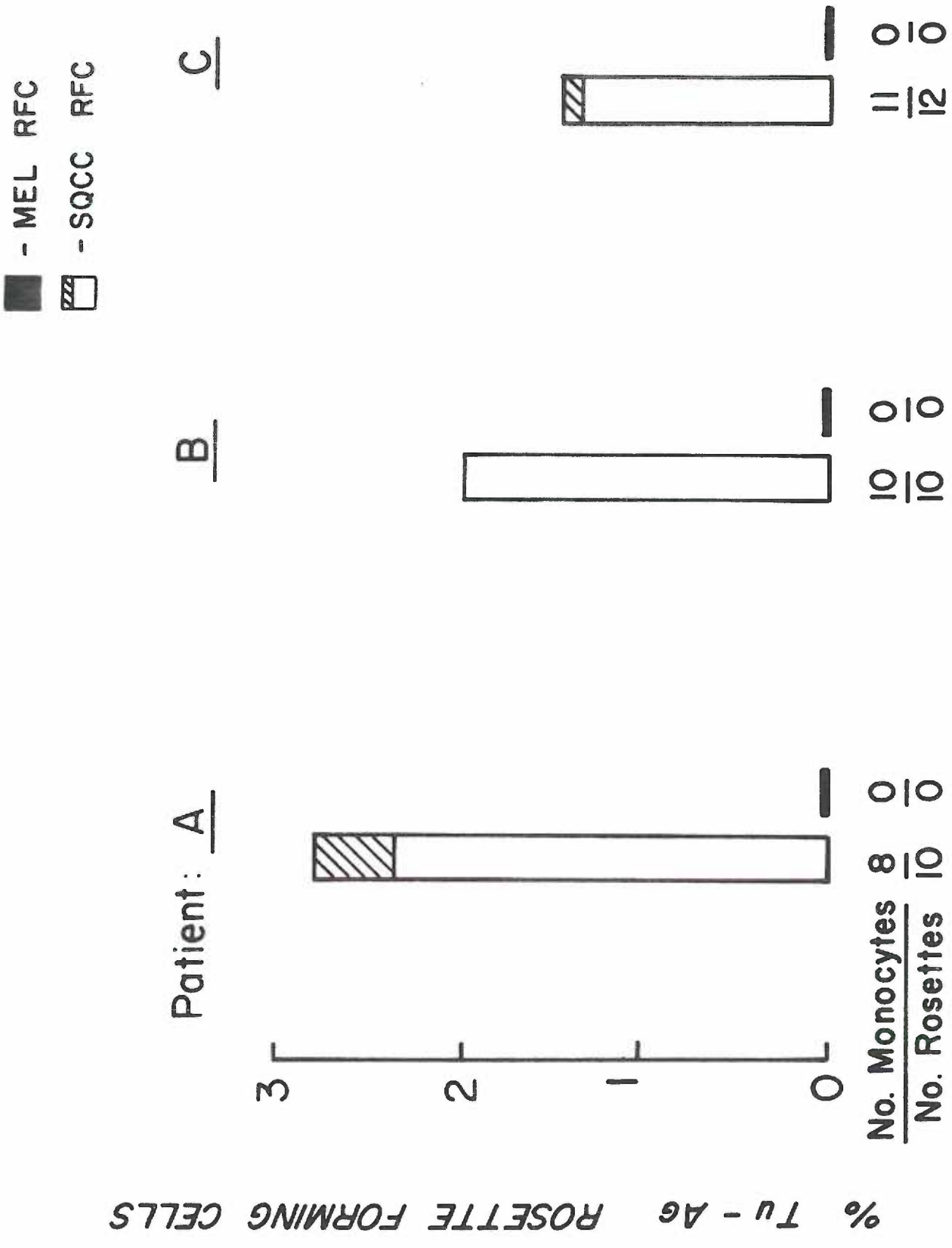
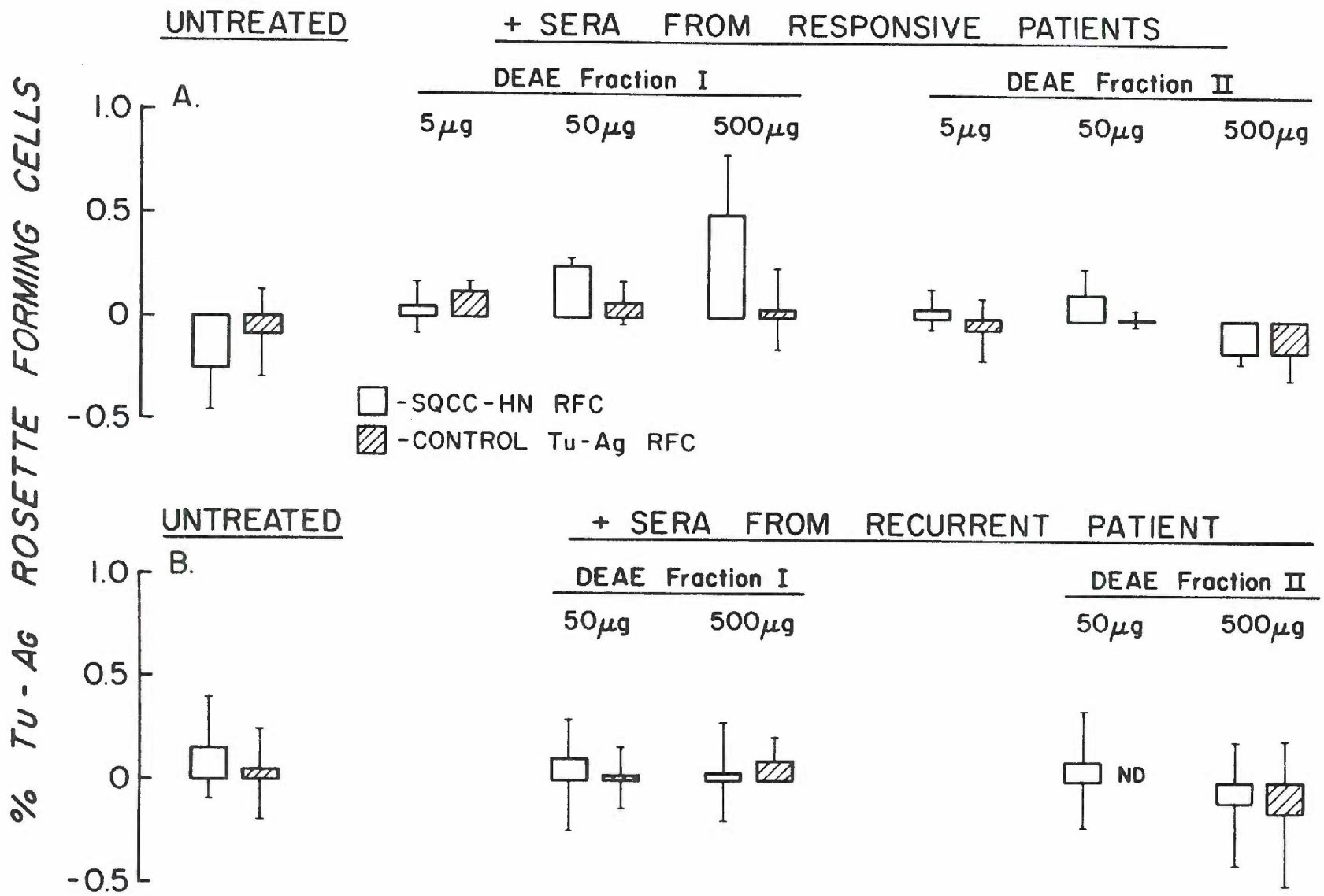

■ - MEL RFC
▨ - SQCC RFC
% Tu - Ag ROSETTE FORMING CELLS
3
2
1
0
Patient: A
B
C
No. Monocytes 8 10 0 10 10 0 11 12 0
No. Rosettes 10 10 0 12

Figure 10: Acquired rosette forming response after incubation with serum fractions from responsive and recurrent tumor patients. Pooled sera from responsive primary or recurrent tumor patients were fractionated into IgG rich (Fraction I) and IgG depleted (Fraction II) fractions by DEAE chromatography. Each fraction is preincubated with normal donor cells at the indicated concentrations at 4 C for 30 minutes to determine arming capacity for tumor specific rosette formation.


UNTREATED
+ SERA FROM RESPONSIVE PATIENTS
DEAE Fraction I
DEAE Fraction II
5μg
50μg
500μg
5μg
50μg
500μg
A.
1.0
0.5
0
-0.5
-SQCC-HN RFC
-CONTROL Tu-Ag RFC
UNTREATED
+ SERA FROM RECURRENT PATIENT
B.
DEAE Fraction I
DEAE Fraction II
50μg
500μg
50μg
500μg
1.0
0.5
0
-0.5
ND
% Tu-Ag ROSETTE FORMING CELLS

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Manuscript 3

MYELIN BASIC PROTEIN BINDING CELLS IN ACTIVE MULTIPLE SCLEROSIS

Abstract

A sensitive antigen-specific rosetting technique was used to enumerate blood cells that bind myelin basic protein (BP) in MS patients and controls. Sixteen of 23 MS patients in exacerbation but only 7 of 48 patients in remission formed elevated numbers of rosettes. Five of these 7 latter patients had recovered from an exacerbation within four months of the laboratory evaluation. Eight of 20 patients with progressive MS had BP rosette-forming cells, all being subjects with disease for > 5 years. None of 15 normal volunteers and one of 8 neurologic controls had BP rosette-forming cells. These results suggest that BP binding cells in MS are confined to patients in acute exacerbation or within four months of activity and those who have had progressive disease for at least five years.

Introduction

Multiple Sclerosis (MS) is characterized by primary demyelination of the central nervous system (CNS). Although the cause of initial demyelination is unknown, the myelin breakdown characteristically occurs within the MS "plaque," (34) and leads to the release of myelin fragments which have been observed within nearby phagocytic cells and in the cerebrospinal fluid (7,35). It has been suggested that exposure of these myelin fragments can immunize the host, stimulating an autoimmune attack against the CNS and leading perhaps, to clinical dysfunction (10,34).

Myelin is comprised of many components including lipids and basic protein (BP), a structural protein which constitutes about 40% of the total myelin protein (19). BP and several of its proteolytic fragments are highly immunogenic and when injected with Freunds complete adjuvant into various animal species, induce a paralytic disease, Experimental Allergic Encephalomyelitis (EAE) (24). Because of the immunogenic and potentially encephalitogenic properties of BP in humans, much attention has been focused on the immune response to BP in patients with paralytic demyelinating disease - especially those with MS.

Although one might expect an immune response to CNS components (including BP) in MS patients with active demyelination, skin test reactivity has not been demonstrated as yet (21,28). In vitro measurement of cellular immune reactivity to BP has produced conflicting results: several investigations have been unable to demonstrate MIF release (3,20,23,27) or lymphocyte transformation

(3,18) to neural tissue antigens. Others, however, have observed MIF production (1,25), lymphocyte transformation (8,15), increases in active T rosettes (14) or neurocytotoxicity (4) in response to CNS antigens. The specificity of these responses for MS is questionable (11,13,25,26) since other demyelinating conditions can evoke similar immune responses. However, several investigations have correlated immune responsiveness to BP with periods of clinically active MS (2,22,23).

Skin test response, lymphokine release and cellular proliferation are established correlates of immune reactivity, and all depend upon a single primary event: interaction of immune cells with specific antigen. Detection of this primary interaction (enumeration of ABC) has been successfully demonstrated in animals (9,33), but this methodology has had limited application in man because of technical difficulties (11). Recently, Tong, et al (31) developed a technique for automated cytofluorographic enumeration of antigen-binding cells. This procedure identifies peripheral cells which form specific "rosettes" with antigen-conjugated erthrocytes.

The present communication describes a clinical application of the rosetting technique described above (31): the enumeration of mononuclear cells from MS patients which can bind directly to basic protein-conjugated erythrocytes. This paper will demonstrate that most MS patients currently in exacerbation or those recently recovering from such a clinical episode have circulating mononuclear cells capable of binding specifically to BP-conjugated erythrocytes. Patients in remission, however, rarely exhibit BP binding

cells. Our results suggest the active disease process is associated with a measureable immune response which is subsequently suppressed or abrogated during periods of remission.

MATERIALS AND METHODS

Patient Population

Ninety MS patients and eight controls with other neurologic diseases were diagnosed and followed at the Portland VA Medical Center and in two major neurology clinics at the University of Oregon Health Sciences Center. Each patient was evaluated and assigned a neurological grade according to the criteria devised by Swank (29). A definite diagnosis of MS required a history of recurrent symptoms and neurological findings pointing to at least two distinct CNS lesions. MS patients were subdivided into exacerbating/remitting or progressive (lack of clearly definable relapses and remissions) stages of disease.

Blood samples from patients described above or from 16 normal volunteers were coded, and evaluation of rosette-forming cells to BP or human serum albumin (HSA) was carried out without prior knowledge of the donor's clinical status. The results were analyzed for significant increases in BP-specific rosette-forming cells, and the code was broken weekly to determine correlation of clinical status with test results.

The Rosette Forming Cell Assay

a. Separation of red cells and mononuclear cells.

Heparinized peripheral venous blood (10 ml) was obtained from patients or controls and mixed with 2 ml 6% dextran (MW > 250,000 Daltons) and incubated at 37 C for 30

minutes. The cell rich supernatant was washed twice at 160 x g for 10 minutes and resuspended in 10 ml RPMI 1640. This suspension was gently layered over 5 ml Ficoll-Hypaque gradient in a 15 ml conical tube and centrifuged at 400 x g for 30 minutes. The mononuclear cells at the interface were carefully aspirated with a pasteur pipette, washed twice and resuspended in RPMI 1640 to a final concentration of 2 x 10^6 cells/ml.

b. Preparation of antigen-conjugated red blood cells (RBC).

Chromium chloride conjugation (13): Washed RBC's (2.5% v/v in RPMI 1640, pH 7.2) were mixed with equal volumes of 0.15 mg/ml human basic protein (obtained from Dr. Marian Kies, NIH) or human serum albumin, and 0.01% $CrCl_3$ (made up in PBS at least one week previously). After four minutes incubation at room temperature, the cells were washed 3x and resuspended at 0.25% v/v in RPMI 1640 pH 7.2.

c. Quantitation of antigen-specific rosette-forming cells.

An equal volume of mononuclear cells (2 x 10^6 ml) was added to 0.25 ml of antigen-conjugated autologous red cells (0.25%) and incubated for 60 minutes or overnight at 4 C. At the time of assay, 4.5 ml (0.1 mg/100 ml) of acridine orange (Allied Chemicals Co.) in cold RPMI 1640 was added to stain cell nuclei, and the tubes were gently inverted to suspend the cells.

Aliquots of 0.25 ml were introduced into a Bio/Physics Cytofluorograf 6100A. This instrument can distinguish nucleated cells from individual and clumped red cells by

means of a laser beam (wavelength, 4880A) which causes the acridine orange stained nuclei to fluoresce in the green region (5000A). In addition, rosetted and nonrosetted nucleated cells can be distinguished by light scatter, the degree of scatter depending on the size of the particles. These two parameters (fluorescence and scatter) are visualized and the cell populations electronically quantitated on the Y and X axes of a storage oscilliscope. The percentage of antigen-binding cells was evaluated by carrying out triplicate determinations on 1-5 x 10^4 cells in 3-5 separate tubes containing mononuclear cells and HBP- or control antigen (HSA)-conjugated erythrocytes. The mean $\pm$ standard deviation for the BP and control responses were evaluated and were compared for significant differences by Student's t test.

d. Inhibition of antigen-specific rosettes.

Mononuclear cells (0.25 ml at a concentration of 2 x 10^6 cells/ml) were incubated with antigens (optimal inhibiting concentrations were determined for each antigen) dissolved in 0.25 ml of RPMI 1640. The mixture was diluted ten fold and incubated at 37 C for 30 minutes. The cells were then washed thrice. After subsequent incubation with antigen-conjugated RBCs (as above) the percentage specific RFC was calculated from the formula:

% specific rosette-forming cells (RFC) to BP conjugated RBC =

% RFC with control antigen preincubation minus HBP preincubation

RESULTS

Initial Observations

In initial studies, the percent rosette-forming cells from a group of MS patients and controls were assessed with a negative control (HSA) and to several recall antigens including tuberculin (PPD) and measles virus preparations. Antigen concentrations were based on our previously published data (31) using HSA-, KLH-, and PPD-conjugated erythrocytes. The responses of these patients and controls varied according to their previous exposures: patients with PPD reactivity had elevated levels of PPD binding cells, and most persons tested had specific rosette-forming cells to measles antigen as expected.

In further studies, the specificity of the cell binding reaction to myelin BP was assessed in 12 persons by inhibiting rosette formation with free antigen. Cells from MS patients with active disease (presumed BP reactive) were preincubated with unconjugated BP for one hour before carrying out the rosetting procedure. In each case, the preincubation step with unconjugated BP significantly decreased ($p < 0.01$) the percentage of cells which bound the erythrocyte conjugated HBP, whereas preincubation with the control antigen (HSA) had no effect. The specificity of the inhibition was further documented since preincubation with unconjugated HBP had no effect on PPD or KLH-binding cells. Neither the MS nor normal donor produced significant rosettes with HSA-RBC. In the MS patient the specific increase in rosette-forming cells to BP-RFC compared to BP preincubated rosette-forming cells is significant ($p < 0.01$).

Basic Protein Binding Cells in Patients With Exacerbating Remitting Type MS:

Seventy diagnosed MS patients with histories of exacerbating and remitting disease were evaluated for HBP rosette-forming cells. Twenty-two patients were evaluated during an exacerbation and one additional patient was evaluated twice during an exacerbation and once during a subsequent remission. The remaining 47 patients were evaluated during a clinical remission. Sixteen of the 23 patients with clinical activity at the time of examination had a significant increase in HBP-specific rosette-forming cells, whereas only 7 of 48 patients in remission were positive (Fig. 1). However, 5 of the 7 patients in remission who were positive had had clinically active disease within four months of the evaluation (data summarized in Table 1). The only sequential evaluation showed BP positive reactivity while the patient was in active disease, but showed no response when the patient was in a subsequent remission.

Basic Protein Binding Cells in Patients With Progressive Type MS:

Eight of the 20 patients with progressive MS had a positive test for BP binding cells (Fig. 1, Table I). Of the six patients with 4 years of disease, none had HBP binding cells whereas 8 of 14 with >5 years of disease had significantly elevated levels of HBP binding cells.

Basic Protein Binding Cells in Patients Without MS and in Normal Controls:

Blood samples from eight patients with neurologic complaints other than MS, and from sixteen normal controls were coded and assessed in random fashion with MS samples. The non-MS neurological control group consisted of 2 patients with stroke and one each with optic neuritis amyotrophic lateral sclerosis, nerve entrapment, migraine headaches, brain tumor, and Parkinson's disease. None of the 16 normal volunteers were positive and only one of the eight non-MS patients (a patient with nerve entrapment) showed significantly elevated levels of BP-binding cells (Fig. 1).

Discussion

The antigen-specific rosette-forming cell assay described previously (31) has allowed us to measure peripheral BP-binding cells in MS patients. The results presented above indicate a striking relationship between BP recognition (as measured by the rosette-forming cell assay) and active disabling disease in patients with exacerbating-remitting type MS. Just as striking is the rapid disappearance of measurable BP-binding cells during periods of clinical remission. Only 7 of the 48 patients in remission had BP binding cells, and 5 of these had had recent clinical activity (≤ 4 months).

Our inability to detect BP binding cells during remission may be due to the disappearance of these cells from the circulation, or to the functional loss of blocking (either specifically or non-specifically) of BP receptors by free BP or its fragments (35). Other possibilities have not been excluded. The underlying mechanisms responsible for the loss of BP-binding activity may have important therapeutic implications, especially if the BP binding cells are involved in a pathogenic mechanism. The BP-binding cell has not as yet been identified, but studies are in progress to determine cell type(s) (eg, B cell, T cell, macrophage armed with cytophilic antibody) and cell function(s) (eg, phagocytic ability, lymphokine release, or cytotoxicity in neural tissue cultures).

The mechanisms which regulate the presence or absence of BP-binding cells in patients with progressive MS are also in need of clarification. The apparent absence (or functional loss) of BP-binding cells in the first four years of progressive disease may be

due to chronic myelin breakdown and the release of excessive free BP, which could result in saturation of cellular BP binding sites. Perhaps changes in the rate of BP breakdown (and its release into the CSF) during later phases (5 years) of the disease might allow subsequent detection of BP-binding cells. Other explanations of these data have not been excluded.

The encephalitogenic potential of basic protein in animals has inspired several lines of research which have attempted to implicate this myelin structural protein in human paralytic demyelinating disease. However, the inconsistancy in demonstrating immune reactivity to BP in MS patients and the inability to distinguish whether or not immune reactivity to BP is a primary factor in the pathogenesis of the disease has prevented a clear understanding of its role in MS.

The methods used currently to detect immune reactivity to BP require a) a primary specific interaction of an immune cell with BP and b) secondary events such as proliferation (lymphocyte transformation), lymphokine release (MIF test), cytotoxicity, or an increase in T cells which can form active rosettes with sheep erythrocytes. Assessment of immunity to BP by tests which require amplification may be influenced by low numbers of responsive or recruitable cells or deficiencies in noncellular components.

The antigen-specific rosette-forming cell assay that we have developed has both theoretical and practical applications to MS. a) This test measures the primary interaction of cells with BP and does not require secondary amplification steps. b) The test

appears to be specific for BP since preincubation with unconjugated BP but not HSA, reduced BP rosette-forming cells. c) The test has statistical and technical advantages over autoradiographic techniques requiring visual enumeration of rosette-forming cells: cytofluoragraphic analysis allows 1-4 x 10^4 cells to be enumerated in triplicate for each of 3-5 replicate tubes for each variable. The degree of precision inherent in the cytofluorography analysis cannot be achieved by visual enumeration of rosette-forming cells.

Several points regarding the nature of this test remain to be illucidated. In most instances, a background level of rosette-forming cells could be measured to both HSA and BP, as well as other antigen-conjugated erythrocytes. This background may be due to nonspecific rosette formation by macrophages or by "sticky" erythrocytes, and is undoubtedly influenced by machine settings and erythrocyte concentration (32). In some instances, the percent rosette-forming cells to BP was significantly lower than those to HSA. Although the source of this variation is not known, it is possible that using a control antigen such as histones (which have a charge similar to BP) may alleviate this problem.

It is perhaps coincidental that approximately 70% of patients with active disease have BP binding cells and that from 54-72% MS patients have the HLA-Dw2 determinant (compared to only 18% of the normal population, 16,17). It has been suggested that this high incidence of the Dw2 marker may indicate a predominant form of MS which is controlled by a closely linked "MS gene" (30). Patients without the Dw2 marker may have segregated the "MS gene" from Dw2 or

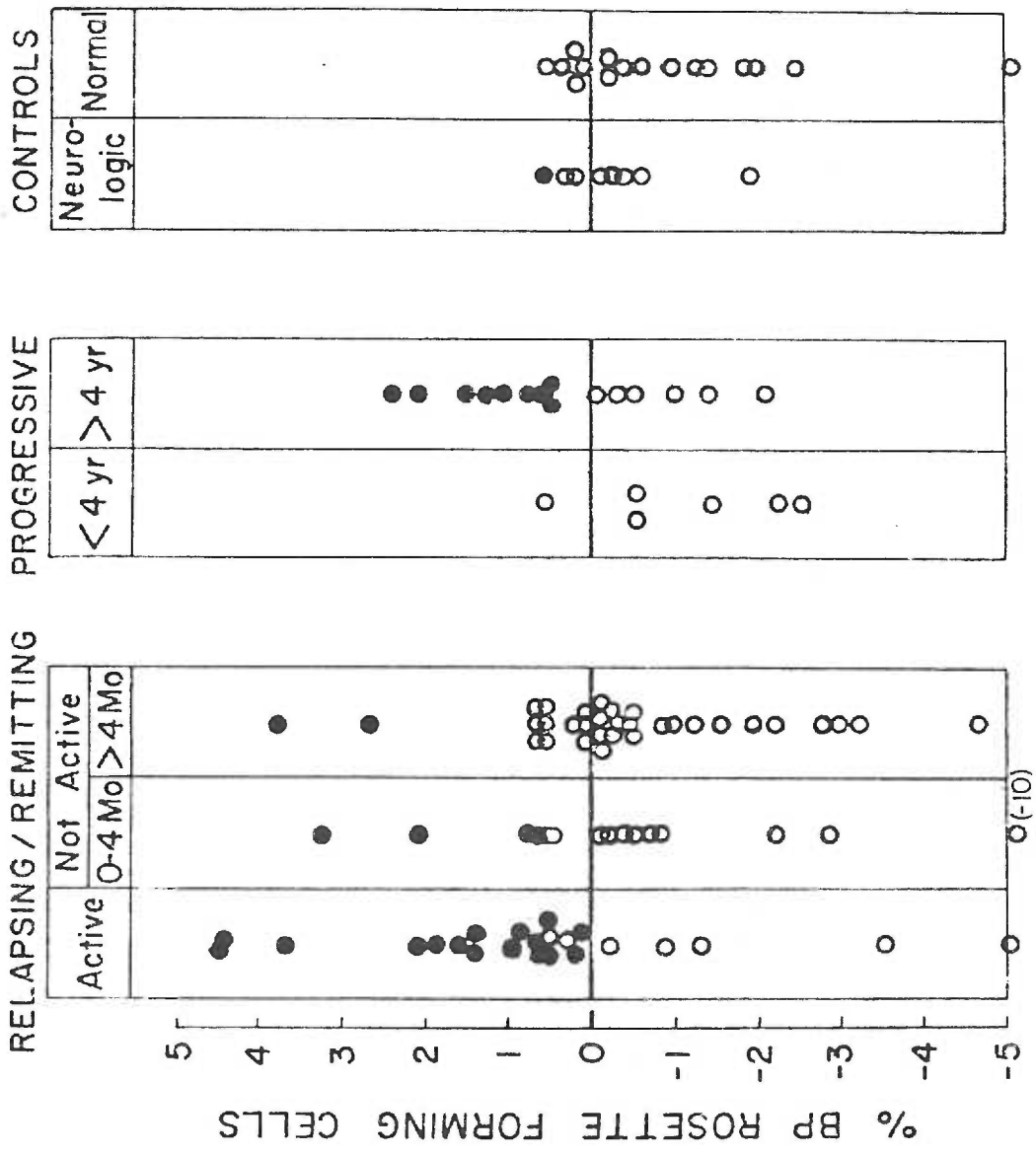
may have a different form of MS altogether.

It will be important to determine whether or not the HBP recognition is HLA linked: it is conceivable that BP recognition could be controlled, a) through an immune response gene which causes a breakdown in self tolerance, b) through a susceptibility gene which allows an immunocyte to be infected by a persistant virus leading to the expression of receptors to BP or cross-reactive antigens, or c) by other mechanisms, including specific or non-specific suppressor cells.

Table I. BP-RFC IN PATIENTS WITH MS

Clinical Status	Duration of Current	% Positive Response to BP-RFC (# Positive/# Tested)
Exacerbation		
	< 1 Mo	55 (6/11)
	1-2 Mo	83 (5/6)
	> 2 Mo	83 (5/6)
Remission		
	< 1 Mo	67 (2/3)
	1-2 Mo	25 (1/4)
	2-4 Mo	25 (2/8)
	4-8 Mo	0 (0/8)
	8-12 Mo	0 (0/8)
	> 12 Mo	12 (2/17)
Progressive		
	0-4 Yr	0 (0/6)
	5-10 Yr	57 (4/7)
	>10 Yr	57 (4/7)

Figure 1. Scatter diagram indicating the % BP rosette forming cells (BP-RFC) in patients with relapsing/remitting and progressive MS, as well as neurologic and normal controls. Solid circles indicate that the % BP-RFC was significantly elevated over control RFC ($p < 0.05$ by Student's t test).


% BP ROSETTE FORMING CELLS
5
4
3
2
1
0
-1
-2
-3
-4
-5
(-10)
RELAPSING / REMITTING
Active
Not Active
0-4 Mo
>4 Mo
PROGRESSIVE
< 4 yr
> 4 yr
CONTROLS
Neuro-logic
Normal

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Manuscript 4

ASSESSMENT OF THE MECHANISM OF THE LEUKOCYTE ADHERENCE INHIBITION TEST

ABSTRACT

This study was designed to elucidate the mechanism of the leukocyte adherence inhibition (LAI) test in man. To identify the reactive cell types, enriched leukocyte populations (dextran-separated leukocytes and Hypaque-Ficoll isolated mononuclear cells and neutrophils, as well as rosette-isolated B- and T-lymphocytes) were tested for leukocyte adherence in the absence of serum to tumor-specific antigens. LAI reactivity was not restricted to any of the enriched populations, suggesting the involvement of multiple cell types. Attempts to demonstrate soluble lymphocyte factors in the LAI mechanism have been uniformly negative. In contrast, factors in serum of immune donors were able to arm naive cells to be specifically responsive. This suggests a role for serum factors in the mechanism of LAI reactivity and partially explains the participation of multiple cell types in the responses observed. In additional studies, we could not document a correlation between the magnitude of the dermal test (delayed cutaneous hypersensitivity) and the magnitude of the LAI response in patients with squamous cell carcinoma of the head and neck. In 34 of 54 of these patients, there was agreement between the two tests (both positive, 27 of 54; both negative, 7 of 54). In the remaining 20 patients, the dermal test was 5mm while the LAI test was negative (< 30% inhibition).

INTRODUCTION

The LAI test developed by Halliday and Miller (5) and modified by others (1,6,7,9,12,13), apparently measures specific immune responses to tumor extracts in humans. However, there is considerable disagreement among several laboratories concerning the mechanism of the LAI test. Maluish and Halliday (10), Powell et al, (12), and Holt et al, (8) suggest that the LAI test is mediated by a soluble T-cell factor and that the indicator cells include lymphocytes and/or neutrophils. Grosser et al (4), and others (3,6,11) contend that T-cell factors are not involved and that LAI reactivity is mediated by cytophilic antibody. Additionally, their evidence implicates circulating monocytes as both the antigen-responsive and indicator cells.

Burger et al. (1) have utilized the LAI assay to assess tumor-specific immunity in patients with melanoma, squamous carcinoma, and other types of cancer. In the following report, we describe experiments which support the presence of a serum arming factor and the involvement of a variety of cell types in the LAI reaction. Additionally, we present data which examine the relationship between skin test response and LAI reactivity in squamous cell carcinoma patients.

MATERIALS AND METHODS

Tumor Extract

Tumor tissues were obtained within 4 hours of excision from melanoma, squamous cell carcinoma, and neuroblastoma patients with histologically proven cancer. Tumor antigen extraction with 3 m KCI has been described in detail elsewhere (1).

Experimental Subjects

Experimental subjects were selected according to LAI responses to melanoma, squamous carcinoma, or neuroblastoma extracts. Patients were tumor-free for at least 3 months after therapeutic intervention and were considered immunocompetent by their ability to respond to one or more common microbial skin test antigens or mitogenic agents *in vitro* and/or *in vivo*. Nonimmune donors were healthy adults who had no previous contact with tumor extracts or patients and had negative LAI values to the tumor extracts.

Fractionation of Mononuclear and Polymorphonuclear Cells

Heparinized (10 units of heparin per ml) blood (5 ml) was combined with 1 ml of 6% dextran (M.W.>250,000) and incubated in an inverted plastic syringe for 30 min. at 37 degrees. The cell-rich supernatant fluid was centrifuged at 160 g for 10 min, resuspended in twice the original volume of RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), and washed in RPMI 1640 at 160 g. The

washed cells were incubated in 2 ml of cold 0.85% NH_4Cl for 10 min.at 4 C to lyse erythrocytes. Medium (10 ml) was added to the suspension to reestablish isotonicity, and the cells were centrifuged at 160 x g for 10 min. and were resuspended at 5 to 10 x 10^6 cells/ml.

E-Rosette Separation Procedure

Fresh SRBC (Prepared Media Inc., Tualatin, Oregon) were washed 3 times immediately before use and adjusted to a final concentration of 1% (v/v) in RPMI 1640. E-rosettes were prepared by mixing equal volumes of 1% SRBC and mononuclear cells at 5 x 10^6/ml in a 50-ml conical tube as described by Dean et al. (2). The mixture was immediately centrifuged at 200 g for 5 min. and incubated at 4C for 1 hour. After gentle resuspension, the reaction mixture was gently layered over a Ficoll-Hypaque gradient at 4 C and centrifuged at 400 g for 30 min. Both the unrosetted interphase and the rosetted pellet fractions were washed again and resuspended at 5 x 10^6 /ml. The rosetted lymphocytes were recovered after incubation in 2 ml of 0.85% NH_4Cl for 10 min. at 4 C to lyse erythrocytes. Medium (10 ml) was added to the suspension to reestablish isotonicity, and the cells were washed twice at 160 g for 10 min. and resuspended at 5 x 10^6 cells/ml in RPMI 1640.

EAC-Rosette Separation Procedure

EAC-rosettes were prepared by a modification of the procedure described by West and Herbeman (14). Sheep erythrocytes were washed

3 times and resuspended at a concentration of 1% (v/v) in RPMI 1640. An equal volume of RPMI 1640 containing trypsin at 1 mg/10 ml was added, and typsinization was carried out at 37 C for 30 min. The typsinized SRBE were washed 3 times and resuspended at the original volume in PBS. An equal volume of 19S rabbit anti-SRBC at a 1:20 dilution (courtesy of Dr. Gerrie Leslie, Department of Microbiology and Immunology, University of Oregon Health Sciences Center, Portland, Oregon) was added, and the mixture was incubated for 30 min. at 37 C. The erythrocyte antibody mixture was washed twice with PBS at 160 x g for 10 min. After resuspension to the original volume, an equal volume of human AB serum at a 1:300 dilution was added as a source of complement, and the mixture was incubated at 37 for 30 min. The erythrocyte antibody complement complexes were washed twice with PBS and then resuspended in RPMI 1640 to their original volume.

Equal volumes of erythrocyte antibody complement and the mononuclear cells (at 5 x 10^6/ml) were mixed and left undisturbed at room temperature for 2 hours to form EAC-rosettes. The EAC-rosettes and the unrosetted cells were recovered by Ficoll-Hypaque fractionation by the same procedure described above for E-rosettes.

Leukocyte Adherence Inhibition

The LAI assay used was a variation of the method described by Hallidan and Miller (5) and modified by others (1, 6, 7, 9, 12, 13).

Preparation of Donor Cells: LAI was carried out using dextran-sedimented leukocytes and mononuclear and PMN fractions, as well as

T- and B-enriched and T and B depleted fractions as prepared by E- and EAC-rosettes (described above).

Preincubation Step: For each antigen or antigen dilution to be tested, 1.5 to 3.0 x 10^6 cells in 0.15 ml were added to a plastic tube (Falcon 2054) containing 0.15 ml of antigen and 0.3 ml of medium, and the suspension was incubated at 37 C for 30 min. As a negative control, 2.5 x 10^6 cells in 0.25 ml were combined with 0.75 ml medium in a plastic tube and incubated under the same conditions.

Adherence Step: The preincubated cell suspensions were transferred with plastic-tipped Eppendorf pipets (Brinkman, Westbury, N.Y.) to wettable plastic tubes (Falcon 3033; Bioquest, Cockeysville, Md.) to test for leukocyte adherence. Four replicates of control cells and 2 replicates of test cells (0.2 ml/tube) were prepared from each preincubated tube. The number of cells in 2 of the control tubes were determined on a Coulter particle counter (Coulter Electronics Inc., Hialeah, Fla.) after residual erythrocytes were eliminated by lysis. This count established the total number of cells before the adherence process began. The total remaining control and test samples were further incubated for 1 to 2 hours at 37 C to allow adherence. These samples were then diluted in 10 ml Isoton II (Coulter Diagnostics, Hialeah, Fla.) and the cells were counted as above. The LAI was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Nonadherent cells}_{(\text{test - control samples})}}{\text{Total control cells} - \text{Nonadherent cells}_{\text{control}}} \times 100$$

Inasmuch as LAI values were usually less than 20% in control subjects and in patients with tumors of different histological types, responses of 20 to 30% were considered questionable, and those greater than 30% were considered positive.

Quest for Soluble Mediators of LAI Reactivity

To look for mediators released by immune cells, 0.15 ml immune cells was incubated with appropriate concentrations of 0.15 ml antigen and medium as described in the preincubation step above. Supernatant fluids of these mixatures were obtained by centrifugation at 200 g for 10 min. Control supernatants were generated in a similar manner by incubating immune cells without antigen. The supernatants were transferred to plastic tubes containing 0.15 ml recipient cells and further incubated for 20 min. at 37 C . The incubated mixtures were then treated in the same manner as described in the adherence step above to determine the percentage of LAI recipient cells.

Mixing of Leukocytes from Immune and Nonimmune Donors

Mononuclear cells from immune and nonimmune donors were prepared by Ficoll-Hypaque fractionation as described. After reconstitution of the cells in medium, they were mixed in the following proportions of immune to nonimmune cells: 100:0, 90:10, 75:25, 50:50, 25:75, 10:90, 1:99, and 0:100. Each mixture was independently tested for LAI reactivity.

Evaluation of Serum Factors for Effects on LAI Reactivity

Serum was evaluated for either arming or blocking effects on LAI reactivity using a preincubation step. To assess arming, serum (in a final dilution of 1:2) was incubated with LAI nonreactive cells for 30 min. at 37 C prior to the adherence step in the standard LAI procedure. The incubation mixture was washed (160 x g for 10 min.), and the cells were tested for LAI reactivity as described. To assess blocking, serum (in a final dilution of at least 1:2) was incubated with LAI responsive cells as described for arming experiments. The cells were washed and the LAI test was conducted as described above.

RESULTS AND DISCUSSION

Cells Involved in the LAI Test.

Although tumor-specific LAI reactivity can be demonstrated in both tumor patients and cancer-free immune donors by the modified LAI assay, the nature of the responding/reacting cells involved is still a controversial issue. In the following experiments, enriched cell populations from previously identified immune donors were used to attempt to define the reactive cell types. Responses from 3 donors to melanoma extract were measured using dextran-separated leukocytes and Ficoll- Hypaque - fractionated mononuclear and polymorphonuclear cells. The mean LAI response (± S.E.) of each fraction from at least 4 concomitant determinations is presented in Figure 1. In each cell fraction tested, LAI reactivity to melanoma was retained. However, the Ficoll-Hypaque-separated mononuclear cells elicited higher mean LAI reactivities (p 0.05 in Donors 1 and 2) than did the dextran-separated leukocytes. Mean responses of the polymorphonuclear fractions, on the other hand, were consistently lower than those of unfractioned leukocytes, but this difference was only significant ($p < 0.05$) in Donor 1. When mononuclear and polymorphonuclear cells were reconstituted according to their respective percentages after Ficoll-Hypaque fractionation, the LAI reactivity observed in unfractionated leukocytes was restored. In all 3 experiments, the differences in LAI reactivity in the reconstituted and unfractionated cells were not statistically signigicant. Antigen specificity was retained by all of the cell fractions

tested, since no significant responses were detected to the control antigen, a squamous carconoma extract.

Since the mononuclear cell fractions appeared to produce an enhanced LAI response, we examined the LAI reactivity of sub-populations which had been depleted of T- or B-cells. The results of 1 of 4 such studies using cells from a melanoma-immune donor are presented in Figure 2. Depletion of T-cells by E-rosettes (Bar E) or of B-cells by EAC-rosettes (Bar G) did not reduce or abolish LAI responsiveness. The LAI response was retained in the rosetted B-cell populations (Bar F) and was significantly elevated (p 0.05) in the E-rosetted T fraction. However, there was no corresponding elevation in LAI response in the T-enriched fraction after B-cells were removed by EAC rosetting (Bar G). These results cannot clearly distinguish whether the increased response by E-rosetted T-cells implicates T-cells as indicator/responder cells in this assay or simply represents artifacts created by the E-rosetting procedure.

Soluble Mediators in the LAI Test

. With the observations above the (a) LAI reactivity is observed in all of the cell populations examined and (b) greater than 40% of the cells participated in the glass-adherence inhibition phenomenon (data not shown), it seemed likely that the LAI response involved more than a single type of responder-indicator cell. To determine whether soluble mediators which could activate bystander cells were involved in the reaction, supernatants obtained after preincubating immune cells with the appropriate antigens were

assessed for adherence inhibition activity on nonimmune cells. In 5 experiments using supernatants from melanoma-immune cell, little or no change in adherence inhibition of normal cells was observed (Table 1). These data suggested that the LAI response was not mediated by soluble factors which could be detected in this fashion. However, the requirement of cell-to-cell contact or the possible participation of a short-lived factor which requires immediate absorbtion by adjacent cells could not be excluded. To address this issue, we examined the LAI reactivity of nonimmune mononuclear cells mixed with various proportions of immune cells. In each of 3 experiments (Figure 3), the LAI response was assessed using mixtures of nonimmune cells containing 1, 10, 25, 50, 75, 90, and 100% immune cells. In all cases, the extent of LAI response could be accounted for by the percentage of immune cells added to the mixture. When the data were evaluated by linear regression analysis, the percentage of LAI showed a positive correlation with the percentage of immune cells present ($r=0.97$, 0.92, and 0.98, respectively), which was highly significant ($p < 0.01$) in all cases. The linear relationship indicates that the cells are responding in an independent fashion with little synergistic effect. Thus, the LAI responses measured in the cell pools were probably mediated by direct participation of immune cells with little or no recruitment of nonimmune cells. We considered the additional possibility that a soluble substance secreted by immune cells in limited quantities mediated the inhibition reaction in the mixing experiments. If this were the case, the percentage of LAI would still be directly

correlated to the percentage of immune cells (as was observed), but the nonadherent cells could be recruited from immune and nonimmune populations. However, preliminary mixing experiments using selectively labeled cell populations suggested that nonimmune cells were not recruited into the reaction.

From the experiments above, it is likely that multiple cell types are involved in our modified version of the LAI test. Cell fractions enriched in mononuclear cells (> 95% lymphocytes) and polymorphonuclear cells retain LAI reactivity, and mononuclear subfractions are still LAI-responsive even after T- or B-cell depletion. The involvement of many cell types and the participation in the LAI test by 40 to 75% of the total added cells in all of the enriched fractions examined do not support the contention of Marti et al. (11) that the monocyte is the sole reactive cell type. Contrary to reports demonstrating the presence of soluble mediators from immune lymphocytes (8,10,12) our experiments using immune supernatants or direct mixtures of immune with nonimmune cells did not result in detection of mediators of LAI reactivity.

Serum Factors in the LAI Tests

Due to the participation of a high percentage of cells of multiple types in the LAI reaction, we investigated the possibility of serum factors (antibody) in the mechanism of adherence inhibition. Serum from LAI-responsive subjects armed cells from LAI-negative individuals to respond to tumor antigens in a specific fashion (Table 2). The arming factor in some sera was shown to be

relatively potent since dilutions as high as 1:100 were still effective. Since the serum arming effects appeared to be specific, it is likely that immunoglobulin is involved in the LAI mechanism. This factor may be similar to the arming factor previously described by Marti et al. (11). In addition, Howell and Goldrosen (personal communication) found that "arming" of normal mouse peritoneal cells by "hyperimmune" artisera led to strong tumor-specific LAI responses. Together with the observations above concerning multiple cell types and the lack of soluble factors, a mechanism similar to the phenomenon now referred to as antibody-dependent cellular cytotoxicity (in that null cells, monocytes, and lymphocytes become "armed" and can act as effector cells) is suggested.

Although serum from responsive patients routinely armed normal cells to be specifically responsive, serum from LAI-negative patients did not. Moreover, serum from these patients inhibited LAI reactivity of responsive cells. The specificity of the LAI-blocking sera was investigated by using cells which were responsive to both melanoma and squamous cell carcinoma antigens. The blocking sera inhibited LAI reactivity of both autologous and heterologous cells to squamous antigen, but not melanoma antigen (Table 3). This serum and all other LAI-blocking sera tested did not inhibit mitogen-induced lymphocyte proliferation (data not shown).

The role of blocking factors in the mechanism of the LAI test may be an important consideration. It seems reasonable that because of technological differences between labortories some investigators

may be measuring the combined effects of cells and serum factors, whereas others could be measuring cellular effects alone. This could partially account for discrepancies between laboratories on the question of the persistence or decline of LAI responses in various clinical settings.

Correlations of LAI with Dermal Testing

Burger et al. (1) previously published a strong correlation between LAI reactivity and dermal testing to melanoma antigen (1). In patients with squamous cell carcinoma of the head and neck, although both DCH reactions and LAI tests appeared to reflect tumor specificity, a correlation between the magnitude of the dermal test (DCH) and the magnitude of the LAI response could not be documented. In addition, in 34 of 54 of these patients there was agreement between the 2 tests (both positive, 27 of 54; both negative, 7 of 54). In the remaining 20 patients, the dermal test was > 5mm while the LAI test was negative (< 30% inhibition). This is not completely surprising since this is an attempted correlation between an in vivo (DCH) and an in vitro (LAI) assessment of reactivity. One consideration should be that the positive dermal tests in the patients with negative LAI's may be false positives. An additional consideration is that the LAI test may not be measuring cell-mediated phenomenon. If this is the case, absolute agreement with DCH reactions or in vitro correlates of DCH reactivity (leukocyte migration inhibition, etc.) could not be expected.

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Effects of Supernatants from Immune Cells on LAI Reactivity

Experiment	Responding cells	% LAI reactivity to Melanoma extract	Squamous cell carcinoma extract	Neuroblastoma extract
1.	Immune cells	62	23	-[a]
	Nonimmune cells	32	16	-
	Supernatants immune + nonimmune cells	34	30	-
2.	Immune cells	62	23	-
	Nonimmune cells	22	56	-
	Supernatant immune + nonimmune cells	20	63	-
3.	Immune cells	56	-	6
	Nonimmune cells	5	-	0
	Supernatant immune + nonimmune cells	13	-	3
4.	Immune cells	82	0	-
	Nonimmune cells	22	18	-
	Supernatant immune + nonimmune cells	32	10	-

[a]: not done

Table 2

Arming of Normal Cells with Serum from LAI Responders

Test[a] cells	Serum	% LAI to Melanoma antigen 4 ug/test	2 ug/test	1 ug/test	Squamous antigen (4 ug/test)
RS	None	0	0	0	
	Auto[b] (1:2)[c]	12	0	0	
	Mel-A (1:2)	36	15	3	
	Mel-B (1:2)	55	6	3	
	Mel-C (1:2)	60	60	10	
RS	Auto (1:2)	0	0	0	0
	Mel-C (1:10)	50	22	3	0
	(1:50)	44	18	11	13
	(1:100)	54	30	9	0
AT	Auto (1:2)	12	12	0	0
	Mel-C (1:2)	57	33	11	6
	(1:100)	40	40	16	0
	(1:1000)	30	26	7	0

[a]Cells from normal donors RS and AT

[b]Auto, autologous serum; Mel-A, -B, and -C, sera from melanoma patients A, B and C respectively.

[c]Numbers in parentheses, serum dilution.

Table 3

Blocking of LAI Reactivity from Patients with Negative LAI Values

Patient cells	Serum	% of LAI	
		Melanoma antigen	Squamous antigen
EY (SQ)[b]	None	77	58
	EY (SQ)	71	50
	DL (SQ)	77	14
DL (SQ)	None	100	55
	DL (SQ)	95	16
	EY (SQ)	92	61

[a]DL serum will not block phytohemaglutinin responsiveness, and phytohemagglutinin-blocking sera will not block LAI reactivity (EY) cells).

[b]SQ, squamous cell carcinoma patient.

Figure 1 : LAI response of 3 melanoma-immune donors (melanoma reactivities, left; and squamous cell carcinoma reactivities, right). (□): dextran sedimentation leukocytes; (▨): Ficoll-Hypaque mononuclear fraction; (▥): Ficoll-Hypaque PMN fraction, (■): reconstituted leukocyte fraction.

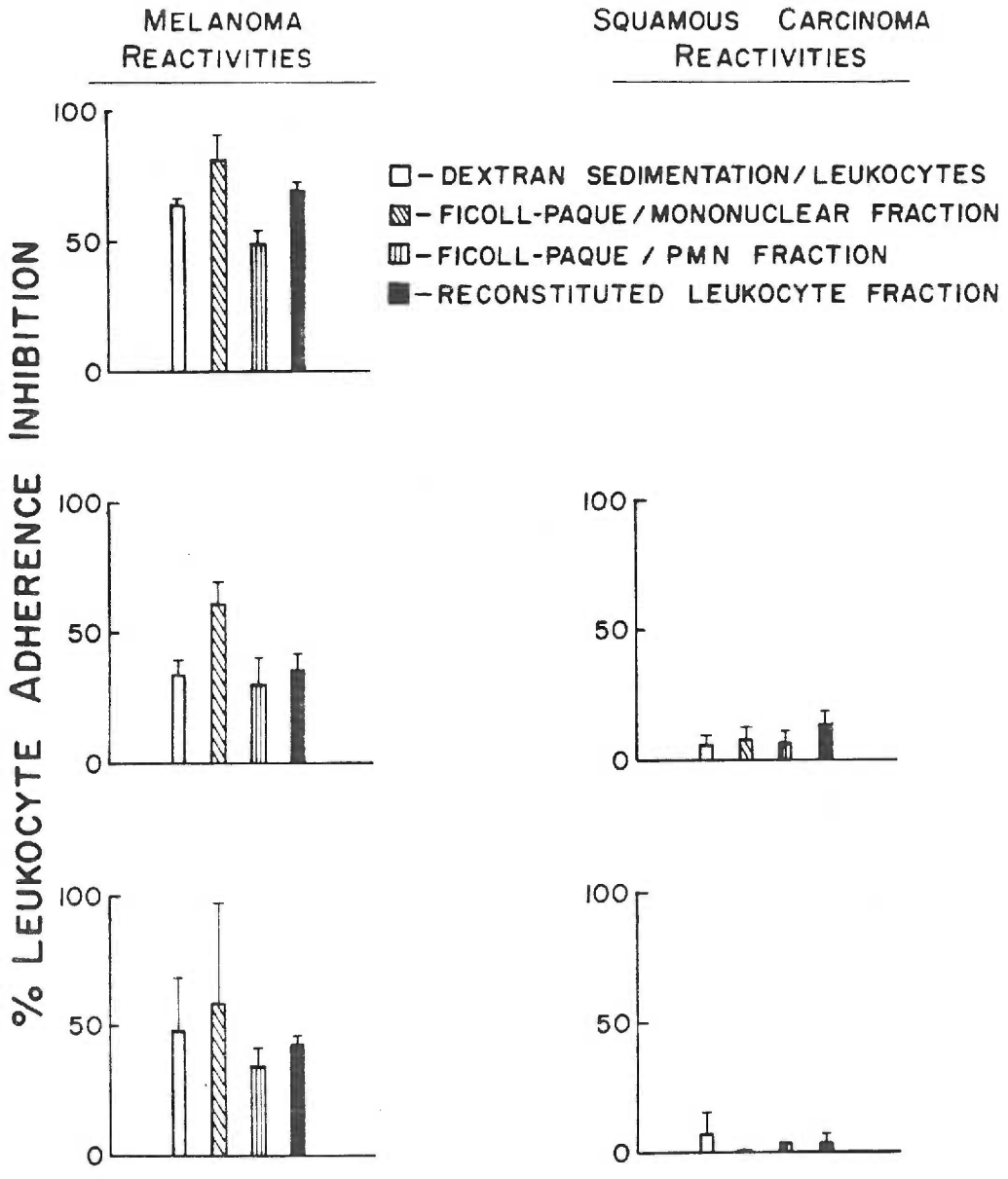
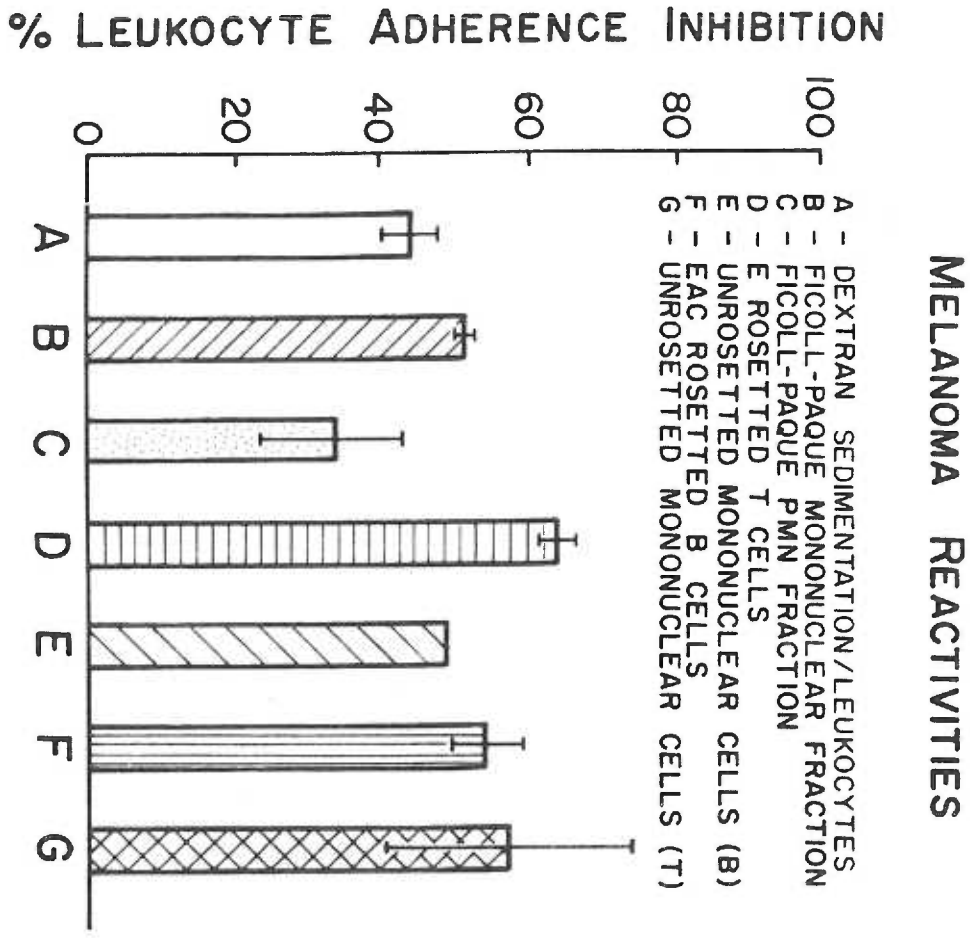
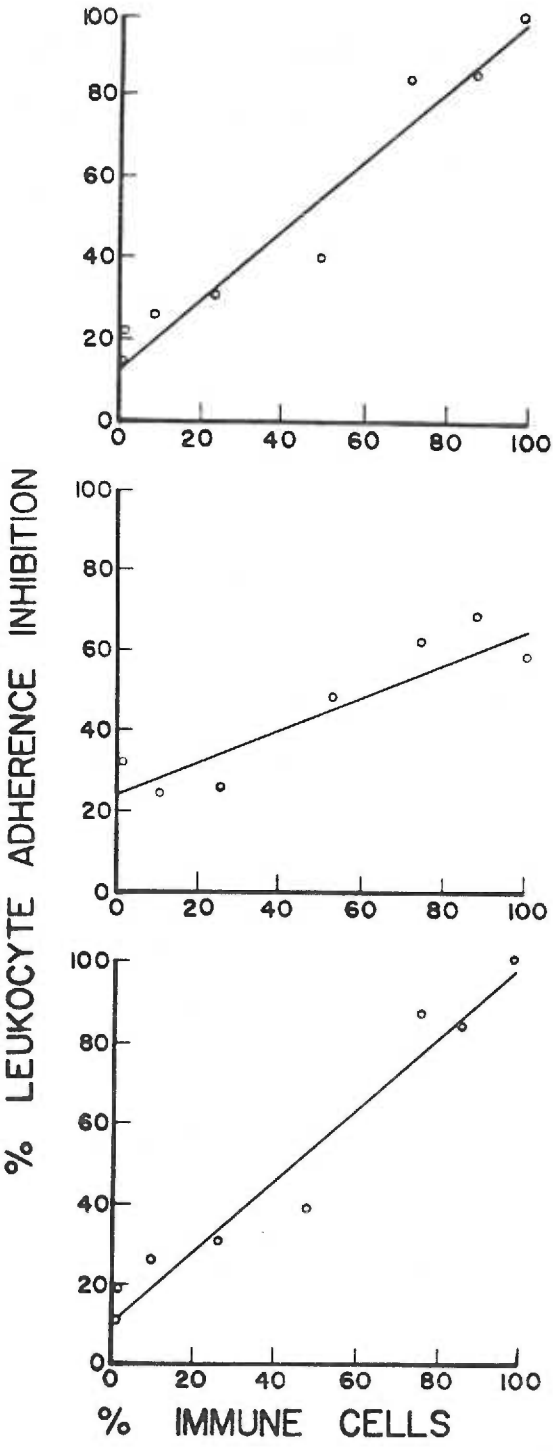

MELANOMA REACTIVITIES
SQUAMOUS CARCINOMA REACTIVITIES
□ – DEXTRAN SEDIMENTATION / LEUKOCYTES
▨ – FICOLL-PAQUE / MONONUCLEAR FRACTION
▥ – FICOLL-PAQUE / PMN FRACTION
■ – RECONSTITUTED LEUKOCYTE FRACTION
% LEUKOCYTE ADHERENCE INHIBITION
100
50
0

Figure 2: LAI response of enriched leukocyte fractions (melanoma reactivities). A. dextran sedimentation leukocytes; B. Ficoll-Hypaque mononuclear fraction; C. Ficoll-Hypaque PMN fraction; D. E-rosetted T-cells; E. unrosetted mononuclear cells (B-cells); F. EAC-rosetted B cells; G. unrosetted mononuclear cells (T-cells).



MELANOMA REACTIVITIES

Figure 3: Linear regression analyses of percentage of LAI response compared to percentage of immune(responsive) cells.


% LEUKOCYTE ADHERENCE INHIBITION
% IMMUNE CELLS
0
20
40
60
80
100

DISCUSSION AND SUMMARY

I have developed a rosette forming cell technique for routine evaluation of antigen binding cell response in humans. The use of laser cytofluorometry facilitates evaluation of a donor's antigen specific rosette forming response to a panel of antigens. Triplicate sampling of the rosette forming response (per 10^4 cells) to each antigen can be evaluated within a short period of time. The % of rosette forming cell detected in this manner correlated with that determined by conventional method of microscopic enumeration (Manuscript 1). The cytofluorometric technique,however, represents a less strenuous and statistically advantageous approach compared to the conventional techniques.

Greater than 90% (19/21) of PPD or KLH immunized donors evaluated have detectible antigen specific rosette forming cells (RFCs) to the relevant antigen (Manuscript 1). Reactivity is restricted to the antigen to which the donor was immunized, and specific rosette formation is not demonstrated among control donor cells, after background correction. Specificity can be additionally demonstrated by inhibition of the RFC response by preincubation with the relevant antigen, but not by control antigens.

Tumor specific rosette formation is detected in tumor patients using this technique (Manuscript 2). Autologous erythrocytes are conjugated with various 3M KCl tumor antigen extracts with previously demonstrated functional specificity (Manuscript 4). Patients with primary malignancy of squamous cell carcinoma of the head and neck (SQCC-HN) examined in this manner showed a high

frequency of responsiveness (70%, 22/30) to tumor extract of the same type, but did not respond to other tumor antigen extracts. Conversely, reactivity among melanoma patients is restricted to melanoma antigen extract, but not to SQCC-HN or any other tumor antigens (Appendix D). SQCC-HN specific rosette formation is inhibited by preincubation with the same tumor antigen, but not by other tumor antigen extracts. These observations indicate that the rosette forming assay measures tumor specific recognition, instead of reactivity against contaminating antigens, such as carcino-embryonic or HLA antigens common to tumor antigen extracts (11,12).

The in vivo relevance of the tumor specific rosette forming response is reflected by the dichotomy of responsiveness among patients at different stages of malignancy. Contrary to the responsiveness of treated SQCC-HN patients that have remained tumor free (RFC'+' 5/6; 83%,) or those with untreated primary malignancy (RFC'+'22/30; 73%), recurrent patients with the same malignancy are unreactive (RFC'+' 0/9; 0%). While it is not known if unresponsiveness is the cause or result of tumor recurrence, it appears that these patients have lost the capacity of tumor specific recognition, as measured in this assay. In longitudinal studies on one patient, tumor specific rosette formation can be demonstrated before and after resection of a primary malignancy, but was absent upon tumor recurrence after 6 months.

Rosette formation as detected in this assay is largely mediated by cytophilic antibodies. PPD or KLH specific rosette formation, as well as tumor specific rosette forming response can be effectively

inhibited by anti- human Ig antibodies. In addition, PPD or KLH specific antibodies can arm normal donors for antigen specific rosette formation. However, tumor specific responsiveness to tumor antigens is lost after 24 hour cell culture (possibly due to receptor turnover) but can be restored after incubation with whole serum or an IgG rich serum fraction from the same or other responsive donors. This mechanism may explain why antigen specific RFC response is only observed in immunized donors in this assay, since reactivity is dependent upon the generation of humoral antibodies .

In both the conventional antigen system as well as the tumor system, the cytofluorometric technique primarily detects antigen recognition by cytophilic antibody armed monocytes. T and B lymphocytes also participate in specific rosette formation, but they constitute only a minor proportion of the total RFCs detected (Paper 1 & 2). Detection of lymphocyte rosettes can be improved by optimizing incubation conditions (4,5) such as adopting longer periods of incubation or higher Ag-RBC concentrations, but these changes also resulted in high background levels during cytofluorometric determination that interfered with proper data intepretation.

Tumor specific rosette formation as detected in this assay may represent tumor recognition by a monocyte subpopulation (Manuscript 2). After 24 hour cell culture (which allows shedding of surface receptors) followed by autologous serum incubation, RFC response was restored to levels measured prior to culture. In no instance, however, did this treatment improve RFC response, even though one

might expect that after culturing more monocytes with free Fc receptors would be available for arming. This may be an important consideration, since other studies (16) have suggested that antibody armed monocyte infiltration significantly retards tumor development. The demonstration of antibody dependent monocyte rosette formation may represent the recognition phase of a monocyte subpopulation that can mediate tumor rejection response.

Unresponsiveness in recurrent tumor patients may be due to defects in both humoral and monocyte functions (Manuscript 2). Patient cells remained unresponsive after 24 hour cell culture, and did not acquire tumor specific rosette forming reactivity even after incubation with responsive patient sera. In one instance, incubation with KLH did not result in acquired KLH-RFC response either. In addition, whole sera, or IgG serum fractions from recurrent patients could not arm normal cells for tumor specific recognition. Although only a small number of recurrent patients have been examined so far (4), each is invariably defective in both humoral and monocyte functions as measured by the RFC assay.

The RFC assay has also been utilized to evaluate myelin basic protein (MBP) reactivity (Manuscript 3) among patients with multiple sclerosis (MS). Rosette formation to MBP was observed in a high percentage of MS patients (70%, 16/23) in exacerbation. On the contrary, only a low frequency (16%, 5/31) of responsiveness was observed among patients in remission. Preliminary studies indicate that MBP recognition is also mediated by cytophilic antibodies directed against the autologous protein. These observations are

indirect evidence that disease exacerbation is an autoimmune manifestation, as suggested by other studies (1,14).

The development of the cytofluorometric rosette forming cell assay as described in this thesis has allowed direct examination of immune recognition by donors immune to conventional antigens. In addition, studies on tumor-bearing and MS patients indicate that this assay can offer valuable information regarding disease staging and tumor rejection mechanisms. Future experiments will be directed towards understanding the in vivo relevance of antibody mediated antigen recognition by monocytes as detected in this assay, as well as its relationship to other more commonly utilized assays, such as LAI, DCHR or cellular cytotoxicity (2,6). Recent studies have indicated that peripheral blood monocytes are heterogenous and comprised of subpopulations that mediate regulatory (7,9,10) or effector (3,8,13,15) functions. It will be of interest to determine if detectible tumor specific, or MBP specific RFCs are restricted to a particular subpopulation or function. The examination of more patients with recurrent malignancy is also needed to determine the exact nature of their immunological defects. In addition, patients with other malignancies, such as breast carcinoma or melanoma can be examined to ascertain the applicability of this assay in assessing tumor specific rosette formation.

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Appendix I. Evaluation of experimental parameters in the cytofluorometric rosette forming assay

In order to optimize the detection of rosette forming cells by cytofluorometry, experiments were performed to evaluate the various parameters of the rosette forming assay.

To establish the optimal concentration of Ag-RBC that can be used for specific rosette forming cell detection, donor cells were preincubated with 0.025%, 0.25% and 2.5% of appropriate Ag-RBCs (Table I). At high concentration of preincubating Ag-RBCs (2.5%) a high level of antigen specific rosette forming cells can be observed cytofluorometrically (Expt.I & II). However, due to the high concentration of Ag-RBC present in the samples, a high background to control Ag-RBCs was also observed. A large part of this background is likely to be caused by coincidence (electronic signal overload) and does not represent true rosette formation. This high background also precludes proper interpretation of the % antigen specific rosette forming cells detected, since a portion of this is likely to be comprised of electronic 'noise'. A major portion of background observed can be eliminated by decreasing the concentration of preincubating Ag-RBCs; as shown in Expts. III & IV using 0.025% Ag-RBCs. In some cases, however, preincubation with this concentration of Ag-RBC also resulted in a loss of sensitivity of the assay, such that antigen specific rosette forming cells could no longer be detected (Expt.I & II). The use of 0.25% Ag-RBC represents

TABLE I

Antigen Specific Rosette Formation with Various Concentrations of Ag-RBCs

Donor reactivity	Concentration Ag-RBCs	% rosette formation[a]		% antigen specific rosette forming cells[b]	
		PPD	KLH	PPD	KLH
I. PPD(-) KLH(+)	0.25 %	3.96 ± 0.22	8.89 ± 0.08		4.49[c]
	0.025 "	2.76 ± 0.18	2.52 ± 0.35		(0.24)
II. PPD(+) KLH(-)	0.25 %	11.77 ± 0.25	9.52 ± 0.11	2.25[c]	
	0.025	2.50 ± 0.07	2.50 ± 0.05	(0)	
III.PPD(+) KLH(-)	2.5 %	24.08 ± 0.47	20.40 ± 0.18	3.68 [c]	
	0.25	2.57 ± 0.13	1.83 ± 0.15	0.74 [c]	
	0.025	0.95 ± 0.07	0.31 ± 0.04	0.64 [c]	
IV. PPD(+) KLH(-)	2.5 %	46.10 ± 0.2	41.50± 1.31	3.35 [c]	
	0.25	8.72 ± 0.26	6.53± 0.30	1.45 [c]	
	0.025	0.81 ± 0.25	0.51± 0.13	0.17 [c]	

[a] % rosette formation to KLH and PPD is determined using KLH-RBCs and PPD-RBCs respectively at the indicated concentration

[b] % antigen specific rosette forming cells = $\%\ RFC_{\text{test Ag-RBCs}} - \%\ RFC_{\text{control Ag-RBCs}}$

[c] ( ): values are statistically significant by Student's t test ($p < 0.05$)

an optimal concentration for antigen specific rosette forming cell detection; for while a substantial amount of background was generated at this red cell concentration, it retains the sensitivity of specific RFC detection of higher incubation concentrations.

Early studies have reported the presence of antigen binding cells to conventional antigens in unimmunized humans and animal systems (1,4). Although we have demonstrated that the background level of rosette forming cells to control antigens is not antigen specific, it is important to establish the nature of background detected cytofluorometrically. Rosetted samples were acridine orange stained and then evaluated by fluorescent microscope and cytofluorometric analysis (Table II). Since the cytofluormetric technique depends upon the enlarged integral size of rosettes for their identification, the possibility that a background is due to red and white blood cell clumping was also considered. In five separate experiments where rosetted samples were examined in parallel by cytofluorometry and fluorescent microscopy, the background detected in electronic analysis can be shown to be largely due to contaminating white/red cell aggregates. A low level of RFCs to control antigens can be observed in the microscope against control Ag-RBCs, but these RFCs were not inhibited by antigen preincubation. It is thus likely that the background rosette formation is not antigen dependent and probably represents nonspecific adherence to treated red cell surfaces. It is also important to note that antigen specific rosette forming cells can be

TABLE II

Microscopic Identification of Background Observed during Cytofluorometric Analysis

Donor reactivity	% antigen specific RFC[a] determined by		% Background determined by[b]	
	Fluorescent[c] microscopy	Cytofluorometry	Fluorescent microscopy	Cytofluorometry
I. PPD(-) KLH(+)	0.40	0.62	5.3 (0.2)	4.58 ± 0.14
II. PPD(-) KLH(+)	0.4	0.52	7.8 (0.6)	7.49 ± 0.16
III. PPD(+) KLH(-)	0.3	0.65	5.6 (0.3)	6.06 ± 0.23
IV. PPD(-) KLH(-)	0	0	3.3 (0)	2.73 ± 0.25
V. PPD(-) KLH(-)	0	0	4.3 (0.3)	4.18 ± 0.09

[a] % antigen specific RFC = % $RFC_{test\ Ag}$ - % RFC_{HSA}

[b] % Background determined cytofluorometrically represents "rosette formation" using HSA-RBCs. % cell clumps, ie WBC or WBC/RBC aggregate as well as background rosette (values in paranthesis) in rosetted sample after incubation with HSA-RBC is evaluated and is represented as the background value indicated.

[c] 300-400 cells (nucleated WBC as identified after acridine orange staining) are counted to determine the %

observed in all instances in the immune donor. Minor differences could be observed between the level of antigen specific rosettes determined cytofluormetrically and microscopically, but a correlation can be established between the two techniques by using a larger sampling size (Manuscript I).

Reports on T lymphocyte extraction using sheep erythrocytes rosetting technique have suggested an improved yield with extended period of incubation (2,3). In order to determine if the detection of antigen specific rosette formation can be enhance by long incubation periods, the level of antigen specific rosette forming cells was examined after 1 and 12 hours of incubation (Table III). Both PPD as well as KLH specific rosette forming cells can be readily detectible after an hour's incubation with Ag-RBCs, and overnight incubation did not result in an enhancement of rosette formation.

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TABLE III

Effect of Incubation Time on Antigen Specific Rosette Formation

Donor Reactivity	Incubation period	% antigen specific rosette forming cells[a]	
		PPD	KLH
I. PPD(+) KLH(+)	1 hour	1.23 ± 0.30	1.53 ± 0.08
	12 hours	1.62 ± 0.15	1.43 ± 0.15
II. PPD(+) KLH(-)	1 hour	0.65 ± 0.07	
	12 Hours	0.77 ± 0.09	

[a] % antigen specific rosette forming cells = $\%\ RFC_{\text{test Ag-RBC}} - \%\ RFC_{\text{HSA-RBC}}$

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Appendix II. Influence of specific antibodies on specific rosette formation

Studies on the mechanism of antigen specific rosette formation detected cytofluormetrically have indicated the involvement of cytophilic antibodies (Manuscript I). Experiments were performed to determine if antibodies can be demonstrated in rosette forming cells using fluorescein-conjugated anti-human immunoglobuin reagents (Table I). By using direct immunofluorescence technique, a major portion of rosette forming cells were stained with the fluorescent goat anti-human-Ig immunogloublins (82 $\pm$ 23 %). Since goat immunoglobulins do not bind to human Fc receptors, these experiments suggest that antigen specific rosette forming cells are comprised predominantly of immunoglobulin bearing cells.

Previous experiments have shown that normal donors can acquire antigen specific rosette forming response following incubation with specific antibodies (Manuscript I). In order to establish that the arming phenomenon is solely mediated by immunogloublin-mononuclear cell interaction and not due to other protein activators present in the antibody extract, specific antibody fractions was treated with various concentrations of goat anti- human-Ig immunoglobulins . The arming capacity of the treated fractions were compared with untreated antibody fractions (Table II). Although the arming ability of KLH antibody can be consistently demonstrated in the untreated fractions (Expt.I & II), pretreatment of the fractions with 50ug or

TABLE I

FLOURESCENT STAINING OF ROSETTE FORMING CELLS OF IMMUNE DONORS

USING FLUORESCINE CONJUGATED GOAT ANTIHUMAN IMMUNOGLOBULIN

DONOR REACTIVITY	INCUBATION WITH	TOTAL RFCs[a]	%G Hu-Ig stained
KLH(+) PPD(+)[c]	autologous RBC	0	0
	HSA-RBC	0	0
	PPD-RBC	1.33	75% (6/8)
	KLH-RBC	1.0	100% (6/6)
KLH(+) PPD(+)[d]	autologous RBC	0	0
	HCH-RBC	0	0
	PPD-RBC	.64	50% (2/4)
	KLH-RBC	.93	100% (6/6)

determined microscopically, value represents mean of two determinations of RFCs per 300 nucleated cells examined.

direct staining with fluorescein conjugated goat anti- human immunoglobulin (G Hu-Ig) incubation at 4 C , 30 min . No. represents % RFCs stained.

total mononuclear cells stained ,10%

total mononuclear cells stained; 18%

TABLE II

Demonstration of Antigen Specific Rosette Forming Cells of Normal Donors after Incubation with Specific Antibody or Antibody- antiantibody Complexes

Donor reactivity	Preincubation with	% KLH specific rosette forming cells[c]	
		Cytofluorometry	Microscopy
I. PPD(-) KLH(-)	none	(-0.07 + 0.29)	0
	100 ug KLH Ab[a]	1.91 + 0.26	0.96
	100 ug KLH Ab - 50 ug G HuIg[b]	0.74 + 0.28	0.99
	100 ug KLH Ab -100 ug G HuIg	0.86 + 0.30	0.33
II.PPD(-) KLH(-)	none	(-0.32 + 0.20)	
	100 ug KLH Ab	0.93 + 0.13	1.39
	100 ug KLH Ab- 50 ug G HuIg	0.13 + 0.08	0.97
	100 ug KLH Ab-100 ug G HuIg	(-0.07 + 0.25)	0.30
	100 ug KLH Ab-200 ug G HuIg	(-0.02 + 0.18)	0.30

[a] KLH specific antibodies (KLH Ab) are extracted by affinity chromatography from immune donor sera.

[b] KLH antibodies was complexed with the indicated concentration of goat anti-human-Ig (G HuIg) by incubation at 37 C for 30 minutes. Reacted mixture is then used for donor cell preincubation

[c] KLH specific rosette forming cells = % $RFC_{KLH\text{-}RBCs}$ - % $RFC_{HSA\text{-}RBCs}$

100ug of anti-antibody resulted in a decrease in the arming ability of the anibody fraction (Expt.I). Similarly, cells from donor II could no longer form antigen specific rosettes if the preincubating antibody fractions have been pretreated with 100 and 200 ug of antiantibody. These observations confirm previous data which demonstrate the role of cytophilic antibodies in antigen specific rosette formation.

Appendix III. Role of monocyte in tumor specific rosette formation

Our study involving donors responsive to conventional antigens (Manuscript I) indicates that antigen specific rosette formation are primarily mediated by cytophilic antibody arming of monocytes. To evaluate the participation of monocytes , the tumor specific rosette forming response of patients with squmaous cell carcinoma of the head and neck was evaluated before and after monocyte depletion by G-10 passage. Four patients tested in this manner (Table I) demonstrated tumor specific rosette forming response to squamous antigen extract but not to control tumor antigen extract before monocyte depletion. After G-10 passage, tumor specific rosette forming response can no longer be demonstrated. In all cases, monocyte depletion by G-10 also resulted in total loss (100%) of rosette forming cell response, thus indicating that monocytes are the predominant cell population in tumor specific rosette formation.

To characterize further the cell type involved in tumor specific rosette formation, rosetted samples from responsive patients were enriched for rosette forming cells by isopycnic centrifugation with Ficoll-paque. Enriched rosetted samples were either esterase or acridine stained and examined under the light microscope. Morphological identification confirmed monocyte depletion data and revealed a predominant involvement of monocytes in tumor specific rosette forming cells (Table II). Five

TABLE I

Effect of Monocyte Depletion on Tumor Specific Rosette Formation

Patient/ staging[a]	Tu-Ag RBC	% Tumor specific rosette forming cells[b]		% RFC depletion[d]
		before monocyte depletion	after monocyte depletion [c]	
I. A.S. ($T_2N_0M_0$)	SQCC-HN	1.06 + 0.13	(-0.09 + 0.13)	100
	Mel	(0.03+0.21)	(-0.17 + 0.23)	-
II. W.N. ($T_1N_0M_0$)	SQCC-HN	0.42 + 0.08	(-0.08 + 0.07)	100
	Mel	(0.09+ 0.10)	(-0.03 + 0.14)	-
III. X.M. ($T_3N_3M_1$)	SQCC-HN	1.55 + 0.29	(0.01 + 0.40)	100
	Mel	(0.01+ 0.09)	(0.19 + 0.19)	-
IV. X.P. ($T_1N_0M_0$)	SQCC-HN	0.18 + 0.04	(0.12 + 0.55)	100
	Mel	(0.28 + 0.31)	(0.22 + 0.54)	-

[a] % Antigen specific RFCs = % $RFC_{\text{test Ag-RBCs}}$ - % $RFC_{\text{HSA-RBC}}$

[b] rosetted samples were stained with acridine orange (final concentation 0.1 mg%) and examined for green fluorescence. Three hundred cells were examined to determine the % RFCs and WBC clumps in each sample

[c] % background in cytofluorometry represents % RFCs to HSA-RBCs in each determination;
% background determined by fluorescent microscopy represents the sum of cell clumps and background rosettes found in samples preincubated with HSA-RBCs. Values in parentheses are background rosettes found in the sample.

TABLE II

Morphological Identification of Tumor Specific Rosette Forming Cells

Patient / staging	Tu-Ag-RBC[b]	% Antigen specific RFCs in enriched fraction[a]	Identity of rosette forming cells	
			% Monocytes	% Lymphocytes
I. M.C. ($T_3N_3M_1$)	SOCC-HN	1.5	92 (11/12)[c]	8 (1/12)
	Mel	0	-	-
II. P.O. ($T_2N_0M_0$)	SOCC-HN	1.1	100(8/8)[c]	0 (0/8)
	Mel	0	-	-
III. F.R. ($T_2N_0M_0$)	SOCC-HN	2	75 (6/8)[d]	25 (2/8)
	Mel	0	-	-
IV. C.R. ($T_3N_0M_0$)	SOCC-HN	2.8	80 (8/10)[d]	20 (2/10)
	Mel	0	-	-
V. C.O. ($T_2N_1M_1$)	SOCC-HN	2	100(10/10)[d]	0 (0/10)
	Mel	0	-	-

[a] rosetted samples were enriched for rosette forming cells by isopycnic centrifugation with Ficoll Paque (400 g, 30 minutes) at 4 C.

[b] patients were tested either with squamous cell carcinoma (SOCC-HN) or melanoma(Mel) extracts conjugated to autologous erythrocytes.

[c] monocyte is identified after esterase staining.

[d] monocytes are identified after acridine orange staining

patients with squamous cell carcinoma of the head and neck formed antigen specific rosette forming cells to squamous antigen extract but not control tumor (melanoma) antigen extracts. In all five determinations tumor specific rosette forming cells were predominantly (90 $\pm$ 10 %) esterase positive mononuclear cells bearing monocyte morphology. The participation of lymphocytes was relatively minor, ranging from 0-10% from experiment to experiment.

Appendix IV. Tumor specific rosette forming response in melanoma patients.

A high frequency of patients with primary malignancy of squamous cell carcinoma of the head and neck have elevated levels of tumor specific rosette forming cells as detected by the cytofluorometric technique (Manuscript II). The RFC response appears to be specific, since these patients do not respond to 3M KCl tumor antigens of the other histological types, including melanoma and colon carcinoma. The interpretation of specificity, however, requires that these control antigens are reactive when tested with patients of the same histological tumor.

In this study, preliminary experiments were performed to determine if tumor specific rosette forming response can be detected among melanoma patients. The same protocol as used in evaluating patients with squamous cell carcinoma was adapted, using 3M KCl extracts of melanoma and squamous cell carcinoma for specificity control. Five melanoma patients as well as their close contacts (spouses) demonstrated tumor specific rosette forming reponse to melanoma extract, but not to squamous cell antigen extract. Similar to the results reported previously (manuscript II), one squamous cell carcinoma patient tested in this manner responded to the squamous antigen but not to melanoma antigen. Healthy donors with no prior exposure to either tumor did not form tumor antigen rosettes, although they demonstrated KLH specific rosettes.

TABLE I

Tumor Specific Rosette Forming Cells in Cancer Patients

Cell donor	Donor sensitivity by LAI test			% antigen specific rosette formation[a]		
	Melanoma antigen	KLH	SQCC-HN antigen	Melanoma antigen	KLH	SQCC-HN antigen
1. Melanoma	+	-	-	2.0[b]	(0)	ND
2. Melanoma	+	-	-	1.4[b]	ND	(0)
3. Melanoma	+	-	-	0.7[b]	ND	(0)
4. Melanoma	+	-	-	0.5[b]	ND	(-0.06)
5. Contact	+	+	-	0.9[b]	1.1[b]	ND
6. Contact	+	+	-	1.5[b]	1.1[b]	ND
7. Control	-	+	-	(0)	1.2[b]	(0)
8. Control	-	+	-	(0)	1.6[b]	(0)

[a] % antigen specific rosette forming cells = % $RFC_{\text{test Ag-RBC}}$ - % $RFC_{\text{HSA-RBC}}$

[b] : values are statistically significant from control values by Student's t test; ($p < 0.01$)

These preliminary results indicate that the rosette forming cell assay can be used to detect tumor specific recognition in melanoma patients. It is unlikely that the rosette formation is directed against contaminating antigens common to many tumor antigen extracts (such as carcinoembryonic antigens), since these patients did not respond to another control tumor antigen extract. The number of melanoma patients examined so far is small, and further evaluations will be required to determine the applicability of the rosette forming cell assay to detect speicific tumor recognition in melanoma as well as patients with other types of malignancies.

Appendix V. Statistical methods for data analysis

A. Cytofluorometrically determined rosette forming response to test (KLH, PPD or Tu-Ag conjugated RBCs) and control (HSA-RBCs) is compared using Mann-Whitney (2) and Student t (1,3) statistical analysis.

Mean response to a given antigen is evaluated using triplicate or quadruplicate sampling. The reported % RFCs for each sample is based on the mean of at least 3 observations of the % RFCs per 10^4 nucleated cells. Replicate means are used to determine the mean RFC response ($\pm$ S.D.) to a given antigen. As an example, the KLH- (relevant test antigen) and HSA- (control antigen) rosette forming cell response determined in this manner (Fig. 2, P.83) is shown below.

Antigen	Samples	% RFC / 10^4 cells[a] (x)	Sample mean[b] ($\bar{x}$)	Mean Response $\bar{X}$[c] $\pm$ S.D.
A. HSA	1	3.26, 2.70, 3.04	3.00	
	2	3.13, 2.78, 2.94	2.95	2.94 $\pm$ 0.07
	3	3.26, 2.96, 2.39	2.87	
B. KLH	1	4.07, 3.69, 3.70	3.82	
	2	3.55, 3.73, 3.79	3.69	3.76 $\pm$ 0.07
	3	4.12, 3.85, 3.34	3.77	

[a] determined cytofluorometrically.

[b] $\bar{x} = \dfrac{x_1 + x_2 + x_3 + \ldots . x_n}{n}$

[c] $\bar{X} = \dfrac{\bar{x}_1 + \bar{x}_2 + \bar{x}_3}{N}$; where N is the sampling size.

[d] S.D.(σ) $= \sqrt{\dfrac{\Sigma(\bar{X} - \bar{x})}{df}}$, where df = N - 1.

The Mann Whitney U. is a powerful nonparametric statistic for comparing population small groups. Based on sampling sizes of 3, two groups are statistically different (at a one-tailed 5% error rate) if there is no overlap in the sampling values between the two populations. In the above example, the mean RFC response to KLH is considered significantly higher than HSA.

Differences between sample groups are corroborated by Student's t test. Although sample sizes tend to be small, the Student's t test is appropriate since sampled means are always normally distributed and on a continuous scale (1,2,3).

Antigen	% Rosette forming response	Difference between the Means (Δ)[a] + S.D.
A. HSA	2.94 $\pm$ 0.07 (3)	
B. KLH	3.76 $\pm$ 0.07 (3)	0.82 $\pm$ 0.05

[a] Differences between the means (Δ) = $\bar{X}_B - \bar{X}_A$.

S.D. fo differences between the means $(S_{\bar{X}_A - \bar{X}_B}) = \sqrt{s^2 \left(\frac{N_A + N_B}{N_A N_B}\right)}$; where

s^2 (pooled variance) $= \frac{\sum(\bar{X}_A - \bar{x}_A)^2 + \sum(\bar{X}_B - \bar{x}_B)^2}{df_A + df_B}$, $df_A = N_A - 1$; $df_B = N_B - 1$

$$= \frac{0.0086 + 0.0086}{4} = 0.0043.$$

therefore

$$S_{\bar{X}_B - \bar{X}_A} = \sqrt{0.0043 \left(\frac{N_A + N_B}{N_A N_B}\right)}$$

$$= \sqrt{(0.0043)\frac{6}{9}} = 0.05$$

t value for differences between the means (Δ)

$$= \frac{\bar{X}_B - \bar{X}_A}{S_{\bar{X}_B - \bar{X}_A}}$$

$$= \frac{0.82}{0.05} = 16.4$$

This value is compared to the t values for df = 4 ($df_A + df_B$); where $t > 2.77, 4.60, 8.61$ are significant at 95%, 99% and 99.9% confidence levels. In this example, t = 16.4, indicating that the differences betwen the means is statistically significant ($p < 0.001$)

In this thesis, donors are considered responsive to conventional or tumor antigens if the % rosette formation is significantly elevated over background (determined with HSA-RBC) at $\alpha/2 \leq 0.05$ or determined by both Mann Whitney and Student's t tests

B. In evaluating the spcific rosette forming response to tumor antigens, proper interpretation of whether a patient exhibited a positive or negative response is dependent upon a) differences between the means of rosette formation to Tu-Ag and control (HSA), and b) variances of each of the determinations. In some cases, high variances have precluded a large difference between the means (Fig. 2, P.138) to be statistically significant ($p < 0.05$). In this study, over 60 determinations of rosette formation to HSA and SQCC-HN (Table III) are analysed to determine the frequency distribution as well as the 95% confidence limits of variance in each data pool.

Paired t analysis of variance of HSA (X) and SQCC-HN (Y) rosette formation indicates that distribution of variances are similar (Table IV). Mean of variance of X is 0.20, Y, 0.22.

TABLE III

Variances of pooled data from HSA and SQCC-HN rosette formation evaluation

HSA (X)		SQCC-HN (Y)	
0.6400	0.1900	0.4000	0.1500
0.1000	0.1000	0.0600	0.2100
0.1900	0.1500	0.2100	0.0200
0.6000	0.2300	1.1500	0.0700
0.2100	0.1200	0.2700	0.3600
0.1600	0.1300	0.0500	0.6900
0.0100	0.0200	0.1300	0.6500
0.1400	0.1300	0.0100	0.1600
0.1600		0.1900	0.2800
0.0500		0.0300	0.0300
0.0200		0.6200	0.8500
0.2800		0.2100	0.1000
0.3400		0.2500	0.2200
0.0900		0.3800	0.1700
0.2200		0.6400	0.0100
0.3900		0.4600	0.2300
0.2300		0.3800	0.0500
0.1000		0.2800	0.1400
0.0600		0.1000	0.1400
0.0800		0.2300	
0.5600		0.2200	
0.2100		0.1500	
0.1200		0.0100	
0.3500		0.4100	
0.2400		0.0800	
0.3800		0.2400	
0.0100		0.1500	
0.0100		0.1100	
0.1200		0.0500	
0.2800		0.1900	
0.0400		0.1700	
0.2800		0.0600	
0.0300		0.1200	
0.1700		0.1200	
0.0100		0.0500	
0.0400		0.1900	
0.1100		0.1700	
0.3500		0.0700	
0.3100		0.2100	
0.3800		0.2800	
0.2600		0.2200	
0.1400		0.2200	
0.3200		0.0100	
0.0400		0.0500	
0.2300		0.4300	
0.0300		0.0800	
0.7300		0.0500	
0.1900		0.3800	
0.5600		0.1900	

DESCRIPTIVE STATISTICS

	X SAMPLE	Y SAMPLE
N =	57	68
MEAN =	0.204210526316	0.224117647059
VARIANCE =	0.0286783834586	0.0447111501317
STD DEV =	0.169346932239	0.211450112631
DATA MIN =	0.01	0.01
DATA MAX =	0.73	1.15
DATA RANGE =	0.72	1.14
STANDARD ERR OF MEAN =	0.022430549132	0.0256420926155
COEFFICIENT OF VARIATION =	82.9276214571	94.3478192842
SKEWNESS =	1.21305692777	2.02188456486
KURTOSIS =	4.15392440907	7.99561087312

POOLED VARIANCE =0.0374116791261

T-TEST

T TEST OF EQUALITY OF MEANS = -0.573114937422
WITH 123 DEGREES OF FREEDOM

SIGNIFICANCE LEVELS:
ONE TAIL TEST = 0.28381
TWO TAIL TEST = 0.56762

Similarly, variance distribution of the two data pools is very similar (Fig.1 & 2). In both cases, distribution is not normally distributed (skewness = 1.2 & 2.0 respectively). The frequency of distribution analysis also indicates that observations with high variances (5.1 in X and 7.5 in Y) are likely to be extreme observations (1) and can be rejected during data analysis.

The mean and distribution of variance in each data pool is not statistically different form one another ($p = 0.57$). This suggests that variance observed in rosette formation with HSA and SQCC-HN is inherent in the assay, and likely to be independent of the antigens used.

1. Suedcor, G.W. and Cochran, W.G. "Statistical Methods". P. 103 Iowa State U. Press, Iowa. 1972.
2. Siegal, S. "Nonparametric Statistics for Behavioral Science". Pp. 116-127. McGraw Hill, N.Y. 1956
3. Steel, R.G., Torrie, J.H. "Principles and Procedures of Statistics" McGraw Hill, N.Y. 1960

Figure 1: Distribution frequency of variance of pooled HSA-rosette formation evaluation

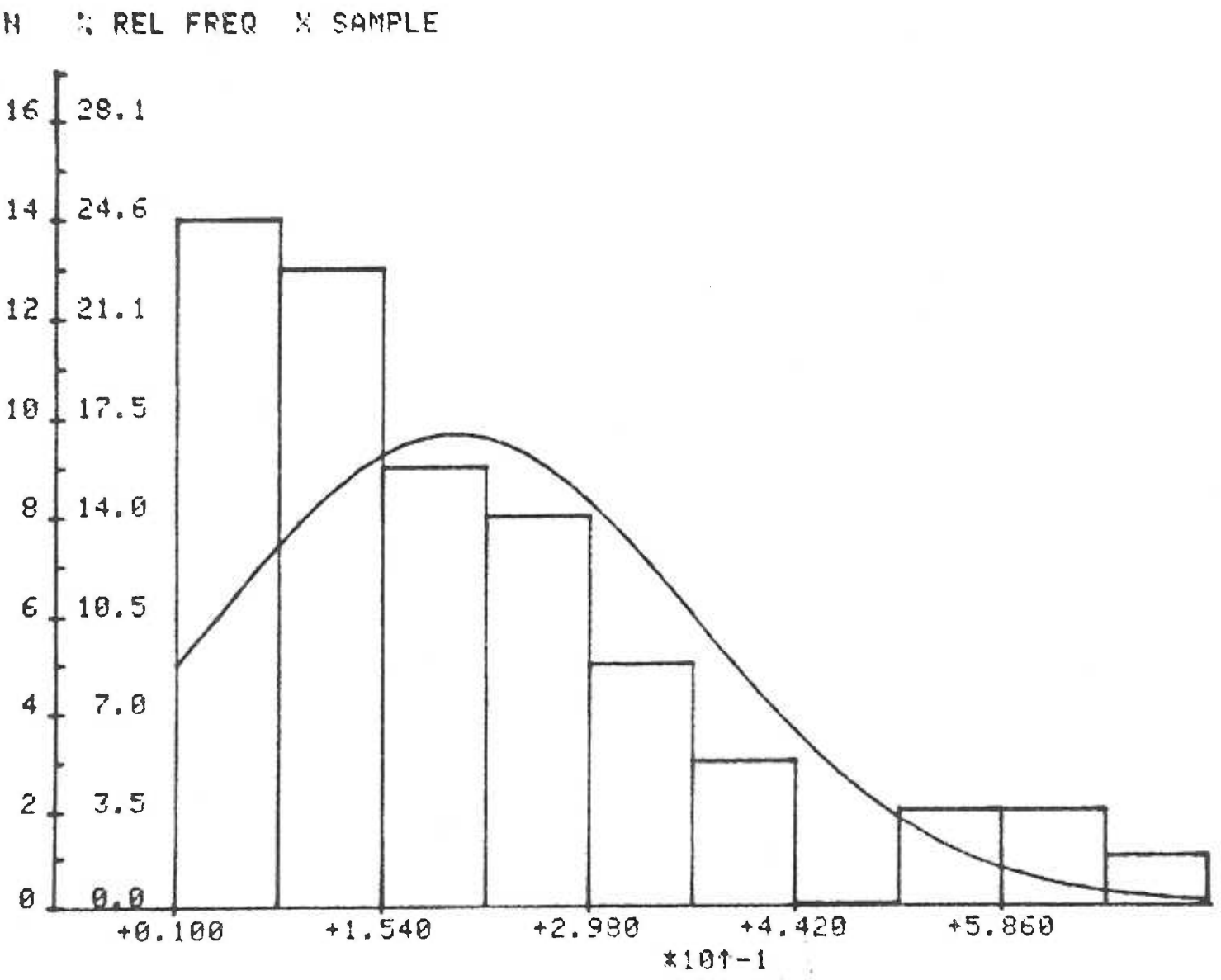
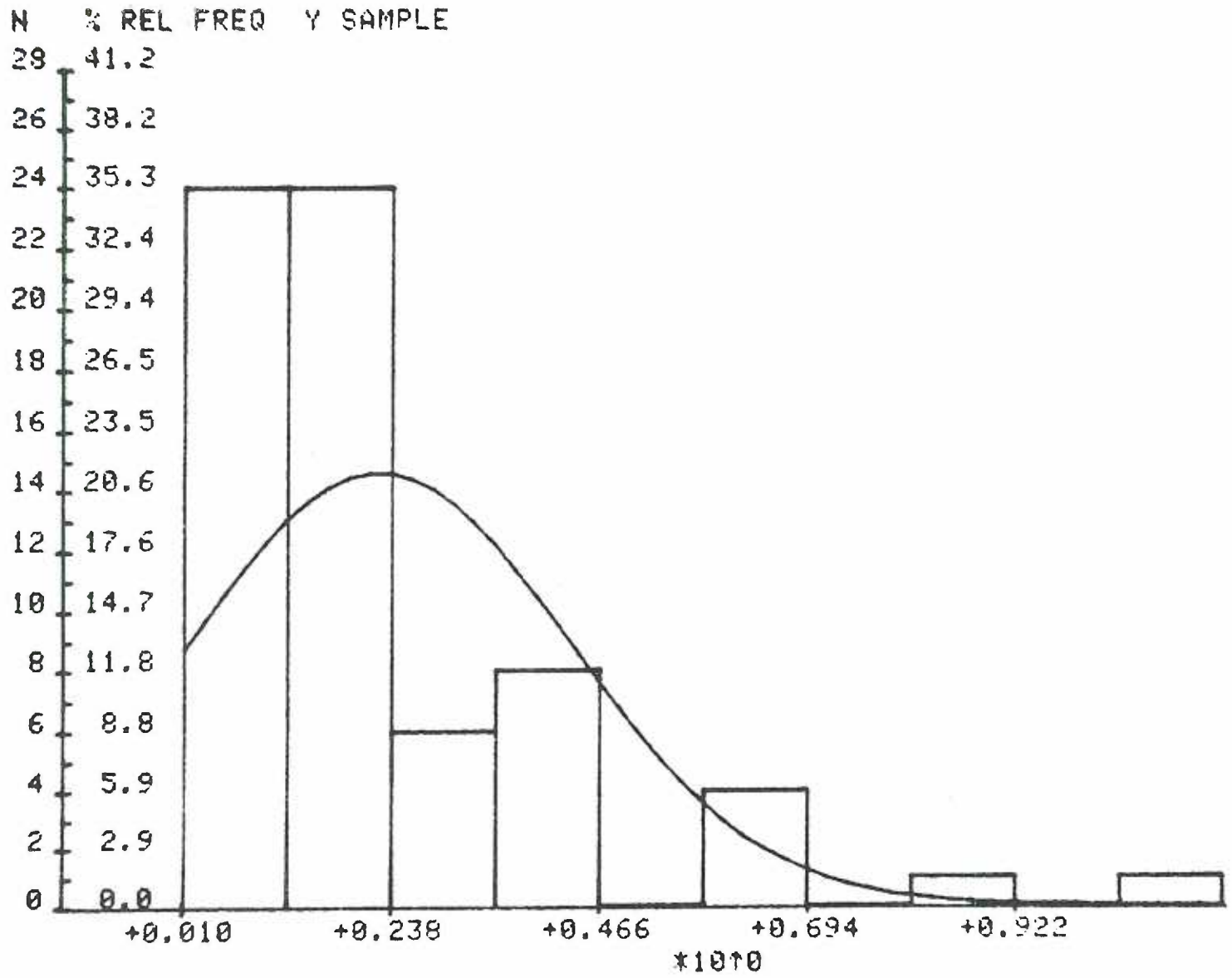

N % REL FREQ % SAMPLE
16
14
12
10
8
6
4
2
0
28.1
24.6
21.1
17.5
14.0
10.5
7.0
3.5
0.0
+0.100
+1.540
+2.980
+4.420
+5.860
*10↑-1

Figure 2: Distribution frequency of variance of pooled SCCC-HN rosette formation evaluation


N
% REL FREQ
Y SAMPLE
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
41.2
38.2
35.3
32.4
29.4
26.5
23.5
20.6
17.6
14.7
11.8
8.8
5.9
2.9
0.0
+0.010
+0.238
+0.466
+0.694
+0.922
*10↑0

ABBREVIATIONS

Å	Angstrom
ABC	antigen binding cell
Ag	antigen
anti-Ig	anti-immunoglobulin
BSA	bovine serum albumin
CC	colon carcinoma
ADCC	antibody dependent cellular cytotoxicity
CFA	complete Freund's adjuvant
CFM	cytofluorometry
CMI	cell mediated immunity
CRI	cross reacting idiotype
DEAE	diethylaminoethyl
df	degree of freedom
DNP	dinitrophenol
FACS	fluorescent activated cell sorter
HBP	human basic protein
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSA	human serum albumin
HSF	horse spleen ferritin
KLH	keyhole limpet hemocyanin
LAI	leukocyte adherence inhibition
LPS	lipopolysaccharide
MBP	myelin basic protein
MIF	migration inhibition factor

m-Ig	membrane immunoglobulin
Mel	melanoma
MS	multiple sclerosis
NCS	newborn calf serum
NK	natural killing
PPD	purified protein derivative, tuberculin
R	correlation coefficient
RBC	red blood cell
RFC	rosette forming cell
RPM	revolutions per minute
RPMI	Roswell Memorial Park Institute
S.D.	standard deviation
SQCC-HN	squamous cell carcinoma of the head and neck
SRBC	sheep red blood cell
TBS	Tris-buffered saline
TD	T - dependent
TGAL	synthetic polypeptide{(Tyr-Glu)-Ala-Leu}
Thy	thymus associated antigen
TI	T independent
TIGAL	iodine derivatized TGAL
TNP	trinitrophenol
WBC	white blood cell

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