# SURFACE IMMUNOGLOBULINS, T-CELLS AND SERUM IMMUNOGLOBULINS IN PATIENTS WITH BRUTON'S AGAMMAGLOBULINEMIA AND ADULT ACQUIRED HYPOGAMMAGLOBULINEMIA

by

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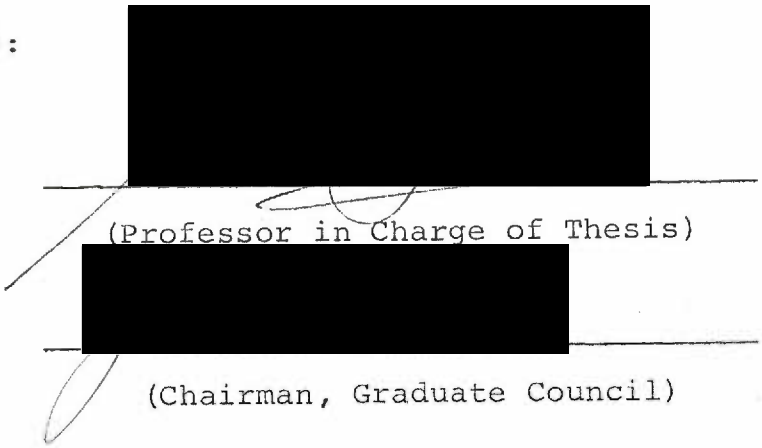
A THESIS
Presented to the Department of Microbiology & Immunology
and the Graduate Division of the University of Oregon
Health Sciences Center in partial fulfillment of the
requirements for the degree of
Master of Science

APPROVED:



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ACKNOWLEDGEMENTS

I want to express my gratitude to the many persons, family and friends whose aid and influence made this thesis possible. Special recognition is due to Dr. Gerrie A. Leslie, Professor in charge of this thesis, for the instruction, guidance and support given through the past three years; to Dr. Bernard Pirofsky, for supplying the blood samples, as well as criticisms and overall interest; to Drs. Marvin Rittenberg and Dennis Burger for their suggestions and criticisms; to Ms. Teresa Ralston for skillful technical assistance; and to Ms. Rita Moran and Ms. Kristine Kuchenbecker for the typing of this manuscript.

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INTRODUCTION

Through the immunofluorescent techniques developed by Coons a few decades ago (1,2), it was possible to show the presence of immunoglobulins on the surface of B-lymphocytes (3,4,5) and lend support to the hypothesis first proposed by Ehrlich (6) that they act as cell receptors for antigens.

In early studies the three major classes of human immunoglobulins were demonstrated on the cell membrane of lymphocytes (3,7-13). IgG was in most instances the predominant surface immunoglobulin. Subsequently, Van Boxel et al. (14) showed the presence of IgD on the membrane of lymphocytes from adults. Recent studies of human cord (15) and adult blood lymphocytes (16,17) and lymphocytes from patients with chronic lymphatic leukemia (18,19) have shown that IgD and IgM are the major classes of membrane immunoglobulins, whereas lymphocytes bearing IgG and IgA determinants are reduced to less than 1% when the reagents used for their detection are $F(ab')_2$ fragments of the conjugates (20).

In contrast to its frequent appearance on B cells, IgD represents only about 0.25% of the total serum immunoglobulins and its antibody function there is not clear. Antibody activities such as reactivity with skin, polymorphonuclear leukocytes or mast cells (21), ability to activate the complement cascade, presence in secretions and ability to cross the placenta are, for the most part, negative for this immunoglobulin (22,23). However, recent reports have shown IgD antibody in

some patients that are chronically exposed to certain antigens (24-26) and some IgD myeloma proteins are able to activate the alternate complement pathway (27). IgD has now been found in nasal secretions (28), saliva, cerebrospinal fluid and amniotic fluid (Leslie and Teramura, 1976, in preparation). The major function of IgD may however be as a lymphocyte receptor (16).

The prominance of IgD as a cell surface immunoglobulin, in view of the low level found in serum, has awakened a great deal of interest in determining the biological function(s) of this immunoglobulin as:

1) humoral antibody
2) antigen recognition molecules
3) a "trigger" mechanism in lymphocyte differentiation
4) markers for the type and specificity of antibodies that the progeny of the cell will produce if stimulated
5) a mechanism for including tolerance in a cell when exposed to the antigen.

Previous studies (11,29-32) have demonstrated the presence of surface immunoglobulins on the lymphocytes of some agammaglobulinemic patients. However, we undertook this study with the purpose of correlating the number of IgD and IgE bearing cells, the number of IgA, IgG and IgM bearing cells and the concentration of serum immunoglobulins. To further evaluate the immunological status of these patients T-lymphocytes were

quantified by rosetting with sheep red blood cells (SRBC).

It is important to point out here that the ability of Fc receptors on B-lymphocytes (and possibly activated T-cells) to bind IgG has probably been responsible for the wide range of values reported for membrane immunoglobulin-bearing lymphocytes found by various investigators. This is particularly true in the case of IgG-bearing cells. We circumvented this problem by using fluorescein conjugated $F(ab')_2$ fragments of anti-$\gamma$ and $\alpha$ (20).

IgG is by far the immunoglobulin present in the highest concentration in the serum, consequently it is the most liable to be present in minute amounts contaminating the isolated and washed cells. Such contaminating IgG would tend to form immune complexes when the fluorescein-conjugated anti-$\gamma$ antiserum is added. Complexes which, in turn, could be bound through the Fc receptor to the membrane of the cell; if the conjugate is prepared with the intact antibody molecule. Such possibility is avoided if the Fc portion of the antibody molecule is removed. Hence the need to use $F(ab')_2$ fragments coupled to fluorescein for the anti-$\gamma$ antiserum.

The results of this study show that Bruton's agammaglobulinemic patients can have normal levels of B-cells. Adult acquired hypogammaglobulinemic patients present a wide range in B-cell values, from virtually no B-cells to normal limits. In both groups of patients IgM and IgD are predominant immuno-

globulins on the membrane of the lymphocytes. In both groups of patients, when B-cells are demonstrable, IgE is present on their membranes in normal ranges. Neither the Bruton's nor the acquired hypogammaglobulinemics exhibit a consistent correlation between the levels of serum immunoglobulin and the number of lymphocytes bearing that immunoglobulin on the surface. Most of the patients in each group show normal percentages of T-cells.

# MATERIALS & METHODS

## I. Patients

Table I gives the patient population, their age, sex, clinical diagnosis and how long they have been diagnosed and under prophylactic treatment with modified serum immunoglobulin (M-SIG). The passive immunoglobulin therapy was given to the patients as monthly doses of modified tetanus immunoglobulin (human) in 0.3 M glycine (Cutter Laboratories, Inc., Berkeley, California); and administrated intramuscularly, intravenously or by both routes, depending on the particular susceptibility of each patient to adverse reactions. Intravenous therapy consisted of infusing 150 mg/Kg of body weight of the M-SIG. Intramuscular injections of approximately 100 mg/Kg of body weight were given. This preparation of modified immunoglobulin was shown to have 0.5 μg/ml IgD by radioimmunoassay (Leslie, G.A., unpublished, 1976). Healthy donors (staff from our departments) were used as normal controls for the immunofluorescence studies.

## II. Purification of Human Serum IgS Used to Prepare Antisera

IgA was purified from an IgG-IgA myeloma serum by ion exchange chromatography on a DEAE-cellulose column. A linear elution gradient was used with 0.015 Tris buffer pH 8.0 as

starting buffer and a 0.3 M Tris buffer pH 8.0 as the limit buffer. Fractions containing from 0.3 to 2.1 mg/ml IgA were pooled and further purified by gel filtration over a Sephadex G-200 column (Pharmacia, Piscataway, N.J.).

IgG was obtained from a pool of sera from normal donors. First, the globulin fraction was obtained by precipitation with 16% W/V $Na_2SO_4$ performed at room temperature and with constant stirring. Two subsequent precipitations with 14% W/V $Na_2SO_4$ under the same conditions followed the preceding step. This fraction was dialysed against 0.015 Tris buffer pH 8 and applied to a DEAE-cellulose column equilibrated with the same buffer. The protein eluted with the starting buffer (0.015 M Tris, pH 8) was dialysed against Sephadex buffer (0.01 M Tris, 0.14 M NaCl, 0.001 M EDTA, 0.001% Na Azide, pH 7.4), assessed for purity by double diffusion and immunoelectrophoresis and was lyophilized. This protein preparation was used to produce rabbit anti-human IgG.

IgM was isolated from the serum of a patient with Waldenstrom's macroglobulinemia. The globulin fraction was precipitated with 50% saturated $(NH_4)_2SO_4$ for 30 min at 4° C with constant stirring. The globulins were reprecipitated twice with 45% saturated $(NH_4)_2SO_4$, dialysed for 48 hours against four changes of 500 ml of Sephadex buffer, and filtered through a Sephadex G-200 column. The IgM-rich peak was recycled over the same column. This protein was used to

TABLE I

Patient Population and Clinical Data

| Patient | Age | Sex*** | Clinical Diagnosis | Time Under Treatment |
|---|---|---|---|---|
| MS | 10 | M | Bruton's | 1½ years (IV)* |
| JB | 7 | M | Bruton's | 5 years (IV) |
| KO | 18 | M | Bruton's | 1 year (IV) |
| SM | 18 | M | Bruton's | 10 years (IV) |
| TF | 25 | M | Bruton's | 2 years (IV) |
| CK | 30 | M | Bruton's | 17 years (IV + IM) |
| DC | 18 | F | Adult Acquired | 9 years (IV) |
| SK | 31 | F | Adult Acquired | 4 years (IM)** |
| JL | 36 | F | Adult Acquired | 7 years (IV) |
| MM | 37 | F | Adult Acquired | 3 years (IV) |
| BY | 46 | F | Adult Acquired | 8 months (IV) |
| CS | 50 | F | Adult Acquired | 1½ years (IV) |
| GK | 72 | F | Adult Acquired | 6 years (IV) |
| JR | 46 | M | Adult Acquired | 6 months (IM) |
| AB | 50 | M | Adult Acquired | 2 years, 4 months (IV) |
| VC | 53 | M | Adult Acquired | 8 months (IV) |

* Intravenously

** Intramuscularly

*** M - Male; F - Female

prepare rabbit anti-human IgM.

IgD was purified from an IgD-myeloma serum. The globulin fraction of the serum was obtained by precipitation with $(NH_4)_2SO_4$ at a final concentration of 50%, performed at $4^oC$ for 30 min and with constant stirring. This protein fraction, after being dialysed against Sephadex buffer, was applied to a Sephadex G-200 column. The IgD-rich peak obtained was recycled over the same column. The recycled fractions that contained most of the IgD activity as assessed by double diffusion in agarose gel were pooled and concentrated. This concentrate was used to produce the anti-human IgD antiserum.

III. Antisera

Antisera to human IgG, IgA, IgM and IgD were raised in rabbits. Anti-IgG, anti-IgM and anti-IgA were prepared by injecting into the footpads of rabbits, 2-5 mg of the protein emulsified in complete Freund's adjuvant (CFA) at day 0. Two weeks later they were boosted with the same amount of protein emulsified in CFA, subcutaneously. They were bled for the first time at day 21 and were bled twice a week thereafter over a period of one month. At day 30 they were again boosted with the same dose of protein emulsified in incomplete Freund's adjuvant (IFA). They were bled twice from the ear artery over a period of one week and exsanguinated by cardiac puncture. Anti-human IgD antiserum was prepared by injecting animals with 350 µg of the purified IgD myeloma protein emulsified in CFA.

Inoculations were given subcutaneously and in multiple sites ( 10 sites). One month later they received a boost of 114 μg in IFA, given intramuscularly and subcutaneously. They were bled twice a week over a period of two months, were boosted at two months with 20 μg/animal in IFA subcutaneously and bled starting one week later twice a week for two more weeks.

The antisera were rendered monospecific for the appropriate heavy chain (α, γ, μ, or δ) by passage over immunoadsorbent columns not possessing that particular immunoglobulin. Immunoadsorbents were prepared by activating Sepharose-4B with CNBr (33). (1 gr CNBr dissolved in 10 ml distilled water was added per 10 ml of washed and packed Sepharose 4B.) The reaction mixture was immediately brought to pH 11 with 2M NaOH and kept at that pH by the continuous addition of NaOH until the pH stabilized at 11 (approximately 8-10 min). The reaction was terminated by filtration and washing with 2 liters of chilled distilled water, followed by washing with 2 liters of chilled 0.5 M $NaHCO_3$ pH 9. The activated packed Sepharose was resuspended in an equal volume of 0.5 M $NaHCO_3$ pH 9 buffer. Protein dissolved in the same buffer at a concentration of 20 mg/ml was added to the activated Sepharose at a ratio of 2-3 mg of protein/ml of packed Sepharose. The suspension was stirred at $4^{o}$ C for 16 hr. The conjugated Sepharose was poured into a column, allowed to settle and washed with Sephadex

buffer (0.01 M Tris, 0.14 M NaCl, 0.001 M EDTA, 0.001% Na azide, pH 7.4) until no absorbance (280 nm) could be detected in the filtrate. Before using the immunoadsorbent for the purification of antisera, and in order to minimize antigen release from it under the elution conditions, the immunoadsorbant was washed with the eluting agent, 3 M NaSCN (34,35).

The monospecificity of the antisera was assessed by immunoelectrophoresis and by double diffusion in agarose. Fluorescein labelling of the antisera was performed according to the method of Wood et al. (36). The IgG fraction of the antisera was isolated by precipitating the globulin fraction of the rabbit serum with 50% saturated $(NH_4)_2SO_4$ at $4^{\circ}$ C, followed by ion exchange chromatography in a DEAE-cellulose column, equilibrated in 0.01 M phosphate buffer pH 7.2. The IgG-rich fraction of the rabbit antiserum obtained through elution of the column with 0.01 M phosphate buffer pH 7.2 as starting buffer, and at a concentration of 20 mg/ml in 0.15 M NaCl was brought to pH 9.5 at room temperature by the addition of 0.1 N NaOH. FITC (25 µg/mg of protein) was added to a weighing bottle in which the labelling was to be performed. The appropriate volume of protein was then added. Small magnetic bars were used to effect stirring. During the first hour of reaction, the pH was maintained between 9.0 and 9.5. The reaction was then allowed to continue at $4^{\circ}$ C for 17 hr. At the end of the coupling period, the unbound FITC was removed by passage of the mixture over a Sephadex G-25 column. The conjugate was then dialysed against 0.01 M phosphate buffer pH 7.2 and chromatographed on a DEAE-cellulose column equilibrated with the same buffer. Step wise elu-

tions with 0.03 M, 0.05 M and 0.1 M phosphate buffers pH 7.2 were performed.

The fluorescein:protein molar ratio was calculated using the nomogram published by The and Feldkamp (37). Conjugates having F/P ratios between 1.7 and 3.0 were chosen. Each conjugated antiserum had to be tested and when dilutions were required, they were diluted with 2% bovine serum albumin in PBS (phosphate buffered saline = 0.01 M phosphate, 0.15 M NaCl pH 7.0).

$F(ab')_2$ fragments of the anti-$\gamma$ and anti-$\alpha$ antisera were used for making the fluorescent conjugates for these specificities. These were obtained by pepsin digestion at pH 4 of the DEAE-cellulose purified IgG fractions of the rabbit antisera (38). The IgG fractions were dissolved in 0.1 M acetate buffer pH 4 to give a 1 to 3% solution. Crystalline pepsin was dissolved in a small aliquot and pre-warmed at 37°C, then added to the substrate. The enzyme:substrate ratio was 1:100 (1 mg of enzyme for every 100 mg of IgG).

The reaction mixture was incubated at 37° C for 18-24 hr and the reaction was stopped by the addition of solid Tris salt to give a pH of 8.0. Purification of the $F(ab')_2$ fragments was achieved by gel filtration through a Sephadex G-200 column.

Fluorescein labelled antisera specific for $\kappa$ and $\lambda$ light chains, for $\varepsilon$ heavy chains, as well as a polyvalent anti-immuno-

globulin antiserum with specificities for $\alpha$, $\gamma$, $\mu$, $\kappa$ and $\lambda$ were obtained from Meloy Laboratories, Springfield, Va. Their specificity was assessed by double diffusion in agarose gel.

IV. Lymphocyte Isolation

Lymphocytes were isolated from defibrinated peripheral blood by centrifugation on a hypaque-ficoll gradient, according to the method of Boyum (39): 30 ml of peripheral blood was defibrinated by gentle stirring for 10 min at room temperature in a flask containing from 10 to 20 glass beads $N^{O}$ 3000 and measuring 3 mm in diameter. The defibrinated blood was diluted 1:2 with Hank's minimum essential medium (Hank's MEM, Grand Island Biological Co.) and two volumes were layered on top of one volume of either "Ficoll-Paque" (Pharmacia, Piscataway, N.J. each 100 ml contains an aqueous sterile solution of 5.7 g Ficoll 400 + 9.0 g Diatrizoate sodium) or a Hypaque Ficoll mix consisting of 10 parts of 34% aqueous solution Hypaque (Winthrop Labs, N.Y.) plus 24 parts of an aqueous solution of 8% Ficoll (Pharmacia, Piscataway, N.J.). The tubes were centrifuged at 400 x G for 30 min at room temperature. The layer of white cells at the interphase was collected with sterile Pasteur pipettes and washed three times with Hank's MEM. Cells were counted and resuspended in the same medium at a concentration of $1 \times 10^{7}$ cells/ml. Viability of the cells was determined by trypan blue exclusion: 0.1 ml

of a cell suspension of 1 x $10^5$ cells/ml was mixed with 0.1 ml of a 0.3% solution of trypan blue in physiological saline. Dead cells take the dye, whereas live cells remain unstained. At least 200 cells were counted.

V. Immunofluorescent Staining

0.05 ml of the purified lymphocytes at a concentration of 1 x $10^7$ cells/ml were centrifuged at 400 x G for 10 min at 4° C in 6 x 50 mm test tubes. The supernatant was removed and the cell button was resuspended in 0.05 ml of the appropriately diluted conjugated antiserum and incubated for 30 min in the dark in an ice bath. After washing three times with cold Hank's MEM, the cell pellet was resuspended in a drop of medium and deposited on a slide and mounted in one drop of buffered glycerol (10% 0.1 M phosphate buffer, pH 9 plus 90% glycerol). Cells were counted under a Zeiss microscope equipped for reflected light excitation (reflector 510, excitation filter 440-490 nm, barrier filter 520). At least 300 cells were counted.

VI. Regeneration Studies

In order to corroborate the specific binding of the conjugates to the cell receptors, the lymphocytes were treated with papain, according to the method of Cross (40): Peripheral blood lymphocytes were resuspended at a concentration of 1.5 x $10^7$ cells/ml in RPMI-1640 medium (Grand Island Biological Co.) containing 1 mg/ml papain, which had been pre-activated by

incubation with 0.01 M cysteine for 1 hr at 37° C, and 100 μg/ml DNAse (used to prevent gelation due to released DNA), and incubated at 37° C for 20 min. After incubation the cells were washed three times with cold RPMI-1640 medium and the viability assessed by trypan blue exclusion. Aliquots of cells were stained for surface immunoglobulins and the remaining cells were incubated at 37° C at a concentration of $2 \times 10^6$ cells/ml in RPMI-1640 medium supplemented with 0.025 M Hepes and 10% FCS. After six hr of incubation the cells were washed three times, assessed for viability and the presence of surface immunoglobulin-bearing cells.

## VII. Quantitation of Serum Immunoglobulins

Serum immunoglobulin concentrations in the immunodeficient patients were quantified by radial immunodiffusion using commercial plates from Helena Laboratories (Beaumont, Texas). Their levels of sensitivity were: 1.5 mg/dl for IgG, 6 mg/dl for IgM, 5 mg/dl for IgA, and 5 mg/dl for IgD. IgE levels were determined by radioimmunoassay using the Phadebas™ commercial kit from Pharmacia (Piscataway, N.J.). The level of sensitivity for this assay was 5 U/ml.

## VIII. T-cell Rosettes

T-cell concentrations were assessed by rosetting the patients' lymphocytes with sheep red blood cells (SRBC), es-

sentially as described by Wybran et al. (41): 0.2 ml of lymphocytes at a concentration of 1 x $10^6$ cells/ml in medium-199 (M-199) supplemented with 10% FCS were added to round bottomed tubes (12 x 75 mm). 0.2 ml of SRBC at a concentration of 1 x $10^7$ was added and the cells mixed. The mixture was incubated at 37° C for 15 min and then centrifuged at 200 x G for 5 min at 4° C. The supernatants were not discarded, and the tubes were incubated for 24 hr at 4° C, then the cells were carefully resuspended and the number of rosetted and non-rosetted lymphocytes were counted. A rosette is defined as a lymphocyte having at least five SRBC attached to its cell membrane.

# RESULTS

Table II shows the results obtained when lymphocytes from normal donors were examined for surface immunoglobulins. Approximately 14% of the lymphocytes were positive, with values ranging between 10% and 23%, as judged by staining with a polyvalent antiserum. The total sum of the cells positive for each heavy chain approaches very closely the value of the polyvalent antiserum (16.3 ± 4.7% vs. 14.3 ± 3.6%). However, when the number of cells staining with anti-$\kappa$ and anti-$\lambda$ are added, the sum is somewhat lower than the values obtained with the polyvalent antiserum or with the total of the values for the heavy chains.

Cells from ten immunodeficient patients were stained for surface immunoglobulins using FITC labelled $F(ab')_2$ anti-$\alpha$ and $\gamma$, and FITC labelled IgG anti-$\alpha$ and $\gamma$. The results are presented in Table III. The sum of the values obtained with the anti-H chain antisera approaches more closely those obtained as a result of the addition of $\kappa + \lambda$ values, when $F(ab')_2$ anti-$\alpha$ and $\gamma$ are used in place of IgG anti-$\alpha$ and $\gamma$.

In Table IV are summarized the results obtained when lymphocytes from six X-linked Bruton's agammaglobulinemic patients were examined for surface immunoglobulins and for T-cells. As shown in Table III, the values for $\alpha$- and $\gamma$-bearing cells are reduced to less than 1% when $F(ab')_2$ anti $\alpha$ and $\gamma$ reagents are used. On the basis of these observations we must assume that the group presented in Table IV consists of individuals

with no positive cells, which is the case of patient MS; patients with very low numbers of immunoglobulin-bearing cells, as in patients TF, KO and JB; and patients with numbers of positive cells that range within the limits of normal donors (patients CK and SM).

Table IV also shows that with the exception of one patient (TF) this group has normal levels of T-cells, as reported by other investigators (42,43).

Lymphocytes from ten adults with acquired hypogammaglobulinemia were examined for the presence of surface immunoglobulins of the seven different isotypes and for T-cells. The results are presented in Table V.

This group of patients also presents a variability in their membrane immunoglobulins that ranges from very low levels of positive cells, as in the case of patient VC, to normal quantities of Ig-bearing cells, as in the cases of patients AB and CS. The number of T-cells ranges between 61-84%, which is similar to the values obtained for normal controls.

Since several of the patients had substantial numbers of Ig-bearing lymphocytes it was important to show that these positive cells were not due to cytophilic absorption of immunoglobulins which the patients received monthly. In order to investigate this possibility we carried out experiments in regenerating the immunoglobulin receptors on cells from a normal donor and from an immunodeficient patient, by incubat-

TABLE II

Surface Immunoglobulins on PBL's* from Normal Donors Determined by Immunofluorescence

| Donor | % Positive Cells Stained with Anti- δ | μ | ε | γ | α | κ | λ | Poly | Sum H-Chains | Sum L-Chains |
|---|---|---|---|---|---|---|---|---|---|---|
| AS | 4 | 6 | 1 | 3 | 2 | 8 | 4 | 14 | 16 | 12 |
| SB | 6 | 3 | 1 | 5 | 1 | 4 | 4 | 16 | 16 | 8 |
| TP | 8 | 5 | .5 | 2 | 1 | 8 | 6 | 15 | 16.5 | 14 |
| GH | 5 | 6.5 | 1.5 | 2 | .5 | 4 | 6 | 10 | 15.5 | 10 |
| GT | 3 | 8 | 2 | 1 | 2 | 9 | 3 | 16 | 17 | 12 |
| GL | 6 | 6 | 2 | 3 | .5 | 6 | 7 | 14 | 17.5 | 13 |
| JO | 4 | 5 | 1 | 1 | .5 | 5 | 5 | 12 | 11.5 | 10 |
| PM | 2 | 5 | .5 | 20 | .5 | 5 | 4 | 23 | 28 | 9 |
| DO | 3 | 5 | 1 | 1.5 | .5 | 4 | 4 | 11 | 11 | 8 |
| DL | 3 | 9 | .5 | 1 | .5 | 8 | 4 | 12 | 14 | 12 |
| Mean | 4.5 | 5.8 | 1.1 | 4 | 1 | 6.1 | 4.8 | 14.3 | 16.3 | 10.8 |
| SD | 1.9 | 1.7 | .6 | 5.7 | .6 | 2 | 1.2 | 3.6 | 4.7 | 2.1 |

* Peripheral blood lymphocytes.

TABLE III

Comparison of the Effects of Staining Lymphocytes from Immunodeficient Patients with FITC**-labelled $F(ab')_2$ Fragments vs. FITC-labelled Intact IgG

Against γ and α Specificities
% Positive Cells by Membrane Immunofluorescence

| Patient | γ | γ'* | α | α'* | Poly | Sum δ,μ,γ,α,ε | Sum δ,μ,ε,γ',α' | Sum κ+λ |
|---|---|---|---|---|---|---|---|---|
| MM | 6 | <1+ | 2.5 | <.5 | ND++ | 15.5 | 7.5 | 7.5 |
| DC | 1 | <1 | 1 | <.5 | ND | 6 | 4.5 | 4.5 |
| AB | 13 | <1 | 7 | <.5 | ND | 33 | 13.5 | 14 |
| CS | 4 | <1 | 1.5 | <.5 | ND | 16.2 | 11.2 | 12.5 |
| BY | 2.5 | <1 | 3.5 | <.5 | ND | 15.8 | 10.3 | 7.5 |
| MS | 2.5 | <.5 | 1 | <.5 | ND | 3.5 | 0 | 0 |
| GK | 1 | <.5 | 1 | <.5 | 10 | 10.5 | 9 | 9 |
| TF | 1 | <.5 | 1 | <.5 | 2 | 4 | 2.5 | 1 |
| VC | <1 | <.5 | 1 | <.5 | 2 | 2.5 | 1.5 | <1 |
| JL | 3 | <.5 | 2 | <.5 | 10 | 10.3 | 5.3 | 4 |
| Mean | 3.45 | 0.25 | 2 | .5 | 6 | 11.73 | 6.53 | 6.05 |
| SD | ±3.75 | ±0.26 | ±2.01 | 0 | ±4.61 | ±9.14 | ±4.49 | ±4.91 |

* γ' and α' stand for FITC-labelled $F(ab')_2$ fragments of rabbit IgG anti-human γ and α chains, respectively.

+In order to compute the additions, an arbitrary value of 0.5% was given for < 1% and <.5%, respectively.

++ND = Not Determined

**FITC = Fluorescein Isothiocyanate

TABLE IV

Surface Immunoglobulin-bearing Cells and T-cells in Sex-linked Agammaglobulinemic Patients

| Patient | Surface Igs (% Positive Cells) | | | | | | | | | T-cells(%)** |
|---|---|---|---|---|---|---|---|---|---|---|
| | δ | μ | ε | γ | α | κ | λ | Poly | κ+λ | |
| MS | 0 | 0 | 0 | 2.5 | 1 | 0 | 0 | ND° | 0 | 75 |
| TF | <1 | <1 | 1.5 | 1 | <1 | <1 | <1 | 2 | 1 | 49 |
| KO | <1 | 0 | 0 | 0 | 0 | <1 | <1 | 2 | 1 | 64 |
| CK | 8 | 5 | 1.5 | 3.5 | <1 | 11 | 3 | 16 | 14 | 71 |
| JB | <1 | <1 | <1 | 3 | 0 | 1 | <1 | 1 | 1.5 | 70 |
| SM | <1 | 8 | <1 | 1 | <1 | 8 | 2 | 11 | 10 | 76 |
| Mean | 1.66 | 2.33 | 0.66 | 1.83 | 0.41 | 3.5 | 1.08 | 6.4 | 4.58 | 67.5 |
| SD | ±3.1 | ±3.37 | ±0.68 | ±1.36 | ±0.38 | ±4.75 | ±1.15 | ±6.73 | ±5.9 | ±10.01 |

** SRBC's rosettes

o Not determined

ing them at 37° C cells after they had been treated with 1 mg/ml of papain. Under the conditions employed, papain removed the surface immunoglobulins from approximately 80% of the cells without affecting the cell viability as judged by trypan blue exclusion.

As shown in Table VI, after six hours incubation at 37° C, in both individuals, more than 75% of the cells had regenerated their immunoglobulin receptors. Since several of the patients had substantial numbers of Ig-bearing cells it was also important to correlate the serum concentration of each immunoglobulin with the percentage of cells bearing the corresponding immunoglobulin. This comparison is presented in Table VII.

There is no correlation between the levels of immunoglobulins in the serum and the percentages of lymphocytes bearing immunoglobulins on their membranes. Patients diagnosed as being Bruton's agammaglobulinemics, e.g. patient TF has 62 mg/dl of IgD in his serum, but less than 1% of his lymphocytes are positive for membrane-IgD. Patients KO and JB, who also have less than 1% IgD-positive cells, have undetectable amounts of IgD; while patient CK, who has undetectable amounts of IgD in his serum, has 8% mg/dl of serum IgM, but the former has only less than 1% IgM-bearing cells, while the latter has 5%. Patients MS, KO and JB have undetectable amounts of serum IgM.

When the values for serum IgE in this group of patients

TABLE V

Surface Immunoglobulin-bearing Cells and T-cells in Adult Acquired Hypogammaglobulinemic Patients

| Patient | Surface Immunoglobulins (% Positive Cells) | | | | | | | | | % T-cells** |
|---|---|---|---|---|---|---|---|---|---|---|
| | δ | μ | ε | γ | α | κ | λ | Poly | κ+λ | |
| MM | 3 | 3.5 | <1 | 6 | 2.5 | 3.5 | 4 | ND* | 7.5 | 72 |
| DC | 1.5 | 2 | <1 | 1 | 1 | 1.2 | 3.3 | ND | 3.5 | 84 |
| CS | 3 | 6.2 | 1.5 | 4 | 1.5 | 8 | 4.5 | ND | 12.5 | 76 |
| BY | 5.3 | 3.5 | 1 | 2.5 | 3.5 | 1.5 | 6 | ND | 7.5 | 70 |
| GK | 5 | 2 | 2 | 1 | <1 | 6 | 3 | 10 | 9 | 61 |
| JL | 2.3 | 2.5 | <1 | 3 | 2 | 3 | 1 | 10 | 4 | 76 |
| SK | 1.5 | 1.5 | <1 | <1 | <1 | 2 | 2 | 5 | 4 | 76 |
| AB | 5 | 6 | 2 | 13 | 7 | 6 | 8 | ND | 14 | 63 |
| VC | <1 | <1 | <1 | <1 | <1 | 0 | <1 | 2 | 0.5 | 75 |
| JR | 2 | 3 | <1 | 1 | 1 | 3.3 | 1.5 | 5 | 4.8 | 74 |
| Mean | 2.9 | 3.07 | 0.95 | 3.25 | 2 | 3.45 | 3.38 | 6.4 | 6.73 | 72.7 |
| SD | ±1.68 | ±1.84 | ±0.64 | ±3.86 | ±2.01 | ±2.51 | ±2.34 | ±3.5 | ±4.22 | ±6.72 |

* Not determined

** SRBC's rosettes

TABLE VI

Effect of Papain Treatment on PBL's* from a Normal Donor and an Immunodeficient Patient and the Subsequent Regeneration of Surface Immunoglobulins after 6 hr Incubation at 37°C

| Donor | Treatment of Cells | % Cell Viability | % Positive Cells with Anti- δ | μ | Polyvalent |
|---|---|---|---|---|---|
| Control | None | 99 | 8 | 6 | 15 |
| | Papain, No regeneration time | 98 | 1 | 1 | 3 |
| | Papain, 6 hr regeneration time | 89 | 7 | 6 | 14.5 |
| Patient | None | 97 | 8 | 7 | 17 |
| | Papain, No regeneration time | 96 | 1 | 2 | 5 |
| | Papain, 6 hr regeneration time | 89 | 6 | 6 | 13 |

*PBL's = Peripheral Blood Lymphocytes

TABLE VII

Comparison Between the Levels of Serum Igs* and the Surface Igs-bearing Lymphocytes in Immunodeficient Patients

| Patient | Surface Igs (% positive cells) γ | α | μ | δ | ε | Serum Igs (mg/dl) G | A | M | D | E† |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal++ | 4 (5.7) | 1 (0.6) | 5.8 (1.7) | 4.5 (1.9) | 1.1 (0.6) | 1160 | 200 | 99 | 2.17 | 258** |
| MS | 2.5 | 1 | 0 | 0 | 0 | 200 | - | - | - | 10 |
| TF | 1 | <1 | <1 | <1 | 1.5 | 400 | - | 30 | 62 | 45 |
| KO | 0 | 0 | 0 | <1 | 0 | 150 | - | - | - | 6.5 |
| CK | 3.5 | <1 | 5 | 8 | 1.5 | 242 | - | 30 | - | 10 |
| JB | 3 | 0 | <1 | <1 | <1 | 334 | - | - | - | 10 |
| SM | 1 | <1 | <1 | 8 | <1 | 140 | - | 9 | ND | 6.5 |
| AB | 13 | 7 | 6 | 5 | 2 | 210 | - | 12 | - | <5 |
| VC | <1 | <1 | <1 | <1 | <1 | 240 | - | 11 | - | 50 |
| JR | 1 | 1 | 3 | 2 | <1 | 360 | - | 26 | - | 12 |
| MM | 6 | 2.5 | 3.5 | 3 | <1 | 699 | - | 9 | - | 13 |
| DC | 1 | 1 | 2 | 1.5 | <1 | 233 | - | 9 | - | <5 |
| CS | 4 | 1.5 | 6.2 | 3 | 1.5 | 375 | - | 49 | - | <5 |
| BY | 2.5 | 3.5 | 3.5 | 5.3 | 1 | 392 | 25 | 65 | - | <5 |
| GK | 1 | <1 | 2 | 5 | 2 | 483 | 7.5 | 55 | - | <5 |
| JL | 3 | 2 | 2.5 | 2.3 | <1 | 334 | - | - | - | <5 |
| SK | <1 | <1 | 1.5 | 1.5 | <1 | 210 | - | - | - | <5 |

++ Normal values are the mean of ten determinations. Numbers in parentheses are standard deviations.
* Igs = immunoglobulins
\+ Values for IgE are reported in Units/ml.
** Normal values for serum IgG, IgA and IgM were obtained from Stiehm and Fudenberg (44); normal values for IgD and IgE were obtained from Luster, Leslie and Bardana (26b).

were examined we found that there is no correlation between these values and the number of IgE-bearing cells. Patients MS, CK and JB have 10 U/ml of serum IgE, but the first one has no detectable IgE-bearing cells, the second has 1.5% IgE positive cells and the last one has less than 1% positive cells. KO and SM, who have 6 U/ml serum IgE, have different values for their corresponding IgE-bearing cells: no IgE positive cells for KO, while SM has less than 1% positive cells. Patient TF, who has as many IgE-bearing cells as does patient CK, has normal levels of serum IgE (45 U/ml).

In patients with Adult Acquired Hypogammaglobulinemia, none of the ten patients have detectable amounts of serum IgD, although their IgD-bearing cells range from less than 1% to 5.3%. When the precentages of IgM-bearing lymphocytes are examined in this group, it is observed that patients AB and CS, who have 6% and 6.2% positive cells, have serum IgM values of 12 mg/dl for the former and 49 mg/dl for the latter. Patients BY and GK have the highest values of serum IgM within the group (65 and 55 mg/dl, respectively); however they have only intermediate percentages of IgM-bearing cells (3.5 and 2% respectively). Patient VC's IgM-bearing cells amount only to less than 1%. His serum IgM value is 11 mg/dl.

This group of patients also lacks a direct correlation between the number of IgE-bearing lymphocytes and the values of serum IgE: patients AB, DC, CS, BY, GK, JL and SK have

less than 5 U/ml of serum, but patients AB and GK have 2% IgE-bearing cells, while DC, JL and SK have less than 1% IgE-bearing cells. Patients CS and BY have 1.5 and 1% respectively. Patients VC, JR and MM all of whom have all less than 1% IgE-bearing cells, have 50, 12 and 13 U/ml, respectively.

# DISCUSSION

The emphasis in modern clinical immunology has recently shifted toward attempts to relate the immune response in various disorders to basic mechanisms of cellular immunology. In this context recognition of two major lymphocyte cell types, T- and B-cells, has served as a convenient focal point for examination of the immune system.

Since B-cells have been directly related to humoral antibody formation, the distribution of various B-cell receptors has also been proposed as a means of classifying various types of humoral immune deficiency diseases.

Immunodeficient patients constitute a very challenging and important study group, not only because the basis of their disease is often difficult to ascertain, but because they can provide insight into a better understanding of the ontogeny of immunoglobulins and the pathways of lymphocyte differentiation.

Winchester et al. (20) showed that Fc receptors of certain B-cells can bind exogenous IgG. That this phonomenon may have been responsible for the high concentration of IgG-bearing cells in some early studies was corroborated in our work. Cells from ten immunodeficient patients were examined by surface immunofluorescence and it was found that a considerable reduction, from a mean value of 3.5% to 0.3%, in the percentage of IgG-bearing cells was seen when fluorescein

labelled $F(ab')_2$ fragments of anti-$\gamma$ was used.

Since our anti-$\alpha$ antiserum was giving us values higher than the normals previously reported for IgA-bearing cells in peripheral blood, we prepared a fluorescein conjugate of $F(ab')_2$ anti-$\alpha$. When this conjugate was used a reduction from a mean value of 2% to less than .5% was observed for IgA positive cells. This difference was probably due to the presence of immunoglobulin aggregates present in our anti-$\alpha$ antiserum.

These observations were further supported by the fact that in most normal donors and immunodeficient patients, the addition of $\kappa$ and $\lambda$ positive cells approaches more closely the value obtained by adding the percentages of positive cells for the $\mu$, $\delta$, and $\varepsilon$ isotypes (assuming that the values for the $\alpha$ and $\gamma$ positive cells are less than 1% for each isotype) than the value obtained by the addition of the percentages for the five types of H-chain specificities (using the intact IgG antibody molecules for the $\gamma$ and $\alpha$ specificities). This latter value in turn approaches more closely the value given by the polyvalent antiserum, which is expected if it is assumed that a polyvalent antiserum has intact antibody molecules directed against the human $\gamma$ specificity.

The demonstration of B-cells by immunofluorescence in sex-linked agammaglobulinemic patients is not new. Siegal

et al. (11), Aiuti et al. (30) and Gajl-Peczalska et al. (31) have reported the presence of membrane immunoglobulin-bearing lymphocytes in Bruton's agammaglobulinemic patients. Furthermore, similar findings by Cooper et al. (29,32) suggested the possibility that this disease is not uniform, and they coined the term "agammaglobulinemia with B lymphocytes" to describe this particular variation of the entity. Similarly, Gajl Peczalska et al. (31) classify them under the category of "Infantile X-linked Agammaglobulinemia Type II". In our experiments we found some individuals with low numbers of B-cells but in contrast with previous reports, we found X-linked agammaglobulimic individuals with approximately normal levels of B-cells. IgD and IgM were the major surface Ig's represented but occasionally IgE was also present.

Adult acquired hypogammaglobulinemia patients represent a very heterogeneous group and reports of affected individuals bearing no surface Ig positive cells (45), very low levels of surface Ig positive cells (7,8) and possessing normal levels (7,29) have been published. Within our group of patients there are individuals that fit in each of these categories (31). However, the majority of patients would be classified in the "slightly lower than normal" category. In this group IgM and IgD constituted the major surface Ig's. IgE was present on their lymphocytes within the range found for normal donors. The presence of normal numbers of IgE-

bearing cells in those X-linked agammaglobulinemic patients that have B-cells, as well as in all the adult acquired hypogammaglobulinemic patients could suggest a separate pathway of cell differentiation and maturation for this particular immunoglobulin, as proposed by Dwayer et al. (46). To further investigate this point, we tried to correlate the levels of serum immunoglobulins and the percentages of Ig-bearing cells of the various isotypes. No obvious relationship was found since patients having relatively high numbers of IgM, IgD or IgE bearing cells presented both, comparatively high and low levels of the respective immunoglobulin in the serum. The reciprocal was also true. However, the observation that all immunodeficient patients examined in this study had low levels of IgE, is in variance with the previous findings and hypothesis of Dwayer et al. (46). Our data however, are in agreement with the results of Van der Giessen et al. (47); and in accordance with the experiments by Manning et al. (48), who were able to suppress IgE formation in mice by treatment with anti-$\mu$ antiserum.

Our data would suggest that sex-linked agammaglobulinemia can manifest itself in at least two different ways: (a) sex-linked agammaglobulinemia with no B-cells, in which the genetic defect seems to be located in structural genes as reported by Yount et al. (49) and (b) sex-linked agammaglobulinemia with B-cells, in which the genetic defect seems to involve

regulatory genes. Acquired hypogammaglobulinemia, consisting of a heterogeneous group of patients, is more likely to implicate not only regulatory genes as reported by Rivat et al. (50), but also some extrinsic regulatory mechanisms as observed by Waldmann et al. (51) who showed that T-cells from hypogammaglobulinemic patients could suppress the differentiation of Ig-bearing lymphocytes.

Finally, our experiments showed that the T-cell population in our group of immunodeficient patients is not significantly altered from the levels reported for normal donors by other workers (41). This is in accordance with other previous functional (8) and quantitative (42) studies. Such observations suggest that in those patients where the B-cell population is reduced, there is a proliferation of null cells. A possible exception is patient TF, who had a reduced number of T-cells and a very elevated level of serum IgD. According to the special report of a W.H.O. committee (52), there is a group of X-linked agammaglobulinemic patients in which high levels of IgM are seen coupled with variable degrees of T-cell aberation. It seems therefore, that patient TF might be a variation of this form, in which there is no aberation of the serum IgM, but serum IgD is abnormal. Further studies of patients like TF may shed some light on the still dark pathways of B-cell differentiation and ontogeny of immunoglobulins.

# SUMMARY

We have shown that Bruton's agammaglobulinemic patients can have normal levels of B-cells. Adult acquired hypogammaglobulinemic patients present a wide range in B-cell values, from virtually no B-cells to normal limits. In both groups of patients IgM and IgD are predominant immunoglobulins on the membrane of the lymphocytes. In both groups of patients, when B-cells are demonstrable, IgE is present on their membranes in normal ranges. Neither the Bruton's nor the acquired hypogammaglobulinemics exhibit a consistent correlation between the levels of serum immunoglobulin and the number of lymphocytes bearing that immunoglobulin on the surface. Most of the patients in each group show normal percentages of T-cells.

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