

Suppressor thymus-derived lymphocytes in fungal infection.

J D Stobo, ... , R E Van Scoy, P E Hermans

J Clin Invest. 1976;57(2):319-328. <https://doi.org/10.1172/JCI108283>.

Research Article

Thymus-derived lymphocyte (T-cell) function, as determined in vivo by cutaneous reactivity to several antigens and in vitro by responsiveness to mitogens and antigens, was assessed in 14 patients infected with a variety of fungal organisms. While all patients manifested a normal frequency of peripheral blood T cells, only seven patients reacted to at least one of the antigens used for cutaneous testing and demonstrated normal in vitro T proliferative responses. Three patients exhibited cutaneous anergy but normal in vitro T-cell reactivity while four patients demonstrated persistent anergy and marked in vitro T-cell hyporeactivity which was independent of activity of infection, concurrent medication, or any associated disorders. The marked diminution of in vitro T-cell reactivity noted for these later four patients was not due to a deletion of antigen- or mitogen-reactive cells. Thus, patients' cells which had been initially cultured for 7 days without any mitogenic or antigenic stimulus and which were subsequently washed and recultured with phytohemagglutinin, concanavalin A, or histoplasmin demonstrated a marked increase in their responsiveness. Moreover, this reactivity noted for recultured cells could be suppressed by a nonphagocytic, nonadherent, nonimmunoglobulin-bearing, sheep red blood cell rosette-forming population of cells isolated from the fresh peripheral blood mononuclear cells of the same patient. While these "regulator" T cells were capable of suppressing T-proliferative responses to antigens and mitogens, they did [...]

Find the latest version:

<https://jci.me/108283/pdf>



Suppressor Thymus-Derived Lymphocytes in Fungal Infection

JOHN D. STOBO, SIGRUN PAUL, ROBERT E. VAN SCOY, and
PAUL E. HERMANS

*From the Departments of Immunology and Medicine, Mayo Medical School
and Foundation, Rochester, Minnesota 55901*

ABSTRACT Thymus-derived lymphocyte (T-cell) function, as determined in vivo by cutaneous reactivity to several antigens and in vitro by responsiveness to mitogens and antigens, was assessed in 14 patients infected with a variety of fungal organisms. While all patients manifested a normal frequency of peripheral blood T cells, only seven patients reacted to at least one of the antigens used for cutaneous testing and demonstrated normal in vitro T proliferative responses. Three patients exhibited cutaneous anergy but normal in vitro T-cell reactivity while four patients demonstrated persistent anergy and marked in vitro T-cell hyporeactivity which was independent of activity of infection, concurrent medication, or any associated disorders. The marked diminution of in vitro T-cell reactivity noted for these later four patients was not due to a deletion of antigen- or mitogen-reactive cells. Thus, patients' cells which had been initially cultured for 7 days without any mitogenic or antigenic stimulus and which were subsequently washed and recultured with phytohemagglutinin, concanavalin A, or histoplasmin demonstrated a marked increase in their responsiveness. Moreover, this reactivity noted for recultured cells could be suppressed by a nonphagocytic, nonadherent, nonimmunoglobulin-bearing, sheep red blood cell rosette-forming population of cells isolated from the fresh peripheral blood mononuclear cells of the same patient. While these "regulator" T cells were capable of suppressing T-proliferative responses to antigens and mitogens, they did not diminish pokeweed mitogen-induced immunoglobulin synthesis by normal bone marrow-derived lymphocytes. Patients in whom suppressor "T" cells were found were at risk for relapsing, disseminated fungal infection.

Dr. Stobo is a senior investigator of the Arthritis Foundation.

Received for publication 31 July 1975 and in revised form 6 October 1975.

INTRODUCTION

Several lines of evidence suggest that cell-mediated immunity or thymus-derived lymphocyte (T-cell)¹ function plays a crucial role in host defense against infection with fungal organisms. Thus, individuals manifesting defects in T-cell-mediated immunity which occur either with malignancy or which result from various therapeutic regimens appear to be at risk for fungal infections (1). Moreover, patients with localized or disseminated fungal infection do manifest deficiencies in T-cell reactivity although it is difficult to decide whether the defect precedes or is secondary to the infection (2, 3). Finally, individuals manifesting congenital defects in T-cell function are at risk for at least localized fungal infection when compared to patients manifesting isolated defects in bone marrow-derived lymphocyte (B cell) or humoral immunity (4, 5).

In delineating abnormalities of T-cell-mediated immunity which may be implicated in the susceptibility to fungal infection, it is no longer tenable to consider only intrinsic abnormalities which may occur in effector cell populations. Compelling evidence now exists demonstrating that immunologic reactivity at the effector stage is subjected to both suppressive and amplifying influences, with net reactivity then representing a balance between these two regulatory antagonists (reviewed in reference 6). Thus, T-cell hyporeactivity to a given fungal antigen, for example, may not necessarily indicate an absence of T cells capable of reacting to that antigen, but may rather indicate a preponderance of suppressive or a lack of amplifying influences. Within this framework,

¹ *Abbreviations used in this paper:* B cell, bone marrow-derived lymphocyte; Con A, concanavalin A; dThd, thymidine; Ig, immunoglobulin; PHA, phytohemagglutinin; PWM, pokeweed mitogen; PPD, purified protein derivative; SLE, systemic lupus erythematosus; S-RBC, sheep red blood cell; T cell, thymus-derived lymphocyte.

we have investigated the *in vivo* and *in vitro* T-cell reactivity of 14 patients with localized or disseminated infection with a variety of fungal organisms. Our results indicate that marked, persistent T-cell dysfunction is manifest in a portion of these patients. Most importantly, this dysfunction is not due to an absence of T cells capable of reacting to antigens, but rather is due to the presence of a population of T cells which suppresses other, potentially reactive T cells. Moreover, this abnormality appears to place these patients at a high risk for both disseminated and relapsing infection with the same organism.

METHODS

Patients. All patients included in the study were evaluated by and cared for by members of the Division of Infectious Disease at the Mayo Clinic. Urine, peripheral blood, sputum, gastric washes, and other specimens were cultured for bacteria and fungi by the Department of Clinical Microbiology. Cutaneous testing to candida, mumps, trichophyton, purified protein derivative (PPD) (5 tuberculin U), SK/SD, and histoplasmin was performed on all patients. Erythema and induration were measured at 4, 12, 24, and 48 h later with only induration greater than 5 mm present at 48 h in response to candida, mumps, trichophyton, PPD, or histoplasmin or greater than 10 mm in response to SK/SD (10 U of streptokinase, 2.5 U of streptodornase) scored as positive reactivity.

Cell cultures. Mononuclear cell populations containing less than 5% granulocytes were obtained from heparinized peripheral blood by sedimentation over Ficoll-Hypaque (7). These cells were washed and resuspended in RPMI 1640 medium which had been supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mmol/ml), and fetal calf serum (5%; all from Grand Island Biological Co., Grand Island, N. Y.) which had been heated at 56°C for ½ h. To 1.5×10^6 cells placed in microtiter wells was added three concentrations (4.5, 2.25, and 1.13 µg/ml final concentration) of phytohemagglutinin (PHA: Wellcome Reagents Ltd., Beckenham, England), concanavalin A (Con A; two times crystallized, ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio; 18, 9, and 4.5 µg/ml final concentration), histoplasmin (Parke, Davis & Company, Detroit, Mich.; which had been dialyzed exhaustively against phosphate-buffered saline and placed in culture at a final dilution of 1/100, 1/200, and 1/400), preservative-free *Candida albicans* (Hollister-Stier Laboratories, Downers Grove, Ill.; used at a final dilution of 1/800, 1/1,600, 1/3,200), or medium only. The cultures were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37°C for 72, 96, or 120 h. For mitogen reactivity, 1 µCi of tritiated thymidine (³H]dThd; 2 Ci/mmol, New England Nuclear, Boston, Mass.) was added for the last 12 h of culture and the cells collected, washed, and dried on glass fiber filter pads (Arthur H. Thomas Co., Philadelphia, Pa.) with a custom-made harvester. For antigen reactivity, 1 µCi of ³H]dThd (6.7 Ci/mmol) was added for the last 4 h of culture and the cells collected in a similar fashion. The filters were then placed in 2 ml of Aquasol (New England Nuclear) and the radioactivity measured in a liquid scintillation spectrometer. The arithmetic mean of triplicate samples was determined and the results are expressed as

counts per minute. Unless otherwise specified, results represent maximal mitogen or antigen reactivity.

Lymphocyte subpopulations. Quantitation of T cells was performed by utilizing the sheep red blood cell (S-RBC) rosette technique (8). To 0.1 ml of peripheral blood mononuclear cells ($10\text{--}15 \times 10^6$ cells/ml suspended in RPMI) was added 0.1 ml of washed S-RBC (5×10^8 S-RBC/ml). 50 µl of human AB serum which had been absorbed with S-RBC and heated at 56°C for ½ h was added and the mixture incubated at 37°C for 15 min. The cells were then centrifuged at 50 *g* for 5 min, incubated at 4°C for 2 h, and gently resuspended by hand mixing. The frequency of small, round cells binding three or more S-RBCs was enumerated by light microscopy.

To obtain populations of cells enriched for immunoglobulin (Ig)-bearing lymphocytes (B cells) or T cells, S-RBC rosettes were formed as described above. Mononuclear cells binding S-RBC to their surface were then separated from cells not forming rosettes by centrifugation over Ficoll-Hypaque. Cells remaining at the top of the Ficoll-Hypaque as well as cells sedimenting to the bottom of the Ficoll-Hypaque were removed separately, and the S-RBC in each fraction lysed with Tris-buffered ammonium chloride (9). The frequency of B cells in each layer was then determined by utilizing fluorescein-labeled anti-Ig which contained specificities for human κ- and λ-chains (10). The frequency of T cells in each layer was determined by the S-RBC rosette assay.

Depletion of adherent cell populations was obtained by passage of cells over columns prepared by packing plastic syringes with washed nylon wool (LP-1 Leuko Pak, Fenwal Laboratories, Morton Grove, Ill.) equilibrated with 10% fetal calf serum at 37°C. Phagocytic cells were removed from cell populations by incubation of mononuclear cells with carbonyl iron (Technicon Instruments Corp., Tarrytown, N. Y.) followed by passage of the cells through a magnetic field. Effluent populations obtained by either procedure were assayed for their relative frequency of phagocytic cells, as determined by the ingestion of latex particles (Dow Chemical Co., Indianapolis, Ind.) during a 2-h incubation at 37°C, as well as their relative frequency of nonlymphocytic mononuclear cells depicted by staining for cytoplasmic esterase (11).²

Assay of *in vitro* Ig production. Quantitation of the Ig synthesized, *in vitro*, by peripheral blood mononuclear cells was performed as follows. 10^6 cells were cultured for 7 days in quadruplicate, as described above, with two concentrations (6.25 and 1.56 µg/ml) of pokeweed mitogen (PWM, Grand Island Biological Co.). The cultures were terminated by centrifugation and the culture medium assayed for its ability to inhibit the binding of known quantities of radiolabeled IgG to anti-Ig antibody (containing specificities for human κ- and λ-chains) coupled to Sepharose. By utilizing standard curves constructed from the inhibition of binding noted with known concentrations of unlabeled Ig, the amount of Ig present in the culture medium could be determined. The precise details of this technique are presented elsewhere.³

RESULTS

Immunologic and clinical characteristics of patients studied. As indicated in Table I, the 14 patients with fungal infection studied were heterogeneous as regards

² Rosenthal, A. S. Personal communication.

³ Loehnen, C., and J. D. Stobo. Manuscript submitted for publication.

TABLE I
Immunologic Characteristics of Patients with Fungal Infection

| Diagnosis | T cells* | Individuals with positive skin tests† | In vitro lymphocyte reactivity‡ | | | |
|-----------------------|----------|---------------------------------------|---------------------------------|----------------|---------------|--------------|
| | | | 0 | PHA | Con A | Antigen |
| | % | % | | | <i>cpm</i> | |
| Normal (20) | 72±4 | >95 | 1,999±532 | 121,146±16,499 | 85,166±12,842 | 13,692±3,928 |
| SLE (14) | 70±3 | NT | 1,249±338 | 77,319± 8,622 | 67,278± 7,878 | NT |
| Sarcoid (20) | 72±3 | NT | 991±421 | 81,657±11,983 | 58,755±12,134 | NT |
| Fungal infection (14) | | | | | | |
| Group I (7) | 63±3 | 100 | 1,117±308 | 74,570± 6,647 | 55,324± 7,053 | 14,280±3,262 |
| Group II (3) | 72±2 | 0 | 1,731±243 | 107,126±17,919 | 85,004± 5,078 | 8,655±2,998 |
| Group III (14) | | | | | | |
| A | 71±7 | 0 | 1,296±500 | 1,745± 270 | 2,253± 647 | 1,129± 226 |
| B | 71±2 | 0 | 2,031±627 | 3,541± 1,479 | 4,691± 1,129 | 1,233± 252 |
| C | 74±6 | 0 | 400±354 | 13,749± 6,158 | 8,541± 5,661 | 654± 138 |
| D | 84 | 0 | 1,460 | 2,439 | 2,555 | NT |

The number of patients studied is given in parentheses. NT, not tested.

* Determined with the S-RBC rosette assay. Arithmetic mean±SE. For group III, data obtained for the four individual patients is listed separately with "A-D" designating the individual group III patients.

† Percentage of patients positive to at least one of six antigens used for cutaneous testing.

‡ Maximal reactivity in response to several concentrations of PHA, Con A, histoplasmin, and candida antigen. Geometric mean±SE.

their in vivo and in vitro T-cell function and could thus be divided into three groups. Patients in group I demonstrated positive in vivo cutaneous reactivity to one or more of the antigens used for testing as well as a relatively normal in vitro response to mitogens and antigens. Group II patients, while failing to react to any of the antigens used for skin testing, did demonstrate normal in vitro reactivity to PHA, Con A, and histoplasmin or candida antigen. The four patients in group III, however, demonstrated marked deficiencies in their in vivo and in vitro lymphocyte reactivity despite the presence of a relatively normal frequency of peripheral blood T cells. Indeed, the mitogen reactivity noted for these four patients was substantially less than that noted for patients comparably ill with other disorders (systemic lupus erythematosus [SLE] and sarcoidosis).

That the in vitro T-cell hyporeactivity noted for patients in group III did not merely represent some alteration in culture conditions is demonstrated in Fig. 1. Thus, despite the cell density used in culture, the duration of culture or the mitogen concentration used, peripheral blood mononuclear cells from these patients were consistently less reactive than those from normals. Although not shown, similar data was obtained when peripheral blood mononuclear cells from the two patients with histoplasmosis were cultured with various concentrations of histoplasmin antigen. Viability of the cultured cells, as measured by exclusion of trypan blue, as well as the number of cells recoverable at the end of culture, was similar for normal individuals and patients in

group III (75–85% viability, 30–40% cell recovery). Finally, the relative frequency of peripheral blood monocytes, as depicted by cytoplasmic esterase, was similar

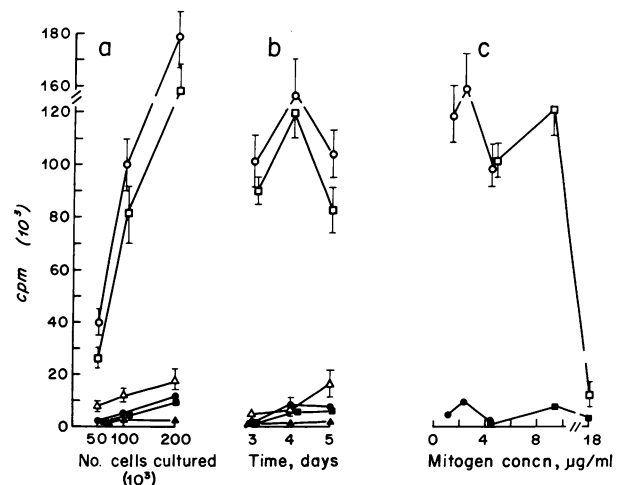


FIGURE 1 In vitro reactivity of lymphocytes from normal individuals (open symbols) and group III patients A and B (closed symbols) to PHA (○), Con A (□), and histoplasmin (△). (a) Various numbers of cells were cultured for 4 days with several concentrations of PHA and Con A or 5 days with several concentrations of histoplasmin. (b) 1.5 × 10⁶ cells were cultured for the indicated times with several concentrations of mitogens and antigens. Data for a and b represent mean maximal reactivities of four experiments±SE. (c) 1.5 × 10⁶ cells were cultured for 4 days with indicated final mitogen concentrations.

for patients in group III ($23 \pm 8\%$) and normal ($21 \pm 6\%$).

Although the infecting organism and mean patient age was similar in all three patient groups, two clinical characteristics distinguished group III from group I and group II patients (Table II). Firstly, three of the four patients in group III demonstrated involvement of more than one organ system and all four patients manifested blood, urine, or bone marrow cultures which were repeatedly positive for the infecting fungal organism. In contrast, involvement of more than one organ system was noted in 40 and 30% of group I and group II patients, respectively. Moreover, only one patient in group I and none of the patients in group II demonstrated positive blood, urine, or bone marrow cultures. Secondly, three of the four patients in group III had two to three episodes each of documented relapsing infection. That is, after appropriate antifungal therapy, these patients lacked either clinical or microbiologic evidence of active infection. However, after intervals ranging from 5 to 12 mo, three patients manifested disseminated infection with the same fungal organism. Relapsing infection was not noted in any patients in group I or group II despite the fact that these patients had been followed for periods ranging from 2 to 3 yr.

It is to be emphasized that the T-cell frequency, cutaneous reactivities, and in vitro mitogen and antigen reactivities included for three of the four anergic patients in group III represent 5-12 determinations performed over a 12- to 18-mo period (data for the fourth patient represents a single determination performed during the absence of active infection). The T-cell hypo-

TABLE II
Clinical Characteristics of Patients with Fungal Infection

| Patient group | Nature of infection* | Mean age yr | No. of patients with relapsing infection |
|---------------|--|----------------|--|
| | | | |
| II | Cutaneous blastomycosis (2) Mucocutaneous candidiasis (1) | 40 ± 17 | 0 |
| III | Disseminated histoplasmosis (2) Disseminated cryptococcosis (2) | 54 ± 2 | 3 |

* Disseminated infection indicates involvement of at least one primary organ system (lung, CNS, etc.) associated with evidence of blood, urine, or other organ infection. Group III patients A and B manifested disseminated histoplasmosis and patients C and D disseminated cryptococcosis.

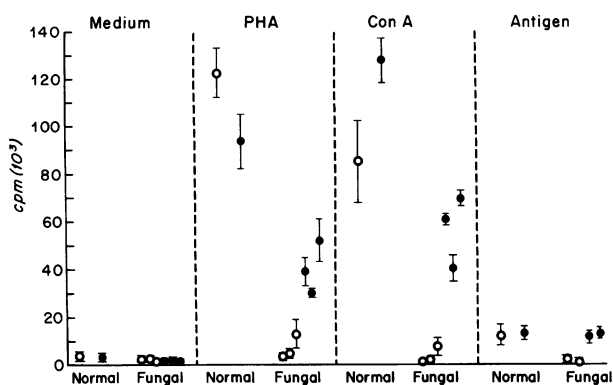


FIGURE 2 Mitogen and antigen reactivity of fresh and cultured cells. Mononuclear cells from normals and group III patients A, B, and C (fungal) were cultured for 7 days without any mitogenic or antigenic stimulus. The cells were then removed and washed and their subsequent reactivity to mitogens and the antigens histoplasmin (patient A and B) measured (\bullet). This reactivity was compared to that noted for cells from the same individual which had not undergone an initial 7-day incubation (fresh, \circ). Values represent mean maximal reactivities \pm SE for five experiments (normal) or three experiments each with individual group III patient. Patients in group I and group II studied in a similar fashion demonstrated reactivities for fresh and recultured cells similar to that noted for normals.

reactivity noted for these patients then was independent of concurrent medication or activity of infection. In only one patient (group I) could an associated granulomatous disease such as sarcoidosis be detected, and in no patient was there evidence of an underlying malignancy. Thus, despite a normal frequency of peripheral blood T cells, 4 of 14 patients with fungal infections manifested severe, persistent defects of in vivo as well as in vitro T-cell reactivity. Moreover, these patients appeared to be at risk for disseminated, relapsing infection. It is these patients that provide the basis for the following immunologic studies.

Effect of culture on T-cell reactivity. Recently it has been possible to demonstrate that T-cell hyporeactivity noted in a portion of patients with collagen vascular disease can be reversed by placing the cells in culture without any mitogenic or antigenic stimulus for 24 h (12-15). Presumably, this is due to the elution from T cells of antibodies which are directed against T-cell surface receptors and thus interfere with the interaction between various stimuli and the cell surface. To determine if the in vitro T-cell hyporeactivity noted for patients in group III was similarly reversible, the following experiments were performed. Peripheral blood mononuclear cells from three of the patients available for study were placed in culture without the addition of any mitogenic stimulus. At various intervals the cultures were removed and the cells and supernatant culture medium

separated by centrifugation. The cells were then washed twice with medium and placed back in culture (recultured) at a concentration of 1.5×10^6 /ml with either medium only or various doses of PHA, Con A, histoplasmin, or candida antigen. The supernatant culture medium was frozen at -20°C for further use. Peripheral blood mononuclear cells from normal individuals or from patients with fungal infection demonstrating normal in vitro T-cell reactivity (group I and group II) were treated in a similar fashion and used as controls. Initial experiments indicated that cells removed 3–4 days after culture demonstrated little change in their subsequent mitogen or antigen reactivity. However, cells from the three patients in group III which had been initially cultured for 7 days and subsequently recultured for 4 days with mitogen or for 5 days with antigens demonstrated an increase in their reactivity. The magnitude of this increase was 4- to 11-fold for PHA and 13- to 41-fold for Con A, when compared to the mitogen reactivities noted for fresh cells from the same patients (Fig. 2). This is contrasted to reactivities noted for recultured cells from either normal individuals or from three patients from group I and one patient from group II. A decrease in the mean responsiveness to PHA and only a 1½-fold increase in the mean responsiveness to Con A was noted for these individuals. Most importantly, while the fresh peripheral blood mononuclear cells from patients in group III with histoplasmosis were unresponsive, in vitro, to histoplasmin, recultured cells from the same patients demonstrated normal DNA synthetic responses to this antigen. Although not shown, the DNA synthetic response of recultured cells from group III patients to candida antigen was also comparable to that noted for recultured lymphocytes from candida-sensitive, normal individuals. Recultured mononuclear cells from normal individuals not demonstrating cutaneous

reactivity to candida or histoplasmin did not react in vitro to these antigens. Unfortunately, changes in specific antigen reactivity which might occur among the recultured mononuclear cells from group III patients with cryptococcal infections could not be determined due to lack of suitable antigen. This increase in mitogen and antigen reactivity could not be accounted for by any significant quantitative change in T cells as the relative frequency of cells capable of forming rosettes with S-RBC was similar for recultured cells ($79 \pm 8\%$) and fresh cells ($72 \pm 4\%$) obtained either from normal individuals or from patients in group III. Moreover, isolation of T- and B-lymphocyte-enriched populations from mononuclear cells which had been initially cultured for 7 days demonstrated that the increased reactivity noted for recultured cells represented reactivity among T cells and not simply acquisition of antigen or mitogen reactivity by B lymphocytes (Table III). Finally, no significant differences in either the total number of cells recovered or the viability of recovered cells was noted when recultured cells obtained from patients in group III or from normal individuals were compared. These findings suggest that T cells capable of reacting to mitogenic or antigenic stimuli do exist among mononuclear cells from patients in group III and that this reactivity can become manifest simply by culturing the cells for 7 days.

Suppression of reactive cultured cells by fresh non-phagocytic, nonadherent, rosette-forming lymphocytes. The near normal T-cell reactivity noted for patients' cultured cells could be explained by the generation of reactive cells, perhaps through some differentiation or maturation process occurring during the 7-day culture. Alternatively, fresh mononuclear cells taken from patients in group III might contain reactive cells which were suppressed in their responsiveness by factors which

TABLE III
Mitogen and Antigen Reactivity of Recultured T and B Cells

| Population | % positive cells | | 0 | PHA | Con A | Histoplasmin |
|--------------|------------------|----|-------|--------|--------|--------------|
| | T | B | | | | |
| "T"-depleted | 15 | 60 | 2,005 | 8,562 | 10,592 | 2,123 |
| "T"-enriched | 92 | 5 | 195 | 48,576 | 54,522 | 13,622 |

S-RBC rosettes were formed by utilizing peripheral blood mononuclear cells from group III patient B which had been cultured for 7 days. Cells binding the S-RBC were then separated from cells not forming rosettes by sedimentation through Ficoll-Hypaque. The S-RBC present in each layer were removed by lysis with Tris-buffered NH_4Cl , and the frequency of T and B cells enumerated as described in Methods. In addition, 1.5×10^5 cells from each layer were cultured with several concentrations of PHA, Con A, or histoplasmin. Results represent the mean maximal reactivities in response to each stimulant observed for two experiments.

dissipated during the culture. If the first possibility were true, admixtures of unreactive fresh peripheral blood mononuclear cells and reactive cultured cells from the same patient should merely result in a simple dilution of the responsiveness of the latter. If, however, cell-associated suppressive influences existed within patients' fresh peripheral blood mononuclear cell populations, such admixtures would result in suppression of the reactivity noted for recultured cells. To delineate between these two possibilities, fresh peripheral blood mononuclear cells or fresh mononuclear cells depleted of adherent or phagocytic cells were mixed with equal numbers of cells from the same patient which had been previously cultured for 7 days (Table IV). These cell mixtures were then assayed for their subsequent reactivity to PHA. Note that when one considers reactivity of each cell population cultured alone, mixtures of fresh and cultured cells manifest a 3- to 4-fold decrease in expected reactivity. This strongly suggests that cell-associated suppressive factors exist within the patients' fresh mononuclear cell populations. Moreover, this suppression was not removed by depletion of monocytes or phagocytes.

Given that the potential reactivity of cultured cells could be suppressed by a nonadherent, nonphagocytic cell, we next determined if this cell was included within either the T- or B-lymphocyte populations. T cells were separated from patients' peripheral blood mononuclear

cells by sedimentation of rosettes through Ficoll-Hypaque, yielding a T-enriched layer consisting of 92% S-RBC rosette-forming, 4% Ig-bearing, and 10% esterase-positive cells. The T-depleted or B-cell layer, on the other hand, demonstrated 10, 50, and 42% of each cell type, respectively. Various numbers of these T- and B-enriched populations were added to various numbers of unfractionated cells from the same patient which had been initially cultured for 7 days. The cell mixtures were then cultured at a constant cell density with mitogens and histoplasmin antigen. Note that addition of increasing proportions of B-enriched cells to reactive cultured cells resulted in mitogen and antigen reactivities which decreased in a relatively linear fashion. Most importantly, this decrease could be predicted by knowing the relative proportions of reactive and unreactive cells (Fig. 3). In contrast, addition of increasing proportions of unreactive, fresh T-enriched populations resulted in reactivities to all three stimuli which were much lower than one would predict on the basis of simple mixing of reactive and unreactive cells. Although the data represented in Table IV and Fig. 3 represent the mean of two experiments performed with cells from one patient in group III, similar data were obtained with two other patients in group III. The fourth patient was not available for such studies.

Thus, these experiments, together with the data dem-

TABLE IV
Inhibition of Cultured, Reactive Cells by Fresh Mononuclear Cell Subpopulations

| Cell populations | Mitogen reactivity | | % positive | | | |
|----------------------------------|--------------------|--------|------------|----|------------|----------|
| | 0 | PHA | T | B | Phagocytic | Esterase |
| | <i>cpm</i> | | | | | |
| Fresh, uncultured: | | | | | | |
| Unfractionated | 1,069 | 6,844 | 73 | 16 | 12 | 21 |
| Column passed | 895 | 6,890 | 89 | NT | 2 | 0 |
| Phagocyte depleted | 738 | 6,693 | 90 | NT | 5 | 3 |
| Cultured | 945 | 22,935 | 78 | 13 | NT | NT |
| Mixtures of cultured cells plus: | | | | | | |
| Fresh, unfractionated | 981 | 7,754 | | | | |
| Fresh, column passed | 1,001 | 6,882 | | | | |
| Fresh, phagocyte depleted | 1,002 | 8,175 | | | | |

Fresh peripheral blood mononuclear cells from group III patient C were passed over nylon wool columns or depleted of phagocytic cells which had ingested colloidal iron. 10^5 cells from each population were cultured, in triplicate, with no mitogen (0) or several concentrations of PHA and compared to that reactivity noted for similar numbers of unfractionated cells or cells which had been initially cultured for 7 days (cultured). In addition, 10^5 cells from each of the fresh, uncultured populations were added to 10^5 cultured cells and the PHA reactivity of these various mixtures compared. The frequency of T cells in each population was determined by utilizing the S-RBC rosette assay, phagocytes by the ingestion of latex beads, and monocytes-macrophages by the presence of cytoplasmic esterase. Results represent the mean maximal PHA reactivity for two experiments. NT, not tested.

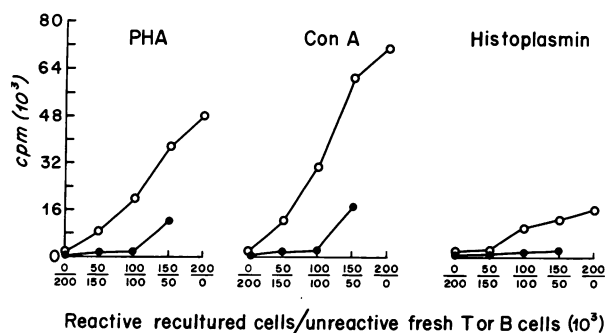


FIGURE 3 Suppression of responsive, cultured cells. Various numbers of B cells (O) or T cells (●) isolated from group III patients' fresh peripheral blood mononuclear cells were added to various numbers of washed cells from the same patient obtained after an initial 7-day culture. The admixtures were then assayed for their reactivity to PHA, Con A, or histoplasmin. Data represents the mean maximal reactivity for two experiments.

onstrating acquisition of reactivity noted for cultured T cells, indicate that the in vitro hyporeactivity noted for mononuclear cells from group III patients is not due to an absence of reactive T cells. Rather, this hyporeactivity represents suppression of these potentially reactive cells by other T cells.

Ability of suppressor T cells to affect Ig synthesis. Waldmann et al. have recently demonstrated that decreased immunoglobulin synthesis noted in a portion of patients with "common variable hypogammaglobulinemia" is due to the suppressive effect of a population of T cells on the proliferative conversion of Ig-bearing B lymphocytes into Ig-secreting plasma cells (16). The group III patients described here demonstrated normal serum Ig levels (mean IgG = 14.6, IgM = 0.91, IgA = 1.5 mg/ml). Moreover, both patients in group III with disseminated histoplasmosis had high titers of complement-fixing antibodies to histoplasmin antigens and precipitating antibodies to H and M antigens. Thus, it would appear that the T cells in these patients which were capable of suppressing the proliferative response of other T cells to mitogens and antigens did not suppress Ig or specific antibody production. To test this hypothesis directly, the effect of peripheral blood mononuclear cells from group III patients on the in vitro synthesis of Ig by normal cells was determined. 10^6 peripheral blood mononuclear cells from several normal individuals, two group III patients, and one patient with late-onset immunodeficiency were cultured alone or in various combinations for 7 days with two concentrations of PWM. At the end of this time the amount of Ig released into the culture medium was assayed (see Methods) and is represented in Fig. 4 as the maximal amount of Ig synthesized per million cells. For the mix-

ing experiments, 10^6 cells from each population were cultured together. Note that the amount of Ig synthesized by group III patients is similar to that synthesized by lymphocytes from normals and is much greater than the Ig synthesized by B cells from the one patient with hypogammaglobulinemia. Admixtures of cells from normal and group III individuals resulted in an amount of Ig synthesized which was near additive. Utilizing similar cell concentrations admixtures of lymphocytes from two normal individuals consistently resulted in Ig production which was only 50–70% of that predicted by summing the amount of Ig produced by each population alone. These findings contrast markedly with the suppression of Ig synthesis noted when mononuclear cells from the patient with hypogammaglobulinemia were added to normal cells.

Thus, while similar relative concentrations of mononuclear cells from group III were capable of suppressing the mitogen and antigen responsiveness of reactive, cultured cells, they did not substantially reduce the amount of Ig synthesized by normal cells.

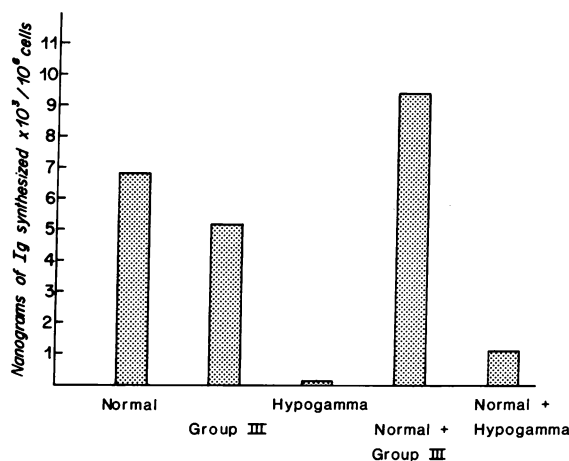


FIGURE 4 Effect of patients' peripheral blood mononuclear cells on Ig synthesis. 10^6 peripheral blood mononuclear cells from three normal individuals, two group III patients (A and C), and one patient with late-onset immunodeficiency (hypogamma) were cultured for 7 days with two concentrations of pokeweed. The amount of Ig synthesized and released into the supernatant culture medium was assayed by utilizing the assay indicated in Methods. In addition, cell mixtures containing 10^6 cells from normal individuals and 10^6 cells from either group III patients or the patient with hypogammaglobulinemia were cultured together with PWM and the Ig synthesis determined. The data is indicated as the mean maximal nanograms of Ig synthesized for three experiments (normal individuals), two experiments for each group III patient, and two experiments for each cell mixture. Admixtures of 10^6 cells from two normal individuals resulted in Ig synthesis which was 50–70% of that predicted by adding the amount of Ig synthesized by each population alone.

DISCUSSION

Recently, there has been a proliferative interest in defining the abnormalities which result in defective immunologic reactivity. Interpretation of such studies must consider data accumulated in both animals and man indicating that net immunologic reactivity, either cellular or humoral, is subjected to a complex set of regulatory influences involving interactions between several populations of immunocompetent cells (6). For example, while T cells are required to cooperate with B cells for the full antibody production to the majority of antigens, T cells can also suppress specific antibody formation (17-19). Similarly, effector cells active in reactions of delayed hypersensitivity are subjected to augmenting and suppressing influences imposed by other T cells, B cells, and macrophages (6, 20-23). Additionally, both T- and B-cell reactivity can be affected by an increasing list of soluble substances which may be produced exogenously by other nonlymphoid cells (24-28).

This variety of mechanisms by which immunologic reactivity may be impaired is reflected by the heterogeneity of immunologic defects detected in patients with fungal infection; some of which are depicted by the three groups of patients indicated here and many of which have been reported previously (2, 3, 29-34). Thus, if one excludes abnormalities resulting either from concurrent disease, such as malignancy, or administration of exogenous drugs such as chemotherapeutic agents, transitory defects in both in vitro and in vivo cell-mediated immunity, abnormalities in in vitro proliferative responses but not in the elaboration of lymphokines, as well as normal proliferative responses accompanied by defects in either release or effect of lymphokines, have all been reported to occur in patients infected with various fungal organisms. Moreover, it is evident from the studies presented here and by others that there is a subpopulation of patients with fungal infection who demonstrate severe, persistent defects of their in vivo as well as in vitro T-cell reactivity (2, 30, 34). The crucial finding demonstrated by this study is that this defect may not result from a lack of potentially reactive T cells but rather may result from a suppression of this reactivity by other non-Ig-bearing, nonphagocytic, S-RBC rosette-forming, and thus presumably T cells. As regards this latter point, we feel relatively confident that it is indeed a thymus-derived lymphocyte which is mediating suppression of T-proliferative responses in group III patients. Thus, populations isolated from patients' fresh peripheral blood mononuclear cells which contained 92% rosette-forming, 4% Ig-bearing, and 10% monocytes did suppress reactivity of cultured cells. Alternatively, populations containing 10% T, 50% B, and 42% monocytes did not. Moreover, populations of fresh mononuclear cells demonstrating a 2- to 7-fold reduction in the relative fre-

quency of esterase-positive or phagocytic cells were not depleted of their ability to suppress reactive cultured cells. Thus, while we have not definitely excluded the possibility that a cell population other than T cells is mediating the suppression, this seems unlikely. However, this does not indicate that another population of cells, perhaps of the monocyte-macrophage series, might be involved in the induction of suppressive T influences.

The data demonstrating acquisition of T-cell reactivity among patient cells during a 7-day culture might reflect elution from T-cell surfaces of inhibiting substances. However, several lines of evidence suggest that this is not so. Firstly, serum from group III patients did not suppress the mitogen or antigen reactivity of normal peripheral blood mononuclear cells.⁴ This suggests that if a soluble substance is responsible for the noted T-cell hyporeactivity, it is not present in large amounts in the serum. Secondly, no increase in the reactivity of patients' cells was noted until at least the 4th day of culture. This is quite different than the elution from T-cell surfaces, during the first 24 h of culture, of antibodies which block T-cell reactivity (14, 15). Finally, we have been able to isolate from the medium obtained from patients' cells cultured for 7 days a soluble material which can suppress mitogen and antigen reactivity of normal lymphocytes. Although a description of the biologic and chemical nature of this material is the subject of a subsequent report,⁴ this inhibitory substance demonstrates the following properties. It has an approximate molecular weight of 1,000, does not contain antigenic determinants detectable on human Ig, and cannot be readily absorbed onto mononuclear cell surfaces. On the basis of these data we suggest that the increased reactivity noted for patients' cells which were placed in culture for 7 days is due to death, metabolic exhaustion, or other alterations occurring among a relatively small population of suppressor T cells. This process is accompanied by a release of the T-associated, soluble suppressor substance into the culture medium. This concept that the regulatory capacity of T cells might change during culture is not new. Indeed, helper and suppressor T-regulatory influences in other systems can be demonstrated to manifest differential survival rates during culture (35). Other, slightly more complex explanations do exist to account for acquisition of reactivity by cultured cells. A rational discussion of these, however, awaits further data dealing with the kinetics for the generation of the soluble suppressor material and the relative ability of the suppressor material to suppress T reactivity of fresh and cultured cells.

The demonstration that peripheral blood mononuclear cells from group III patients did not suppress Ig production by normal B cells suggests that T-mediated regu-

⁴ Stobo, J. D. Manuscript in preparation.

latory mechanisms capable of suppressing cell-mediated (T) and humoral (B) responses may be distinct. Several preliminary experiments not reported here support this. Thus, cell mixtures containing 2/1, 1/1, and 0.5/1 ratios of group III patients' T (obtained by nylon column purification) and normal B cells were not suppressed in their Ig production when compared to cell mixtures containing normal rather than patient's T cells. This then indicates that the failure of group III patient's cells to suppress Ig production in the experiments outlined in Fig. 4 was not simply a manifestation of using too few suppressor T cells. That B cells of group III patients are not inherently resistant to suppressive T influences is suggested by data demonstrating that T cells from a patient with acquired hypogammaglobulinemia can suppress Ig production by group III patients' B cells tenfold. The persistence of apparently normal humoral immune reactivity in these patients may play an important role in their surviving the fungal infection. Thus, while the T-cell defect may hinder the ability of the patients to completely clear the organism, thus predisposing them to relapsing infections, intact humoral responses to the organisms may provide sufficient "immunity" for them to cope with active infection. Additionally, the persistence of normal B-cell reactivity may account for the relative lack of other phenomena in these patients such as recurrent viral infections and the occurrence of malignancy which have been associated with T-cell defects.

Several other obvious questions concerning the regulatory defect in these patients remain unanswered. For example, factors leading to the generation of suppressor T cells, specifically as they relate to possible interactions between exposure to certain antigens and genetic factors governing immune responsiveness, are conjectural. In this regard, while the noted suppression was "non-specific" in that T-proliferative responses to a variety of stimuli were suppressed, this does not imply that the suppressive regulatory influences were not induced by specific fungal antigens. Thus, while suppressive T cells might be generated in response to a specific antigenic challenge, soluble factors mediating the suppression might act irrespective of the antigen specificity of the reacting cells. Moreover, it is not evident whether or not the defect precedes or results from the fungal infection. Finally, whether the heterogeneity in immunologic reactivity noted for the three patient groups delineated here, as well as that noted among patients described elsewhere, represents quantitative rather than qualitative differences in basic defects, as suggested previously, is not known (3).

Thus, defective in vivo and in vitro T-cell reactivity noted in a subpopulation of patients with a fungal infection appears to be due to an abnormality in the regu-

lation of reactive T cells rather than to a depletion of these cells. While these studies raise several important questions concerning the regulation of immunologic reactivity in general, they also serve to emphasize the importance of abnormalities of immunoregulation in clinical disorders. Indeed, we have now been able to demonstrate that T-cell hypofunction noted in a variety of disorders, including collagen vascular disease, combined immunodeficiency disorders, and generalized viral infections, is not due to deletion of reactive T cells but rather is due to a preponderance of cell-associated suppressive regulatory forces. Such studies are important not only in advancing our knowledge of the regulation of immune reactivity, but also for providing rationale for therapy aimed at correcting the defect.

ACKNOWLEDGMENTS

We would like to thank Mrs. Barbara Christian for expert technical assistance. In addition, the support and cooperation of the members of the Division of Infectious Disease is gratefully appreciated.

This work was supported by U. S. Public Health Service grant A. I. 12054.

REFERENCES

1. Nelson, N. A., H. L. Goodman, and H. L. Oster. 1957. The association of histoplasmosis and lymphoma. *Am. J. Med. Sci.* **233**: 56-65.
2. Diamond, R. D., and J. E. Bennett. 1973. Disseminated cryptococcosis in man. Decreased lymphocyte transformation in response to cryptococcus neoformans. *J. Infect. Dis.* **127**: 694-697.
3. Valdimarsson, H., J. M. Higgs, R. S. Wells, M. Yamamura, J. R. Hobbs, and P. J. L. Holt. 1973. Immune abnormalities associated with chronic mucocutaneous candidiasis. *Cell. Immunol.* **6**: 348-361.
4. Biggar, W. D., H. T. Meuwissen, and R. A. Good. 1971. Successful defense against *Histoplasma capsulatum* in hypogammaglobulinemia. *Arch. Intern. Med.* **128**: 585-587.
5. Salvin, S. B., R. D. A. Peterson, and R. A. Good. 1965. The role of the thymus in resistance to infection and endotoxin toxicity. *J. Lab. Clin. Med.* **65**: 1004-1022.
6. Gershon, R. K. 1974. T cell control of antibody production. *Contemp. Top. Immunobiol.* **3**: 31-40.
7. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **21 Suppl.** **97**: 77-89.
8. Jondal, M., G. Holm, and H. Wigzell. 1972. Surface markers on human T and B lymphocytes. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. *J. Exp. Med.* **136**: 207-215.
9. Boyle, W. 1968. An extension of the ⁵¹Cr release assay for the estimation of mouse cytotoxins. *Transplantation (Baltimore)*. **6**: 761-764.
10. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface θ or immunoglobulin determinants. *J. Exp. Med.* **138**: 71-88.
11. Li, C. Y., K. W. Lam, and L. T. Yam. 1973. Esterases in human leukocytes. *J. Histochem. Cytochem.* **21**: 1-12.

12. Wernet, P., and H. G. Kunkel. 1973. Antibodies to a specific surface antigen of T cells in human sera inhibiting mixed leukocyte culture reaction. *J. Exp. Med.* **138**: 1021-1026.
13. Williams, R. C., R. B. Lies, and R. P. Messner. 1973. Inhibition of mixed leukocyte culture responses by serum and γ globulin fractions from certain patients with connective tissue disorders. *Arthritis Rheum.* **16**: 597-605.
14. Wernet, P. R. Thoburn, A. Moore, and H. G. Kunkel. 1974. Blockage of responder and stimulator lymphocyte surface structures and the shedding phenomenon mediated by human antilymphocyte sera in tissue culture. In Proceedings of the Eighth Leukocyte Culture Conference. Academic Press, Inc., New York. 543-552.
15. Winfield, J. B., R. J. Winchester, P. Wernet, S. M. Fu, and H. G. Kunkel. 1975. Nature of cold-reactive antibodies to lymphocyte surface determinants in systemic lupus erythematosus. *Arthritis Rheum.* **18**: 1-8.
16. Waldmann, T. A., M. Durm, S. Broder, M. Blackman, R. M. Blaese, and W. Strober. 1974. Role of suppressor T cells in pathogenesis of common variable hypogammaglobulinaemia. *Lancet.* **2**: 609-613.
17. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells—a model for the two-cell theory of immunocompetence. *Transplant. Rev.* **1**: 92-113.
18. Horiuchi, A., and B. H. Waksman. 1968. Role of the thymus is tolerance. VIII. Relative effectiveness of non-aggregated and heat aggregated bovine gammaglobulin injected directly into lymphoid organs of normal rats in suppressing immune responsiveness. *J. Immunol.* **101**: 1322-1332.
19. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.* **15**: 1-94.
20. Ha, T. Y., and B. H. Waksman. 1973. Role of the thymus in tolerance. X. "Suppressor" activity of antigen-stimulated rat thymocytes transferred into normal recipients. *J. Immunol.* **110**: 1290-1299.
21. Uhr, J. W., and G. Moller. 1968. Regulatory effect of antibody on the immune response. *Adv. Immunol.* **8**: 81-127.
22. Sjögren, H. O., I. Hellström, S. C. Bansal, and K. E. Hellström. 1971. Suggestive evidence that the "blocking antibodies" of tumor-bearing individuals may be antigen-antibody complexes. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 1372-1375.
23. Kirchner, H., T. M. Chused, R. B. Herberman, H. T. Holden, and D. H. Lavrin. 1974. Evidence of suppressor cell activity in spleens of mice bearing primary tumors induced by Maloney sarcoma virus. *J. Exp. Med.* **139**: 1473-1487.
24. Contractor, S. F., and H. Davies. 1973. Effect of human chorionic somatomammotrophin and human chorionic gonadotrophin on phytohemagglutinin-induced lymphocyte transformation. *Nat. New Biol.* **243**: 284-286.
25. Yachnin, S. 1975. Fetuin, an inhibitor of lymphocyte transformation. *J. Exp. Med.* **141**: 242-256.
26. Murgita, R. A., and Tomasi, T. B. 1975. Suppression of the immune response by α -fetoprotein. *J. Exp. Med.* **141**: 269-286.
27. Kamrin, B. B. 1959. Successful skin homograft in mature nonlittermate rats treated with fractions containing alpha-globulins. *Proc. Soc. Exp. Biol. Med.* **100**: 58-61.
28. Occhino, J. C., A. H. Glasgow, S. R. Cooperband, J. A. Mannick, and K. Schmid. 1973. Isolation of an immunosuppressive peptide fraction from human plasma. *J. Immunol.* **110**: 685-694.
29. Catanzaro, A., L. Spitler, and K. M. Moser. 1974. Immunotherapy of coccidioidiomycosis. *J. Clin. Invest.* **54**: 690-701.
30. Mendes, N., C. C. Musati, R. C. Leao, E. Mendes, and C. K. Naspitz. 1971. Lymphocyte cultures and skin allograft survival in patients with South American blastomycosis. *J. Allergy.* **48**: 40-45.
31. Alford, R., and R. A. Goodwin. 1973. Variation in lymphocyte reactivity to histoplasmin during the course of chronic pulmonary histoplasmosis. *Am. Rev. Respir. Dis.* **108**: 85-91.
32. Kirkpatrick, C. H., R. R. Rich, and J. E. Bennett. 1971. Chronic muco-cutaneous candidiasis: model building in cellular immunity. *Ann. Intern. Med.* **74**: 955-978.
33. Newberry, W. M., J. W. Chandler, T. B. Y. Chin, and C. Kirkpatrick. 1968. Immunology of the mycoses. I. Depressed lymphocyte transformation in chronic histoplasmosis. *J. Immunology.* **100**: 436-443.
34. Graybill, J. R., and R. H. Alford. 1974. Cell-mediated immunity in cryptococcosis. *Cell. Immunol.* **14**: 12-21.
35. Taussig, M. J. 1974. Demonstration of suppressor T cells in a population of "educated" T cells. *Nat. New Biol.* **248**: 236-238.